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# Association of Serum 25-Hydroxyvitamin-D Level with Carotid Artery Intima Media Thickness in Indian Patients of Type-2 Diabetes Mellitus

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**Original Research Article**

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**Abstract:** Vitamin D deficiency is common in Indian population which has a high prevalence of cardiovascular disease. We aimed to find the association of serum 25-hydroxyvitamin D(25-OH-D) level with Carotid artery Intima Media Thickness(CIMT)-a marker of subclinical atherosclerosis, in type-2 diabetics, a high risk group for cardiovascular disease. Type-2 Diabetes Mellitus patients attending to OPD of PGIMER & Dr. RML Hospital, New Delhi, were enrolled in the study. Winter serum 25-OH-D levels were measured by enzyme immunoassay using IDS 25-OH-D EIA kit. Vitamin D deficiency was defined as serum 25-OH-D level less than 50 nmol/L.CIMT was measured by B-mode ultrasonography, on Philips HD 11 machine with transducer L12-3 MHz. CIMT >0.8 mm was considered abnormal. Multivariate linear regression analysis was used to identify independent predictors of increased CIMT.100 patients were studied (55 men and 45 women)with mean age 52.9 ± 11.1 years and mean duration of diabetes 9.2 ± 6.2 years. The prevalence of vitamin D deficiency was 71% in study group. Increased CIMT was seen in 25% patients. Serum 25-OH-D level was associated inversely with CIMT on univariate regression analysis ($r^2$=0.61, p-value<0.001). On multivariate regression analysis, low serum 25-OH-D level was found to be independent predictor of increased CIMT (p-value<0.001) even after adjustment for risk factors showing association with CIMT on univariate analysis (p value<0.005,relaxed upto p value<0.2) which included serum 25-OH-D level, sex, smoking, HbA1c and HDL cholesterol. This regression model accounted for 65%($r^2$=0.65) of total variance in CIMT. Serum 25-OH-D level has an independent inverse association with Carotid Intima Media Thickness-a marker of subclinical atherosclerosis.

**Keywords:** Serum 25-OH-D, CIMT, Type 2 Diabetes, Hypovitaminosis D.

## INTRODUCTION

Vitamin D sufficiency is primarily related to bone integrity. It is essential not only for bone development in children and maintenance of bone in adults, but also for the prevention of osteoporosis and fractures in elderly [1]. Low levels of vitamin D represent a problem of global dimension [2]. There is accumulating experimental and clinical data suggesting that serum levels of 25-hydroxyvitamin-D(25-OH-D) is associated with cardiovascular morbidity and mortality, type-2 Diabetes Mellitus(DM) and metabolic syndrome [1,3]. However, the data showing association of vitamin D deficiency with cardiovascular disease(CVD) or atherosclerosis is not consistent [1,3,4]. Carotid Intima Media Thickness (CIMT) is a well accepted surrogate marker of atherosclerosis and provides a non-invasive method for the risk assessment of cardiovascular diseases [5].

Several studies have demonstrated low serum 25-OH-D levels in populations across India [6-8]. However, Indian studies regarding association of Vitamin D with cardiovascular disease are scarce. So, in patients of type-2DM, a group in whom the incidence of cardiovascular diseases are very high, we aimed to find the association between serum 25-OH-D level and CIMT in a representative sample of type-2 diabetic patients. We hypothesised that vitamin D deficiency is associated with increased thickness of carotid intima media. Proposed objective was to study the serum 25-OH-D levels in type 2 diabetic patients and to see the association of serum 25-OH-D levels with CIMT.

## MATERIALS AND METHODS

100 consecutive type-2 DM patients attending to OPD in Dr. RML Hospital, New Delhi between

November 2011 to February 2013 were recruited for study after written informed consent. The study protocol was approved by the institutional review board and ethical committee.

**Inclusion criteria**

Adult patients >18 years age with type 2DM.
Type 2 diabetes mellitus was defined as-
-ADA criteria [9]
-Patients receiving treatment for type-2DM

**Exclusion criteria**

Patients with the conditions likely to influence vitamin D level or CIMT were excluded:

1. Patients with recent history of acute illness
2. Patients with eGFR<60 ml/min
3. Patients with serum SGPT >3 times upper normal limit or serum bilirubin >2 mg/dl
4. Patients taking medications likely to affect vitamin D levels (Vitamin D supplements, Calcium, Parathormone, Thyroxine, Anticonvulsants, Cholestyramine and Orlistat)
5. Pregnancy
6. Known case of malabsorption syndrome
7. Known case of cardiovascular disease (MI/CABG or PTCA/CHF/Typical angina/ Stroke)

Detailed history and clinical examination was done as per standard protocol for each subject. Medication use data was obtained for use of statins, anti-diabetic drugs, anti-hypertensive drugs, vitamin D supplements, aspirin and other drugs through questionnaire and pill reviews. In anthropometric examination, standing height, body weight, waist circumference (lowest abdominal girth measured with a standardized tape between subcostal margin above and iliac crest below with patient in standing position) was measured and Body Mass Index(BMI) was calculated. Blood pressure measurements were taken twice in both arms (by a single observer with a standard aneroid sphygmomanometer) five minutes apart and a mean was taken.

Samples for blood investigations were collected after an overnight fasting and measurements were done by standard laboratory methods. Investigations included serum 25-OH-D levels, Serum electrolytes, Fasting blood sugar, Kidney Function Tests, Liver Function Tests, Lipid Profile,HbA1c ,C-reactive protein, Urinary Albumin Creatinine Ratio.

Serum 25-OH-D levels were measured by an enzyme-immunoassay using IDS 25-OH-D EIA kit. Calibrators, controls and samples were diluted with biotin labelled 25-OH-D.The diluted samples were incubated in microtitre wells which were coated with a highly specific sheep 25-OH-D antibody for 2 hours at room temperature before aspiration and washing. Enzyme (horseradish peroxidase)labelled avidin, was added which used to bound selectively to complexed biotin and following a further wash step, colour was developed using a chromogenic substrate (TMB).The absorbance of the stopped reaction mixtures were read in a microtitre plate reader, colour intensity developed being inversely proportional to the concentration of 25-OH-D.The sensitivity, defined as the concentration corresponding to the mean minus 2 standard deviations of 10 replicates of the zero calibrator, was 5 nmol/L.

CIMT was measured by B-mode ultrasonography on Philips HD 11 by transducer L12-3 MHz by a single radiologist who was blind to the clinical characteristics of the patients. We confined the IMT measurements to common carotid artery (CCA) of both left and right side. To access CCA-IMT, we focused on far-wall IMT, which is defined as the distance between the leading edge of the lumen-intima interface and the leading edge of the media-adventitia interface. Far wall IMTs of both CCAs was measured at three sites(thickest point, and at sites 1cm upstream and downstream). The mean IMT of the carotid arteries was assessed. The mean CCA-IMT was defined as the mean IMT of the right and the left CCAs calculated from 3 measurements on each side. If uniform intimal thickening in the CCA was found, we measured IMT every 1 cm from the bifurcation to the end of CCA, and then took three measurements at the thickest point,1cm upstream and 1 cm downstream [10]. CIMT >0.8 mm was considered abnormal [11] (figure 1).

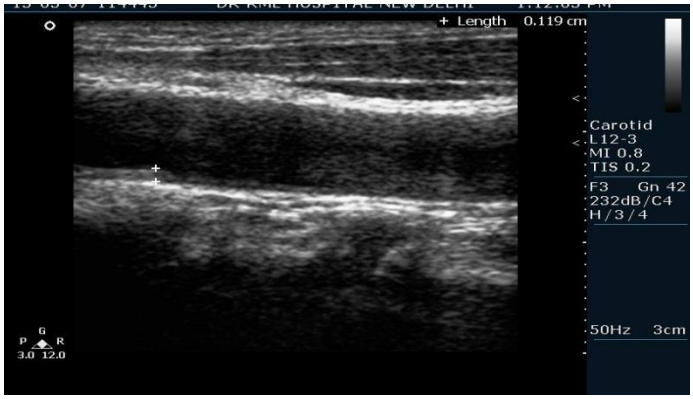


**Fig-1: Carotid Intima Media Thickness by B mode ultrasonography**

Sample size was calculated by using formula:
$n = Z^2\sigma^2 / (error)^2$

Where, n = Required sample size, Z = Confidence level at 95% (standard value of 1.96), σ = Standard deviation(0.15 in this study), e= Absolute precursor error(±0.03 in this study) Hence, sample size was:
$n = [(1.96)^2 \times (0.15)^2 ] / (0.03)^2 = 96.04 \approx 100$

## STATISTICS

Statistical tests were performed by SPSS version 22.Continuous data were presented as mean ± standard deviation and categorical data as proportions. The differences were assessed using Student's t test for continuous variables and chi square test for categorical variables. P-value <0.05 was considered significant. Univariate linear regression analysis was carried out to find association of various risk factors with CIMT as a dependent variable. Multivariate regression analysis was used to evaluate independent predictors of CIMT.

## RESULTS

Out of the total of 100 patients 55 were men and 45 were women. The age of the patients ranged from 25 to 80 years with a mean age of 52.9±11.1 years. The duration of diabetes ranged from 1 to 25 years with a mean of 9.2±6.2 years. More than half the patients(68%) were hypertensive and few(22%) smoked.35% of the patients were overweight (BMI 25-29.9) and 18% were obese(BMI ≥ 30).Values of HbA1c <7% was found in only 9% patients and 91% patients had HbA1c ≥7%.This indicated a lower rate of target glycaemic control and was associated with long duration of diabetes (9.2±6.5years) and lesser use of insulin (39%) in this population. HDL and LDL cholesterol had mean of 36.1±10 mg/dl and 94.9±29 mg/dl respectively. Prevalence of vitamin D deficiency in this population of type-2 diabetes was 71%.There was no significant difference in serum 25-OH-D levels between males and females. 25% of patients had CIMT >0.8 mm. No significant difference was seen in CIMT values between males and females.

On the basis of vitamin D status, patients were divided in two groups. Patients with serum 25-OH-D levels <50 nmol/L were considered vitamin D deficient [4,12].
Group 1: serum 25-OH-D levels <50 nmol/L
Group 2: serum 25-OH-D levels ≥50 nmol/L

**Table1: Baseline characteristics of patients according to vitamin D status.**

| **Variable** | **Hypovitaminosis D** (25-OH-D levels < 50 nmol/L) | | |
|---|---|---|---|
| | With | Without | *p*– value |
| N | 71 | 29 | – |
| Sex (M/F) | 38/33 | 17/12 | 0.64 |
| Age (years) | 52.6 ± 11.3 | 53.48 ± 8.3 | 0.74 |
| BMI (kg/m$^2$) | 25 ± 3.7 | 25.8 ± 4 | 0.34 |
| Waist circumference (cm) | 88.5 ± 6.8 | 91.3 ± 7.2 | 0.08 |
| Diabetes duration (years) | 8.9 ± 6.4 | 10 ± 6.7 | 0.45 |
| Diet only (n) | 10 (14%) | 7 (12%) | 0.22 |
| OHAs only (n) | 33 (46%) | 10 (34%) | 0.27 |
| Insulin treatment | 28 (39%) | 11 (38%) | 0.88 |
| Current smokers | 13 (18%) | 9 (31%) | 0.16 |
| Statin users | 28 (39%) | 11 (40%) | 0.61 |
| Aspirin users | 18 (25%) | 8 (27%) | 0.81 |
| Anti-hypertensive users | 50 (70%) | 18 (62%) | 0.41 |
| Blood pressure (mmHg)<br>Systolic<br>Diastolic | <br>134.5 ± 11.9<br>83.2 ± 8.4 | <br>134 .6 ± 11.8<br>82.9 ± 7.6 | <br>0.96<br>0.86 |
| HbA1c (%) | 8.5 ± 1.2 | 8.2 ± 1.2 | 0.18 |
| Triglycerides (mg/dl) | 138 ± 55 | 125 ± 18 | 0.20 |
| HDL cholesterol (mg/dl) | 36.1 ± 9.2 | 36.2 ± 12.7 | 0.94 |
| LDL cholesterol (mg/dl) | 97.5 ± 28.6 | 88.7 ± 29.6 | 0.18 |
| e-GFR (ml/min/m$^2$) | 99 .5 ± 27.6 | 94.6 ± 25.5 | 0.40 |
| Calcium (mg/dl) | 8.8 ± 0.6 | 8.9 ± 0.7 | 0.76 |
| CRP (mg/dl) | 2.6 ± 2.4 | 2.7 ± 2.1 | 0.81 |
| Micro or Macro-albuminuria (n) | 52 (66%) | 24 (83%) | 0.12 |
| ATP III Metabolic Syndrome (n) | 49 (69%) | 18 (62%) | 0.57 |
| CIMT (mm) | 0.77 ± 0.10 | 0.54 ± 0.10 | <0.001 |

Data are mean ± SD, unless otherwise indicated. Differences were assessed by unpaired t-test (for continuous variables) and by the Chi-square test (for categorical variables).

In these 2 groups, baseline characteristics of patients including age, sex, BMI, waist circumference, diabetes duration, diet, OHA-only treatment, insulin treatment, smoking history, statin use, aspirin use,

antihypertensive use, systolic and diastolic blood pressures,HbA1c levels, Triglyceride levels, HDL cholesterol levels, LDL cholesterol levels, e-GFR, serum calcium, CRP, micro or macro albuminuria, presence or absence of metabolic syndrome were comparable as shown in table 1.

Mean CIMT of group 1 was 0.77±0.10mm while that of group 2 was 0.54±0.10mm.The results demonstrated that patients with vitamin D deficiency had markedly greater CIMT than their vitamin D non-deficient counterparts (p-value<0.001).

Univariate linear regression analysis was applied with CIMT as dependent variable and serum 25-OH-D, age, sex, smoking, diabetes duration, BMI, Metabolic Syndrome, statin/aspirin/antihypertensive use, mean SBP, CRP, HbA1c, eGFR, HDL cholesterol, LDL cholesterol, urine albumin creatinine ratio and serum calcium as independent variables as detailed in table 2.

**Table 2: Univariate regression analysis between CIMT and other variables**

| Independent predictor of CIMT | $r^2$ | *p*- value |
|---|---|---|
| Serum 25-OH-D level | 0.61 | <0.001 |
| Age | 0.000 | 0.83 |
| Sex | 0.016 | 0.19 |
| Smoking | 0.017 | 0.18 |
| Diabetes duration | 0.000 | 0.75 |
| BMI | 0.007 | 0.38 |
| Statin use | 0.000 | 0.96 |
| Aspirin use | 0.000 | 0.84 |
| Antihypertensive use | 0.005 | 0.45 |
| Mean SBP | 0.001 | 0.69 |
| CRP | 0.000 | 0.78 |
| HbA1c | 0.025 | 0.116 |
| eGFR | 0.000 | 0.93 |
| HDL cholesterol | 0.021 | 0.14 |
| LDL cholesterol | 0.002 | 0.64 |
| urine albumin creatinine ratio | 0.000 | 0.75 |
| Metabolic Syndrome | 0.009 | 0.33 |
| Serum Calcium | 0.000 | 0.77 |

It demonstrated that CIMT had a significant negative correlation with serum 25-OH-D levels{$r^2$=0.61,p-value<0.001}as shown in a scatter plot (figure 2).

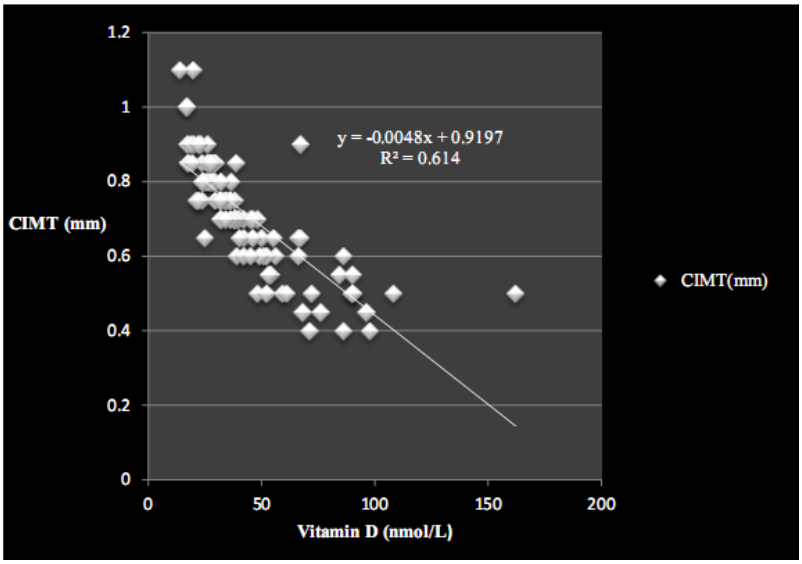


**Fig-2: Scatter plot showing linear inverse relation between serum 25-OH-D level and CIMT**

Other variables showed no such correlation with CIMT. Further, multivariate regression analysis was done with CIMT as dependent variable and variables showing association with CIMT on univariate analysis(p-value<0.005, relaxed upto p value<0.2) [13] which included serum 25-OH-D levels,sex,smoking,HbA1c and HDL cholesterol as independent variables. It also showed that 25-OH-D levels were independently associated with CIMT{$r^2$=0.65,p-value<0.001}(table 3).

**Table 3: Multivariate linear regression analysis.**

| Dependent variable | Independent variables | *p*-value |
|---|---|---|
| CIMT | Serum 25-OH-D levels | <0.001 |
| | Sex | 0.13 |
| | Smoking | 0.68 |
| | HbA1c | 0.04 |
| | HDL | 0.04 |
| $r^2$ model | 0.65 | |

Thus, the study showed a strong independent inverse association between 25-OH-D level and CIMT. Incidentally, HbA1c level(p-value=0.04) and HDL level(p-value=0.04) were other variables found to be associated with CIMT.

## DISCUSSION

We found that decrease in serum 25-OH-D level was strongly associated with increase in CIMT(p-value<0.001) and this association was independent of typical risk factors and confounding variables for CVD. It was also seen that the prevalence of vitamin D deficiency was high (71%) in Indian diabetics.

Recent studies have suggested that vitamin D deficiency can be related to development of diabetes and cardiovascular disease. Indian studies are scarce on this association. Serum 25-OH-D level has been widely used in research to determine the vitamin D status [14]. This is due to its long half-life(~3 weeks) and because the 25-hydroxylation step is not tightly regulated thus reflecting body stores of vitamin D [1]. Serum 25-OH-D is relatively stable and not much influenced by the diet, life style and mobility [15]. On the other hand, paradoxically, levels of $1,25(OH)_2D$ are often normal in severe vitamin D deficiency. Therefore its measurements are not accurate reflection of vitamin D stores and normally not used to diagnose vitamin D deficiency in patients of normal renal functions [14]. This study was desiged to assess the association of vitamin D deficiency with CIMT which had been well accepted as a surrogate marker of subclinical atherosclerosis [16].

100 consecutive type 2 diabetic patients visiting to OPD in RML Hospital, New Delhi, a tertiary care hospital catering to a large population of north India, were enrolled. The mean age of study population and duration of diabetes were compatible with previous studies and was typical of diabetic patients attending to tertiary care hospital. Due to increased duration of diabetes, patients were more likely to develop the complications including CVD.22% of the patients were smokers .68% of the patients were hypertensive, which is compatible with high prevalence of hypertension in type 2 diabetic patients and suggestive of more cardiovascular risk.

The prevalence of vitamin D deficiency[serum 25-OH-D level<50 nmol/L] in a population of type 2 diabetes in this study was high (71%) which was compatible with the prevalence studies done in India and abroad [2,6,17]. Vitamin D deficiency was found more prevalent in age group of 21-40 years, a bit unusual for a working and physically active age group.

CIMT >0.8 mm was considered abnormal [11]. CIMT values were found higher in younger age group 21-40 years as compared to older age groups. This might be related to higher prevalence of vitamin D deficiency in this age group.

Statistical analysis done in 2 groups [vitamin D deficient and vitamin D non-deficient] inferred that patients with vitamin D deficiency had markedly greater CIMT than vitamin D non-deficient group. Other baseline characteristics had no significant difference between these two groups.

Simple univariate linear regression analysis with CIMT as a dependant variable and serum 25-OH-D as independent variable concluded that, CIMT has a significant negative correlation with serum 25-OH-D level(p-value<0.001). Multivariate regression analysis showed that serum 25-OH-D level was independently associated with CIMT even after adjustment for multiple confounding factors. The conclusion was a strong inverse association between serum 25-OH-D level and CIMT.

Targher G *et al* [1] in 2006 in Italy, studied serum 25-hydroxyvitamin D concentrations and CIMT among type 2 diabetic patients. Baseline characteristics of patients used were comparable to those used in present study. They found that among type 2 diabetic patients, those with vitamin D deficiency had a marked

increase in CIMT(1.10±0.15 vs 0.87±0.14 mm, p=0.001) when compared to those with vitamin D sufficiency. Result in present study is similar and rather association is even stronger [1]. Targher *et al* [1] found that 25-OH-D level was independently and inversely associated with CIMT on multivariate analysis which included age, BMI, diabetes duration, fibrinogen, HbA1c, LDL cholesterol, calcium, e-GFR, medications (statins, antihypertensive, aspirin, antidiabetic drugs), sex, smoking and metabolic syndrome as variables. It also showed that male sex, age, smoking and fibrinogen were also independently associated with CIMT, which was not seen in present study. This may be due to different ethnicity, smaller study population and different clinical patterns of the patients [1].

The findings from a population-based cohort of older adults, in a study by Reis J in 2009, suggested a potential role for vitamin D in the development of subclinical atherosclerosis [3].

The North Manhattan Study by Carelli AL *et al* [4] in 230 community dwelling adults was carried out in 2011. They also found that serum 25-OH-D was inversely associated with CIMT(p=0.05) and maximal carotid plaque thickness (p=0.03).This study also assessed indices of mineral metabolism including serum calcium, paratharmone and 1,25-OH-D in patients. However, parathormone and 1,25-OH-D could not be done in present study due to logistic and financial reasons [4].

Whereas, Sachs MC in a study in 2013 could not find evidence linking impaired vitamin D metabolism with increased subclinical atherosclerosis. The study group was younger and had type 1 diabetes unlike our patients [18].

Levels of serum 25-OH-D showed multiple associations with established and emerging cardiovascular risk factors but were not independently related to measures of carotid IMT in a study by Deleskog A *et al* [19].

Hui Ma *et al* in the Shanghai Changfeng study in 2014 found that serum 25-OH-D levels were associated with carotid atherosclerosis in normotensive and euglycemic Chinese postmenopausal women [20].

Chen RH et al in a recent study in Shanghai, China also found that serum vitamin D level is significantly and independently associated with carotid atherosclerosis in patients with Type2 DM [21].

This strong inverse association between serum 25-OH-D level and CIMT(a marker of preclinical atherosclerosis), as evidenced by present study and supported by many other studies mentioned above, suggests that vitamin D deficiency might be an underestimated, novel risk factor for cardiovascular disease among type 2 diabetic patients.

Though the exact mechanism is still not clearly known, the following potential, biochemical mechanisms can be attributed to the protective effects of vitamin D against atherosclerosis. Vitamin D can inhibit various aspects of inflammation which have been established as a key pathogenic mechanism in atherosclerosis. It can exert an anti proliferative effect on vascular smooth muscle cells and myocardial cell hypertrophy and proliferation, which underlies the pathogenesis of congestive heart failure. Further, vitamin D can improve insulin secretion and resistance, which is thought to play a causal role in atherosclerosis and also can act as a negative endocrine regulator for the renin angiotensin system, which itself plays an important independent role in hypertension and cardiovascular health.

## LIMITATIONS

The study was cross-sectional and had no controls. This study had a small population which might result in Type-II error or β error. Also, this study was restricted to a north Indian population of Delhi visiting to a single centre. As parathyroid hormone and $1,25(OH)_2D$ measurements were not done in this study, their possible effects could not be evaluated. Further, on the basis of this study, it is not possible to say whether vitamin D deficiency and atherosclerosis have a cause and effect relationship. Hence, bigger, controlled and prospective studies should be done to further establish this association.

## CONCLUSIONS

This study concluded that:

- There is a significant inverse association between serum 25-OH-D level and CIMT, a marker of subclinical atherosclerosis, in patients of type 2 diabetes mellitus.
- This association is independent of conventional risk factors.
- Prevalence of vitamin D deficiency is high in type 2 diabetic patients.

Though this study is a preliminary study, in view of its significant observation, more data should be collected on the possible protective effect of vitamin D supplementation on cardiovascular disease in type 2 diabetics. This might prove to be a very cost effective way of reducing cardiovascular disease(CVD) in type 2 diabetic patients in particular and community in general.

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