doi: 10.4183/aeb.2016.150

RELATIONSHIP OF OXIDATIVE STRESS TO URINARY ANGIOTENSIN CONVERTING ENZYME 2 IN TYPE 2 DIABETES MELLITUS PATIENTS

C.I. Bondor[1], A.R. Potra[2,*], C.C. Rusu[2], D. Moldovan[2], S.D. Bolboacă[1], I.M. Kacso[2]

"Iuliu Hatieganu" University of Medicine and Pharmacy, [1]Medical Informatics and Biostatistics, [2]Nephrology, Cluj-Napoca, Romania

Abstract

Context. Angiotensin converting enzyme 2 (ACE2) is highly expressed in the kidney and cleaves angiotensin II to Angiotensin (1–7), annihilating the deleterious effects of angiotensin II which is known to be a strong activator of oxidative stress.

Objective. We aimed to evaluate the relationship of oxidative stress to urinary ACE2 (uACE2) in type 2 diabetes mellitus (T2DM) patients.

Design. We included consecutive normo or microalbuminuric T2DM patients in an observational transversal study. Routine laboratory investigations, plasma malondialdehyde (MDA, fluorimetric thiobarbituric method) as a marker of prooxidant capacity and superoxide dismutase (SOD, cytochrome reduction method) and catalase (CAT) activity (in erythrocyte lysate by the modification of absorbance method) as two measures of serum antioxidant capacity and uACE2 (ELISA method) were assessed.

Results. MDA showed a negative correlation with SOD (r=-0.44, p=0.001), CAT (r=-0.37, p=0.006), uACE2 (r=-0.33, p=0.016) and a positive correlation with glycated haemoglobin (HbA1c) (r=0.49, p<0.001) and associated cardiovascular disease (r=0.42, p=0.001). CAT as also positively correlated to uACE2 (r=0.29, p=0.037). SOD was also negatively correlated with glycemia (r=-0.71, p<0.001) and HbA1c (r=-0.53, p<0.001). Patients with lower MDA (when divided according to median value of 3.88 nmol/mL) had higher uACE2 57.15(40.3-71.2) pg/mL compared to 38.5(31.8-45.95) pg/mL in patients with higher MDA (p<0.001). In multivariate logistic regression uACE2 was the only predictor for MDA above or below its median (OR=0.94, 95%CI[0.90-0.98], p=0.002).

Conclusion. Increased prooxidant serum capacity is associated with lower uACE2 levels in T2DM patients.

Key words: type 2 diabetes mellitus, oxidative stress, urinary ACE2.

INTRODUCTION

Oxidative stress is defined as a state of overproduction of reactive oxygen species (ROS) and other free radicals, which exceeds the buffering capacity of the antioxidant defences. This leads to an imbalance between ROS production and their destruction, determining oxidative damage, cell dysfunction and death (1, 2). Superoxide dismutase (SOD) and catalase (CAT) are two of the key enzymes which help to maintain the equilibrium within this system (3). Malondialdehyde (MDA) on the other hand is a recognized marker o prooxidant capacity (3).

It is generally accepted that diabetes is associated with increased oxidative stress. Recent evidence suggests that oxidative stress has not only a pivotal role in development of β cell dysfunction and insulin resistance of diabetes (4), but also in occurrence of diabetic complications, including nephropathy (5 - 8).

On the other hand, in diabetes there is an over-activation of angiotensin converting enzyme – Angiotensin II (Ang II) – angiotensin 1 receptor axis, involved in the progression of diabetic kidney disease. In the kidney Ang II promotes vasoconstriction, stimulates water and sodium reabsorption, cell proliferation, inflammation, fibrosis and also enhances oxidative stress (9). Ang II is known to be a prooxidant peptide which increases superoxide production by the activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (10), one of the key enzymes involved in ROS generation (11). Recently new elements of the renin angiotensin system have been described, such as angiotensin converting enzyme (ACE) 2 which decreases Ang II levels, thus maintaining a balance within this system. ACE2 is monocarboxypeptidase involved in Ang II catabolism, which removes the single amino acid from the C-terminus and generates Angiotensin (Ang) 1-7. Ang 1-7 binds to its Mas receptor and induces the release of nitric oxide from endothelial cells, balancing the effects of Ang II (10), thus exerting renoprotective effects. Several experimental studies addressed the

*Correspondence to: Alina Ramona Potra, "Iuliu Haţieganu" University of Medicine and Pharmacy Cluj, Nephrology, 3-5 Clinicilor street, Cluj-Napoca, 400006, Romania, E-mail: alinalen@yahoo.com

issue of direct relation between ACE2 and oxidative stress (10, 12 -16), ACE2 deficiency being associated with increased ROS production.

Experimental studies have shown that ACE2 is expressed in the kidney at different levels, especially in proximal tubules, but also in mesangial glomerular cells and podocytes (17). It seems that ACE2 is released into the urine from proximal tubular cells by proteolytic shedding of its ectodomain (18). ACE2 expression is downregulated in different pathologies, decreased ACE2 activity being associated with high blood pressure, diabetes and oxidative stress (13), while increased levels of ACE2 have been shown to be renoprotective (10, 19).

To our knowledge no clinical study to evaluate the relation between oxidative stress and ACE2 was published. We aim to assess, for the first time to our knowledge, the relationship between oxidative stress and urinary ACE2 (uACE2) in type 2 diabetes mellitus (T2DM) patients with incipient diabetic kidney disease.

MATERIAL AND METHODS

Patients

We conducted an observational transversal study in which we included consecutive T2DM patients evaluated in outpatients setting of the "Mihai Manasia" Clinic of Nephrology Cluj. Inclusion criteria were presence of T2DM and presence of a signed informed consent. Exclusion criteria were patients with urinary albumin to creatinine ratio (UACR) > 300mg/g, estimated glomerular filtration rate (eGFR) < 30 mL/min/1.73m^2, presence of clinical/biological signs of active systemic infection, patients with known malignancies or autoimmune diseases.

Methods

For each patient, data related to personal and medical history were recorded: age, duration of T2DM, chronic medication (oral antidiabetics, insulin, ACE inhibitors, Ang II receptor antagonists, and statins), presence of known diabetic retinopathy, peripheral diabetic neuropathy and other comorbidities. Clinical assessment included the measurement of arterial blood pressure, waist circumference, height and body weight. After overnight fasting for about 12 hours, venous blood and fresh morning urinary spot were collected. The biological evaluation included routine laboratory tests: serum creatinine, glucose, total cholesterol, high-density lipoprotein (HDL) cholesterol, triglycerides (automated enzymatic colorimetric spectrophotometric method), HbA1c and C-reactive protein (immunoturbidimetric method), albuminuria from morning urinary spot (immunoturbidimetric method), urinary creatinine (automated enzymatic colorimetric method). Specific determinations were performed at the Immunology Department of Cluj -Napoca County Hospital. Plasma MDA was determined by fluorimetric method using thiobarbituric acid test (20) and values were expressed in mmol/mL. SOD levels were determined on erythrocyte lysate using cytochrome C reduction method as previously described (21). The enzyme activity was expressed as U/mg protein, one unit of SOD was defined as the enzyme activity that inhibits the rate of reduction in cytochrome C by 50 %. CAT activity was determined as previously described (22) in erythrocyte lysate by the modification of absorbance at 240nm; values were expressed in units/mg protein. Urinary ACE2 levels were quantified by ELISA method using commercial ELISA kits (Abbexa Ltd, Cambridge, UK) according to the protocol provided by the supplier. The minimum detection limit was 3.3 pg/mL, 0.7 pg/mL sensitivity, the intra-assay coefficient of variation (CV) <10% and the inter-assay CV<12. The patients' urine used for these determinations was stored at -70 °C until it was analysed.

T2DM was diagnosed according to the American Diabetes Association criteria (23). eGFR was estimated according to Chronic Kidney Disease Epidemiology Collaboration equation (CKD- EPI) (24, 25). Urinary albumin to creatinine ratio was calculated. Low-density lipoprotein (LDL) cholesterol was calculated according to Friedwald equation (26).

We considered that the patients had diabetic kidney disease if their UACR was more than 30mg/g or if their eGFR was less than 60mL/min/1.73m^2.

We evaluated the presence of associated cardiovascular disease (CVD) assessing the records of acute myocardial infarction, coronary revascularization, cerebrovascular ischemic disease, peripheral arterial disease or heart failure; for each of these conditions one point was assigned resulting in cardiovascular disease score (CVDSc), ranging from zero to a maximum of five.

The study was approved by the Ethical Committee of "Iuliu Haţieganu" University of Medicine and Pharmacy Cluj-Napoca and is in accordance with revised ethical standards of Declaration of Helsinki.

Statistical analysis

Statistical analysis was performed using the

Statistical Package for Social Sciences (SPSS) software (version 15, SPSS, Chicago, IL, USA). Continuous variables are presented as the mean±standard deviation (SD) for normally distributed data or as the median (25^{th} percentile – 75^{th} percentile) for non-normally distributed parameters. For the comparison of the means, variables were tested by Student t test or Mann-Whitney test, when appropriate. Proportions were compared using the chi-squared test or Fisher Exact test. Pearson's correlation coefficient or Spearman's correlation coefficient were used to assess the linear, respectively nonlinear relationship between two quantitative or categorical variables. Multiple logistic regression analysis (Forward method) was performed to analyse which factors can predict the apartenence to two subgroups: less / higher than median value of MDA, CAT and SOD in order to emphasize the relationship between MDA, CAT, SOD and all other variables. Odds ratio (OR), 95% confidence interval (CI) for OR of the significant variables entered in the model were presented. Multiple linear regression analysis (Stepwise method) was used to assess the linear association between the dependent variable and the independent ones. Due to the presence of outliers, the UACR was transformed with natural logarithm before the linear regression. The coefficient B, 95%CI of B, standard error and standardized beta coefficient of each significant variable which entered the model were presented.

Table 1. Descriptive clinical and biological characteristics of the studied subjects

Parameters	Patients (n=53)
Male n (%)	34 (64.20)
Age (years)	64.98±10.62
Diabetes duration (years)	8.00 (6.00-15.00)
Waist circumference (cm)	108.49±13.55
BMI (kg/m^2)	32.07±5.96
Total cholesterol (mg/dL)	189.68±45.83
HDL cholesterol (mg/dL)	44.55±12.53
Triglycerides (mg/dL)	140.00 (103.00-215.50)
LDL cholesterol (mg/dL)	106.80±42.15
CRP (mg/dL)	0.32 (0.18-0.48)
Serum glucose (mg/dL)	142.62 (126.19-173.15)
HbA1C (%)	7.45 (6.63-8.98)
UACR (mg/g)	15.36 (4.35-36.02)
eGFR (mL/min)	89.30 (67.86-99.50)
MDA (nmol/mL)	4.00±1.59
SOD (U/mg)	619.30±139.92
CAT (U/mg)	3.13 (2.60-3.46)
uACE2 (pg/mL)	43.50 (35.15-63.65)

Legend: n- number of patients, BMI – body mass index, HDL- high density lipoprotein, LDL- low density lipoprotein, CRP – C reactive protein, HbA1c - glycated haemoglobin, UACR- urinary albumin to creatinine ratio, eGFR - estimated glomerular filtration rate, MDA - malondialdehyde, SOD - superoxide dismutase, CAT- catalase, uACE2 - urinary angiotensin converting enzyme 2. Values are expressed as mean ± standard deviation for normally distributed variables and median (interquartile range) for non-normally distributed ones.

RESULTS

Characteristics of the study population

Fifty-three T2DM patients with normo or microalbuminuria were included in the study. Their median eGFR of 89.30 (67.86-99.50) mL/min was near the normal range. Clinical and biological characteristics of the studied subject are presented in Table 1.

The following chronic complications of diabetes were detected: 22 patients (41.5%) had diabetic kidney disease, 25 patients (47.2%) had peripheral neuropathy and 21 patients (39.6%) had retinopathy. Twenty-two patients (41.5 %) had at least one CVD in their medical history: 9 patients (17 %) peripheral arterial disease, 10 patients (18.9%) had history of stroke, 7 patients (13.2%) had history of myocardial infarction, 6 patients (11.3%) had revascularization and 13 patients (24.5%) had heart failure. Hypertension was present in 46 patients (86.8%). Chronic medication of the studied sample is depicted in Table 2.

LnUACR significantly correlated with eGFR (r=-0.27, p=0.047) and CRP (r=0.44, p=0.001). Even if in the univariate analysis only eGFR and CRP were significantly correlated with lnUACR, in the multivariate regression model uACE2 (B=0.012, p=0.004) and CAT (B=-0.206, p=0.023) were also found to be significant determinants for lnUACR (after adjusting for CRP (B=0.452, p<0.001) and eGFR (B=-0.007, p=0.014)).

Evaluation of the main determinants of the oxidative stress

In our T2DM patients, MDA showed a negative correlation with SOD (r=-0.44, p=0.001), CAT (r=-

Table 2. Chronic medication of the study population

Medication	N (%)
ACEI or ARBs	42 (79.2)
Statins	34 (64.2)
Other HTA drugs	36 (67.9)
Oral antidiabetic drugs	25 (47.2)
Insulin	30 (56.6)
Diet	5 (9.4)

Legend: N (%) - number of patients (percent), ACEI- angiotensin-converting enzyme inhibitors, ARBs- angiotensin II receptor blockers, HTA – hypertensive, Diet - without any antidiabetics medication. Values are expressed as numbers (percentage).

Table 3. Comparison of the patients according to MDA median (3.90 nmol/mL) in the study group

	MDA<3.90 nmol/mL (n=26)	MDA≥3.90 nmol/mL (n=27)	p
Male n (%)	18.00 (69.20)	16.00 (59.30)	0.449
Age (years)	65.27±9.71	64.70±11.61	0.887
Diabetes duration (years)	8.50 (6.00-12.00)	8.00 (6.00-18.00)	0.810
Waist circumference (cm)	107.73±14.91	109.22±12.34	0.693
BMI (kg/m^2)	31.22±6.29	32.89±5.63	0.313
Total cholesterol (mg/dL)	180.00 (153.00-219.00)	203.00 (155.00-229.50)	0.520
HDL cholesterol (mg/dL)	45.27±10.44	43.85±14.43	0.685
Triglycerides (mg/dL)	132.50 (94.00-199.00)	142.00 (105.00-223.00)	0.444
LDL cholesterol (mg/dL)	107.74±39.80	105.90±45.03	0.915
CRP (mg/dL)	0.25 (0.17-0.36)	0.37 (0.20-0.61)	0.301
Serum glucose (mg/dL)	136.79 (125.95-180.12)	143.81 (128.63-168.33)	0.896
HbA1C (%)	7.00 (6.50-8.30)	7.82 (7.00-9.80)	**0.030**
eGFR (mL/min/1.73m^2)	92.27 (71.57-102.35)	83.11 (52.59-94.84)	0.255
UACR (mg/g)	18.08 (4.06-72.60)	13.09 (4.88-26.39)	0.226
MDA (nmol/mL)	2.67±0.71	5.28±1.06	
SOD (U/g)	654.21±98.12	585.68±165.85	**0.028**
CAT (U/g)	3.39 (2.53-3.49)	2.91 (2.68-3.38)	0.378
uACE2	57.15 (40.30-71.20)	38.50 (31.80-45.95)	**<0.001**
CVDSc n (%)	0.00 (00.00-0.00)	1.00 (0.00-1.50)	**0.002**

Legend n - number of patients, BMI - body mass index, HDL- high density lipoprotein, LDL- low density lipoprotein, CRP - C reactive protein, HbA1c - glycated haemoglobin, eGFR - estimated glomerular filtration rate, UACR- urinary albumin to creatinine ratio, MDA - malondialdehyde, SOD - superoxide dismutase, CAT- catalase, uACE2 - urinary angiotensin converting enzyme 2, CVDSc – cardiovascular disease score. Values are expressed as mean ± standard deviation for normally distributed variables and median (interquartile range) for non-normally distributed ones.

Table 4. Multivariate linear regression for parameters of oxidative stress

Dependent variable	Independent variable	Unstandardized Coefficients		Standardized Coefficients	P	95% Confidence Interval for B	
		B	Std. Error	Beta		Lower Bound	Upper Bound
	HbA1c	0.309	0.109	0.347	**0.007**	0.089	0.528
MDA	CAT	-0.533	0.235	-0.277	**0.028**	-1.006	-0.061
	Serum glucose	-2.220	0.351	-0.580	**<0.001**	-2.925	-1.515
SOD	MDA	-32.581	8.048	-0.371	**<0.001**	-48.753	-16.409
	Diabetes duration	4.979	1.745	0.260	**0.006**	1.472	8.486
CAT	MDA	-0.183	0.066	-0.363	**0.007**	-0.316	-0.051
uACE2	MDA	-3.410	1.531	-0.298	**0.030**	-6.484	-0.336

Legend: MDA - malondialdehyde, HbA1c - glycated haemoglobin, CAT- catalase, SOD - superoxide dismutase, uACE2 - urinary angiotensin converting enzyme 2. Independent variables: age, diabetes duration, waist circumferences, BMI, total cholesterol, HDL-cholesterol, LDL cholesterol, serum glucose, HbA1c and CRP.

0.37, p=0.006), uACE2 (r=-0.33, p=0.016) and a positive correlation with HbA1c (r=0.49, p<0.001) and CVDSc (r=0.42, p=0.001). When analysing patients with diabetic kidney disease (n=22), the correlation between MDA and uACE2 was even higher (r=-0.49, p=0.020).

Patients with lower MDA (when divided according to median value of 3.90 nmol/mL) had higher uACE2 57.15 (40.3-71.2) pg/mL compared to 38.5 (31.8-45.95) pg/mL in patients with higher MDA (p<0.001). In Table 3 comparison of patients with MDA levels less or higher than median (3.90 nmol/mL) is presented.

In multivariate logistic regression (Stepwise model) with MDA as dependent variable (less than median *vs.* higher than median) and all the other variables as independent variables only uACE2 (OR=0.94, 95%CI [0.90-0.98], p=0.002) entered the regression model as significant predictor for MDA.

When studying determinants of CAT, except for the correlation with MDA, CAT was positively correlated to uACE2 (r=0.29, p=0.037). In multivariate logistic regression age (OR=1.09, 95%CI[1.01-1.18], p=0.028), MDA (OR=0.56, 95%CI[0.35-0.89], p=0.015) and UACR (OR=0.98, 95%CI[0.96-0.99], p=0.022) but not uACE2 were significant predictors of CAT.

In what concerns determinants of SOD,

except for the correlation with MDA, SOD was negatively correlated with glycemia (r=-0.71, p<0.001) and HbA1c (r=-0.53, p<0.001). MDA (OR=0.51, 95%CI[0.30-0.86], p=0.012) and glycaemia (OR=0.95, 95%CI[0.93-0.98], p=0.001) entered in the multivariate logistic regression model as significant predictors for SOD.

Multivariate linear regression models (Stepwise) for MDA, SOD, CAT, uACE2 as dependent variables and age, diabetes duration, waist circumferences, BMI, total cholesterol, HDL-cholesterol, LDL-cholesterol, glycaemia, HbA1c and CRP as independent variables are presented in Table 4. There was no significant difference in the levels of MDA, SOD, CAT, and uACE2 based on chronic medication (insulin, oral antidiabetics drugs, statins, angiotensin converting enzyme, Ang II receptor antagonists). Neither the presence nor the absence of hypertension, diabetic nephropathy or diabetic neuropathy influenced the oxidative stress markers or uACE2 levels.

DISCUSSION

In our T2DM patients we found an inverse relationship between plasma MDA and uACE2. Multivariate logistic regression confirmed that uACE2 is the main determinant of MDA. Patients with lower uACE2 level exhibit higher oxidative stress, compared to those with higher uACE2 levels. Interestingly, in multivariate linear regression the importance of uACE2 as determinant of MDA was outweighed by glycated HbA1C and CAT. However, a strong relationship between uACE2 and MDA is illustrated by the fact that MDA is the only significant determinant of uACE2 in multivariate linear regression (Table 3).

Previous experimental studies suggested direct relation between ACE2 and oxidative stress (12); ACE2 knockout mice have higher levels of oxidative stress in kidney tissue (13), this link being also evident in CVD (10). In a model of chronic obstructive pulmonary disease it was suggested reduced ACE2 mRNA expression is associated with increase MDA levels and increased ROS generation, while ACE2 administration inhibits oxidative stress (14). Other experimental diabetes studies which evaluated the relationship between oxidative stress and ACE2 (15, 16), showed that kidney ACE2 overexpression exerts protective effects in preserving endothelial function by reducing oxidative stress (16). Other experimental data suggests that the development of diabetic nephropathy is mediated at least in part by increased oxidative stress and lower ACE2 generation (15). Administration of recombinant human ACE2 in mice prevents Ang II induced renal oxidative stress and tubulointerstitial fibrosis (27, 28). All these data are in accordance with our findings regarding the significant correlation between MDA and uACE2 in patients with diabetic kidney disease.

Increased oxidative stress might be a consequence of an alteration in the antioxidant defence system, being known that under normal circumstances SOD and CAT are free radical scavenging enzymes involved in the defence against oxidative injury. As expected, in our study decreased circulating levels of SOD and CAT were associated with increased MDA levels.

We found a direct relation between CAT and uACE2 but in multivariate regression CAT was not an independent determinant of uACE2. Also, SOD was not directly linked to uACE2. This suggests that uACE2 does not directly correlate with antioxidant defence. Our findings are in agreement with data from a recent experimental study that showed that SOD and CAT in kidney was not different in ACE2 knockout mice, compared to controls (12). These ACE2 knockout mice exhibited nevertheless higher oxidative stress, and the authors suggested that increased oxidative stress could be a result of higher ROS production and not of an alteration in antioxidant production. However, in another experimental diabetic nephropathy model in Zucker diabetic fatty rats with decreased antioxidant capacity in the kidney, Ang 1-7 administration restored SOD and catalase activity in the kidney (29).

When analysing patients with higher MDA levels we also observed that they are associated with higher incidence of CVD. Recent reports suggest that oxidative stress is involved in different CVD (2, 30), but the direct relation between markers of oxidative stress and CVD is still a subject of research (31). Several studies linked oxidative stress with atherosclerosis and incident coronary artery disease (32). In line with these data, we observed that lower antioxidant defence, expressed as lower SOD levels, was associated with increased incidence of CVD. Oxidative stress is related to cardiovascular disease especially when the antioxidant capacity is insufficient to reduce ROS generation or other free radicals (33).

It seems that increased oxidative stress due to an imbalance between free radicals production and antioxidant production is related to hyperglycaemia and poor glycaemic control. Chronic hyperglycaemia

leads to increased mitochondrial overproduction of ROS causing tissue damage (6, 34) and is associated with increased oxidative stress markers (35). Lipid peroxidation (evaluated by plasma MDA levels) was linked with poor diabetes control (36). In line with these data, we found a direct correlation between MDA and HbA1c levels. Also SOD was inversely related to glycaemia and HbA1C, suggesting that poorly controlled T2DM patients exhibit a lower antioxidant defence.

In our T2DM patients we found a direct correlation between lnUACR and uACE2, CAT and uACE2 being one of the main predictors of lnUACR. There is growing evidence that there is a decreased antioxidant capacity in diabetes patients (37, 38) and that the imbalance between oxidation and antioxidation might precede the development of renal lesions in patients with diabetes. Recent findings suggest that the antioxidant capacity weakens in parallel with the severity of microalbuminuria in T2DM patients (39). This is in line with our finding regarding the inverse correlation between CAT activity and microalbuminuria in our patients.

The shortcoming of our study is the relatively low number of patients included. We were not able to draw conclusions about causality because our study was transversal. The interesting results of our research could be a starting point for further larger transversal and prospective studies in order to establish the importance of the relationship between oxidative stress and uACE2 in the development and progression of diabetic kidney disease.

Conflict of interest

The authors declare that they have no conflict of interest concerning this article.

Acknowledgment

This work was published under the research contract no. 1495/2/28.01.2014 financed by "Iuliu Haţieganu" University of Medicine and Pharmacy Cluj-Napoca.

References

1. Giustarini D, Dalle-Donne I, Tsikas D, Rossi R. Oxidative stress and human diseases: origin, link, measurement, mechanisms, and biomarkers. Crit Rev Clin Lab Sci 2009; 46(5-6):241-281.
2. Kayama Y, Raaz U, Jagger A, Adam M, Schellinger IN, Sakamoto M, Suzuki H, Toyama K, Spin JM, Tsao PS. Diabetic Cardiovascular Disease Induced by Oxidative Stress. Int J Mol Sci 2015; 16(10):25234-25263.
3. Dalle-Donne I, Rossi R, Colombo R, Giustarini D, Milzani A. Biomarkers of oxidative damage in human disease.Clin Chem 2006; 52(4):601-623.
4. Nowotny K, Jung T, Höhn A, Weber D, Grune T. Advanced glycation end products and oxidative stress in type 2 diabetes mellitus. Biomolecules 2015; 5(1):194-222.
5. Tiwari BK, Pandey KB, Abidi AB, Rizvi SI. Markers of Oxidative Stress during Diabetes Mellitus. J Biomark 2013; 2013:378790.
6. Giacco F, Brownlee M. Oxidative stress and diabetic complications. Circ Res 2010; 107(9):1058-1070.
7. Singh DK, Winocour P, Farrington K. Oxidative stress in early diabetic nephropathy: fueling the fire. Nat Rev Endocrinol 2011; 7(3):176-184.
8. Fujita H, Fujishima H, Chida S, Takahashi K, Qi Z, Kanetsuna Y, Breyer MD, Harris RC, Yamada Y, Takahashi T. Reduction of renal superoxide dismutase in progressive diabetic nephropathy. J Am Soc Nephrol. 2009; 20(6):1303-1313.
9. Mizuiri S, Ohashi Y. ACE and ACE2 in kidney disease. World J Nephrol 2015; 4(1):74-82.
10. Rabelo LA, Alenina N, Bader M. ACE2-angiotensin-(1-7)-Mas axis and oxidative stress in cardiovascular disease. Hypertens Res 2011; 34(2):154-160.
11. Jandeleit-Dahm K, Cooper ME. Hypertension and diabetes: role of the renin-angiotensin system. Endocrinol Metab Clin North Am 2006; 35(3):469-490.
12. Ortiz-Melo DI, Gurley SB. Angiotensin converting enzyme 2 and the kidney. Curr Opin Nephrol Hypertens 2016; 25(1):59-66.
13. Wysocki J, Ortiz-Melo DI, Mattocks NK, Xu K, Prescott J, Evora K, Ye M, Sparks MA, Haque SK, Batlle D, Gurley SB. ACE2 deficiency increases NADPH-mediated oxidative stress in the kidney. Physiol Rep 2014; 2(3):e00264.
14. Xue T, Wei N, Xin Z, Qingyu X. Angiotensin-converting enzyme-2 overexpression attenuates inflammation in rat model of chronic obstructive pulmonary disease. Inhal Toxicol. 2014; 26(1):14-22.
15. Chang SY, Chen YW, Chenier I, Tran Sle M, Zhang SL. Angiotensin II type II receptor deficiency accelerates the development of nephropathy in type I diabetes via oxidative stress and ACE2. Exp Diabetes Res 2011; 2011:521076.
16. Zhang Y, Liu J, Luo JY, Tian XY, Cheang WS, Xu J, Lau CW, Wang L, Wong WT, Wong CM, Lan HY, Yao X, Raizada MK, Huang Y. Upregulation of Angiotensin (1-7)-Mediated Signaling Preserves Endothelial Function Through Reducing Oxidative Stress in Diabetes. Antioxid Redox Signal. 2015; 23(11):880-892.
17. Ye M, Wysocki J, William J, Soler MJ, Cokic I, Batlle D. Glomerular localization and expression of Angiotensin-converting enzyme 2 and Angiotensin-converting enzyme: implications for albuminuria in diabetes. J Am Soc Nephrol 2006; 17(11):3067-3075.
18. Xiao F, Zimpelmann J, Agaybi S, Gurley SB, Puente L, Burns KD. Characterization of angiotensin-converting enzyme 2 ectodomain shedding from mouse proximal tubular cells. PLoS One 2014; 9(1):e85958.
19. Oudit GY, Herzenberg AM, Kassiri Z, Wong D, Reich H, Khokha R, Crackower MA, Backx PH, Penninger JM, Scholey JW. Loos of angiotensin-converting enzyme-2 leads to late development of angiotensin II-dependent glomerulosclerosis. Am J Pathol 2006; 168(6):1808-1820.
20. Conti M, Morand PC, Levillain P, Lemonnier A. Improved fluorometric determination of malondialdehyde . Clin Chem 1991; 37(7):1273-1275.
21. Diaconu R, Macarie A, Orasan R. Analysis of oxidative stress in sun-exposed and unexposed skin. Int J Bioflux Soc 2014; 6(3):153–157.
22. Pippenger CE, Browne RW, Armstrong D. Regulatory antioxidant enzymes. In: Methods in Molecular Biology, vol. 108: Free Radicals and Antioxidant Protocols , 1st ed . Totowa NJ: Humana Press Inc., 1993:299-311.
23. American Diabetes Association. (2) Classification and diagnosis of diabetes. Diabetes Care 2016; 39(S1):S13-S22.
24. Inal BB, Oguz O, Emre T, Usta M, Inal H, Altunoglu E,

Topkaya C. Evaluation of MDRD, Cockcroft-Gault, and CKD-EPI formulas in the estimated glomerular filtration rate. Clin Lab 2014; 60(10):1685-1694.
25. Vučić Lovrenčić M1, Radišić Biljak V, Božičević S, Prašek M, Pavković P, Knotek M. Estimating glomerular filtration rate (GFR) in diabetes: the performance of MDRD and CKD-EPI equations in patients with various degrees of albuminuria. Clin Biochem 2012; 45(18):1694-1696.
26. Fukuyama N1, Homma K, Wakana N, Kudo K, Suyama A, Ohazama H, Tsuji C, Ishiwata K, Eguchi Y,Nakazawa H, Tanaka E. Validation of the Friedewald Equation for Evaluation of Plasma LDL-Cholesterol. J Clin Biochem Nutr 2008; 43(1):1-5.
27. Zhong J, Guo D, Chen CB, Wang W, Schuster M, Loibner H, Penninger JM, Scholey JW, Kassiri Z, Oudit GY. Prevention of angiotensin II-mediated renal oxidative stress, inflammation, and fibrosis by angiotensin-converting enzyme 2. Hypertension 2011; 57(2):314-322.
28. Batlle D, Wysocki J, Soler MJ, Ranganath K. Angiotensin-converting enzyme 2: enhancing the degradation of angiotensin II as a potential therapy for diabetic nephropathy. Kidney Int 2012; 81(6):520-528.
29. Giani JF, Burghi V, Veiras LC, Tomat A, Muñoz MC, Cao G, Turyn D, Toblli JE, Dominici FP. Angiotensin-(1-7) attenuates diabetic nephropathy in Zucker diabetic fatty rats. Am J Physiol Renal Physiol 2012; 302(12):F1606-1615.
30. Gómez-Marcos MA, Blázquez-Medela AM, Gamella-Pozuelo L, Recio-Rodriguez JI, García-Ortiz L, Martínez-Salgado C. Serum superoxide dismutase is associated with vascular structure and function in hypertensive and diabetic patients. Oxid Med Cell Longev 2016; 2016:9124676.
31. Elnakish MT, Hassanain HH, Janssen PM, Angelos MG, Khan M. Emerging role of oxidative stress in metabolic syndrome and cardiovascular diseases: important role of Rac/NADPH oxidase. J Pathol 2013; 231(3):290-300.
32. Chotimol P, Saehuan C, Kumphune S. Correlation between cardio-ankle vascular index and biomarkers of oxidative stress. Scand J Clin Lab Invest 2016; 76(2):105-111.
33. Leopold JA. Antioxidants and coronary artery disease: from pathophysiology to preventive therapy. Coron Artery Dis 2015; 26(2):176-183.
34. Fiorentino TV, Prioletta A, Zuo P, Folli F. Hyperglycemia-induced oxidative stress and its role in diabetes mellitus related cardiovascular diseases. Curr Pharm Des 2013; 19(32):5695-5703.
35. Aouacheri O, Saka S, Krim M, Messaadia A, Maidi I. The investigation of the oxidative stress-related parameters in type 2 diabetes mellitus. Can J Diabetes 2015; 39(1):44-49.
36. Fatani SH, Babakr AT, NourEldin EM, Almarzouki AA. Lipid peroxidation is associated with poor control of type-2 diabetes mellitus. Diabetes Metab Syndr 2016; pii: S1871-4021(15)30061-8. [Epub ahead of print]
37. Singh DK, Winocour P, Farrington K. Oxidative stress in early diabetic nephropathy: fueling the fire. Nature Reviews Endocrinology 2011; 7(3):176-184.
38. Fujii M, Inoguchi T, Sasaki S, Maeda Y, Zheng J, Kobayashi K, Takayanagi R. Bilirubin and biliverdin protect rodents against diabetic nephropathy by downregulating NAD(P)H oxidase. Kidney Int 2010; 78(9):905-919.
39. Shao N, Kuang HY, Wang N, Gao XY, Hao M, Zou W, Yin HQ. Relationship between Oxidant/Antioxidant Markers and Severity of Microalbuminuria in the Early Stage of Nephropathy in Type 2 Diabetic Patients. J Diabetes Res 2013; 2013:232404.