Original Article

VEGF-A and cardiac autonomic function in newly diagnosed type 2 diabetes mellitus: A cross-sectional study at a tertiary care center

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Abstract

Introduction: Cardiac autonomic neuropathy (CAN) is a key complication of type 2 diabetes mellitus (Ty2DM). Vascular endothelial-derived growth factor (VEGF-A) plays a key role in diabetic macrovascular and microvascular complications. It is shown to be elevated in diabetic neuropathy and has the potential to serve as a biomarker in Ty2DM. We evaluated VEGF-A levels and cardiac autonomic function in newly diagnosed Ty2DM patients. **Materials and Methods:** Forty-four newly diagnosed patients (with symptoms within 1 year from the date of recruitment) were included in the study. Cardiac autonomic function was assessed using heart rate variability (HRV) and Ewing's battery tests. Ewing's scores were computed and tabulated. VEGF-A levels were estimated using enzyme-linked immunosorbent assay (ELISA). **Results:** The patients demonstrated normal responses to the reactivity tests. Ewing's scores were 0 (0-0) and 0 (0-0) for sympathetic and parasympathetic parameters, respectively. The autonomic tone was impaired as assessed by HRV parameters. VEGF-A levels were elevated (308.3 ± 167.2 pg/mL) when compared with the previous literature. **Discussion:** Impaired tone with normal reactivity was suggestive of early stage of autonomic neuropathy. Elevated VEGF-A levels may be attributed to a protective action of the factor seen in early stages of neuropathy in Ty2DM. Serial VEGF-A estimation in large cohorts of newly diagnosed diabetics may validate it as a biomarker in CAN seen in Ty2DM.

Keywords: Cardiac autonomic neuropathy, diabetes mellitus, vascular endothelial-derived growth factor

Introduction

Diabetes mellitus (DM) is an epidemic of global magnitude. As per a World Health Organisation's (WHO) report, the burden of diabetes in India was 31.7 million. It is slated to grow further in the years to come.[1,2] DM is associated with multiple complications such as neuropathy, nephropathy, and retinopathy. Among these, diabetic neuropathy is an important cause of both morbidity and mortality in patients of DM.

One of the common manifestations of diabetic neuropathy is cardiac autonomic neuropathy (CAN). It may manifest as benign symptoms, such as resting tachycardia in the early stages, to sudden cardiac death in chronic cases. The mechanisms governing CAN are primarily due to metabolic insult and activation of pro-oxidant factors such as polyol pathway, Protein Kinase C, advanced glycosylation end products, and hexosamine pathway.[3,4]

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VEGF-A (Vascular Endothelial derived growth factor) has been shown to play a pivotal role in complications arising due to DM, especially diabetic retinopathy and nephropathy. VEGF-A has been isolated from serum and intraocular fluids of patients with diabetic retinopathy. In addition, its levels have been reported to reflect the stage of diabetic neuropathy in patients of Type 2 diabetes mellitus (Ty2DM). Therefore, VEGF-A has the potential to serve as a biomarker for CAN in Ty2DM patients.

However, we did not come across any report that has explored the relationship between VEGF levels and the extent of CAN in Ty2DM. Therefore, we undertook the present study to see the levels of VEGF-A in newly diagnosed Ty2DM patients.

Materials and Methods

The study design was a cross-sectional observation. Ethical clearance was obtained from Institute Ethics Committee before commencing the study. The study was approved by the Ethics Committee vide letter number AIIMS/IEC-2017/840 dated 24th May 2017. The sample size was calculated assuming $\alpha = 0.05$ and confidence interval of 95%. Ty2DM patients were recruited from the Department of Endocrinology and Metabolism at our institute as per criteria proposed by American Diabetes Association.[5] All the patients recruited in the study were "newly diagnosed"—patients had clinical symptoms of DM within *1 year* from the date of recruitment in the study.

Informed written consent was obtained from all participants before inclusion in the study. Patients reported to the Autonomic function laboratory, Department of Physiology on the morning of the test.

Autonomic function testing

The assessment of autonomic function was performed during forenoon hours (09:00 am to 01:00 pm) at the autonomic function laboratory at our institute. The temperature of the lab was maintained in the thermoneutral zone and the environment was noise-free to allay any undue anxiety. Patients reported to the laboratory 2 h after light breakfast. Abstinence from tea, coffee, and nicotine was ensured on the day of the test. Post arrival to the lab, patients were requested to void their bladder. The study protocol was explained in detail and supine rest of 10 min was provided.

Disposable adhesive Ag-AgCl ECG electrodes were attached in Lead II configuration after cleaning the skin with alcohol-based swabs. Respiratory movements were recorded by attaching a strain transducer around the 4th intercostal space. Data were sampled at 2000 Hz using Bionomandix® module wirelessly coupled to Biopac MP 150® system (Biopac Systems Inc., United States of America). Bandpass filter was applied to the ECG signal in real time. ECG and respiratory movements were acquired throughout the autonomic assessment.

Evaluation of autonomic function was done in two domains:

1. Autonomic tone: assessed using heart rate variability (HRV)
2. Autonomic reactivity: assessed by Ewing's battery of five tests

HRV was assessed using the ECG record obtained during supine rest. Lead II ECG was recorded in supine position for 5 min. Data were visually inspected for presence of any artifacts or ectopics. HRV analysis was done using aHRV Nevrokard™ software (Nevrokard Kiauta, Slovenia) and the following parameters were computed as per recommendations of European Task Force.[6]

A. Time-domain indices
   1. SDNN: standard deviation of RR intervals
   2. RMSSD: root mean square of the standard deviation of all RR intervals
   3. pNN50: percentage of RR intervals varying by more than 50 ms than the previous interval
B. Frequency-domain indices
   1. Total Power (ms^2)
   2. LF power (n.u.): representing the influence of sympathetic component
   3. HF Power (n.u.): representing the influence of parasympathetic component

The autonomic tone was assessed using the Ewing's standardized battery of five tests, namely, deep breathing test (DBT), Valsalva maneuver (VM), isometric exercise test, cold pressor test, and lying-to-standing test (LST). RR intervals and Blood pressure changes were quantified for these tests as per standard protocols described in the literature, and Ewing's score was computed.[7-9] Blood pressure measurements for all patients were performed by a single observer to avoid interobserver bias.

Biochemical estimation

As a part of routine work-up, HbA_{1c} and Vitamin D levels were also estimated. Because Vitamin D may independently act as a confounding factor for the pathogenesis of autonomic neuropathy, patients with Vitamin D deficiency were excluded from the study.

VEGF-A estimation

Blood samples were collected using aseptic precautions from antecubital vein post performance of autonomic function assessment. Blood was collected in Vacutainer™ serum separator tubes containing spray-coated silica and clot activator and serum separator gel for serum separation. Post centrifugation, serum was pipetted using micropipette and stored in sterile Eppendorff™ tubes at −20°C for VEGF-A estimation at a later date. VEGF-A was estimated using Sandwich ELISA method using kit supplied by Diaclone™ (Diaclone SAS, Besancon Cedex, France). VEGF-A levels were estimated using linear regression curve which was plotted by using standards supplied by the manufacturer with the ELISA kit.

Statistical analysis

Values were tabulated in a spreadsheet program (Microsoft Excel™, Microsoft Corporation, United States of America).

Values were assessed for Gaussian fit using Kolmogorov–Smirnov test. Values were expressed as Mean ± SD or Median (Interquartile range) depending on the distribution of data. P <0.05 was considered as significant.

Results

Out of 53 patients enrolled in the study, nine patients were Vitamin D deficient. These patients were excluded from the analysis. Therefore, VEGF-A was estimated for 44 patients and data analysis was done for these patients only (n = 44). Mean age and BMI of the patients were 41.93 ± 6.73 years and 25.62 ± 4.36 kg/m^2, respectively. As discussed previously, all patients were newly diagnosed and had clinical symptoms of diabetes within 1 year from the time of inclusion in the study.

Representative records of respiratory movements and ECG for LST, DBT, and VM for a 42-year-old male diabetic patient are shown in Figures 1–3, respectively.

Mean VEGF levels were 308.3 ± 167.2 pg/mL. The Autonomic tone and reactivity parameters are summarized in Tables 1 and 2, respectively. While the patients had normal reactivity parameters, the autonomic tone was impaired as assessed by frequency-domain parameters.

Discussion

DM is associated with both micro- and macro-vascular complications. Of these, microvascular complications—neuropathy, retinopathy, and nephropathy—are associated with significant morbidity and mortality. Diabetic neuropathy has been traditionally attributed to "metabolic" or "vascular" insult.[10-13] Recently the implication of Schwann cells in the pathogenesis of diabetic neuropathy has also come to light.[13,14] The incidence of diabetic neuropathy varies according to the duration of disease and the rigorousness of good glycemic control.[3] In addition, age has been proposed as an accelerant that may hasten the pathological process. In the Indian context, limited reports are available that have described the incidence of diabetic neuropathy in Indian population. The incidence ranges from 15–29% in such reports.[15,16]

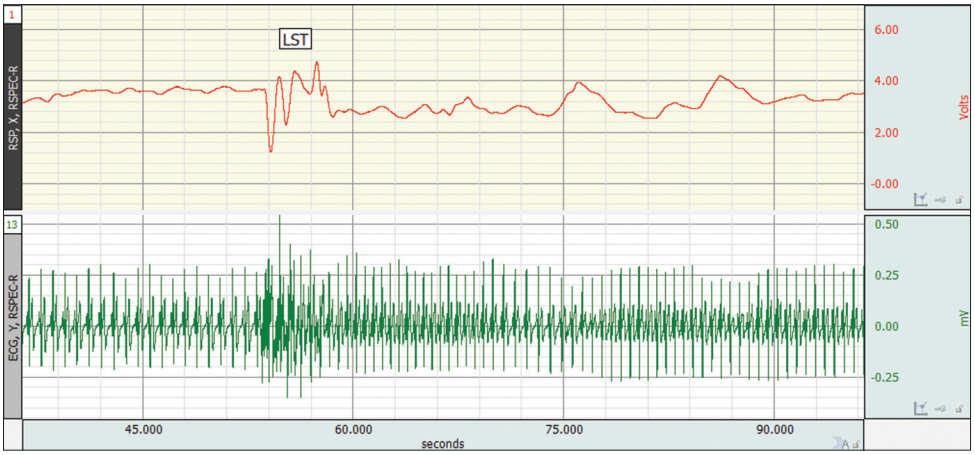

Figure 1: Representative record of lying-to-standing test for a 42-year-old male diabetic patient. The upper channel shows respiratory movements and the lower channel shows Lead II ECG record. The maneuver of standing from lying down posture is marked using the Label LST. Blood pressure was monitored using sphygmomanometer, and 30:15 ratio was calculated from the ECG record

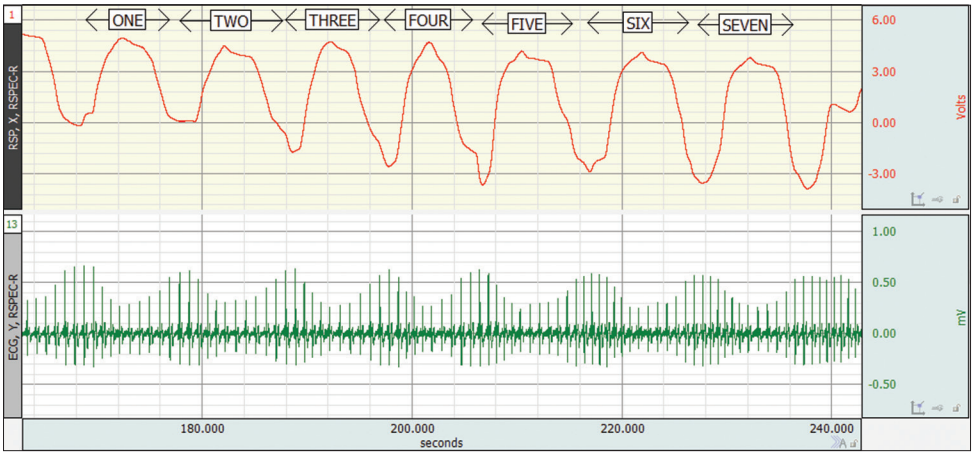

Figure 2: Representative record of Deep breathing test (DBT) for a 42-year-old male diabetic patient. The upper channel shows respiratory movements and the lower channel shows Lead II ECG record. The individual cycles are labeled using boxes containing text labels from one to seven, respectively. The last six cycles were chosen for computation. ΔHR and E: I ratio was calculated using the above record

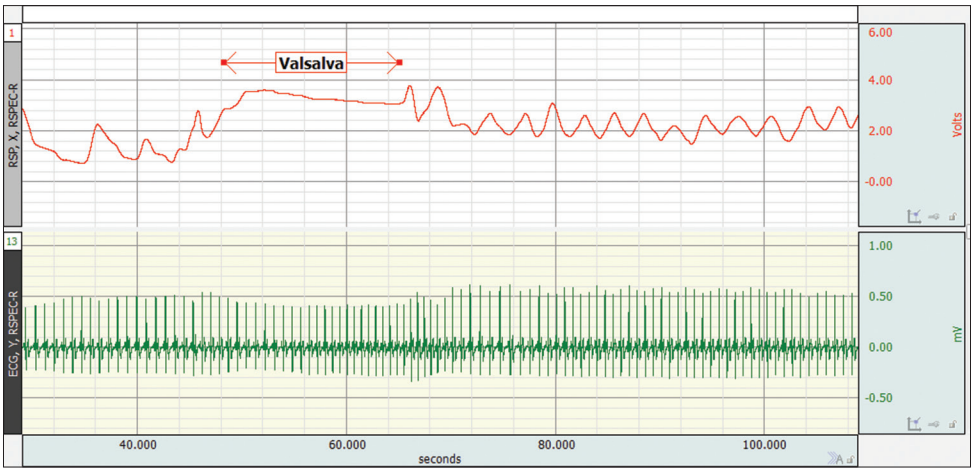

Figure 3: Representative record of Valsalva maneuver (VM) for a 42-year-old male diabetic patient. The upper channel shows respiratory movements and the lower channel shows Lead II ECG record. The duration of expiratory effort for VM is depicted by the box labeled Valsalva flanked by arrows on either side. Valsalva ratio (VR) was calculated as the ratio of longest RR interval after the strain period (up to 30 s) divided by the shortest RR interval during the strain

Table 1: Patient characteristics: anthropometric and biochemical parameters

Parameter	Values
Age	41.93±6.73 years
BMI	25.62±4.36 kg/m^2
HbA1c	9.30±2.35%
VEGF levels	308.30±167.20 pg/mL

Values expressed as Mean SD or Median (Interquartile range) depending on the Gaussian fit

Table 2: Autonomic reactivity and tone parameters

S. No.	Parameter	Values
Autonomic tone (HRV)		
1.	SDNN	27.52 (19.77-40.48)
2.	RMSSD	18.66 (10.58-30.61)
3.	Total power (ms^2)	436.1 (223.2-1215)
4.	LF Power n.u.	50.99±17.18
5.	HF Power n.u.	38.00±14.84
6.	LF/HF ratio	1.455 (0.758-2.185)
Autonomic reactivity (Ewing's battery of tests)		
7.	Δ HR (DBT)	19.7±7.12 beats/min
8.	E: I ratio	1.29±0.11
9.	VR	1.81±0.44
10.	30:15 ratio	1.33±0.23
11.	Δ SBP (LST)	5 (0-9.5) mmHg
12.	Δ DBP (HGT)	20 (18-26) mmHg
13.	Δ DBP (CPT)	14 (12-18) mmHg
14.	Ewing's Score: Sympathetic	0 (0-0)
14.	Ewing's Score: Parasympathetic	0 (0-0)

Values expressed as Mean SD or Median (Interquartile range) depending on the Gaussian fit

VEGF-A has emerged as a candidate molecule for pathogenesis in diabetic complications. The role of VEGF in retinopathy is well established. Elevated intraocular VEGF levels have been proposed to be positively related to the incidence of diabetic retinopathy.[17] In addition, VEGF has been proposed to be an important factor in the pathogenesis of diabetic nephropathy. These reports have led to the exploration of VEGF antagonists as a modality for the treatment of diabetic complications. VEGF gene transfer has been proposed as a modality for diabetic neuropathy.[18]

In addition to its role in vasculogenesis and angiogenesis, VEGF has been shown to play a neuroprotective role in both the central and peripheral nervous systems.[19,20] Ischemic neural damage seen in animal model of diabetes is associated with elevation of VEGF levels.[21] The role of VEGF in diabetic neuropathy has attracted interest in the scientific community. However, there are few reports that corroborate this relationship. Quattrini *et al.*[22] looked into the association between VEGF and intraepithelial nerve fiber density in patients with diabetic neuropathy. They reported an inverse relationship between VEGF levels and the level of severity of diabetic neuropathy coupled with loss of intraepidermal nerve fibers. Another report by Deguchi and colleagues[23] explored serum VEGF levels and extent of diabetic polyneuropathy in 220 diabetes patients. They observed elevated levels of serum VEGF in patients with diabetic neuropathy, particularly those with active clinical symptoms. They proposed that there is a reduction in VEGF levels with the progression of neuropathy. However, we did not come across the reports that have explored serum VEGF levels with respect to CAN in DM.

We observed autonomic functions of 44 Ty2DM patients along with serum VEGF-A levels. Mean HbA1c levels were 9.30 ± 2.35%, which were well above the cut off proposed by American Diabetic Association. The autonomic tone was reduced, whereas reactivity parameters were normal. This was consistent with previous well-documented reports of impaired HRV in DM.[24] HRV parameters have been shown to be affected earlier than reactivity and reduced HRV is a well-known predictor of morbidity and mortality in Ty2DM. Our data is suggestive of early stages of autonomic neuropathy in diabetes, probably because our patient population was newly diagnosed (clinical symptoms within 1 year from the day of recruitment in the study).

Serum VEGF levels have been estimated in the Indian population by several groups. Mean serum VEGF levels in our study were elevated when compared to previous reports(308.3 ± 167.2 pg/mL versus 96.66 ± 37.35 pg/mL and 113.33 ± 10.84 pg/mL).[25,26] This data may seem contradictory to the previous literature but can be explained based on the following hypothesis.

Diabetic neuropathy progresses from asymptomatic (subclinical) stage to the symptomatic stage with the progression of disease. Factors that may facilitate the process include poor glycemic control coupled with duration of disease and patient's age. As proposed by Deguchi *et al.*,[23] VEGF may play a protective role preventing the transition from asymptomatic to symptomatic stage. Autonomic neuropathy usually follows a similar temporal course as polyneuropathy in patients of Ty2DM. Therefore, we propose that VEGF levels may also demonstrate the same temporal trend for autonomic neuropathy as they do for generalized neuropathy.

There are some limitations to our study. Recruiting newly diagnosed Ty2DM patients without any comorbidities was a difficult task responsible for the low sample size of our study. In addition, serial follow-up to ascertain the conversion of subclinical autonomic neuropathy to overt stage was not possible due to time constraints and the cross-sectional design of our study.

Conclusion

Based on our findings, we propose that newly diagnosed patients of Type 2 diabetes mellitus in early stages of cardiac autonomic neuropathy may have elevated Serum VEGF levels. These levels may decline with the progression of CAN from latent to the overt stage. However, this proposal requires experimental validation by following large cohorts of newly diagnosed Ty2DM patients over a long period.

Early screening of CAN in Ty2DM patients is of clinical significance from a primary care physician perspective. Such screening can be easily performed by using HRV in conjunction with Ewing's battery tests. Our findings hint toward the presence of elevated serum VEGF-A levels in early CAN in the patient population. Therefore, role of VEGF-A as a biomarker for initial stages of CAN in Ty2DM may be explored by future studies. If validated, the biomarker may serve as a valuable adjunct to the standard battery of autonomic function tests being utilized for the diagnosis and prognosis of autonomic neuropathy in DM patients.

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Declaration of patient consent

The authors certify that they have obtained all appropriate patient consent forms. In the form, the patients have given their consent for their images and other clinical information to be reported in the journal. The patients understand that their names and initials will not be published and due efforts will be made to conceal their identity, but anonymity cannot be guaranteed.

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Conflict of interest

There is no conflict of interest.

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