

Monitoring of Glycation, Oxidative Stress and Inflammation in Relation to the Occurrence of Vascular Complications in Patients with Type 2 Diabetes Mellitus

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Short title: Monitoring of glycation, oxidative stress and inflammation

Summary

The study aimed to evaluate if the monitoring of advanced glycation end products (AGEs), advanced oxidation protein products (AOPP), lipoperoxides (LPO) and interleukin-6 (IL-6) in plasma could help to predict development of diabetic complications (DC). Clinical and biochemical parameters including AGEs, AOPP, LPO and IL-6 were investigated in patients with type 2 diabetes mellitus (DM2) with (+DC) and without (-DC) complications. AGEs were significantly higher in both diabetic groups compared to controls. AGEs were also significantly higher in group +DC compared to -DC. AGEs significantly correlated with HbA1c. We observed significantly higher AOPP in both diabetic groups in comparison with controls, but the difference between -DC and +DC was not significant. LPO significantly correlated with BMI. IL-6 were significantly increased in both diabetic groups compared to controls, but the difference between -DC and +DC was not significant. There was no significant correlation between IL-6 and clinical and biochemical parameters. These results do not exclude the association between IL-6 and onset of DC. We suggest that the measurement of not only HbA1c, but also AGEs may be useful to predict the risk of DC development in clinical practice. Furthermore, the measurement of IL-6 should be studied as adjunct to HbA1c monitoring.

Key words: advanced glycation, oxidative stress, inflammation, diabetic complications, type 2 diabetes mellitus

Introduction

Over the last few years the number of people with type 2 diabetes mellitus (DM2) has massively increased, becoming one of the most important public health challenges globally. Diabetic complications (DC) are now a global health problem without effective therapeutic approach (Singh *et al.* 2013). Recently, major advances have been made in understanding the mechanisms that are involved in the pathogenesis of DM2 (Barbarroja *et al.* 2012).

Chronic hyperglycemia is a major etiological factor causing both micro- and macrovascular lesions associated with DM2 (Paneni *et al.* 2013). Numerous biochemical changes initiated by hyperglycemia directly influence cellular function and lead to abnormal vascular remodeling and the development of vascular DC (Wadham *et al.* 2007). Long-term hyperglycemia is linked with early, intermediate and advanced glycation, glycoxidation, oxidative stress and low-grade inflammation (Williams and Nadler 2007; Aronson 2008; Negre-Salvayre *et al.* 2009; Garcia *et al.* 2010; Navale and Paranjape 2013). However, the inter-relationship between advanced glycation, oxidative stress, inflammation and the progression of vascular DC have not been fully understood (Bigagli *et al.* 2012). Levels of early glycation product - glycated hemoglobin (HbA1c) predict potential diabetic micro- and macrovascular DC (Zoungas *et al.* 2012; Rácz *et al.* 2012; Higgins 2013). Higher HbA1c levels are also associated with higher levels of markers of inflammation (De Rekeneire *et al.* 2006).

Elevated levels of glucose accelerate the formation of advanced glycation end-products (AGEs). AGEs are produced through the nonenzymatic glycation and oxidation of proteins, lipids and nucleic acids (Jakuš and Rietbrock 2004; Giacco and Brownlee 2010). These compounds derive from both endogenous and exogenous sources, and take effect at both intracellular and extracellular level. AGEs excess is one of the most important mechanisms involved in the pathophysiology of chronic vascular DC. An excessive AGE

formation has been demonstrated in the newly disclosed biochemical pathways involved in the microvascular pathobiology of DM2 (Chen *et al.* 2013; Chilleli *et al.* 2013). AGEs are also believed to have a key role in the development and progression of cardiovascular disease in patients with DM2 (Hegab *et al.* 2012). Via their chief signaling receptor-the AGE-specific receptor (commonly abbreviated as RAGE) AGEs generate reactive oxygen species (ROS) and activate inflammatory signaling cascades (Yan *et al.* 2008; Yamamoto and Yamamoto 2013). RAGE has a dual function and it is involved in both AGE removal and AGE-induced cell activation (Manigrasso *et al.* 2013 accepted by Trends Endocrinol Metab, Motawi *et al.* 2013). Cell activation can occur via receptor-mediated generation of ROS through both mitochondrial and cytosolic pathways involving the electron transport chain and nicotinamide adenine dinucleotide phosphate-oxidase (NADPH oxidase), respectively. ROS can trigger pro-inflammatory signaling pathways causing mitogen-activated protein kinase-dependent (MAPK-dependent) activation of transcription factors such as nuclear factor kappa B (NF- κ B) and consequent increased cytokine and adhesion molecules expression, induction of oxidative stress, and an increase in cytosolic ROS (Calder *et al.* 2011, Piarulli *et al.* 2013).

From markers of oxidative stress advanced oxidation protein products (AOPP) have emerged as a novel class of inflammatory mediators. AOPP are the dityrosine-containing and cross-linking protein products formed during oxidative stress (Kalousová *et al.* 2002). In DM2 the formation of AOPP is induced by intensified glycoxidation processes, oxidant-antioxidant balance, and coexisting inflammation (Piwowar *et al.* 2007). Data suggest that chronic accumulation of AOPP in plasma may promote renal inflammation in DM2 patients probably through activation of renal NADPH oxidase (Shi *et al.* 2008) and are associated with increased susceptibility to develop diabetic retinopathy (Al-Shabrawey and Smith 2010; Chen *et al.* 2013).

Other markers of oxidative stress are lipoperoxides (lipid hydroperoxides) (LPO). LPO are generated from polyunsaturated fatty acids and represent primary end products of the lipoperoxidation. Lipoperoxidation is a cascade of events resulting from the action of ROS on polyunsaturated fatty acids present in cell membranes (Bandeira *et al.* 2013). Circulating LPO levels have been shown to be significantly elevated in association with myocardial ischemia and cardiovascular risk factors (Walter *et al.* 2008). Lipoperoxidation is increased in DM2 patients and may be related to modulation of the inflammatory response (Bastos *et al.* 2012; Yadav and Ramana 2013).

Inflammation is thought to mediate pancreatic β -cell dysfunction and the development of insulin resistance (Williams and Nadler 2007). Insulin resistance leads to a perturbation in the lipid homeostasis, cytokines, and adipokines production, resulting in increased systemic inflammation, with higher levels of inflammatory markers such as C-reactive protein (CRP), tumor necrosis factor- α (TNF- α), and interleukin 6 (IL-6) (Garcia *et al.* 2010; Pedicino *et al.* 2013). Elevated serum IL-6 levels have been found to be associated with insulin resistance and increased risk of DM2 (Wang *et al.* 2013). In chronic hyperglycemia, cytokines infiltrate vascular tissues and inhibit function and repair. Studies in recent years have shown that inflammation, and more specifically pro-inflammatory cytokines, are the determinants in the development of micro- and macrovascular DC (Mora *et al.* 2006; Jeffcoate *et al.* 2006; Mocan *et al.* 2006; King 2008; Wu *et al.* 2012; Navale and Paranjape 2013). Numerous efforts have gained way to understanding the mechanisms of the inflammatory response, however, effective treatments have still not emerged (Nguyen *et al.* 2012).

The aim of the present study was evaluated if monitoring of circulating HbA1c, plasma levels of AGEs, AOPP, LPO and IL-6 in patients with DM2 could help to predict the DC development.

Materials and Methods

Patients and the sample collection

The study was approved by Local Ethical Committee. Blood, serum/plasma were obtained from 81 patients (49 men, 32 women; age: 63 ± 9.6 years) suffering from DM2, regularly attending the II. Internal Medicine Clinic, Comenius University in Bratislava, Slovakia. Venous blood samples were collected after 12-hours overnight fasting. Within 1 h of collection, blood was centrifuged (1200 x g, 10 min), serum and plasma (EDTA as an anticoagulant) were obtained. Aliquoted biological samples were frozen for later investigation. Blood plasma and serum were kept at - 80 °C.

Criteria for inclusion/exclusion of patients were as follows: included patients were nonpregnant, and nonlactating women, without cancer and without mental health problems (senile dementia and Alzheimer's disease, among others) and without secondary diabetes. Twenty-six patients were without diabetic complications (-DC group; 13 men and 13 women; age: 58.3 ± 9.6 years) and 55 patients were with micro- or macrovascular complications (+DC group; 36 men and 19 women; age: 65.2 ± 8.8 years). Microvascular complications included diabetic retinopathy, nephropathy and neuropathy. Diabetic retinopathy was diagnosed through fundus examination performed by an ophthalmologist, while nephropathy was defined on the basis of at least two values of albumin/creatinine ratio > 30 mg/g (NICE Clinical Guideline 73 2008). Diabetic neuropathy was confirmed by electromyography (EMG) exploration using the conductivity assessment of sensor and motor fibers of peripheral nerves. Macrovascular complications were defined as follows: myocardial infarction (documented history of myocardial infarction, and/or electrocardiographic and echocardiographic evidence of myocardial necrosis); stroke (documented history of previous stroke requiring hospitalisation, including minor stroke); peripheral artery disease (history of gangrene, or ankle-brachial index < 0.7 with signs of atherosclerosis of arteries of lower limbs

at the echo-colour Doppler examination) (Carman and Fernandez 2000, Khan *et al.* 2008). From all enrolled patients, 38 were treated with only oral anti-diabetic agents (OAD), 8 with OAD and were on a diet, 22 with combination insulin and OAD and 10 only with insulin. Furthermore, 56 patients were also being treated with lipid or cholesterol lowering drugs and 61 with antihypertensive drugs (Table 1). 20 healthy blood volunteers without diabetes or other metabolic diseases (8 men and 12 women; age: 54.2±6.6 years) were used as controls. 19 diabetic patients and 2 controls were smokers. None of diabetic patients and controls were taking any additional antioxidants and individually antioxidant supplements. All participants were fully informed and gave written consent.

Methods

Clinical and anthropometric assessment

Resting systolic (SBP) and diastolic (DBP) blood pressure levels were measured in the left arm in the sitting position by sphygmomanometer (Omron M3, Intellisense, Japan). Body weight and height were measured without shoes and with light clothing using a digital weighing and measuring station with automatic body mass index (BMI) calculation (kg/m², SECA 764, Germany). Waist circumferences were measured in cm with an inelastic tape to the nearest mm, with the subject in a standing position.

Determination of basic biochemical parameters

Basic biochemical parameters were measured by using standard biochemical procedures at the Biochemical Clinical Laboratory of the University Hospital, Comenius University, Bratislava; and the results were identified with the use of reference ranges in this laboratory. Fasting blood glucose was measured by commercially available kit by glucose oxidase method using Hitachi 911 Analyzer (Roche Diagnostics, Switzerland). HbA1c was determined by HPLC (DiaStat, Bio-RAD, USA) in fair capillary blood using the IFCC calibration. Serum total cholesterol (TC), triacylglycerols (TAG), LDL-cholesterol (LDL) and HDL-cholesterol

(HDL) were estimated from serum enzymatically and colorimetrically using automatic analyser Cobas Integra 400 (Roche, Switzerland). Serum creatinine was estimated using an enzymatic method (Vitros 250, Johnson and Johnson Company, USA).

Determination of AGEs in plasma

Determination of AGEs in plasma (i.e. some fluorescent products from the family of AGEs) was based on the spectrofluorimetric detection according to Henle *et al.* (1999) and Munch *et al.* (1997) in modification Kalousová *et al.* (2001). Blood plasma was diluted 1:40 with PBS pH 7.4 and fluorescence intensity was recorded at the emission maximum at 440 nm upon excitation at 345 nm (spectrofluorimeter Perkin Elmer LS 45). Fluorescence intensity was expressed in AU/g protein.

Determination of AOPP in plasma

AOPP were determined in the plasma using the method previously described by Witko-Sarsat *et al.* (1996) and modified by Kalousová *et al.* (2001). AOPP were quantified by spectrophotometry (GEN5 Elisa from Biotek, USA) at 340 nm after incubation of samples with acetic acid using chloramin T with potassium iodide for the calibration curve. Concentration of AOPP in the sample was expressed in $\mu\text{mol/l}$.

Determination of LPO in plasma

LPO in plasma were determined as described by El Saadani *et al.* (1989) and modified by Sumegová *et al.* (2006). Determination is based on the ability of peroxide to oxidize iodide (I⁻) to iodine (I₂). Iodine in the reaction mixture then reacts with excess iodide to form I₃ with the absorption maximum at a wavelength of 365 nm. Absorbance was measured in a narrow cell in the dual-beam spectrophotometer PharmaSpec Shimadzu UV-1700 at 365 nm. All results were presented in nmol/ml of sample.

Determination of IL-6 in plasma

Plasma levels of IL-6 were determined using Fluorokine MAP cytokine multiplex kit designed for use with Luminex® 100™ analyser produced at R&D Systems (Minneapolis, MN, USA) according to the manufacturer's instructions. The cytokine was analysed using xMap™ technology which combines the principle of a sandwich immunoassay with fluorescent-bead-based technology allowing multiplex analysis of up to 100 different analytes in a single microtiter well (Vignali 2000). Analysis of data was done using four-parametric-curve fitting (Little 2004). Results were presented in pg/ml.

Statistics

Clinical and biochemical parameters of the patients and controls were compared using the Student's t-test and were expressed as means±S.D. values. Results without normal distribution were statistically processed by Kruskal-Wallis test and the Conover Inmann post hoc test. Normality of data distribution was tested using the Shapiro-Wilk test. The correlation between measured parameters was examined using the Pearson's correlations test or Spearman's rank correlation test. For evaluation of the relationship between AGEs and clinical and biochemical parameters a multivariate linear regression analysis was used. For statistical analysis we employed the statistical program StatsDirect v. 2.3.7 (StatsDirect Sales, Sale, UK). For graphical representation of data we used Excel 2000 (Microsoft Co.). P values less than 0.05 were accepted as being statistically significant.

Results

Comparison of clinical and biochemical parameters

Patients in -DC group had significantly higher BMI in comparison with controls (-DC vs. controls: 32.18 ± 6.01 vs. 28.41 ± 5.06 kg/m², $p=0.020$). Patients in +DC group had significantly higher waist circumference in comparison with controls (+DC vs. controls: 104.9 ± 13.5 vs. 96.9 ± 13.99 cm, $p=0.028$) (Table 1).

Patients in +DC group had significantly higher SBP in comparison with controls (+DC vs. controls: 154 ± 20 vs. 131 ± 19 mm Hg, $p<0.0001$) and in comparison with -DC group (+DC vs. -DC: 154 ± 20 vs. 143 ± 21 mm Hg, $p=0.015$). We found significantly lower DBP in +DC group in comparison with controls (+DC vs. controls: 79 ± 9 vs. 85 ± 12 mm Hg, $p=0.011$) (Table 2).

We observed significantly higher fasting blood glucose (FBG) and HbA1c levels in both diabetic groups in comparison with controls (+DC vs. controls: FBG 8.86 ± 2.81 vs. 5.42 ± 0.68 mmol/l, HbA1c 6.07 ± 1.43 vs. 3.4 ± 0.39 %; -DC vs. controls: FBG 8.73 ± 2.15 vs. 5.42 ± 0.68 mmol/l, HbA1c 5.80 ± 1.47 vs. 3.4 ± 0.39 %, all $p<0.0001$), but the differences between -DC and +DC groups were not significant (Table 2).

TC levels were significantly lower in both diabetic groups in comparison with controls (+DC vs. controls: $p=0.003$, -DC vs. controls: $p=0.023$). LDL levels were significantly lower in all diabetic groups in comparison with controls (all $p<0.0001$). We found significantly decreased HDL levels in both groups compared to controls (+DC vs. controls: $p=0.033$, -DC vs. controls: $p=0.027$) (Table 2).

Creatinine levels were significantly higher in +DC group in comparison with controls (+DC vs. controls: $p=0.007$) and in comparison with -DC group (+DC vs. -DC: $p=0.022$) (Table 2).

The plasma AGE levels were significantly higher in both diabetic groups in comparison with controls (+DC vs. controls: 209.66 ± 33.51 vs. 144.31 ± 27.68 AU/g proteins, $p<0.0001$; -DC vs. controls: 176.09 ± 29.35 vs. 144.31 ± 27.68 AU/g proteins, $p=0.003$). AGEs was also significantly higher in +DC group vs. -DC 209.66 ± 33.51 vs. 176.09 ± 29.35 AU/g proteins, $p<0.0001$) (Table 2, Fig. 1).

We observed significantly higher plasma AOPP levels in both diabetic groups in comparison with controls (+DC vs. controls: 124.90 ± 39.21 vs. 57.97 ± 10.97 $\mu\text{mol/l}$, -DC vs.

controls: 124.51 ± 41.35 vs. 57.97 ± 10.97 $\mu\text{mol/l}$, in both $p < 0.0001$), but the difference between -DC and +DC was not significant (Table 2).

We found no significant differences in plasma LPO levels between -DC and +DC patients in comparison with controls. Also the difference between -DC and +DC patients was not significant (Table 2).

IL-6 levels were significantly increased in both diabetic groups in comparison with controls (+DC vs. controls: 1.74 ± 0.14 pg/ml vs. 1.36 ± 0.12 pg/ml, $p = 0.048$, -DC vs. controls: 1.75 ± 0.19 pg/ml vs. 1.36 ± 0.12 pg/ml, $p < 0.05$). The difference between -DC and +DC was not significant (Table 2, Fig. 2).

Analysis of treatment showed that patients which were treated with OAD and OAD in combination with diet had significantly decreased levels of AGEs in comparison with patients which were treated with insulin in combination with OAD (195.34 ± 36.16 resp. 164.80 ± 32.75 vs. 214.41 ± 34.23 AU/g prot.; $p < 0.05$). Patients treated with OAD in combination with diet had significantly decreased levels of AOPP in comparison with patients which were treated only with OAD (101.64 ± 43.91 vs. 135.27 ± 40.89 $\mu\text{mol/l}$; $p = 0.041$). Patients treated by hypolipidemic treatment had lower TC, TAG and LDL in comparison with controls (TC: 4.59 ± 0.52 vs. 5.35 ± 0.86 mmol/l; TAG: 1.49 ± 0.72 vs. 1.91 ± 1.49 mmol/l; LDL: 2.55 ± 0.76 vs. 3.76 ± 0.79 mmol/l); $p < 0.05$). Patients without hypolipidemic treatment had higher LPO in comparison with controls (55.27 ± 20.67 vs. 43.95 ± 15.48 nmol/ml; $p < 0.05$).

Correlations between clinical and biochemical parameters

The plasma AGE levels significantly correlated with BMI ($r = 0.400$, $p = 0.007$), with FBG ($r = 0.524$, $p = 0.0001$), with HbA1c ($r = 0.477$, $p = 0.0005$) (Fig. 3) and with TAG ($r = 0.295$, $p = 0.0422$) in +DC group, but not in -DC group and in controls.

The plasma LPO levels significantly correlated with BMI ($r = 0.438$, $p = 0.003$) (Fig. 4) and with waist circumference ($r = 0.438$, $p = 0.0033$) only in +DC group. LPO levels

significantly correlated with FBG in both diabetic groups and in controls (-DC: $r= 0.528$, $p=0.0081$, +DC: $r=0.337$, $p=0.0108$, controls: $r=0.596$, $p=0.019$). LPO levels significantly correlated also with TAG in both diabetic groups and in controls (-DC: $r= 0.600$, $p=0.0026$, +DC: $r=0.371$, $p=0.0132$, controls: $r=0.875$, $p<0.0001$). LPO levels inversely correlated with HDL in -DC group and in controls (-DC: $r=-0.510$, $p=0.0151$, controls: $r=-0.521$, $p=0.0462$).

The plasma AOPP levels significantly correlated with FBG in -DC group ($r=0.484$, $p=0.0374$), but not in +DC group or in controls.

There was no significant correlations between IL-6 and clinical and biochemical parameters.

Evaluation of the relationship between AGEs and clinical and biochemical parameters was performed using a multivariate linear regression analysis in which AGEs were included as the dependent variable. All variables listed in Tables 1 and 2 were considered as independent variables. The significant predictors of AGEs were HbA1c ($\beta=0.507$; $p=0.0001$) and TAG ($\beta=0.336$; $p=0.004$) in all diabetic patients ($R^2=0.38$), HbA1c ($\beta=0.514$; $p=0.0013$) and TAG ($\beta=0.389$; $p=0.011$) in +DC patients ($R^2=0.47$), and HbA1c ($\beta=0.539$; $p=0.0256$) in -DC patients ($R^2=0.41$).

Discussion

Chronic microvascular DC, including retinopathy, neuropathy, nephropathy, and macrovascular disease, are the major cause of morbidity and mortality in DM2 patients (Nolan *et al.* 2011; Chilelli *et al.* 2013). Dysfunction of the key cells responsible for vascular function can be induced by increased cellular concentrations of glucose during hyperglycemia. This can activate multiple pathways of biochemical dysfunction leading to increased glycation and oxidative stress and inflammation with subsequent direct tissue damage (Yan *et al.* 2008;

Al-Shabrawey and Smith 2010; Nguyen *et al.* 2012; Vlassara *et al.* 2012; Motawi *et al.* 2013; Yamamoto and Yamamoto 2013).

We have found only a few studies investigating clinical and biochemical parameters together with parameters of glycation, glycooxidation, oxidative stress and inflammation of patients with DM2 regard to the presence of DC.

HbA1c has achieved importance in diabetes because of its value in the evaluation of glucose control in clinical practice and its relation to long-term microvascular complications. This was shown for DM2 patients in the United Kingdom Prospective Diabetes Study (UKPDS Group 1998). The relationship between HbA1c and macrovascular disease appears to be more complex. A large body of observational studies, including the UKPDS, has documented increased incidence of cardiovascular and peripheral vascular disease with increased HbA1c. However ACCORD trial (Action to Control Cardiovascular Risk in Diabetes) no significant improvements in cardiovascular outcomes were achieved with further improvements in HbA1c (ACCORD Group 2008).

HbA1c has gained acceptance as an accurate index of long-term blood glucose control. Longitudinal studies have shown that good metabolic control, reflected by stable HbA1c level, can prevent or postpone micro- and macrovascular and other complications or slow down the progress of DC (Zoungas *et al.* 2012; Penno *et al.* 2013). In the present study, we observed significantly higher HbA1c levels in both diabetic groups in comparison with controls, but the difference between -DC group and +DC group was not significant. Therefore it would be beneficial to find also additional predictors of DC.

The preclinical and clinical studies of the last years demonstrated a close participation of AGEs in all of the micro- and macrovascular complications of diabetes, especially in type 2 diabetes mellitus (Chilelli *et al.* 2013). In accordance with some other studies we detected

significantly increased plasma AGEs in +DC patients compared to controls (Kilhovd *et al.* 1999; Kiuchi *et al.* 2001; De la Maza *et al.* 2012; Kerkeni *et al.* 2012; Bansal *et al.* 2013).

In our study the plasma AGE levels in diabetic patients +DC were significantly higher in comparison with -DC group. In contrast, Abou-Seif and Youseff (2004) investigated not significant differences in AGEs between +DC and -DC patients. We suppose that the possible explanation is that in study of Abou-Seif and Youssef (2004) were studied only OAD treated patients and patients with microvascular complications. However, in our study we have included patients treated also with insulin and with macrovascular complications. We found the significant correlation between plasma AGE levels and HbA1c only in +DC patients. No similar associations were found in -DC patients. Bansal *et al.* (2013) also showed positive correlation between plasma AGE levels and HbA1c in DM2 patients with vascular DC.

Studies evaluating relationship between DM2 and oxidative stress, especially in micro- and macrovascular complications in detail are discussed in review of Bandeira *et al.* (2013). In our study AOPP levels showed the most expressive raise in plasma of +DC and -DC diabetic patients compared to controls, but no significant differences between -DC and +DC groups were observed. Others studies also reported about no significant AOPP differences between DM2 patients with micro- or macrovascular complications (Kalousová *et al.* 2002; Abou-Seif and Youssef 2004). Kaneda *et al.* (2002) suggested that AOPP is an independent risk factor for coronary artery disease. According Piwowar *et al.* (2007) plasma AOPP levels seem to be considered as a useful marker to estimate oxidative protein damage in diabetic patients. Gradinaru *et al.* (2013) in their study suggested that AOPP appear to be important biomarker for evaluating the association between diabetes and atherosclerotic disorders. They excluded patients with BMI > 25 kg/m², lipid-lowering therapy or using insulin treatment. In our study these criteria were not excluded and we suppose that they could influence our results.

Dyslipidemia, which affects almost 50% of DM2 patients, is a cardiovascular risk factor characterized by elevated TAG levels, low HDL levels, and a preponderance of LDL particles. Number of lipid lowering agents, including statins, fibrates, niacin and bile acid sequestrants, are available to target normalization of the entire lipid profile. Despite use of combination and high-dose lipid lowering agents, many DM2 patients did not achieve lipid target (Vaverková 2000; Niemeijer-Kanters *et al.* 2001; Mooradian 2009; Vijayaraghavan 2010; Kaefer *et al.* 2012). In our study TC, LDL and HDL levels were significantly lower in both diabetic groups in comparison with controls. TAG levels were similar to controls. Long-term treatment of DM2 (oral antidiabetic agents, insulin, lipid or cholesterol lowering drugs and antihypertensive drugs) optimize lipid profile (Hayashi *et al.* 2006; Mooradian 2009; Sánchez-Quesada *et al.* 2012) and seems to be probably helpful to prevent lipoperoxidation. In our study LPO levels in diabetic patients were similar to controls. We found the close relationship between plasma LPO with TAG in all groups. We found also significant correlation between LPO and BMI in +DC patients. Also in study of Chittar *et al.* (1994) were LPO significantly influenced by BMI in diabetic patients with DC. LPO also positively correlated with TAG and BMI in obese subjects (Hadwan *et al.* 2011).

In individuals with impaired glucose tolerance, DM2 or the metabolic syndrome, serum IL-6 levels were found to be higher compared with those with normal glucose tolerance (Pickup *et al.* 1997; Müller *et al.* 2002). Many clinical studies suggest that acute hyperglycemia can result in elevated levels of circulating pro-inflammatory cytokines, in particular TNF- α , IL-6 and IL-18 (Esposito *et al.* 2002; Wellen and Hotamishigil 2005; van den Oever *et al.* 2010). Tulathar *et al.* (2012) found impact of glycemic status as indicated by HbA1c on inflammation process in DM2 patients. The pro-inflammatory cytokine IL-6 may modulate the onset and progression of DC (Galasseti *et al.* 2006; Tunes *et al.* 2010). While the role of IL-6 and other pro- and anti-inflammatory cytokines in the evolution of diabetes is

still incompletely defined, the evidence of Galassetti *et al.* (2006) suggests that the simultaneous presence of diabetes may result in inappropriately elevated IL-6 levels, with potentially negative implications on the onset and progression of DC. Experimental, clinical and epidemiological studies suggest that some processes related to low-grade inflammation may be relevant to diabetic micro- and macroangiopathy (Zozulinska and Wierusz-Wysocka 2005). In our study were found significantly increased IL-6 levels in both diabetic groups compared to controls, but no significant correlation between circulating IL-6 and parameters of early and advanced glycation and oxidative stress. However, these results do not exclude the association between IL-6 and onset of DC. Furthermore, the measurement of IL-6 in plasma should be studied as adjunct to HbA1c monitoring.

We suggest that the measurement not only HbA1c, but also fluorescent AGEs in plasma may be useful to predict the risk of DC development at a primary care setting. The spectrofluorimetric technique for AGEs and spectrophotometric technique for markers of oxidative stress (AOPP, LPO) used in our study are simple, fast and inexpensive so they can be applicable on large sample size analysis in current laboratory practice, for evaluating glycative and oxidative stresses. We were searching for valuable biomarker for evaluation of the risk of DC development. From this point of view among used markers we propose measurement of fluorescent AGEs as valuable biomarker in clinical practice for prediction of diabetic complications. Furthermore, the measurement of AOPP, LPO and IL-6 in plasma should be studied as markers of oxidative stress and inflammation, but not to predict of DC development.

Conflict of Interest

There are no conflicts of interest.

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Table 1 Baseline characteristic of diabetic patients and controls

| Parameter | DM2 patients (n = 81) | - DC group (n = 26) | + DC group (n = 55) | Controls (n = 20) |
|---|----------------------------------|--------------------------------|--------------------------------|------------------------------|
| <i>Age (years)</i> | 63±9.6 ³ | 58.3±9.6 ² | 65.2±8.8 ^{1,3} | 54.2±6.6 |
| <i>Weight (kg)</i> | 89.97±16.42 | 92.10±19.17 | 88.97±14.04 | 82.85±19.38 |
| <i>BMI (kg/m²)</i> | 30.89±5.40 | 32.18±6.01 ³ | 30.26±5.02 | 28.41±5.06 |
| <i>Waist circumf. (cm)</i> | 104.9±13.5 ³ | 105.0±13.8 | 104.9±13.5 ³ | 96.9±13.99 |
| <i>DM2 duration (years)</i> | 12.6±7.0 | 8.5±4.4 | 14.6±7.2 | - |
| <i>Smokers (n)</i> | 19 (24) | 6 (23) | 13 (24) | 2 (10) |
| <i>Lipid or TC lowering drugs n (%)</i> | 56 (69) | 16 (62) | 40 (73) | - |
| <i>Antihypertensive drugs n (%)</i> | 61 (75) | 18 (69) | 43 (78) | - |
| <i>Only diet n (%)</i> | 3 (4) | 1 (4) | 2 (4) | - |
| <i>OAD n (%)</i> | 38 (47) | 18 (69) | 20 (36) | - |
| <i>OAD and diet n (%)</i> | 8 (10) | 6 (23) | 2 (4) | - |
| <i>Insulin and OAD n (%)</i> | 22 (27) | 1 (4) | 21 (38) | - |
| <i>Insulin n (%)</i> | 10 (12) | 0 (0) | 10 (18) | - |

DM2 patients - patients with type 2 diabetes mellitus, -DC - diabetic patients without diabetic complications, +DC - diabetic patients with diabetic complications, Controls - control group, BMI - body mass index, DM2 duration - duration of type 2 diabetes mellitus, waist circumf. - waist circumference, TC - total cholesterol, OAD- oral anti-diabetic agents, n - number of subjects per group, % - percentage of subjects per group

Data are presented as means±S.D. of n=81 diabetics patients (-DC n=26, +DC n=55) and n=20 controls. ¹ p<0.05 compared to group – DC, ² p<0.05 compared to group +DC. ³ p<0.05 compared to controls.

Table 2 Biochemical parameters of diabetic patients and controls

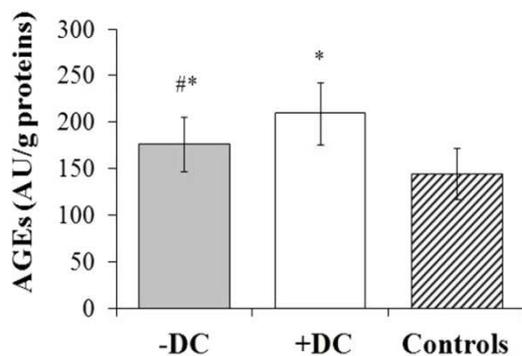
| Parameter | DM2 patients (n = 81) | - DC group (n = 26) | + DC group (n = 55) | Controls (n = 20) |
|-----------------------------|----------------------------------|--------------------------------|--------------------------------|------------------------------|
| <i>SBP (mm Hg)</i> | 151±21 ³ | 143±21 ² | 154±20 ^{1,3} | 131±19 |
| <i>DBP (mm Hg)</i> | 70±10 ³ | 81±12 | 79±9 ³ | 85±12 |
| <i>FBG (mmol/l)</i> | 8.81±2.61 ³ | 8.73±2.15 ³ | 8.86±2.81 ³ | 5.42±0.68 |
| <i>HbA_{1c} (%)</i> | 5.98±1.44 ³ | 5.80±1.47 ³ | 6.07±1.43 ³ | 3.4±0.39 |
| <i>TC (mmol/l)</i> | 4.77±0.93 ³ | 4.83±0.90 ³ | 4.73±0.95 ³ | 5.35±0.86 |
| <i>TAG (mmol/l)</i> | 1.84±1.22 | 1.87±0.83 | 1.82±1.38 | 1.91±1.49 |
| <i>LDL (mmol/l)</i> | 2.68±0.77 ³ | 2.76±0.75 ³ | 2.64±0.78 ³ | 3.76±0.79 |
| <i>HDL (mmol/l)</i> | 1.25±0.29 ³ | 1.22±0.25 ³ | 1.26±0.31 ³ | 1.46±0.42 |
| <i>Creatinine (μmol/l)</i> | 76.66±16.44 ³ | 70.68±11.28 ² | 79.50±17.79 ^{1,3} | 68.65±12.54 |
| <i>AGEs (AU/g proteins)</i> | 199.25±35.68 ³ | 176.09±29.35 ^{2,3} | 209.66±33.51 ^{1,3} | 144.31±27.68 |
| <i>AOPP (μmol/l)</i> | 124.78±39.55 ³ | 124.51±41.35 ³ | 124.90±39.21 ³ | 57.97±10.97 |
| <i>LPO (nmol/ml)</i> | 49.93±18.79 | 54.68±20.72 | 47.50±17.46 | 43.95±15.48 |
| <i>IL-6 (pg/ml)</i> | 1.74±0.11 ³ | 1.75±0.19 ³ | 1.74±0.14 ³ | 1.36±0.12 |

DM2 patients - patients with type 2 diabetes mellitus, -DC - diabetic patients without diabetic complications, +DC - diabetic patients with diabetic complications, Controls - control group, SBP - systolic blood pressure, DPB - diastolic blood pressure, FBG - fasting blood glucose level, HbA_{1c} - glycated hemoglobin, TC - total cholesterol, TAG - triacylglycerols, LDL - low

density lipoproteins, HDL - high density lipoproteins, AGEs - advanced glycation end-products, AOPP - advanced oxidation protein products, LPO - lipoperoxides, IL-6 - interleukin-6, n - number of subjects per group.

Data are presented as means±S.D. of n=81 diabetics patients (-DC n=26, +DC n=55) and n=20 controls. ¹ p<0.05 compared to group - DC, ² p<0.05 compared to group +DC. ³ p<0.05 compared to controls.

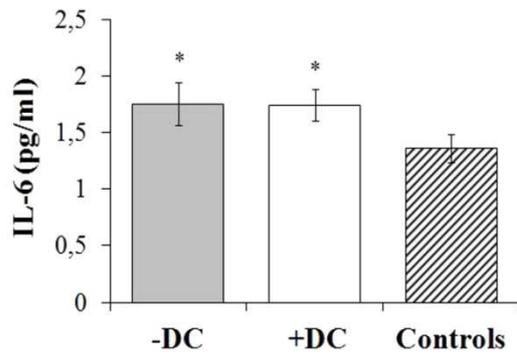
Fig. 1. Comparison of plasma AGE levels in diabetic patients and controls.



Abbreviations: AGEs - advanced glycation end-products, AU - Arbitrary Units, -DC - diabetic patients without diabetic complications, +DC - diabetic patients with diabetic complications.

Values are presented as means±S.D. of n=81 diabetics patients (-DC n=26, +DC n=55) and n=20 controls. #p<0.05 compared to group +DC. *p<0.05 compared to controls.

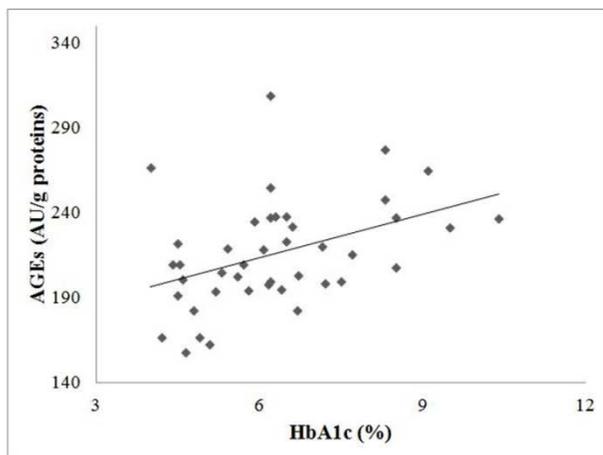
Fig. 2. Comparison of plasma IL-6 levels in diabetic patients and controls.



Abbreviations: IL-6 - interleukin-6, -DC - diabetic patients without diabetic complications, +DC - diabetic patients with diabetic complications.

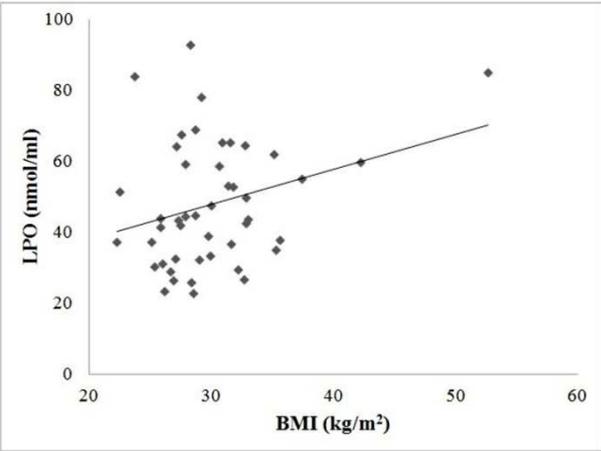
Values are represented as means±S.D. of n=81 diabetics patients (-DC n=26, +DC n=55) and n=20 controls. *p<0.05 compared to controls.

Fig. 3. Correlation between plasma AGEs and HbA1c in +DC group (r=0.477, p=0.0005).



Abbreviations: AGEs - advanced glycation end-products, HbA1c - glycated hemoglobin, AU - Arbitrary Units, +DC - diabetic patients with diabetic complications.

Fig. 4. Correlation between plasma LPO and BMI in +DC group (r=0.438, p=0.003).



Abbreviations: BMI - body mass index, LPO - lipoperoxides, +DC - diabetic patients with diabetic complications.