Assessment of telomerase activity in leukocytes of type 2 diabetes mellitus patients having or not foot ulcer: Possible correlation with other clinical parameters

DIMITRIOS BALTZIS[1,2], EVANGELIA MEIMETI[3], MARIA G. GRAMMATIKOPOULOU[4], MATTHIEU ROUSTIT[5], ELENI MAVROGONATOU[6], DIMITRIS KLETSAS[6], SMARAGDA EFRAIMIDOU[7], CHRISTOS MANES[1], TAXIARCHIS K. NIKOLOUZAKIS[8], JOHN TSIAOUSSIS[8], ARISTIDES M. TSATSAKIS[9], DEMETRIOS A. SPANDIDOS[10], CHRISTINA-MARIA TRAKATELLI[2] and NIKOLAOS DRAKOULIS[3]

[1]Diabetes Center; [2]Third Department of Internal Medicine, Papageorgiou General Hospital, 56403 Thessaloniki; [3]Research Group of Clinical Pharmacology and Pharmacogenomics, Faculty of Pharmacy, School of Health Sciences, National and Kapodistrian University of Athens, 15771 Athens; [4]Department of Nutrition and Dietetics, Alexander Technological Educational Institute, 57400 Thessaloniki, Greece; [5]HP2 Laboratory, University of Grenoble Alpes, 38700 La Tronche Université Grenoble Alpes, France; [6]Laboratory of Cell Proliferation and Ageing, Institute of Biosciences and Applications, National Centre for Scientific Research 'Demokritos', 15341 Athens; [7]Department of Hematology, Papageorgiou General Hospital, 56403 Thessaloniki; [8]Laboratory of Anatomy-Histology-Embryology, [9]Department of Forensic Sciences and Toxicology, [10]Laboratory of Clinical Virology, Medical School, University of Crete, 71003 Heraklion, Greece

Received September 20, 2017; Accepted December 25, 2017

DOI: 10.3892/etm.2018.5798

Abstract. Telomerase is the enzyme that maintains telomere length by adding telomeric repeats after each cell division. Numerous metabolic factors such as obesity, insulin resistance or physical inactivity have been associated with shortened telomeres. In the present study, we assessed telomerase activity in diabetic patients having or not foot ulcer. A total of 90 adult patients with type 2 diabetes mellitus (T2DM) were studied. Patients were allocated into two groups according to the absence or presence of active foot ulcers as follows: Non-ulcer group (N=58) and ulcer group (N=32). Our data revealed that the patients with diabetic ulcers had significantly greater waist circumference and neuropathy disability score, while exhibiting lower telomerase activity, indicating the possible existence of a common clinical profile among ulcer-bearing diabetic patients. Validation of our findings by extending the study in larger patient groups may contribute to the understanding of T2DM pathophysiology and its main clinical implications.

Correspondence to: Dr Nikolaos Drakoulis, Research Group of Clinical Pharmacology and Pharmacogenomics, Faculty of Pharmacy, School of Health Sciences, National and Kapodistrian University of Athens, Panepistimiopolis, Zografou, 15771 Athens, Greece
E-mail: drakoulis@pharm.uoa.gr

Abbreviations: T2DM, type 2 diabetes mellitus; DPN, diabetic peripheral neuropathy; NDS, neuropathy disability score; TC, total cholesterol; TG, triglycerides; HDL, high-density lipoprotein; LDL, low-density lipoprotein; BMI, body mass index; PCR, polymerase chain reaction

Key words: diabetic peripheral neuropathy, neuropathy disability score, oxidative damage, telomere-diabetes

Introduction

Telomeres are nucleoprotein structures at the end of linear chromosomes containing repetitive DNA sequences (TTAGGG in vertebrates). Their role is to protect chromosome ends from deterioration or fusion with adjacent chromosomes, thus preventing incomplete replication and maintaining protein coding and regulatory elements of the genome (1,2). The enzyme telomerase prevents telomeres from shortening by adding telomeric repeats (2). Several factors, such as increasing age, disease and mortality, have been associated with reduced telomere length (3,4). Telomeric DNA is particularly prone to oxidative damage at the GGG sequence, since exposure to free radicals or oxidants causes DNA damage, including single-strand breaks (5,6). On the other hand, diabetes and metabolic syndrome are associated with oxidative stress (7-9).

Given that a variety of metabolic factors such as obesity (10,11), insulin resistance (12) or physical inactivity (13) have been associated with shortened telomeres, the relationship between type 2 diabetes mellitus (T2DM) and telomerase activity is an issue of intense investigation (14). Today we are in a position to acknowledge a bilateral telomere-diabetes relationship: shorter telomeres may lead to impaired insulin secretion and glucose tolerance in mouse pancreatic β-cells, while chronic hyperglycemia leading to increased oxidative stress attenuates telomerase activity of human leukocytes, results in a reduced telomere length (14-16). As a consequence,

T2DM patients demonstrate shortened telomeres (16,17), with several macro- and micro-vascular complications due to chronic hyperglycemia, such as retinopathy, nephropathy or vascular aging (18-20).

High percentages of patients diagnosed with T2DM have been reported to develop nerve damage (neuropathy), which leads to vascular damage, ulcers and amputation (21,22). Recently, it has been shown that diabetic peripheral neuropathy (DPN) is strongly associated with the risk for myocardial infraction (23). Taking into account that ulcers constitute a major complication of T2DM, the aim of the current study was to assess specific clinical traits, including telomerase activity and neuropathy disability score (NDS), in T2DM patients with or without ulcer.

Materials and methods

The study was conducted in 90 adult patients with T2DM from the Diabetes Mellitus Outpatient Clinic of the 3rd Department of Internal Medicine of Papageorgiou General Hospital in Thessaloniki, Greece. The protocol of the study was approved by the Ethics Committee of Papageorgiou General Hospital and patients provided their informed consent prior to participation complying with the requirements of the Declaration of Helsinki.

Inclusion criteria were the following: Males and females (>18 years of age) with confirmed T2DM diagnosis according to the ADA/IDF criteria (24). Patients having a history of myocardial infarction, stroke, coronary revascularization, cardiac bypass, active or chronic liver or renal disease, lumbar/cervical discopathy, carpal tunnel syndrome, alcohol abuse, inherited neuropathy and vitamin B9/B12 deficiency and patients diagnosed for any autoimmune disease, HIV infection, malignancies, primary neurologic disorders, including previous spinal injury, were excluded. The use of glucocorticoid-, isoniazid- or metronidazole-containing drugs was an extra exclusion criterion. Patients were stratified into a non-ulcer (N=58) and an ulcer (N=32) group based on the absence or presence of active foot ulcers located below the ankle joint.

Fasting blood samples were collected for the assessment of blood lipid levels, including total cholesterol (TC), triglycerides (TG), high-density lipoprotein (HDL) and low-density lipoprotein (LDL), as well as of glycated haemoglobin (HbA1c) and creatinine levels. Body weight, stature and waist circumference were measured for each patient and body mass index (BMI) was calculated.

DPN was evaluated by using the NDS after testing the sensations of pain, touch, cold, and vibration on both legs of the patient and assigning a score according to the level of impaired sensation. An NDS ≥5 was indicative of DPN (23,25).

Telomerase activity assay was performed in the pellets of white blood cells resuspended with CHAPS lysis buffer using the TRAPeze RT Telomerase Detection kit S7710 (Merck KGaA, Darmstadt, Germany), according to the manufacturer's instructions. In detail, the pellets were thawed, resuspended with 200 μl CHAPS and incubated for 30 min on ice. Samples were centrifuged at 12,000 x g for 20 min at 4˚C and the supernatant was aliquoted, snap-frozen in liquid nitrogen and stored at -80˚C until analysis. Samples were further diluted 200-fold and used for the TRAPeze RT Telomerase assay along with a positive control (a telomerase-positive extract provided by the manufacturer also diluted in 200 μl CHAPS), a minus telomerase control (only CHAPS lysis buffer) and a no template control (only nuclease-free water). Heat-treated samples at 85˚C for 10 min also served as negative controls. Polymerase chain reaction (PCR) amplification was performed in 25 μl reactions using 2 μl of sample and 2 units of Titanium Taq DNA Polymerase (Takara Bio, Inc., Otsu, Japan) following the manufacturer's protocol. Telomerase activity was calculated using the generated TSR8 standard curve deduced from TSR8 known concentrations always included in each assay. Protein content was determined with the Protein Determination kit (Cayman Chemical Co., Ann Arbor, MI, USA) and telomerase activity was normalized to μg of protein.

Statistical analysis. Statistical analysis was performed with Statgraphics Centurion software (Statgraphics Technologies, Inc., The Plains, VA, USA). Data were expressed as means ± standard deviation (means ± SD) or as medians ± standard error of the mean (medians ± SEM) when lacking normality in their distribution. Between-group comparisons were performed with ANOVA, followed by post-hoc comparisons performed with Tukey's test or the non-parametric Kruskal-Wallis test for normal and not normal distributions, respectively. Differences were considered significant for $P<0.05$.

Results

Several clinical characteristics of T2DM patients without or with foot ulcers are presented in Table I. The clinical characteristics that were evaluated can be grouped as follows: Demographic characteristics (age and sex), well appreciated risk factors (waist circumference, BMI, smoking history, TC, TG, HDL and LDL levels), prognostic factors (diabetes duration, HbA1c, creatinine levels, NDS) and leukocytes' telomerase activity. Among the clinical characteristics investigated here only waist circumference, NDS and leukocytes' telomerase activity demonstrated statistically significant differences between the two patient groups ($P<0.05$).

In Fig. 1, a graphical representation of the statistical analysis for all clinical parameters tested in the present study between the non-ulcer and ulcer groups is depicted. It is evident that, T2DM patients of the current study with foot ulcer were characterized by a significantly greater waist circumference ($P=0.0382$) and a significantly higher NDS ($P=9.09\text{x}10^{-8}$), accompanied by a significantly lower leukocyte telomerase activity ($P=0.0261$) when compared to the non-ulcer T2DM patients. No other clinical parameters assessed in the present study were statistically different between the two groups of diabetic patients.

Discussion

Diabetic patients who have developed ulcers or have even undergone amputations seem to have a lower life expectancy, which may reach up to 70% (26). On the other hand, short telomere length associated with low telomerase activity has been reported to be an indicator for decreased life expectancy (27).

Table I. Clinical charasteristics of participants allocated to two groups according to the absence or presence of diabetic ulcers.

Characteristic	Total (N=90)	Non-ulcer (N=58)	Ulcer (N=32)	P-value[a]
Age (years)	66.0±9.2	65.3±9.3	67.4±8.8	0.2936
Sex (M/W)	55/35	35/23	20/12	
Diabetes duration (years)	14.0±1.0	12.0±1.2	16.0±1.3	0.0683
Waist circumference (cm)	109.1±12.7	107.1±13.2	112.8±10.8	0.0382
BMI (kg/m^2)	31.3±0.7	30.8±0.8	31.2±1.0	0.0674
Smoking history (Y/N)	17/73	12/46	5/27	
TC (mg/dl)	181.3±39.0	181.8±34.4	176.6±44.1	0.8702
TG (mg/dl)	127.0±7.8	116.5±9.5	121.0±10.3	0.5722
HDL (mg/dl)	43.0±1.1	44.0±1.2	44.0±1.7	0.4887
LDL (mg/dl)	103.5±3.7	102.5±3.9	105.0±5.3	0.8232
HbA1c (%)	7.1±0.1	7.1±0.1	7.1±0.2	0.2672
Creatinine (mg/dl)	0.9±0.1	0.9±0.1	0.9±0.0	0.9696
NDS	10±0.6	5.0±0.7	11.0±0.5	9.09x10^{-8}
Telomerase activity (AU)	1,275.2±156.8	1,573.3±219.7	1,146.7±175.5	0.0261

[a]Estimated with the parametric Tukey's test or the non-parametric Kruskal-Wallis test for normally and not normally distributed variables, respectively. BMI, body mass index; TC, total cholesterol; TG, triglycerides; HDL, high-density lipoprotein; LDL, low-density lipoprotein; NDS, neuropathy disability score; AU, arbitrary units.

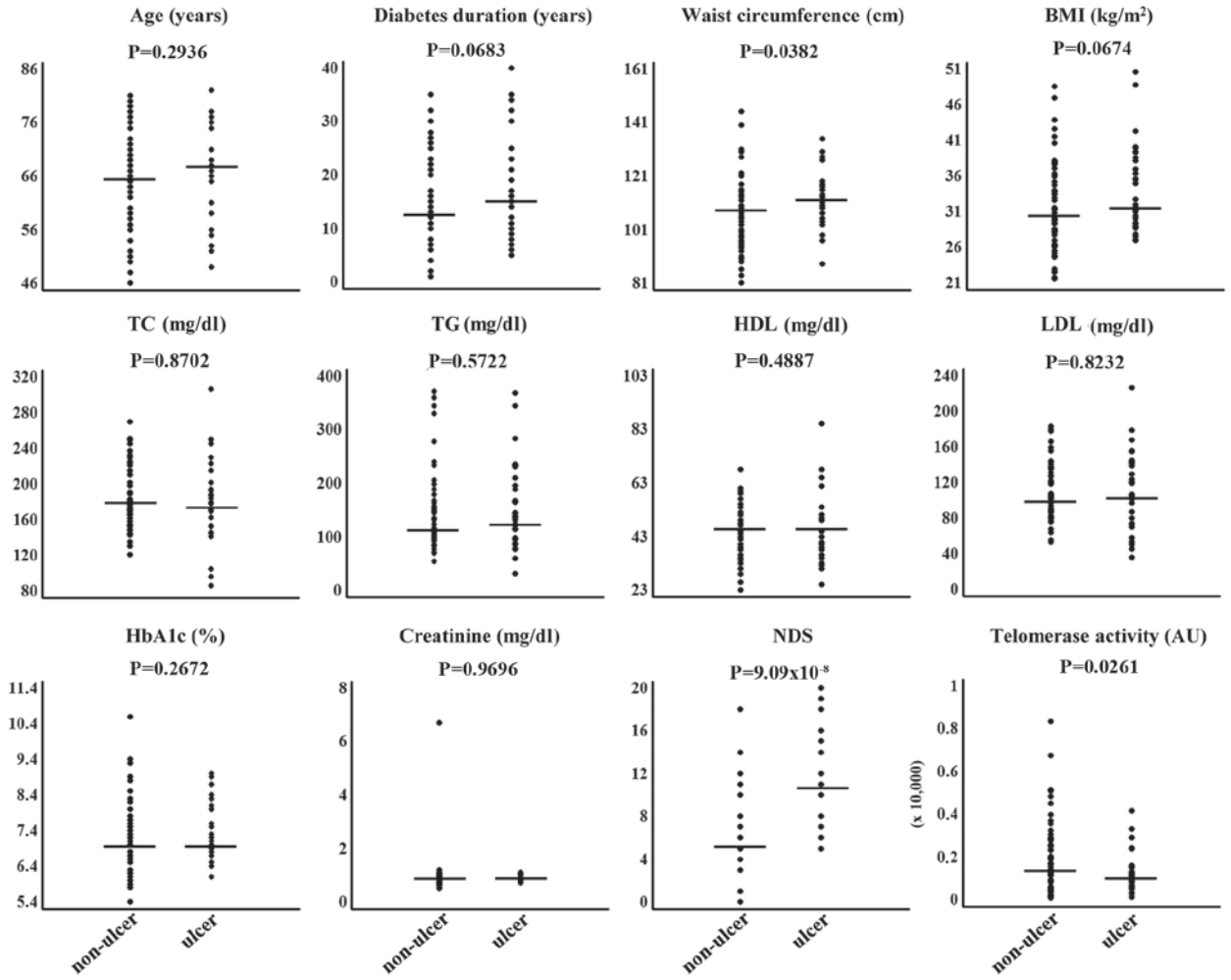

Figure 1. Scatter plots showing all values of clinical parameters investigated in this study for the non-ulcer and ulcer group of T2DM patients. In each graph, dots represent distinct values and horizontal line represents the mean or median, depending on the normality or not of the distribution, respectively. T2DM, type 2 diabetes mellitus; BMI, body mass index; TC, total cholesterol; TG, triglycerides; HDL, high-density lipoprotein; LDL, low-density lipoprotein; NDS, neuropathy disability score; AU, arbitrary units.

By contrast, the ongoing Framingham Heart Study has demonstrated a statistically significant relationship between insulin resistance, oxidative stress, hypertension and telomere length reduction in males (28). However, even though telomerase activity has been often associated with diabetes and some of its complications, a correlation with diabetic ulcers has not been shown thus far (29).

In the current study, we showed that diabetic patients with ulcers present lower telomerase activity in their leukocytes than patients without ulcers irrespective of the disease duration. A decreased leukocyte telomere length and telomerase activity in T2DM patients has been also reported previously (30). In addition, the telomere length can be used as an indicator of hyperglycemia and hyperinsulinemia (17-21,23-25,31-34). Using a sample of T2DM adult men, Murillo-Ortiz *et al* showed that telomere shortening increases with the duration of diabetes, suggesting that the progressive increase in inflammation has an additive direct effect on telomere shortening (35). In addition, telomere length has been shown to be a reliable marker for the prognosis of diabetic kidney disease progression (19) and for retinal endothelial cell senescence (36,37).

Furthermore, we showed that ulcer-bearing T2DM patients were characterized by greater waist circumference and NDS, suggesting a possible interrelationship of these parameters in the clinical outcome of diabetes mellitus. Nevertheless, if patients of this study were segregated based on their NDS, no statistically significant difference of leukocyte telomerase activity was observed between the neuropathy and no neuropathy groups (data not shown).

Thus, our findings may pinpoint to the presence of some common clinical traits in diabetic patients with ulcer, but caution should be taken in the translation and extrapolation of the results due to certain limitations of the study: i) Patient group size is relatively small and thus the study needs to be extended in order to validate our data in larger populations; and ii) a great intra- and inter-group diversity exists in terms of drug treatment for diabetes itself, as well as for existing comorbidities, which may interfere with the measured clinical characteristics. Telomere length, for example, has been reported to be negatively affected by drugs (38,39) and telomerase activity increases with paracetamol administration in rat embryonic liver cells (40).

In conclusion, we have demonstrated that the patients with diabetic ulcers had higher waist circumference and NDS, along with lower leukocyte telomerase activity. Confirmation of these results in a larger patient group could further contribute to the understanding of T2DM pathophysiology.

Competing interests

Demetrios A. Spandidos is the Editor-in-Chief for the journal, but had no personal involvement in the reviewing process, or any influence in terms of adjudicating on the final decision, for this article.

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