Attenuation of Oxidative Stress, Interleukin-6, High-Sensitivity C-Reactive Protein, Plasminogen Activator Inhibitor-1, and Fibrinogen with Oral Vitamin D Supplementation for Six Months in Patients with Type 2 Diabetes Mellitus having Vitamin D Deficiency

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J Lab Physicians

Abstract

Objectives  Type 2 diabetes mellitus (T2DM) associated with oxidative stress and inflammation causes endothelial dysfunction, which promotes cardiovascular risk. Vitamin D with its pleiotropic effect is said to protect against cardiovascular risk. However, with vitamin D deficiency being more prevalent in T2DM, the cardiovascular risk may get compounded.

Materials and Methods  An interventional study was conducted on 100 patients with T2DM having vitamin D deficiency (vitamin D < 20 ng/mL), who were given oral supplementation of 2,000 IU/day of vitamin D for a period of 6 months. Serum vitamin D, biomarkers of oxidative stress, malondialdehyde (MDA), oxidized LDL (OxLDL), ferric reducing ability of plasma (FRAP), biomarkers of inflammation, high-sensitivity C-reactive protein (hsCRP), interleukin-6 (IL-6), plasminogen activator inhibitor-1 (PAI-1), and fibrinogen were measured at baseline and at the end of the third and sixth month of vitamin D supplementation.

Statistical Analysis  Repeated measures analysis of variance (ANOVA) was applied for comparison between baseline and third- and sixth-month data after vitamin D supplementation. Linear regression by generalized estimating equations (GEE), which grouped repeated measures for each subject and accounted for correlations that may occur from multiple observations within subjects, was applied.

Results  Serum vitamin D levels reached normal levels with a significant decrease in OxLDL, hsCRP, IL-6, PAI-1, and fibrinogen levels, with a significant increase in FRAP (p = 0.001) levels at the end of 6 months of vitamin D supplementation. These changes were observed even after correction with glycemic control (HbA1c). However, a significant decrease in MDA was observed only at the end of the sixth month of
Introduction

Oxidative stress (OS) and inflammation are the underlying mechanisms in type 2 diabetes mellitus (T2DM), which cause endothelial dysfunction leading to cardiovascular disease (CVD) risk.\(^1\) Elevation in plasminogen activator inhibitor-1 (PAI-1) levels has been considered as a marker of general endothelial dysfunction with elevated levels reported in diabetic retinopathy, diabetic nephropathy, and coronary heart disease in type 2 diabetes.\(^2\) Vitamin D is now under precise investigation due to the expression of vitamin D receptors (VDR) in body tissues such as endothelial cells, vascular smooth muscle cells, \(\beta\) cells of the pancreas, T helper cells, macrophages, muscles, and adipose tissues. The active form of vitamin D is also produced in endothelial cells through the activity of a specific endothelial \(\alpha\)-hydroxylase on circulating 25(OH) D.\(^3\) A strong and independent association between vitamin D and cardiovascular events such as angina, myocardial infarction, and stroke has been observed.\(^4\) Low serum levels of 1, 25(OH)\(_2\) D predicted acute myocardial infarction and stroke after a 10-year follow-up in an elderly population-based survey.\(^5\) Some cross-sectional studies have shown a relationship between 25(OH)\(_2\) D and markers of inflammation. In the largest report, on more than 15,000 subjects, it was found that 25(OH)\(_2\) D levels less than 53 nmol/L levels were inversely associated with inflammatory biomarker C-reactive protein (CRP).\(^6\) Vitamin D was found to regulate the expression of pro-inflammatory cytokines and adhesion molecules in the vasculature.\(^7\)

Vitamin D is also found to be positively correlated with insulin sensitivity.\(^8\) Indian studies found that the prevalence of vitamin D deficiency (< 20 ng/mL) in South Indian patients with type 2 diabetes mellitus was 83%, with a higher prevalence of vitamin D deficiency found in pre-diabetics and in diabetics with poor glycemic control.\(^9\)–\(^13\) These low vitamin D levels were found to correlate with insulin resistance, risk of development of both type 1 and type 2 DM, hypertension, hyperlipidemia, and CVD.\(^12\) An inverse and independent relationship between circulating 25(OH) D levels and the prevalence of microvascular complications in patients with T2DM was found. Low vitamin D status was reported to be associated with diabetic nephropathy.\(^13\) Vitamin D deficiency could be an indirect risk factor, causing fatal outcomes of the disease linked rather than being the direct cause of fatality. Hence, by itself, vitamin D deficiency and T2DM, are associated with CVD risk independently and when they occur together the risk is compounded.

Subsequent studies that undertook vitamin D supplementation found an improvement in inflammatory biomarkers in patients with heart failure, attenuation of oxidative stress, and inflammation in vitamin D-deficient T2DM patients.\(^14\)–\(^15\) A single dose of vitamin D was found to improve endothelial function and low-grade inflammation in patients with T2DM.\(^16\) Some studies in diabetic patients have reported improvements in clinical parameters such as central glycemia, insulin sensitivity, and lipid profile, with some finding improvement of endothelial function and inflammatory status.\(^17\)–\(^21\) Hence, in the light of these findings, it was postulated that vitamin D with its pleiotropic effects, may be the elixir for the attenuation of disease processes. However, a clinical agreement was not found with reports of no improvement in oxidative stress and inflammation with vitamin D supplementation of 5,000 IU/day for 12 weeks in T2DM patients.\(^22\) Similarly meta-analyses have found no significant changes in inflammatory status with vitamin D supplementation, attributed to differences in vitamin D dosage, ranging from 400 to 200,000 IU/day, different duration of supplementation, ranging from weeks to years, and also due to lack of information on vitamin D status at baseline in the subjects receiving supplementation.\(^23\)

Taking into consideration the variations in available study designs, we undertook the study in subjects with T2DM having vitamin D deficiency. The aim was to observe the effect of oral supplementation of vitamin D of dosage 2,000 IU/day for a period of 6 months on markers of oxidative stress, inflammation, and acute phase reactants. The biomarker levels at baseline were measured and the changes in the levels were studied at two study points, one, at the end of the third month of supplementation and the second, at the end of the study period that is at the end of the 6 months. The effects of vitamin D supplementation at both the study points were compared with baseline levels and also between the two study time points.

Materials and Methods

This interventional prospective study included 100 subjects selected from the patients attending the outpatient Clinic of Endocrinology and Metabolism at Sri Venkateswara Institute of Medical Sciences (SVIMS), Tirupati, Andhra Pradesh, India (► Fig. 1). The subjects were diagnosed with T2DM as per the revised American Diabetic Association (ADA) criteria\(^24\) with a duration of T2DM ranging from 1 to 5 years and
having vitamin D deficiency (vitamin D < 20 ng/mL).\textsuperscript{25} It was ensured that subjects receiving treatment were on oral hypoglycemic agents, were receiving stable statin therapy for a minimum period of 3 months, with stable treated hypertension. Patients with other forms of diabetes (type 1 DM, gestational DM), known history of thyroid disorders, malignancy, cerebrovascular diseases, myocardial infarction, chronic kidney disease, acute and chronic inflammatory diseases, smokers, alcoholic, pregnant and lactating women, patients who were on insulin, corticosteroids and vitamin D or calcium supplementation were excluded from the study. The study was approved by the Institutional Ethics Committee (Human Studies), and was registered in Clinical Trials Registry of India (CTRI/2017/03/008236). The study was conducted in accordance with the principles of the Declaration of Helsinki. Informed written consent was obtained from subjects prior to their enrollment into the study. The subjects were given 90 vitamin D tablets (cholecalciferol) of dosage 2,000 IU and instructed to consume one tablet daily for a period of 3 months. The patients were instructed to hand over the empty tablet strips at the end of the third month for securing proof of regular consumption of the vitamin D supplement. The compliance of supplementation was verified over the telephone on alternate days. At the end of the third month, blood was collected for biochemical analysis, and serum vitamin D levels were measured to check for vitamin D toxicity (> 100 ng/mL). As none of the patient’s vitamin D levels reached the toxic levels at the end of the third month, they were given 90 tablets for the next 3 months of the study period with the same instructions carried forward. The patients were instructed to follow the same routine of daily activities and food intake over the study period and to notify any changes in treatment or routine activities. At the end of 6 months of vitamin supplementation, blood was collected from the subjects for biochemical analysis and measurement of vitamin D levels following which vitamin D supplementation was stopped (\textsuperscript{\textbullet} Fig. 2).

### Sample Collection
After an overnight fast of 8 to 10 hours, 8 mL of venous blood sample was collected, of which 1 mL was transferred into sodium fluoride and potassium oxalate containing tube, 1 mL into sodium citrate anticoagulant containing tube, 1 mL into sodium ethylene diamine tetraacetic acid (Na-EDTA) anticoagulant containing tube, and 5 mL into an additive-free tube. Serum and plasma samples were aliquoted and stored at –80°C in a deep freezer (Thermo Fischer Scientific, Marietta, OHIO 45750) until biochemical analysis.

### Methods
Plasma and serum samples were analyzed immediately for plasma glucose by glucose oxidase peroxidase (GOD-POD) method (Pathozyme Diagnostics, Kolhapur, India) on Syn-chron Unicel Dxc 600 auto analyzer, glycosylated hemoglobin (HbA1C) by ion-exchange high-performance liquid chromatography (HPLC) method (Bio-Rad Laboratories India Pvt. Ltd., Gurgaon) and vitamin D measured as 25 hydroxy cholecalciferol by chemiluminescence method (Beckman system pack, Ireland Inc., Lismeehan, Ireland) on Access 2 auto analyzer, Beckman Coulter, USA. Serum malondialdehyde (MDA) and ferric reducing ability of plasma (FRAP) were measured by spectrophotometric methods\textsuperscript{26,27} on a UV Spectrophotometer (Llantrisant CF728YW, United Kingdom), high sensitive C-reactive protein (hsCRP) (Beckman System Pack, USA) on Synchron Unicel Dxc 600 auto analyzer, Beckman Coulter, USA, plasma fibrinogen by immunoturbidimetry method (Tulip-Quanta, Goa) on Awareness Technology Chemwell Automated EIA and Chemistry analyzer, USA, serum OxLDL, IL-6, and PAI-1 by enzyme-linked immunosorbent assay method (ELISA) (Genxbio, Gurgaon, India) on ELISA reader (Transasia Bio-Medicals Ltd., Mumbai, India) and ELISA Washer (ERBA Diagnostics Mannheim, Germany). Taking into consideration the analyte stability, serum OxLDL was analyzed within 3 months of blood collection.

### Statistical Analysis
Data distribution was studied using Kolmogorov–Smirnov test. Data obtained was expressed as mean ± standard error. The data were converted to percentages with the baseline value taken as 100%. The percentage change in the biomarker levels from the baseline to the end of the third month and from...
the baseline to the end of the sixth month was calculated taking the baseline as 100%. Repeated measures analysis of variance (ANOVA) was used for comparison between baseline and third- and sixth-month data after vitamin D supplementation. Linear regression was performed using generalized estimating equations (GEE), which groups repeated measures for each subject and accounts for correlations that may occur from multiple observations within subjects. The model with the best goodness of fit was selected. A p-value less than 0.05 was considered as statistically significant. All statistical analysis was performed using Statistical Package for the Social Studies (SPSS) windows version 16.0. (SPSS Inc, Chicago, IL, USA), and MedCalc (Version 12.1, Ostend, Belgium) and Microsoft excel spreadsheets.

Results

Table 1 depicts the time-course changes in the parameters compared with the baseline that showed a significant increase in vitamin D levels, decrease in Ox-LDL, IL-6, PAI-1, fibrinogen, and increase in FRAP levels, observed both the end of the third month and sixth month when compared with the baseline. A decrease in MDA and hsCRP levels was observed at the end of the sixth month of vitamin D supplementation when compared with baseline levels and the levels at the end of the third month. The percentage changes from the baseline to the end of the third month and from the baseline to the end of the sixth month were calculated taking the baseline as 100%. A greater percentage of increase in serum levels of vitamin D at the end of the third month and FRAP levels at the end of the sixth month was observed, and a greater percentage of decrease for serum MDA and PAI-1 at the end of the sixth month was observed. The rate of percentage change was double at the end of the sixth month than that observed at the end of the third month for MDA, PAI-1, and FRAP when compared with the baseline.

Table 2 depicts the linear regression analysis performed using the GEE that showed that the time course changes in vitamin D levels showed a significant negative association with Ox-LDL, Hs-CRP, IL-6, PAI-1, and fibrinogen.

Discussion

The improvement in vitamin D levels was progressive with the increase in vitamin D levels observed at the end of the sixth month, being significant when compared with the changes observed at the end of the third month. Normal vitamin D levels were attained at the end of the sixth month.

Table 1 Time course changes in vitamin D levels, oxidant, antioxidant, and inflammatory biomarkers with vitamin D supplementation

| Parameter          | Baseline Mean ± SE (n = 86) | Third month Mean ± SE (n = 86) | Sixth month Mean ± SE (n = 86) | *p-Value *
|--------------------|-----------------------------|--------------------------------|-------------------------------|------------------------
| Vit D (ng/ml)      | 15.28 ± 0.42                | 30.95 ± 0.60†                  | 37.90 ± 0.40†§                | < 0.001                |
| Percentage change compared with baseline | –                            | 120.31 ± 10.01†               | 173.21 ± 12.89               |                       |
| OxLDL (ng/l)       | 2105.8 ± 95.16              | 1217.2 ± 49.30†               | 612.35 ± 25.72§               | < 0.001                |
| Percentage change compared with baseline | –                            | −32.61 ± 3.84                | −65.19 ± 2.28                |                       |
| MDA (µmol/L)       | 3.67 ± 0.08                 | 3.59 ± 0.07 NS                | 2.48 ± 0.07†§                | < 0.001                |
| Percentage change compared with baseline | –                            | −1.08 ± 2.79                 | −29.15 ± 2.53                |                       |
| FRAP (mmol/L)      | 0.51 ± 0.02                 | 0.59 ± 0.01†                 | 0.85 ± 0.01†§                | < 0.001                |
| Percentage change compared with baseline | –                            | 29.03 ± 5.39                 | 82.37 ± 8.75                 |                       |
| HsCRP (mg/dL)      | 1.13 ± 0.07                 | 0.93 ± 0.05 NS                | 0.69 ± 0.05†§                | < 0.001                |
| Percentage change compared with baseline | –                            | −10.03 ± 9.17               | −17.80 ± 8.36                |                       |
| IL-6 (ng/l)        | 7.71 ± 0.21                 | 6.59 ± 0.20†                 | 5.29 ± 0.17†§                | < 0.001                |
| Percentage change compared with baseline | –                            | −14.88 ± 0.80               | −31.62 ± 1.06                |                       |
| PAI-1 (Au/mL)      | 4.54 ± 0.11                 | 3.89 ± 0.09†                 | 2.56 ± 0.06†§                | < 0.001                |
| Percentage change compared with baseline | –                            | −10.98 ± 2.60               | −40.94 ± 1.92                |                       |
| Fibrinogen (mg/dL) | 145.15 ± 3.64               | 130.30 ± 2.95†               | 105.66 ± 2.03§               | < 0.001                |
| Percentage change compared with baseline | –                            | −10.44 ± 1.37               | −26.74 ± 1.31                |                       |

Abbreviations: Au/mL, arbitrary units/microliter; FRAP, ferric reducing ability of plasma; HsCRP, high-sensitivity C-reactive protein; IL-6, interleukin-6; MDA, malondialdehyde; OxLDL, oxidized low density lipoprotein; PAI-1, plasminogen activator inhibitor-1; Vit D, 25 hydroxy cholecalciferol D. Statistical tool used: Repeated measures ANOVA test, followed by Bonferroni’s multiple comparisons. Data are expressed as mean ± standard error. *Statistically significant, †Statistically significant at the end of the third month compared with baseline. ‡Statistically significant at the end of the sixth month compared with the baseline. §Statistically significant at the end of the sixth month compared with the third month (p = 0.001).

NS, Not significant.
Overall, an attenuation of biomarkers of oxidative stress, inflammation, and an improvement in the antioxidant status was observed. The rate of percentage change was double at the end of the sixth month than that observed at the end of the third month for MDA, PAI-1, and fibrinogen was observed both at the end of the third and sixth months when compared with the baseline levels. These changes were progressive as was observed by significant attenuation at the end of the sixth month when compared with the baseline levels. These changes were more significant from the third month onward. Overall, an attenuation of biomarkers of oxidative stress, inflammation, and an improvement in the antioxidant status at the end of 6 months of vitamin D supplementation when compared with baseline levels was observed.

Because glycemic control is known to influence oxidative stress and inflammation, the biomarkers were corrected for HbA1c, the gold standard marker for glycemic control. It was observed that even after correcting, a similar decrease in MDA, Ox-LDL, IL-6, PAI-1, fibrinogen, and an increase in FRAP levels was found. This indicates that the attenuation of oxidative stress and improvement in antioxidant status are due to vitamin D supplementation.

To study the association of time-course changes in vitamin D levels with oxidative stress and inflammation, linear regression analysis was performed using GEE, which groups repeated measures for each subject and account for correlations that may occur from multiple observations within the end of the sixth month was observed, and a greater percentage of decrease for serum MDA and PAI-1 at the end of the sixth month was observed. The rate of percentage change was double at the end of the sixth month than that observed at the end of the third month for MDA, PAI-1, and FRAP when compared with the baseline, indicating that the changes were more significant from the third month onward. Overall, an attenuation of biomarkers of oxidative stress and inflammation, and an improvement in the antioxidant status at the end of 6 months of vitamin D supplementation when compared with baseline levels was observed.

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### Table 2: Association of time-course changes in 25 hydroxyvitamin D levels with oxidant-antioxidant and inflammatory markers

<table>
<thead>
<tr>
<th>Model</th>
<th>Oxidant-antioxidant markers</th>
<th>Inflammatory markers</th>
<th>Interaction of oxidative stress and Inflammation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MDA (µmol/L)</td>
<td>HsCRP (mg/dL)</td>
<td>MDA _FRAP * HsCRP</td>
</tr>
<tr>
<td>Model 1</td>
<td>−4.81 ± 0.45</td>
<td>−5.90 ± 1.00</td>
<td>0.85 ± 0.23</td>
</tr>
<tr>
<td>Model 2</td>
<td>−1.06 ± 0.56</td>
<td>−2.63 ± 1.03</td>
<td>0.85 ± 0.23</td>
</tr>
</tbody>
</table>

Abbreviations: β, coefficient; SE, standard error.
Model 1 = Crude; Model 2 = Generalized estimating equations adjusted for BMI and statin use.
*p-value

(> 30 ng/mL) (<Table 1>). A decrease in the biomarkers of oxidative stress, Ox-LDL, and improvement in antioxidant status, FRAP levels, was observed both at the end of the third and sixth months when compared with the baseline. The attenuation of inflammatory status, IL-6, PAI-1, and fibrinogen was observed both at the end of the third and sixth months when compared with the baseline levels. These changes were progressive as was observed by significant attenuation at the end of the sixth month when compared with the third month (<Table 1>). However, no changes were observed in MDA and Hs-CRP levels at the end of the third month with a decrease observed only at the end of the sixth month of vitamin D supplementation compared with the end of the third month and baseline levels (<Table 1>). These findings indicate that the supplementation of vitamin D is effective if given over a period of 6 months as MDA and Hs-CRP were found to decrease only at the end of the sixth month.

To quantify the changes in the biomarkers at the studied time points, the percentage changes from the baseline to the end of the third month and from the baseline to the end of the sixth month were calculated taking the baseline as 100% (<Table 1>). A greater percentage of increase in serum levels of vitamin D at the end of the third month and FRAP levels at the end of the sixth month was observed, and a greater percentage of decrease for serum MDA and PAI-1 at the end of the sixth month was observed. The rate of percentage change was double at the end of the sixth month than that observed at the end of the third month for MDA, PAI-1, and FRAP when compared with the baseline, indicating that the changes were more significant from the third month onward. Overall, an attenuation of biomarkers of oxidative stress and inflammation, and an improvement in the antioxidant status at the end of 6 months of vitamin D supplementation when compared with baseline levels was observed.

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subjects. The model with the best goodness of fit was selected. This was performed with and without adjusting for the confounding effect of body mass index (BMI) and the use of statin, as both these factors are known to influence oxidative stress and inflammation. The time course changes in vitamin D levels showed a significant negative association with Ox-LDL, Hs-CRP, IL-6, PAI-1, and fibrinogen even after adjusting for BMI and statin use (Table 2).

The presence of both oxidative stress and inflammation in T2DM patients with vitamin D deficiency as observed in this study are well-known CVD risk factors. Hence, the interaction between MDA and FRAP and its association with hs-CRP was studied. Significant association was found between oxidative stress and inflammation even after adjusting for BMI and statin use in T2DM patients having vitamin D deficiency (Table 2). A South Indian study reported a lowering of serum MDA levels and improvement in total antioxidant status in T2DM patients with vitamin D deficiency with oral vitamin D supplementation of 60,000 IU/week for 8 weeks. Meta-analyses of randomized controlled trials with regard to the effect of vitamin D supplementation in diabetic patients have revealed that vitamin D supplementation was found to attenuate inflammation and oxidative stress with improvement in antioxidant status by modifying adipokine concentrations, diminishing pro-inflamatory cytokines such as TNF-α, natriuretic peptide concentrations, and blood pressure. The biological activity of vitamin D is found to be accomplished by its binding to a nuclear vitamin D receptor (VDR) that mediate the regulation of gene transcription of NADPH oxidase, which catalyzes the conversion of oxygen to superoxide, suppresses TNF-α-induced nuclear factor kappa B (NF-κB) activation and thereby prevents further oxidative and inflammatory modifications.

The oxidative stress of T2DM enhances the pro-inflamatory cytokines such as TNF-α, IL-6, and IL-1, which act on the liver stimulating the production of acute-phase proteins, especially hs-CRP. Chronic inflammation promotes endothelial dysfunction, which increases procoagulant factors, and inhibits natural anticoagulant pathways and fibrinolytic activity, leading to a hypercoagulable state in T2DM. Pro-inflammatory and pro-coagulant factors promote vascular smooth cell proliferation and migration in atherosclerotic lesions and cell apoptosis, which result in diabetic cardiovascular complications. The active form of vitamin D acts on VDR of the liver and decreases the production of hs-CRP and prevents the progression of inflammation in T2DM. The results from clinical trials on vitamin D supplementation and its effects on hs-CRP levels reported that 6 out of the 10 trials found a reduced level of circulating hs-CRP after vitamin D supplementation. ROS can directly cause irreversible oxidative modifications of lipids, proteins, or DNA. The ROS brings about the oxidation of LDL converting it to OxLDL, which is subsequently taken up by macrophages contributing to foam cell formation. A further role of Ox-LDL in atherosclerosis could be to initiate and affect inflammatory mediators such as CRP, IL-6, and TNF-α. A positive correlation between CRP and Ox-LDL in humans has been suggested. Vitamin D can suppress foam cell formation by reducing OxLDL uptake by macrophages. Hence, vitamin D deficiency in T2DM patients disturbs the macrophage metabolism and increases foam cell formation, which lead to atherosclerosis and CVD risk. The lowering of oxidative stress by vitamin D supplementation may lead to a decrease in the formation of Ox-LDL. Lower Ox-LDL levels will lead to a decrease in the propensity of foam cell formation and thereby lower the risk of atherosclerosis.

The proposed mechanism of action of vitamin D may be attributed to its effect on systemic inflammation. Improvement in vitamin D levels lowers the production of an upstream inflammatory cytokine IL-6. Reduced IL-6 levels lead to decreased stimulation of the production of acute-phase proteins CRP and fibrinogen from the liver. Elevated fibrinogen levels are associated with a procoagulant environment with increased viscosity of blood due to increased aggregation and reduced deformability of erythrocytes. By lowering CRP levels, multiple effects of CRP are regulated, which include the induction of PAI-1 expression, oxidation, and uptake of LDL by macrophages, which are all downregulated. IL-6 and CRP are associated with cardiovascular risk independent of traditional risk factors.

It can be hypothesized that the inflammation and oxidative stress present in T2DM subjects could be one of the reasons for lowered circulating vitamin D levels due to oxidative catabolism of vitamin D, leading to vitamin D deficiency status, compounded by nutritional deficiencies. Vitamin D deficiency could be an indirect risk factor, causing fatal outcomes of the disease linked by immunosuppressive effects, oxidative stress, and inflammation, rather than being a direct cause of fatality. Hence, using vitamin D supplementation in subjects with T2DM having vitamin D deficiency can be considered as a supportive intervention, which may contribute to the prevention of CVD along with specific treatment modalities of T2DM.

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Conflict of Interest
None declared.

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