

Type 2 diabetes mellitus and vascular complications

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Diabetes mellitus and its complications have imposed a significant burden on public health. High death rates in type 2 diabetes are linked to vascular complications, with the risk of myocardial infarction and stroke tripling in patients with this illness. Oxidative stress can lead to the dysfunction of the advanced glycation end products and the receptor for advanced glycation end products. This study aims to examine potential biomarkers for the early development of vascular complications in type 2 diabetes mellitus. The study included 67 patients with type 2 diabetes mellitus (T2DM) with a male-to-female ratio of 1:1.3. They were divided into two groups based on the presence or absence of macroangiopathic vascular complications. To evaluate the oxidative stress in the studied groups, nitric oxide (NO•) radicals were investigated by electron paramagnetic resonance (EPR) spectroscopy and GPx, eNOS, TNF- α , and TGF- β with ELISA kits following the manufacturer's instructions. All studied parameters were compared with 35 healthy controls.

In summary, the presented results show that high levels of ROS in T2DM and diabetic patients with vascular complications lead to increased levels of NO radicals, depletion of antioxidant enzymes, and increased levels of pro-inflammatory cytokines (TNF- α , TGF- β) which interfere with induced migratory responses and contribute to dysfunction.

Keywords: diabetes mellitus, vascular complications, GPx, eNOS, TNF- α , TGF- β

INTRODUCTION

Diabetes mellitus is still being researched, along with its associated pathologies and complications, as it continues to be the eleventh most common cause of death [1]. About 90% of patients with diabetes have type 2 diabetes mellitus (T2DM), and it affects the economically active population between the ages of 35 - 64 the most. High death rates in T2DM are linked to vascular complications, with the risk of myocardial infarction and stroke tripling in patients with this illness [2]. Diabetes mellitus is also the most frequent cause of acquired blindness, renal failure, and amputation of limbs. Despite significant progress and outstanding achievements in the study of the mechanisms of the development of T2DM and the success of new medicinal products to control glycemia, the complications associated with the disease continue to increase [3]. Therefore, current research is focused on investigating the possible mechanisms for the development of T2DM, such as oxidative advanced glycation of end products (AGE) and of the receptor for advanced glycation end products (RAGE). Levels of AGE and RAGE can

cause inflammation and tissue damage by promoting the expression of vascular cell adhesion molecules, monocyte chemoattractant protein-1, endothelin-1, and plasminogen activator inhibitor-1 (PAI-1), and are involved in damage to vessels and tissues [4]. Superoxide anion, hydroxyl radicals, and hydrogen peroxide are examples of reactive oxygen species (ROS) that mediate oxidative reactions in the development of diabetic cardiovascular complications. The heart contains endogenous antioxidant enzymes that protect against ROS toxicity, including SOD, CAT, GPx, GST, and glucose-6-phosphate dehydrogenase. Primary pathological features of diabetic cardiomyopathy include increased levels of malondialdehyde and markers of oxidative stress, as well as necrosis factor- α (TNF- α) and tumor growth factor (TGF)- β [5 -8]. An increased level of 3-nitro-tyrosine is another indicator of oxidative stress in T2DM [9]. Peroxynitrite (ONOO⁻) and nitrogen dioxide (NO₂) react to form peroxynitrite, which reacts with free tyrosine or proteins to produce 3-nitrotyrosine. This impairs mitochondrial function in the myocardium.

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It should be noted that protein nitration and lipoproteins may also play a direct pathophysiological role in the development of atherosclerosis.

Sirtuin-1 (SIRT1) has a major role in regulating the response to oxidative stress by influencing DNA repair and cell survival. Recent studies suggest that it also regulates cellular glycolytic stress by controlling the glyoxalase system [10]. This is particularly relevant in T2DM, where oxidative stress is one of the primary pathophysiological processes associated with complications. These complications can continue even after carbohydrate metabolism indicators are normalized, leading to vascular complications.

This study aims to examine potential biomarkers for the early development of vascular complications in T2DM.

EXPERIMENTAL

Ethics statement

This work was conducted according to the Declaration of Helsinki, and approved by the Ethics Board, "Clinic for Endocrinology and Metabolic Diseases", UMHAT "Prof. St. Kirkovich" in Stara Zagora, Bulgaria. Written informed consent (2021/2023 MF, TrU, Stara Zagora) was obtained from the patients after hospitalization between January 2021 and August 2023.

Study design and subjects

All reagents were purchased from Merck KGaA, Darmstadt, Germany. The study included T2DM 67 patients, in ratio 1:1.3 male - to - female, and based on the presence or absence of macroangiopathic vascular complications they were divided into two groups (Table 1).

To assess macroangiopathic complications, patients were defined as having experienced a cardiovascular event if they had been diagnosed with coronary heart disease, cerebrovascular disease, and/or peripheral arterial disease. At the time of study entry, 45 (58%) T2DM patients had poor disease compensation. Venous blood was collected at the Clinic for Endocrinology and Metabolic Diseases on the day of the study by venipuncture from a peripheral venous source between 8:00 a.m. and 10:00 a.m. in a vacutainer for serum with a clot activator - 4 mL. The samples collected for the electron paramagnetic resonance (EPR) study were immediately imaged and then frozen at - 80°C for the ELISA assay. About half of the patients with diabetes (58.1 %) were obese. The proportion of patients suffering from ischemic heart disease was 37.6 %. Eleven of the patients (11.8 %) had a history

of cerebrovascular disease. None of the diabetics presented symptomatic lower extremity arterial disease. At study entry (58 %) of T2DM patients with vascular disease had poor disease compensation, defined as HbA1c > 7 % and fasting blood glucose values > 6.1 mmol/L. In terms of therapy, diabetics with good glycemic control (22 patients) took oral hypoglycemic agents (sulfonylureas and biguanides), and in 6 patients biguanides were combined with insulin; 7 patients were on insulin monotherapy. In the subgroup of diabetics with poor glycemic control (n = 45), 15 were treated with oral hypoglycemic agents (sulfonylureas and biguanides); 10 were on insulin therapy, in 20 of whom it was combined with oral medication (insulin plus biguanides). The comparison was made with 35 healthy volunteers, 14 males, and 21 females, with a mean age of 43.32 ± 9.28 years. Only 7 (7.4 %) of the controls had slightly increased body weight (BMI 31- 33). Participants were matched for gender. Patients with and without vascular disease were balanced/matched for gender and age. Table 1 presents the key characteristics of diabetics with concomitant vascular disease and healthy controls.

Electron paramagnetic resonance (EPR) study

All EPR measurements were performed at room temperature on a Bruker BioSpin GmbH, Ettlingen, Germany, equipped with a standard resonator. The EPR experiments were carried out in triplicate. Spectral processing was performed using Bruker WIN-EPR and Sinfonia software, 2009. Based on the methods published by Yoshioka *et al.* [12] and Yokoyama *et al.* [13], the EPR method was developed and adapted for the •NO radical levels estimation.

Enzyme-linked immunosorbent assay

All markers of oxidative stress were measured with ELISA kits following the manufacturer's instructions. The ELISA kits were as follows: Human eNOS (ab241149); Human TNF- α ELISA Kit (ab181421); Human TGF- β ELISA Kit (ab100647).

Statistical analysis

Statistical analysis was performed with Statistica 8, StaSoft, Inc. (Madrid, Spain), and the results were expressed as means ± SE. All data were expressed as means ± SE and obtained by one-way ANOVA, and in the LSD post hoc test, $p > 0.05$ was considered statistically significant. To define which groups were different from each other, LSD post hoc tests were used.

Table 1. Clinical and laboratory data of the studied patients

Variables	Controls (<i>n</i> = 35)	DMT2 (<i>n</i> = 22)	DMVC (<i>n</i> = 45)
Age	43.32 ± 6.45	52.13 ± 4.85	55.75 ± 7.04
Sex (M/F)	14 M/21 F	11 M/11 F	22M/23 F
Disease duration (years)	-	10.7 ± 7.3	12.7 ± 8.6
BMI (kg/m ²), mean ± SD	26.07 ± 3.52	32.11 ± 5.44	33.56 ± 6.21
Blood sugar (mmol/L), mean ± SD	4.98 ± 0.32	8.05 ± 5.11	9.55 ± 4.89
HbA1c%	5.69 ± 0.44	6.37 ± 0.85	9.87 ± 1.27
GFR (mL/min/1.73 m ²)	110.08 ± 9.32	96.39 ± 4.25	89.91 ± 3.01
UAE (mg/day)	1.20 ± 0.35	21.80 ± 1.	24.57 ± 6.85
FBC (mmol/L)	5.31 ± 0.28	6.43 ± 0.89	12.72 ± 5.15
Cholesterol (mmol/L)	4.17 ± 0.3	5.01 ± 0.7	5.47 ± 0.6
Triglycerides (mmol/L)	1.31 ± 0.15	2.13 ± 0.2	2.65 ± 0.2
HDL (mmol/L)	0.91 ± 0.019	1.19 ± 0.04	1.32 ± 0.05
LDL (mmol/L)	2.03 ± 0.11	2.72 ± 0.24	2.96 ± 0.2
CRP (pg/mL)	14.14 ± 0.42	26.68 ± 0.95	51.86 ± 2.79

RESULTS AND DISCUSSION

Clinical and laboratory data for healthy volunteers (controls) diabetes mellitus (T2DM) and diabetes mellitus with vascular complications (DMVC) are presented in Table 1.

GPx antioxidant enzymes' activity in patients with T2DM and controls

Investigation of the intracellular oxidative status involves measurement of antioxidant enzyme activity, which includes GPx activity. The results indicated a statistically significant difference in GPx activity (Fig. 1) in the diabetic group, T2DM compared to healthy controls ($p = 0.0001$) and diabetic patients with vascular complications T2DMVC ($p = 0.003$).

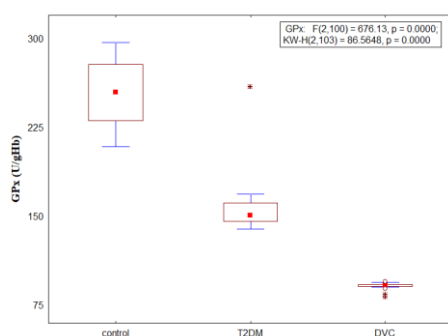


Fig. 1. Activity of antioxidant enzyme GPx in serum samples of diabetic mellitus patients compare to controls and patients with complications. LSD post hoc test, * $p < 0.05$ vs. control; ** $p < 0.05$ vs. DMT2.

Figure 1 presents the reduced activity of the antioxidant enzyme glutathione peroxidase (GPx) in patients with T2DM and vascular complications compared to a control group ($p < 0.05$). The results are consistent with the findings of other studies [14]. The GPx reduced activity was attributed to the

exhaustion of the antioxidant defense system after radical over generation. The autoxidation of glucose in diabetic patients leads to the formation of hydrogen peroxide (H_2O_2). Additionally, H_2O_2 inactivates the antioxidant defense system. The accumulation of H_2O_2 is suggested as a potential explanation for the decreased activity of GPx. Severe oxidative stress, possibly induced by radical over generation, is implicated in the inactivation of GPx. It is interesting to note that GPx, which is usually stable, may become inactivated under conditions of significant oxidative stress. High glucose conditions are mentioned as a factor that can cause the inactivation of GPx through glycation.

The pro-inflammatory cytokine TNF- α and TGF- β levels

The mean value for the tumor necrosis factor (Fig. 2A) in a group with complications of the TNF- α was statistically significantly higher compared to the control groups ($p < 0.05$) and T2DM ($p < 0.05$). Transforming growth factor- β (TGF- β ; Fig. 2B) was statistically significantly higher in the group with vascular complications compared to healthy volunteers ($p < 0.05$) and T2DM ($p < 0.05$). Monocyte-associated inflammatory mediators such as TNF α (Fig. 2A) and CRP (Table 1) have a key role in reducing the risk of cardiovascular diseases in type 2 diabetic conditions [15]. The pathophysiological mechanisms show that the altered monocyte profile precedes the exacerbated secretion of tumor necrosis factor-alpha (TNF- α). The obtained results confirm data reported in the literature [16] that TGF- β is involved and is directly related to diabetic cardiomyopathy which increases the risk of functional and structural abnormalities of the heart. These indicators can be used for the prevention, diagnosis and treatment of diabetes-

related cardiomyopathy [17-19]. Endothelial nitric oxide synthase (eNOS) is regulated by SIRT1. Previous studies have shown that SIRT1 can deacetylate endothelial nitric oxide synthase (eNOS) [20]. Since impaired NO production and vasodilation are associated with diabetes - accelerated endothelial dysfunction, it has been suggested that by regulating SIRT1-mediated eNOS, the vasodilator effects may be controlled [18]. Therefore, information on the levels of NO and eNOS in serum samples of patients with diabetes mellitus is of utmost importance (Fig. 3.).

NO and eNOS levels in serum

NO has been implicated as a major mediator of endothelium-dependent relaxation and together with EDRF (endothelium-dependent relaxing factor), EDCF (endothelium-dependent contracting factor) and EDHF (endothelium-dependent hyperpolarizing factor) plays an important role in the regulation of vascular tone and vasoreactivity, especially in resistance blood vessels where a small change in membrane potential causes a significant change in diameter [21, 22]. A possible mechanism of impaired vasodilation by TNF- α may be through epoxyeicosatrienoic acids (EETs) are synthesized in endothelial cells from arachidonic acid through cytochrome P450 oxygenase [23]. There are various ways in which TNF- α activates NOS. Essentially, it leads to a significant decrease in mRNA levels. Furthermore, the activation of eNOS by TNF- α necessitates the activation of protein kinase B, which is done through the activation of the sphingosine-1-phosphate receptor [24]. The activation of eNOS by TNF- α is associated with an increased production of NO, which provides protective effects against dendritic cell adhesion to the endothelium that TNF- α triggers. Increased TNF- α expression induces ROS production, leading to endothelial dysfunction in type 2 diabetes. TNF- α -related endothelial dysfunction in patho-physiological conditions is associated with excess ROS production and reduced NO bioavailability [25].

Vascular dysfunction or damage induced by aging, smoking, inflammation, trauma, hyperlipidemia, and hyperglycemia are among the myriad risk factors that may contribute to the pathogenesis of many cardiovascular diseases, such as hypertension, diabetes, and atherosclerosis [26]. However, the exact mechanisms underlying the impaired vascular activity remain unclear. The available evidence suggests that inflammatory cytokines play a major role in disrupting both macrovascular and microvascular circulation in vivo and in vitro. Additionally, the AGE/RAGE and NF- κ B signaling pathways are instrumental in promoting TNF- α expression, thereby increasing the production of circulating and local vascular TNF- α . This increase in TNF- α expression leads to the production of ROS, which in turn leads to endothelial dysfunction.

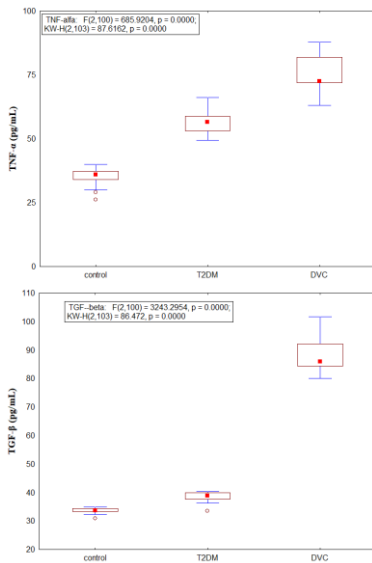


Fig. 2. Pro-inflammatory cytokine levels: (A) TNF- α ; (B) TGF- β in serum samples of diabetic mellitus patients compare to controls and patients with complications; LSD post hoc test, * $p < 0.05$ vs. control; ** $p < 0.05$ vs. T2DM.

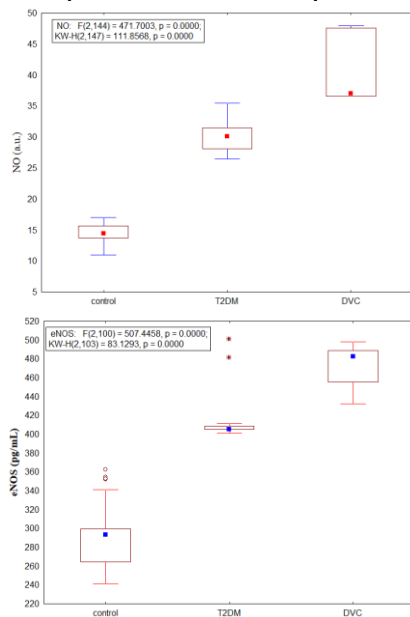


Fig. 3. NO and eNOS levels in serum samples of diabetic mellitus patients compare to controls and patients with complications; LSD post hoc test, * $p < 0.05$ vs. control; ** $p < 0.05$ vs. T2DM.

CONCLUSIONS

In summary, the presented results show that high levels of ROS in T2DM and diabetic patients with vascular complications lead to increased levels of NO radicals, depletion of antioxidant enzymes, and increased levels of pro-inflammatory cytokines (TNF- α , TGF- β) which interfere with monocyte-induced migratory responses and contribute to monocyte dysfunction.

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REFERENCES

1. A. Schmidt, *Arteriosclerosis, Thrombosis, and Vascular Biology*, **38**, (9), e159 (2018).
2. C. González-Juanatey, M. Anguita-Sánchez, V. Barrios, I. Núñez-Gil, J. Gómez-Doblas, X. García-Moll, *J. Clin. Med.*, **12**, 5218 (2023).
3. J. Johnson, R. Jagers, S. Gopalkrishna, A. Dahdah, A. Murphy, N. Hanssen, P. Nagareddy, *ARS*, **36**, 652 (2022).
4. J. Lee, J. Yun, S. Ko, *Nutrients*, **14**, 3086, (2022).
5. Y. Tan, Z. Zhang, C. Zheng, K. Wintergerst, B. Keller, L. Cai, *Nat. Rev. Cardiol.*, **17**, 585 (2020).
6. M. Panayotova, K. Peeva, P. Chakarova, *Pediatrics*, **55** (3), 22 (2018).
7. O. Akpoveso, E. Ubah, G. Obasanmi, *Antioxidants*, **12**, 123 (2023).
8. N. Byrne, N. Rajasekaran, E. Abel, H. Bugger, *Free Radic. Biol. Med.*, **169**, 317 (2021).
9. G. Jakubiak, G. Cieślak, A. Stanek, *Antioxidants*, **11**, 856 (2022).
10. N. Syed, A. Bhatti, P. John, *Antioxidants*, **12**, 1663 (2023).
11. D. Yoshioka, M. Hamada, T. Okamoto, Y. Kobori, Y. Kobayashi, *Photochem. Photobiol. Sci.*, **21**, 1781 (2022).
12. Y. Horizumi, S. Goto, M. Takatsuka, H. Yokoyama, *Mol. Pharmaceutics*, **20**, 2911 (2023).
13. V. Pouresmaeil, A. Al Abudi, A. Mahimid, M. Sarafraz Yazdi, A. Es-Haghi, *Biol. Trace Elem. Res.*, **201**, 617 (2023).
14. S. Ngcobo, B. Nkambule, T. Nyambuya, K. Mokgalaboni, A. Ntsethe, V. Mxinwa, P. Dlodla, *Biomed. Pharmacother.*, **146**, 112579 (2022).
15. S. Kimball, A. Joshi, W. Carson, A. Boniakowski, M. Schaller, R. Allen, J. Bermick, F. Davis, P. Henke, C. Burant, S. Kunkel, *Immunity*, **66**, 2459 (2017).
16. S. Seksaria, S. Mehan, B. Dutta, G. Gupta, S. Ganti, A. Singh, *J. Biochem. Mol. Toxicol.*, **37**, 23330 (2023).
17. M. Panayotova, M. Muhtarov, P. Dragomirova, V. Bangyozov, V. Boeva-Bangyozova, *Gen. Med.*, **20** (3), 47 (2018).
18. T. Meng, W. Qin, B. Liu, *Front. Endocrinol.*, **11**, 568861 (2020).
19. M. Su, W. Zhao, S. Xu, J. Weng, *Antioxidants*, **11**, 108 (2022).
20. M. Muhtarov, V. Boeva-Bangyozova, M. Panayotova, D. Popov, *Gen. Med.*, **21** (1), 64 (2019).
21. F. Nappi, A. Fiore, J. Masiglat, T. Cavuoti, M. Romandini, P. Nappi, S. Avtaar Singh, J. Couetil, *Biomedicines*, **10**(11), 2884 (2022).
22. M. Razan, S. Amissi, R. Islam, J. Graham, K. Stanhope, P. Havel, R. Rahimian, *Biomedicines*, **11**, 1129 (2023).
23. F. Isse, A. El-Sherbeni, A. El-Kadi, *Drug Metab. Rev.*, **54**, 141 (2022).
24. Q. Xiao, D. Wang, D. Li, J. Huang, F. Ma, H. Zhang, X. Ha, *JDC*, 108565 (2023).
25. M. Thomas, M. Coughlan, M. Cooper, *Cell Metabolism*, **35**, 253 (2023).
26. W. Frąk, A. Wojtasińska, W. Lisińska, E. Młynarska, B. Franczyk, J. Rysz, *Biomedicines*, **10**, 1938 (2022).