OPEN

Autonomic Neuropathy and Endothelial Dysfunction in Patients With Impaired Glucose Tolerance or Type 2 Diabetes Mellitus

Bedile Irem Tiftikcioglu, MD, Sule Bilgin, MD, Tarik Duksal, MD, Sukran Kose, MD, and Yasar Zorlu, MD

Abstract: Autonomic neuropathy is one of the most common complications of diabetes mellitus (DM). The etiology of autonomic impairment is not well-understood, yet. There is need for studies to investigate the cause–effect relationships of inflammation and/or endothelial dysfunction and diabetic autonomic neuropathy. Only a few reports have mentioned autonomic neuropathy in individuals with impaired glucose tolerance (IGT), previously. Furthermore, the association between the plasma markers of endothelial dysfunction (von Willebrand factor (vWF), soluble E-selectin) and autonomic neuropathy in patients with IGT or DM has not been studied before. In this study, we aimed to investigate the correlation between plasma markers of endothelial dysfunction and autonomic neuropathy in patients with IGT or type 2 DM (T2DM).

In this case–control study, 25 IGT patients, 25 T2DM patients with autonomic symptoms, and 30 controls were included. Demographical data, HbA1c, vWF, and soluble E-selectin (sE-selectin) levels were analyzed. Sympathetic skin response (SSR) and heart rate variability (HRV) were used as the indicator of autonomic activity.

Plasma levels of HbA1c, vWF, and sE-selectin were higher in patients with IGT than the controls; patients with T2DM had higher levels than both the controls and the patients with IGT. SSR measures were similar among the groups. However, higher number of T2DM patients had absent plantar SSR than controls. HRV analysis at rest revealed lower standard deviation of R-R interval, coefficient of variation of R-R interval, low-frequency (LF) power and total power in patients with IGT and T2DM than the controls. In addition, HRV analysis at deep breathing showed lower high-frequency (HF) power in IGT group. LF:HF ratio was lower in both patient groups at rest. No strong correlation was found between the levels of HbA1c, vWF, sE-selectin, HRV, and SSR measures.

Our results support that endothelial dysfunction is evident in individuals with IGT or T2DM and HRV is impaired in early stages in the course of T2DM. However, increased levels of biomarkers of endothelial damage do not correlate with HRV or SSR. More studies are needed to clarify the disease pathogenesis and its clinical correlates. Impaired HRV in T2DM could be due to mechanisms other than endothelial dysfunction.

(*Medicine* 95(14):e3340)

Abbreviations: BMI = body mass index, CAN = cardiovascular autonomic neuropathy, CV = coefficient of variation of R-R interval, DM = diabetes mellitus, ECG = electrocardiography, FFT = Fast Fourier Transform, HF = high-frequency, HPLC = high-performance liquid chromatography, HRV = heart rate variability, IGT = impaired glucose tolerance, IQR = interquartile range, LF = low-frequency, OAD = oral antidiabetic medication, SAS = survey of autonomic symptoms, SDNN = standard deviation of all NN (R-R) intervals, sE-selectin = soluble E-selectin, SSR = sympathetic skin response, T2DM = type 2 diabetes mellitus, TP = total power, VLF = very low frequency, vWF = von Willebrand Factor.

INTRODUCTION

Autonomic neuropathy is one of the most common complications of diabetes mellitus (DM). Clinical symptoms can be subtle and patients usually attribute them to other issues. These include orthostatic hypotension, resting tachycardia, exercise intolerance, sudomotor dysfunction, erectile dysfunction, constipation, and gastroparesis.[1] Most importantly, cardiovascular autonomic neuropathy (CAN) can lead to silent myocardial infarction, severe morbidity, fatal arrhythmia, and sudden death.[2–4] Several methods have been described to detect autonomic neuropathy.[5,6] The assessment of heart rate variability (HRV) has been a well-accepted method to evaluate the status of autonomic control on the heart.[7] Loss of HRV was found to be an independent predictor of all-cause and cardiovascular mortality in population-based studies.[8,9] In patients with CAN, vagal impairment can lead to a relative predominance of sympathetic activity in the sympathovagal balance. Considerable amount of studies indicated the presence of CAN in patients with diabetes; but, only a few reports have mentioned CAN and impaired HRV measures in individuals with impaired glucose tolerance (IGT).[10–13]

Several mechanisms, including neurovascular insufficiency, metabolic insult to nerve fibers, and autoimmune damage have been implicated in the pathogenesis of DM. Recently, much interest has been directed toward the role of inflammation and endothelial dysfunction in DM. Vinik et al[14] addressed a neuroregulatory role for the autonomic nervous system, which suggests that the peripheral inflammatory response is partly controlled by the central neural circuitry of the autonomic nervous system. In their review, authors also explained the regulatory mechanisms of autonomic nervous system, underlying both the downregulation of induced inflammatory cascade and its potential to initiate and aggravate a proinflammatory response. The etiology of autonomic impairment is not well-understood, yet. The cause–effect relationship between inflammation and/or endothelial dysfunction and diabetic autonomic neuropathy needs to be identified. A few studies have reported that inflammation markers (C-reactive protein, IL-6) and decreased HRV are related in healthy

individuals and diabetic subjects.[15,16] However, the association between plasma markers of endothelial dysfunction (vWF, von Willebrand factor; soluble E-selectin) and impaired HRV in IGT or DM has not been studied before.

The aim of this study was to investigate the correlation between the plasma markers of endothelial dysfunction (i.e., vWF and sE-selectin) and autonomic testing (HRV and SSR, sympathetic skin response) in patients with IGT and diabetic patients with symptoms of autonomic dysfunction.

MATERIALS AND METHODS

Study Population

In this cross-sectional, case–control study, we recruited 25 patients diagnosed with IGT within the last 6 weeks and 25 patients diagnosed with T2DM within the last 2 years who also have autonomic symptoms. All patients were referred consecutively from the Internal Medicine Clinic at our tertiary care medical center. In the control group, 30 age- and sex-matched healthy individuals (hospital staff or relatives of patients) with normal glucose tolerance on standardized oral glucose tolerance test were recruited.

Exclusion criteria were as follows: patients older than 65 years, abnormality in serum tests including complete blood count, liver, kidney and thyroid function tests, serum iron, folate and vitamin B12 levels, history of drug abuse, alcohol intake, toxin exposure, neoplasm, familial or acquired peripheral sensorimotor neuropathy, autoimmune disease, vascular peripheral disease, uncontrolled hypertension, uncontrolled dyslipidemia, congestive heart failure, cardiac arrhythmias, and any other chronic systemic diseases. Also, participants who were taking alpha- or beta-blockers, calcium channel blockers, anticholinergics, sedatives, antidepressant agents were excluded.

All individuals eligible for the study (n = 80) underwent a detailed physical and neurological examination. Demographical data including age, gender, weight, waist circumference, body mass index (BMI) were recorded. The survey of autonomic symptoms (SAS) was used to score the autonomic complaints.[17]

The study protocol was in accordance with the Helsinki declaration of human rights and was approved by the local ethics committee. All patients and controls gave written informed consent to participate in the study.

Analysis of vWF and sE-Selectin

Peripheral venous blood sample for biomarker analysis was collected by atraumatic puncture of the antecubital vein, in the morning, after a rest for 15 minutes, following 12 hours of fasting period. Samples were immediately stored at 4°C for 10 minutes, centrifuged at 1500 rpm for 10 minutes and the extracted plasma was stored at −80°C, at dark until assayed. The presence of vWF (AssayPro LLC, St. Charles, MO) and sE-selectin (eBioscience, San Diego, CA) in plasma was detected by commercially available human-ELISA kits according to the manufacturers' instructions. HbA1c levels were measured using high-performance liquid chromatography (HPLC) method.[18]

Electrophysiological Tests

All recordings were performed by the same clinician blinded to the patients' diagnosis using the automated protocol in Nihon Kohden MEB-9200 electromyography device with autonomic nervous system testing software QP-954BK (Nihon Kohden Co., Tokyo, Japan). Study participants were asked to avoid ingestion of coffee, tea, energy drinks and avoid excessive exercise for 24 hours before testing. All recordings were performed in the morning between 09 AM and 11 AM, 2 hours after a light breakfast. In order to prevent artificial stimulation of sympathetic system, tests were performed in a quiet, semi-darkened room. Room temperature was set at 22 to 24°C. After the placement of surface recording electrodes, all subjects had a rest in supine position for at least 10 minutes to maintain the optimal autonomic balance. They were instructed not to speak or move unnecessarily and their vigilance was controlled by the clinician. Recordings disturbed by agitation, cough, pain, or any discomfort were repeated another day until an optimal recording was achieved.

Heart Rate Variability Analysis

HRV values were measured noninvasively by R-R interval analysis using Autonomic Nervous Testing Software for Neuropack M1 QP-954BK (Nihon Kohden Co., Tokyo, Japan). Filters were set at 1 to 20 Hz, sensitivity was 100 to 200 μV/div and the sweep speed was 50 millisecond/div. Inhibit time was 600 milliseconds. Level trigger was used to acquire waveforms. One recording disk electrode was placed at second intercostal space at the right border of the sternum (cathode) and the other one at the left anterior axial line at the lowest rib (anode). Ground electrode was placed at the right anterior axial line at the lowest rib.

Two recordings were obtained during rest with recording duration of at least 5 minutes (approximately 5–7 minutes) and during controlled deep breathing at a rate of 6 breaths per minute (i.e., 5 seconds of inspiration and 5 seconds of expiration) with recording duration of 10 consecutive cycles. This run was repeated once more after a rest for 5 minutes. Participants were asked not to hyperventilate and the rate of breaths was controlled by the clinician with soft verbal instructions if needed. QRS recordings were reviewed manually for artifacts and optimized for analysis. Recordings with artifacts exceeding 5% of the whole record were renewed.

The standard deviation of the R-R (or normal-to-normal; NN) interval (SDNN) and coefficient of variation of R-R interval (CV = SD ÷ mean R-R interval × 100) were calculated in time domain analysis. Spectral power analysis was performed by nonparametric methods, using Fast Fourier Transform (FFT). Three main spectral components were distinguished in the spectrum: very low frequency (VLF; <0.004 Hz), low-frequency (LF; 0.04–0.15 Hz) and high-frequency (HF; 0.15–0.4 Hz) components. Since, the physiological explanation of VLF band assessed from short-term recordings is not clear; LF and HF components, as well as the total power (TP; ≤0.4 Hz), were taken into account for further analysis.[7]

Sympathetic Skin Responses

SSR was measured noninvasively by SSR test using Autonomic Nervous Testing Software for Neuropack M1 QP-954BK (Nihon Kohden Co., Tokyo, Japan). Filters were set at 0.2 to 1000 Hz, sensitivity was 500 μV/div and the sweep speed was 1 second/div. Patients were in supine position. Recording disc electrodes were placed on palmar and dorsal surfaces of right hand and plantar and dorsal surfaces of right foot. Electrodes on palmar and plantar surfaces were the active electrode, and electrodes on the dorsal surfaces were the reference electrode. Ground electrode was placed on the forearm. Stimulation intensity was 20 mA and the duration was 0.2 milliseconds. In order to obtain the palmar SSR, electrical stimuli were applied to the left median nerve at the wrist line and for the

plantar SSRs, the left posterior tibial nerve was stimulated behind the medial malleolus. To avoid habituation, stimuli were given randomly at a minimum of 30-second intervals. Three recordings were obtained and the one with the shortest latency and highest amplitude was acquired. At least 5 stimuli were given before it was accepted as *absent response* when there was no repeatable deflection of the baseline. Amplitude was measured peak-to-peak and the latency was defined as the interval from the stimulus artifact to the first negative deflection of the response.

Statistical Analysis

Power analysis of the study was performed using the G*Power software version 3.1.9.2 (University of Dusseldorf, Dusseldorf, Germany). A priori analysis with the Mann–Whitney U test indicated that a sample size of at least 26 patients in each group would provide a statistical power of 80% ($\beta = 0.20$ and $\alpha = 0.05$) with the effect size d = 0.80.

Statistical analysis was performed using SPSS v.15.0 for Windows (Statistical Package for Social Sciences, SPSS, Inc., Chicago, IL). Categorical data were analyzed by means of χ^2 test. The Kolmogorov–Smirnov test was used to determine whether continuous variables followed a normal distribution. Normally distributed continuous variables were presented as mean ± SD and range, nonnormally distributed numerical data were presented as median and interquartile range (IQR: 25th–75th percentiles). Since the number of patients in each group was small, multiple group comparisons were performed using nonparametric Kruskal–Wallis test. Post hoc analyses were done using Mann–Whitney U test and the significance level (P-value) was corrected using Bonferroni correction. Linear correlation between the plasma levels of vWF, sE-selectin, HbA1c and the HRV and SSR parameters was tested using Spearman rho correlation test with the cut-off value > 0.600 for strong correlation. P-values <0.05 were considered as significant.

RESULTS

Demographical data are summarized in Table 1. All groups were similar according to age and gender. However, both the patients with IGT and T2DM had significantly higher BMI and waist circumference measurements than the healthy controls. The mean (±SD) duration of disease of the patients with T2DM was 1.0 ± 0.1 years. Neurological examination revealed mild impairment in vibration and position sense in 3 patients with IGT. Eleven patients complained tingling and burning sensation in feet and 7 had mild impairment in position and vibration senses, together with the decrease in Achilles reflex and hypoesthesia in feet. Three patients (12%) were using metformin (OAD; oral antidiabetic medication) in IGT group, whereas this number was 19 (76%) in T2DM group. In addition, 2 patients (8%) were using insulin and 4 (16%) were using both in T2DM group.

The mean levels of HbA1c, vWF, and sE-selectin were significantly higher in IGT patients than the controls. Moreover, patients with T2DM had higher levels than the healthy controls and IGT patients (Table 1).

We found that 1 patient in IGT group (4%) and 3 patients in T2DM group (12%) had loss of response in palmar SSR. Also, 3 patients IGT group (12%) and 6 patients in T2DM group (24%) had no response in plantar SSR. On the contrary, the response rates were 100% in the control group. The comparison of 3 groups for the response rate of plantar SSR revealed a significant difference ($P = 0.019$). The SSR latency and amplitude of both upper and lower limbs are listed in Table 2 for all groups.

Table 3 summarizes the parameters of HRV analysis in patient groups and control subjects. Analysis of short-term recording (during resting) revealed a significant decrease in both time (SDNN and CV) and frequency domains (LF, total power, and LF:HF ratio) in patients with IGT and T2DM than the controls, except the power of HF band in T2DM patients. Similarly, HRV analysis during controlled deep breathing showed significant differences in parameters of time and frequency domains between the groups. Both the patients with IGT and T2DM had lower SDNN, CV, LF, and total power than the controls. Only, but, LF:HF ratio was not different between the groups. Furthermore, no difference was found between the patients with IGT or T2DM in terms of HRV measures.

Finally, there was no significant and strong correlation between the HRV parameters and levels of HbA1c, vWF, and sE-selectin in either patient group (Spearman rho > 0.600). Similarly levels of these biomarkers were not correlated with SSR measures (data not shown).

TABLE 1. Demographical Data of Patients and Healthy Control Subjects Expressed as Mean ± SD or Median (Interquartile Range: IQR)

	Controls (n = 30)	IGT (n = 25)	DM (n = 25)	P Overall	P^* IGT vs Controls	P^* DM vs Controls	P^* IGT vs DM
Age, y[†]	54.5 (11.5)	56.0 (15.0)	54.0 (9.5)	0.786	N/A	N/A	N/A
Sex, n (M/F)	10/20	7/18	11/14	0.481	N/A	N/A	N/A
Weight, kg	74.0 ± 2.0	76.0 ± 2.1	80.0 ± 1.7	**0.027**	0.196	**0.007**	0.193
BMI, kg/m^2	25.3 ± 0.6	28.1 ± 0.6	28.0 ± 0.4	**0.004**	**0.013**	**0.002**	0.473
Waist circumference, cm	88.0 ± 1.9	96.0 ± 2.2	96.0 ± 1.0	**0.002**	**0.004**	**0.003**	0.454
SAS							
No. symptoms[†]	0 (0)	0 (2.5)	4.0 (2.0)	**<0.001**	**0.005**	**<0.001**	**<0.001**
Total impact score[†]	0 (0)	0 (4.5)	10 (4.5)	**<0.001**	**0.005**	**<0.001**	**<0.001**
HbA1c,[†] %	5.20 (0.73)	6.0 (0.55)	7.1 (1.20)	**<0.001**	**<0.001**	**<0.001**	**<0.001**
vWF,[†] pg/dL	12.75 (5.39)	16.94 (16.18)	25.50 (8.16)	**<0.001**	**0.001**	**<0.001**	**0.014**
sE-selectin, pg/dL	10.30 ± 0.78	16.90 ± 1.11	25.80 ± 1.52	**<0.001**	**<0.001**	**<0.001**	**<0.001**

P: significance level, bold indicates significant values <0.05.
*Significance level corrected for multiple comparisons is $P < 0.016$.
[†]Expressed as median (interquartile range: IQR). BMI = body mass index, DM = diabetes mellitus, IGT = impaired glucose tolerance, N/A = not available, SAS = survey of autonomic symptoms, vWF = von Willebrand factor.

TABLE 2. SSR Parameters of Patients and Healthy Control Subjects Expressed as Median (Interquartile Range: IQR)

	Controls	IGT	DM	P
SSR-UL, n (%)				
Latency, ms	1.48 (0.23)	1.37 (0.35)	1.50 (0.29)	0.126
Amplitude, mV	2.05 (1.96)	2.21 (2.79)	2.23 (2.36)	0.707
SSR-LL, n (%)				
Latency, ms	2.07 (0.47)	1.99 (0.36)	2.04 (0.31)	0.132
Amplitude, mV	1.23 (1.23)	1.38 (1.32)	1.02 (1.40)	0.638

P: significance level for comparison of three groups by Kruskal–Wallis test.

DM = diabetes mellitus, IGT = impaired glucose tolerance, SSR-LL = sympathetic skin response-lower limb, SSR-UL = sympathetic skin response-upper limb.

DISCUSSION

The results of this study support the presence of early impairment of autonomic nervous system in patients with T2DM. This is demonstrated by both the survey of autonomic symptoms and electrodiagnostic tests. First of all, a significant number of patients in both patient groups had autonomic symptoms which were revealed by SAS, a questionnaire designed and validated to assess autonomic symptoms in patients with diabetic neuropathy with high sensitivity and specificity.[17] This finding emphasizes the importance of clinical query in high-risk individuals.

Secondly, HRV analysis showed significant differences between patients with IGT and T2DM and healthy control subjects. Analysis of HRV in time and frequency domains reveals substantial information on the balance between sympathetic and parasympathetic innervation of the heart. It has been reported that the time domain measures explore the parasympathetic activity. Spectral measures such as, total power and the power in the HF band reflect the parasympathetic; the power of LF band reflects both sympathetic and parasympathetic activity.[7,19] We found that during rest with normal breathing both patients with IGT and T2DM had lower SDNN, CV, LF power and total power than the healthy controls; all indicating an impairment in parasympathetic activity. The decreased ratio of LF to HF was also in accordance and indicated the sympathovagal imbalance. Furthermore, analysis of the recordings during deep inspiration showed a marked decrease also in HF power, in addition; but, the difference was only statistically significant in IGT group. Due to the decreased powers of both LF and HF bands, the LF:HF ratio was not different between the groups. However, this does not exclude the presence of altered innervation in sympathetic and parasympathetic nerves. Our results were in line with previous studies conducted on diabetic patients.[20,21] To our knowledge, only 2 studies have reported the impaired spectral power analysis of HRV in individuals with IGT, so far.[22,23] Thus, our results could add substantial information to the understanding of CAN and HRV in IGT patients, as well.

HRV analysis can be performed on short (5-minute period) or long-term (24-hour period) electrocardiography (ECG) recordings. Task Force of The European Society of Cardiology and The North American Society of Pacing and Electrophysiology have mentioned the difficulties of physiological interpretation and technical difficulties of long-term recordings.[7] Ectopic beats, arrhythmic events, missing data have been reported as an issue. In previous studies, results obtained from the spectral analysis of long-term and short-term ECG recordings were found to be similar.[24,25] Thus, we used short-term recordings of ECG for HRV analysis, due to the ease of postrecording evaluation of artifacts. HRV has been reported to be maximal during deep breathing at respiratory rates

TABLE 3. HRV Parameters of Patients and Healthy Control Subjects Expressed as Median (Interquartile Range: IQR)

	Controls (n = 30)	IGT (n = 25)	DM (n = 25)	P Overall	P* IGT vs Controls	P* DM vs Controls	P* IGT vs DM
During rest for 5 min							
Time domain							
SDNN, ms	32.80 (20.23)	21.50 (14.25)	21.70 (11.85)	**0.006**	**0.004**	**0.010**	0.778
CV, %	4.80 (1.88)	2.70 (2.35)	2.60 (1.0)	**<0.001**	**0.001**	**<0.001**	0.613
Frequency domain							
LF, ms²	42.75 (24.25)	14.60 (10.45)	13.10 (20.40)	**<0.001**	**<0.001**	**<0.001**	0.641
HF, ms²	17.25 (11.38)	12.20 (12.50)	12.70 (14.70)	0.094	0.021	0.319	0.421
Total power, ms²	82.90 (54.18)	49.70 (29.20)	54.90 (43.45)	**0.001**	**0.001**	**0.002**	0.587
LF:HF ratio	1.96 (1.53)	1.36 (0.70)	1.06 (0.48)	**0.001**	**0.010**	**0.001**	0.056
Deep inspiration recording (10 cycle/min)							
Time domain							
SDNN, ms	51.80 (38.25)	33.10 (12.65)	32.30 (30.40)	**<0.001**	**<0.001**	**0.003**	0.823
CV, %	6.35 (3.7)	4.20 (1.3)	4.10 (4.30)	**<0.001**	**<0.001**	**0.004**	0.838
Frequency domain							
LF, ms²	49.20 (52.08)	36.40 (19.20)	33.90 (42.60)	**0.001**	**0.001**	**0.002**	0.607
HF, ms²	25.35 (23.0)	13.90 (11.45)	12.00 (19.35)	**0.005**	**0.001**	**0.020**	0.823
Total power, ms²	109.45 (92.35)	73.90 (32.55)	69.10 (70.75)	**<0.001**	**<0.001**	**0.001**	0.580
LF:HF ratio	2.32 (1.87)	2.42 (1.42)	2.16 (1.49)	0.225	0.352	0.344	0.093

P: significance level, bold indicates significant values <0.05.

*Significance level corrected for multiple comparisons is <0.016. CV = coefficient of variation of R-R intervals, DM = diabetes mellitus, HF = power of the high-frequency range, IGT = impaired glucose tolerance, LF = power of the low-frequency range, SDNN = standard deviation of all NN intervals.

between 5 and 10 breaths per minute.[26,27] Hence, we evaluated patients with 2 runs of recording; first during rest with normal breathing and second, during deep breathing at a rate of 6 cycles per minute. As the healthy control subjects and the patients in each group were similar in age and gender distribution, analyses were not performed to evaluate the effects of those variables on HRV.

Thirdly, we showed that the plantar SSR of diabetic patients were significantly impaired. SSR is a well-known, simple to record technique to assess the integrity of peripheral sympathetic cholinergic function.[28] The response is only semi-quantitative and highly variable with age and habituation. Therefore, it has been suggested to consider only the absence of response of pathological significance rather than the values of response latency and amplitude.[5] Various SSR abnormalities have been reported in patients with IGT or DM.[29–32] In our study, both the latency and the amplitudes of SSRs were not different among the patient and control subjects. On the contrary, we noticed that the rate of loss of response in plantar SSR was significantly higher in patients with T2DM compared to the controls, a finding that might be suggesting a retarded impairment of sympathetic nervous system during the disease course.

To our knowledge, this is the first study to examine the association of endothelial damage and autonomic neuropathy in patients with IGT or T2DM. The significantly higher levels of both vWF and sE-selectin in patients with IGT or T2DM compared to those of healthy controls, indicates marked endothelial dysfunction in these patients. In addition, the lower spectral power, SDNN, and CV in HRV analysis in the same groups suggests the involvement of CAN in very early phases of the DM. The severity of the HRV impairment in IGT patients was similar to those of with T2DM. This finding emphasizes the importance of early diagnosis and intervention in those patients.

However, we did not find an association between the levels of biomarkers of endothelial dysfunction and the autonomic dysfunction measures. This could be, partially, due to the complex mechanisms in the pathogenesis of diabetic autonomic neuropathy. Indeed, it is still not clear, whether the endothelial dysfunction is the cause or the consequence of diabetic autonomic impairment.

This study is subject to certain limitations. First, HRV might have been influenced by the differences in baseline physical condition of the subjects. Although all participants were informed to avoid excessive physical effort before the evaluation, neither the healthy controls nor the patients were evaluated for their physical exercise capacity and strength. Second, none of the participants were evaluated with clinical depression and anxiety batteries, but only questioned; since subtle mood disorders might also have an effect on the autonomic test results. Third, although questioned and assessed during the physical examination, baseline cardiac status has also not been examined in participants with laboratory tests such as, ECG or transthoracic echocardiography which could reveal subtle cardiac disorders. Fourth, the effects of the medications of patients (e.g., metformin, insulin) could not be excluded. Finally, the size of the study might have been inadequate to demonstrate the possible cause–effect relationship between the biomarkers of endothelial dysfunction and impairments in autonomic system. A prospective study with higher number of patients could yield better information on these aspects.

In conclusion, our results support that endothelial dysfunction is evident in individuals with IGT and T2DM; moreover, HRV is impaired in very early stages of the disease course of DM, namely IGT. However, we found that increased levels of biomarkers of endothelial damage do not correlate with impaired HRV. More studies are needed to clarify the disease pathogenesis and its clinical correlates in diabetic autonomic neuropathy.

REFERENCES

1. Vinik AI, Maser RE, Mitchell BD, et al. Diabetic autonomic neuropathy. *Diabetes Care.* 2003;26:1553–1579.

2. Vinik AI, Ziegler D. Diabetic cardiovascular autonomic neuropathy. *Circulation.* 2007;115:387–397.

3. Suarez GA, Clark VM, Norell JE, et al. Sudden cardiac death in diabetes mellitus: risk factors in the Rochester diabetic neuropathy study. *J Neurol Neurosurg Psychiatry.* 2005;76:240–245.

4. Rizzo MR, Sasso FC, Marfella R, et al. Autonomic dysfunction is associated with brief episodes of atrial fibrillation in type 2 diabetes. *J Diabetes Complications.* 2015;29:88–92.

5. Aminoff MJ. Evaluation of the autonomic nervous system. In: Aminoff MJ, ed. *Aminoff's Electrodiagnosis in Clinical Neurology.* Edinburgh: Saunders; 2012:455–473.

6. Spallone V, Bellavere F, Scionti L, et al. Recommendations for the use of cardiovascular tests in diagnosing diabetic autonomic neuropathy. *Nutr Metab Cardiovasc Dis.* 2011;21:69–78.

7. Heart rate variability. Standards of measurement, physiological interpretation, and clinical use. Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology. *Eur Heart J.* 1996;17:354–381.

8. Wirta O, Pasternack A, Mustonen J, et al. Renal and cardiovascular predictors of 9-year total and sudden cardiac mortality in non-insulin-dependent diabetic subjects. *Nephrol Dial Transplant.* 1997;12:2612–2617.

9. Gerritsen J, Dekker JM, TenVoorde BJ, et al. Impaired autonomic function is associated with increased mortality, especially in subjects with diabetes, hypertension, or a history of cardiovascular disease: the Hoorn Study. *Diabetes Care.* 2001;24:1793–1798.

10. Putz Z, Tabak AG, Toth N, et al. Noninvasive evaluation of neural impairment in subjects with impaired glucose tolerance. *Diabetes Care.* 2009;32:181–183.

11. Putz Z, Nemeth N, Istenes I, et al. Autonomic dysfunction and circadian blood pressure variations in people with impaired glucose tolerance. *Diabet Med.* 2013;30:358–362.

12. Dimitropoulos G, Tahrani AA, Stevens MJ. Cardiac autonomic neuropathy in patients with diabetes mellitus. *World J Diabetes.* 2014;5:17–39.

13. Laitinen T, Lindstrom J, Eriksson J, et al. Cardiovascular autonomic dysfunction is associated with central obesity in persons with impaired glucose tolerance. *Diabet Med.* 2011;28:699–704.

14. Vinik AI, Erbas T, Casellini CM. Diabetic cardiac autonomic neuropathy, inflammation and cardiovascular disease. *J Diabetes Investig.* 2013;4:4–18.

15. von KR, Nelesen RA, Mills PJ, et al. Relationship between heart rate variability, interleukin-6, and soluble tissue factor in healthy subjects. *Brain Behav Immun.* 2008;22:461–468.

16. Lieb DC, Parson HK, Mamikunian G, et al. Cardiac autonomic imbalance in newly diagnosed and established diabetes is associated with markers of adipose tissue inflammation. *Exp Diabetes Res.* 2012;2012:878760.

17. Zilliox L, Peltier AC, Wren PA, et al. Assessing autonomic dysfunction in early diabetic neuropathy: the Survey of Autonomic Symptoms. *Neurology.* 2011;76:1099–1105.

18. Hoelzel W, Weykamp C, Jeppsson JO, et al. IFCC reference system for measurement of hemoglobin A1c in human blood and the national standardization schemes in the United States, Japan, and Sweden: a method-comparison study. *Clin Chem.* 2004;50:166–174.

19. Bernardi L, Spallone V, Stevens M, et al. Methods of investigation for cardiac autonomic dysfunction in human research studies. *Diabetes Metab Res Rev.* 2011;27:654–664.

20. Kudat H, Akkaya V, Sozen AB, et al. Heart rate variability in diabetes patients. *J Int Med Res.* 2006;34:291–296.

21. Migliaro ER, Contreras P. Heart rate variability: short-term studies are as useful as holter to differentiate diabetic patients from healthy subjects. *Ann Noninvasive Electrocardiol.* 2003;8:313–320.

22. Fiorentini A, Perciaccante A, Valente R, et al. The correlation among QTc interval, hyperglycaemia and the impaired autonomic activity. *Auton Neurosci.* 2010;154:94–98.

23. Perciaccante A, Fiorentini A, Paris A, et al. Circadian rhythm of the autonomic nervous system in insulin resistant subjects with normo-glycemia, impaired fasting glycemia, impaired glucose tolerance, type 2 diabetes mellitus. *BMC Cardiovasc Disord.* 2006;6:19.

24. Rottman JN, Steinman RC, Albrecht P, et al. Efficient estimation of the heart period power spectrum suitable for physiologic or pharmacologic studies. *Am J Cardiol.* 1990;66:1522–1524.

25. Berger RD, Akselrod S, Gordon D, et al. An efficient algorithm for spectral analysis of heart rate variability. *IEEE Trans Biomed Eng.* 1986;33:900–904.

26. Freeman R, Cohen RJ, Saul JP. Transfer function analysis of respiratory sinus arrhythmia: a measure of autonomic function in diabetic neuropathy. *Muscle Nerve.* 1995;18:74–84.

27. Freeman R. Assessment of cardiovascular autonomic function. *Clin Neurophysiol.* 2006;117:716–730.

28. Ravits JM. AAEM minimonograph #48: autonomic nervous system testing. *Muscle Nerve.* 1997;20:919–937.

29. Zgur T, Vodusek DB, Krzan M, et al. Autonomic system dysfunction in moderate diabetic polyneuropathy assessed by sympathetic skin response and Valsalva index. *Electromyogr Clin Neurophysiol.* 1993;33:433–439.

30. Niakan E, Harati Y. Sympathetic skin response in diabetic peripheral neuropathy. *Muscle Nerve.* 1988;11:261–264.

31. Koytak PK, Isak B, Borucu D, et al. Assessment of symptomatic diabetic patients with normal nerve conduction studies: utility of cutaneous silent periods and autonomic tests. *Muscle Nerve.* 2011;43:317–323.

32. Isak B, Oflazoglu B, Tanridag T, et al. Evaluation of peripheral and autonomic neuropathy among patients with newly diagnosed impaired glucose tolerance. *Diabetes Metab Res Rev.* 2008;24:563–569.