Original Article

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Biochemistry Section

Serum Vitamin D Levels and Vascular Endothelial Growth Factor in Patients with Type 2 Diabetes Mellitus: A Cross-sectional Study

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ABSTRACT

Introduction: Diabetes Mellitus (DM) is the most common noncommunicable disease and the fifth leading cause of death worldwide. Recent research has demonstrated that low Vitamin D levels and high Vascular Endothelial Growth Factor (VEGF) in middle-aged and elderly populations represent a risk factor for Type 2 Diabetes Mellitus (T2DM).

Aim: To estimate the Vitamin D and VEGF levels in study subjects with T2DM and healthy controls and to correlate the Vitamin D level and VEGF with HbA1c % in study subjects with T2DM.

Materials and Methods: A cross-sectional study was undertaken from October 2014 to November 2016 at the Department of Medicine in collaboration with the Department of Biochemistry, Bangalore Medical College and Research Institute, Bangalore. The study included 50 T2DM subjects on oral hypoglycaemic agents for five years and 50 age and sex-matched healthy controls selected randomly from the general population. In all the study subjects, Random Blood Glucose (RBG), LFT, Renal Function Test (RFT), HBA1c, serum Vitamin D, and serum VEGF were estimated. Student t-test and Chi-square/Fisher-Exact test were used to find the significance of study parameters between cases and controls. Multivariate logistic regression analysis was done to assess the risk factors for DM.

Results: The mean age of the cases was 50.9±9.7 years and of controls was 49.76±7.7 years. Among the 50 cases, 19 (38%) were men and 31 (62%) were women. Among the 50 controls, 27 (54%) were men and 23 (46%) were women. The mean Body Mass Index (BMI) among cases was 27.21±4.59 and in controls was 24.82±2.63 (p-value=0.0016). The mean serum 25(OH) Vitamin D levels in cases were 11.39±3.32 ng/mL and in controls were 28.06±11.14 ng/mL (p-value <0.001). The mean serum VEGF levels in cases were 97.52±16.96 pg/mL and in controls were 56.37±17.74 pg/mL (p-value <0.001).

Conclusion: The present study found that subjects with T2DM have lower serum 25(OH) Vitamin D levels and higher serum VEGF levels than those without T2DM. Serum Vitamin D decreases and serum VEGF-A levels increase with increasing HbA1c%, correlating with vascular complications.

Keywords: Glycaemic control, Hyperglycaemia, Vascular permeability factor, 25-hydroxy Cholecalciferol

INTRODUCTION

DM is a group of disorders characterised by chronic hyperglycaemia associated with disturbances of carbohydrate, fat, and protein metabolism due to absolute or relative deficiency of insulin secretion or its action [1]. DM is one of the most common non-communicable diseases and is the eighth leading cause of death, resulting in 1.5 million deaths worldwide [2]. It is estimated that about 382 million people in the world have diabetes at present, and by 2035, around 592 million people (one adult in 10) will be diabetic [2]. According to the International Diabetes Federation (IDF), India stands second in the world with 65.1 million diabetics [3].

Vitamin D and VEGF levels are found to play a role in the clinical consequences of T2DM: 25-Hydroxyvitamin D (25(OH)D has been shown to be inversely related to Fasting Blood Glucose (FBG) concentrations [4], and Vitamin D has a role in maintaining normal insulin synthesis and secretion [5]. Other evidence has revealed that Vitamin D supplementation increased insulin secretion from the pancreas [4,6]. Additionally, Vitamin D replacement in subjects with impaired glucose tolerance has been shown to decrease insulin resistance [7]. Researchers suggest that maintaining Vitamin D levels might provide protective effects against T2DM and its complications [8].

VEGF is a protein produced by endothelial progenitor cells that stimulates vasculogenesis and angiogenesis. It is a part of the system that restores the oxygen supply to tissues when blood circulation is inadequate [9]. VEGF plays a pivotal role in the retinal microvascular complications of diabetes [10]. VEGF also plays a key role in the development of both Proliferative Diabetic Retinopathy (PDR) and Diabetic Macular Oedema (DME). VEGF has emerged as a major mediator of intraocular neovascularisation, microaneurysm formation, and capillary occlusion with ischaemia, as well as promoting increased vascular permeability [11,12].

Hurskainen AR et al., observed an inverse association between 25(OH)D levels and fasting insulin, fasting glucose, and 2-hour glucose tolerance test, implying that low serum 25(OH)D may be associated with impaired glucose and insulin metabolism [13]. Suzuki A et al., analysed the relationship between serum 25-OHD concentration and the clinical features associated with T2DM. They concluded that microvascular complications and insulin treatment in T2DM patients are associated with the co-existence of hypovitaminosis D [14]. Panou N et al., found that Vitamin D insufficiency may be a poor prognostic factor in patients with advanced diabetic disease, and Vitamin D insufficiency may exert gender-specific effects in the context of T2DM [15].

All the above-mentioned studies have evaluated the role of Vitamin-D and VEGF in increasing the risk of macro and microvascular complications of T2DM. There are no studies that have evaluated Vitamin-D and VEGF levels in study subjects with T2DM on oral hypoglycaemic agents for five years and correlated Vitamin-D levels and VEGF with HBA1c.

Hence, the present study was conducted to evaluate the serum levels of 25(OH)D and VEGF-A and to correlate these parameters with HbA1c in T2DM subjects on oral hypoglycaemic agents for five years.

MATERIALS AND METHODS

A cross-sectional study was conducted in the outpatient Department of Medicine, in collaboration with the Department of Biochemistry, Victoria Hospital and Bowring and Lady Curzon Hospitals attached to Bangalore Medical College and Research Institute, Bangalore, over a period of two years from October 2014 to November 2016. The study was approved by the Institutional Ethics Committee of Bangalore Medical College and Research Institute (vide IEC No: BMC/PG/255/2014-15 Dated 30/11/2014). The procedures followed were in accordance with the ethical standards on human experimentation and with the Helsinki Declaration of 1975 that was revised in 2013. A written informed consent was obtained from all study subjects.

Inclusion criteria: Study subjects with T2DM, aged between 30-70 years diagnosed according to American Diabetes Association criteria {Fasting Blood Sugar (FBS) ≥126 mg/dL and 2-hour Postprandial Blood Sugar (PPBS) ≥200 mg/dL)} [16], on oral hypoglycaemic agents for five years attending the outpatient department were included as cases. Age and sex-matched healthy subjects, selected randomly from the general population, were included as controls.

Exclusion criteria: Subjects with liver and renal dysfunction, cardiovascular and metabolic bone disease, and subjects on Vitamin-D supplementation, pregnant and lactating women were excluded.

Sample size: Sample size was calculated based on study done by Vijay GS et al., [17]. Sample size was calculated using the formula [18]:

$$n=Z^2P(1-P)/d^2$$

Where 'n' is the sample size, z=1.96 (95% confidence interval), p=74.14% (prevalence of Vitamin-D deficiency), d=13% (Absolute precision), dropout rate=10% n=47.98 Approx. 50 in each group.

Thus, 50 cases and 50 controls were included in the study.

Data collection: Data regarding age and gender were collected from all the study subjects. About 5 mL of blood sample was collected from the median cubital vein using aseptic precautions in Ethylene Diamine Tetra Acetic acid (EDTA) tube and in a gel tube. The sample was allowed to clot for 30 minutes and subjected to centrifugation for 10 minutes at 1,000-2,000 x g to separate the serum. The samples were stored in a deep freezer at -80°C until they were processed. HbA1c, FBG, LFT, RFT, Vitamin-D, and VEGF were estimated in all the study subjects. The method of estimation and reference range/cut-off range for all the parameters is given in [Table/Fig-1] [19-25].

STATISTICAL ANALYSIS

Data were entered into MS Excel Version 2016 and analysed using R Software. Descriptive and inferential statistical analysis was carried out in the present study. Results on continuous measurements were presented as mean±Standard Deviation (SD), and results on categorical measurements were presented in numbers and percentages (%). Student t-test (two-tailed, independent) was used to find the significance of study parameters on a continuous scale between two groups: inter group analysis on metric parameters. Chi-square/Fisher-Exact test was used to find the significance of study parameters on a categorical scale between two groups. Pearson Correlation was performed to find the association between Vitamin-D and VEGF with FBG and HbA1c among cases. Multivariate logistic regression analysis was conducted, and odds ratio was calculated to study the risk factors among cases and controls. A p-value of <0.05 was considered significant.

RESULTS

The present study involved 50 T2DM subjects on oral hypoglycaemic agents for five years and 50 age and sex-matched healthy controls randomly selected from the general population. In the present study, the majority of the cases were aged between 41-60 years. The mean age of the cases was 50.9±9.72 years, and for controls, it was 49.76±7.74 years. There was no significant difference in age between cases and controls [Table/Fig-2].

In the present study, Serum 25(OH)D levels were lower than the normal reference values in both cases and controls. The mean Vitamin-D level in cases was in the deficiency range (<20 ng/mL) with 11.39±3.32 ng/mL, and in controls, it was in the insufficiency range (20-30 ng/mL) with 28.06±11.14 ng/mL, with a p-value <0.001. Serum VEGF-A levels were higher than the normal reference values (31-86 pg/mL) in cases with a mean value of 97.52±16.96 pg/mL and within the normal range in controls with 56.37±17.74 pg/mL, with a p-value <0.001 [Table/Fig-3].

A negative correlation was observed between serum 25(OH)D with both FBG and HbA1c in T2DM, which was not statistically significant. A positive correlation was observed between serum

Parameters	Instrument/Principle	Method of estimation	Cut-off range/Normal range/Units	Reference no.
Glycated haemoglobin	Beckmann Coulter AU 480/Photometer	Latex agglutination inhibition assay method	Adults: 4.0-6.2%	[19]
Fasting blood glucose	Beckmann Coulter AU 480/Photometer	Hexokinase(HK): Glucose-6-phosphate dehydrogenase (G6P-DH) method	Adults: 70-105 mg/dL	[20,21]
Liver function tests				
Total bilirubin	Beckmann Coulter AU 480/Photometer	Photometric test	0.3-1.2 mg/dL	[21,22]
Direct bilirubin	Beckmann Coulter AU 480/Photometer	Photometric test	<0.2 mg/dL	[21,23]
Total protein	Beckmann Coulter AU 480/Photometer	Biuret method	6.6-8.3 g/dL	[21,23]
Albumin	Beckmann Coulter AU 480/Photometer	Bromocresol green (BCG) method	3.5-5.2 g/dL	[21,23]
Aspartate Aminotransferase (AST)	Beckmann Coulter AU 480/Photometer	International Federation of Clinical Chemistry (IFCC) method	Men <50 U/L; Women <35 U/L	[21,23]
Alanine Aminotransferase (ALT)	Beckmann Coulter AU 480/Photometer	IFCC method	Men:<50 U/L; Women: <35 U/L	[21,23]
Alkaline phosphatase	Beckmann Coulter AU 480/Photometer	IFCC method	Men: 80-300 U/L; Women: 64-300 U/L	[21,23]
Kidney function tests				
Serum urea	Beckmann Coulter AU 480/Photometer	Urease method	15-45 mg/dL	[21,23]
Serum creatinine	Beckmann Coulter AU 480/Photometer	Jaffe kinetic method	Men: 0.7-1.3 mg/dL; Women: 0.6-1.2 mg/dL	[21,23]
Serum 25(OH)D	Cobas 6000, Roche Diagnostics	Chemiluminescence method	22-32 ng/mL	[24]
Serum VEGF-A	ELISA reader and washer Biorad laboratories	Enzyme linked immunosorbent assay method	31-86 pg/mL	[25]

[Table/Fig-1]: Showing the biochemical parameters [19-25].

Age (years)	Cases			Controls			p-value
	Males	Females	Total	Males	Females	Total	
	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	
30-40	2 (4)	8 (16)	10 (20)	1 (2)	3 (6)	4 (8)	0.068
41-50	3 (6)	12 (24)	15 (30)	15 (30)	12 (24)	27 (54)	
51-60	9 (18)	10 (20)	19 (38)	7 (14)	8 (16)	15 (30)	
61-70	5 (10)	1 (2)	6 (12)	4 (8)	0 (0)	4 (8)	
Total	19 (38)	31 (62)	50 (100)	27 (54)	23 (46)	50 (100)	
Mean±SD	54.95±10.41	48.42±8.52	50.9±9.72	50.78±7.68	48.57±7.80	49.76±7.74	

[Table/Fig-2]: Age and gender distribution among cases and controls.

Parameters	Cases	Controls	t-Score	p-value
Body Mass Index (BMI) (Kg/m^2)	27.21±4.59	24.82±2.63	3.253	0.0016
FBG (mg/dL)	225.50±81.87	97.19±9.40	11.0101	<0.001
HbA1c (%)	8.64±2.32	5.39±0.41	9.7316	<0.001
25(OH)D (ng/mL)	11.39±3.32	28.06±11.14	10.139	<0.001
VEGF-A (pg/mL)	97.52±16.96	56.37±17.74	11.856	<0.001
Urea (mg/dL)	25.36±12.19	24.10±4.99	0.6764	0.500
Creatinine(mg/dL)	0.78±0.28	0.79±0.14	0.2259	0.821
Total bilirubin (mg/dL)	0.50±0.21	0.51±0.22	0.2325	0.816
Direct bilirubin (mg/dL)	0.06±0.078	0.07±0.06	0.718	0.474
Total protein (g/dL)	7.33±0.68	7.35±0.39	0.1804	0.8572
Albumin (g/dL)	4.04±0.55	4.14±0.30	1.1287	0.2618
Globulin (g/dL)	3.29±0.13	3.27±0.09	1.0745	0.2345
ALT (U/L)	25.60±24.32	22.60±9.70	0.8102	0.4198
AST (U/L)	31.77±22.08	34.30±21.54	0.5800	0.5633
ALP (U/L)	188.86±62.97	170±29.09	1.2356	0.2567

[Table/Fig-3]: Comparison of anthropometric and biochemical parameters among cases and controls.

VEGF-A with both FBG and HbA1c in T2DM, which was not statistically significant [Table/Fig-4].

In the study, it is noted from [Table/Fig-5] that serum Vitamin-D decreases and serum VEGF-A increases with worsening glycaemic control, which is statistically significant in the T2DM patients studied.

Parameter pair	Cases	
	r-value	p-value
Serum 25(OH)D (ng/mL) vs FBG (mg/dL)	-0.9196	0.1691
Serum 25(OH)D (ng/mL) vs HbA1c (%)	-0.852	0.1845
VEGF-A (pg/mL) vs FBG (mg/dL)	+0.9408	0.2381
VEGF-A (pg/mL) vs HbA1c (%)	+0.8328	0.1108

[Table/Fig-4]: Correlation of biochemical parameters with FBG and HbA1c among cases.

Parameters		HbA1c <7% n=15 (30%)	HbA1c 7-9% n=17 (34%)	HbA1c >9% n=18 (36%)	p-value
Serum 25 (OH) D (ng/mL)	Mean±SD	10.70±2.58	11.43±3.68	11.92±3.58	<0.001
Serum VEGF-A (pg/mL)	Mean±SD	94.67±20.25	98.83±13.57	98.66±17.56	<0.001

[Table/Fig-5]: Association of biochemical parameters with glycaemic control in cases.

The risk factors for Type II diabetes were studied among cases and controls using multivariate logistic regression analysis, as shown in [Table/Fig-6]. It was found that obesity, defined by BMI ≥25, had an OR of 2.26 (95% CI 1.01 to 5.05, p-value=0.05), poor glycaemic control (HbA1c % more than 8%) had an OR of 18.06 (95% CI 5.29 to 61.75, p=0.97), Vitamin-D deficiency (25-OH-Vitamin-D less than 20 ng/mL) had an OR of 0.84 (95% CI 0.78 to 0.90, p=0.89), and high VEGF-A (more than 46 pg/mL) had an OR of 1.09 (95% CI 1.06 to 1.13, p=0.41), respectively.

Risk factors	Odds ratio (OR)	95% Confidence interval (95% CI)	p-value
Obesity	2.26	1.01 to 5.05	0.05
Poor glycaemic control	18.06	5.29 to 61.75	0.97
Vitamin-D Deficiency	0.84	0.78-0.90	0.89
High VEGF-A	1.09	1.06 to 1.13	0.41

[Table/Fig-6]: Showing multivariate logistic regression analysis to study the risk factors for Type II Diabetes among cases and controls.

DISCUSSION

Fifty T2DM subjects on oral hypoglycaemic agents for five years were evaluated based on their history and biochemical investigations, with special reference to Vitamin-D and VEGF, and correlated with HbA1c.

In the present study, the age of the T2DM subjects ranged from 30 to 70 years, with a mean age of 50.9±9.7 years. Cases and controls were age-matched. The age distribution of the T2DM subjects in the present study was in accordance with previous studies [26,27]. In previous studies, the age range was between 25-68 years, with a mean age of 50.1±13.4 years, and Lee JH et al., in their study, reported an age range of 30-70 years, with a mean of 56.9±8.78 years [28].

Among the cases, 19 (38%) were males and 31 (62%) were females. Cases were gender-matched with controls. Gender distribution is in accordance with the studies of Erem C et al., with 53.34% females and 46.66% males, and Bhargavi SK et al., with 36.66% males and 63.34% females [29,30].

In this study, the cases had a mean FBG value of 225±81.87 mg/dL and 97.19±09.40 mg/dL among controls, with a p-value <0.001, which was statistically strongly significant. The FBG values in cases were higher than the cut-off value of 126 mg/dL, which correlated well with the clinical diagnosis [31].

Glycated haemoglobin is an indicator of both the severity and long-term glycaemic control of DM. It reflects approximately the average blood glucose concentration over the preceding six to eight weeks and is not affected by diet, exercise, insulin therapy, and other drugs. It is a measure of the risk for the development of both micro and macrovascular complications in subjects with T2DM [32]. The mean HbA1c in the cases was 8.64±2.32%, and in controls, it was 5.39±0.41%, with a p-value <0.001. The HbA1c values were higher in cases, which correlated with the clinical diagnosis. The values in this study are in accordance with several studies that have shown an increase in HbA1c levels in diabetics [29,30].

Vitamin-D deficiency seems to predispose individuals to T2DM. A study conducted by Tahrani AA et al., showed that subjects with T2DM or glucose had lower serum Vitamin-D concentrations compared to individuals without diabetes [32]. In the National Health and Nutrition Examination Survey (NHANES) study [33], which assessed insulin resistance, kidney function, and Vitamin-D status of 14,679 subjects, Vitamin-D deficiency was reported to be associated with increased risks of microvascular and macrovascular complications in subjects with T1DM as well as, T2DM. In this study, the mean±SD serum Vitamin-D levels in cases were 11.39±3.32 ng/mL and in controls were 28.06±11.14 ng/mL, with

a p-value <0.001, which is statistically strongly significant. In this study, mean Vitamin-D levels were decreased in cases compared to controls, consistent with the studies of Chiu KC et al., Boucher BJ et al., Song Y et al., and Palomer X et al., [34-37].

Various factors can be attributed to this poor Vitamin-D status among Indians, such as lack of adequate sunlight exposure, darker skin pigmentation, obesity, and predominantly vegetarian dietary habits. A negative Pearson correlation was observed between serum Vitamin-D and FBG, and between serum Vitamin-D and HbA1c in T2DM subjects, which was statistically not significant. The studies conducted by Hurskainen AR et al., Song Y et al., Sun Q et al., Lau SL et al., and Need AG et al., showed that the decrease in Vitamin-D is due to hyperglycemia seen in T2DM subjects [13,36,38-40].

In this study, the mean±SD serum VEGF-A levels in cases were 97.52±16.96, and in controls, they were 56.37±17.74 with a p-value <0.001. This aligns with the studies conducted by Siervo M et al., who demonstrated that VEGF level is increased in diabetic subjects compared to the control group [41]. This increase in VEGF level may be due to increases in response to hypoxia resulting from diabetic microvascular complications and vasculopathy.

Shrikant et al., agree with us, as they found that HbA1c levels in subjects with T2DM showed a positive correlation with VEGF levels, which suggests that VEGF levels increase as HbA1c levels go high (indicating poor long-term control of diabetes), thereby increasing the severity of proliferative DR [42]. They explained that long-term poor control of diabetes causes endothelial damage and hypoxia, leading to increased VEGF, which in turn causes neovascularisation and worsens the state.

Kamba T and McDonald DM showed that the level of VEGF increases with the severity of DR, being higher in subjects with PDR compared to those with non-proliferative DR. VEGF level was increased in diabetic subjects with complications compared to diabetic subjects without complications [43]. They also explained that VEGF plays a key role in the development of both PDR and DME.

In recent years, anti-VEGF agents have emerged as new approaches to the treatment of these devastating diabetic complications. Intravitreal anti-VEGF therapy with bevacizumab (Avastin) is currently being used in clinical practice. Intravitreal injection is an effective anti-VEGF drug for the retina. However, this is an invasive procedure associated with potentially serious complications, such as endophthalmitis or retinal detachment, which may occur in subjects requiring serial treatment over many years. In addition, anti-VEGF drugs could pass into the systemic circulation and may cause hypertension, proteinuria, increased cardiovascular events, and impaired wound healing [44].

Limitation(s)

The results of the present study cannot be generalised to the community, as this study is a cross-sectional study. A prospective study over a longer time duration, involving follow-up of T2DM subjects who go on to develop symptomatic microvascular or macrovascular complications would have been more informative.

CONCLUSION(S)

In the present study, subjects with poor glycaemic control had low vitamin D and high VEGF levels compared to those with good glycaemic control, suggesting that poor glycaemic control was the forerunner of microvascular and macrovascular complications associated with T2DM. Correction of vitamin D deficiency and insufficiency through vitamin D supplementation was associated with an improvement in VEGF levels, suggesting the role of vitamin D in the prevention of complications associated with T2DM. Routine screening of serum vitamin D status and vitamin D supplementation may be an effective public health intervention to improve the vitamin D status of the population, as well as, improve glycaemic control in T2DM subjects and prevent microvascular and macrovascular complications.

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