Plasma Levels of Oxidation Protein Products in Type 2 Diabetic Patients with Nephropathy

[1]Hayat M. Sharada, [1]Mohga S. Abdalla, [1]Ashraf I. Amin, [2]Shaimaa A. El Khouly, [3]Hanaa A. El- Sherif

[1]Chemistry Department, Faculty of Science, Helwan University, Helwan, Egypt.
[2]Clinical Pathology Department, National Institute of Diabetes and Endocrinology, Egypt.
[3]Medical Biochemistry Department, National Research Center, Giza, Egypt.

Abstract: Advanced oxidation protein products (AOPP) are forms of oxidatidatively modified albumin and have recently been investigated as indicators of oxidative stress. They are increased in different disorders, including diabetes mellitus, as a result of hyperglycaemia, oxidative stress and hypoxia. The aim of the present study was to determine the plasma levels of AOPP in the patients with type 2 diabetes mellitus (T2DM) and to estimate its relation and connection with the degree of nephropathy. Plasma levels of AOPP were determined by ELISA Assay in 90 individuals, 60 patients with T2DM and 30 healthy control subjects. The urinary albumin/creatinine ratio (ACR) was used as the reference to define the stage of kidney dysfunction by the assessment of the degree of albuminuria. T2DM patients were divided into three groups according to the value of ACR; twenty patients with normoalbuminuria, twenty three patients with microalbuminuria and seventeen patients with macroalbuminuria. There was a significant increase in the mean levels of total cholesterol and triglycerides in both normoalbuminuria and macroalbuminuria groups compared to the control group. A significant increase in the mean levels of triglycerides and HDL-cholesterol was found in microalbuminuria group compared to the control group. Diabetic patients had significantly higher levels of AOPP in comparison with the control group. AOPP was increasing progressively and significantly from normoalbuminuria, through microalbuminuria to macroalbuminuria. In the current study, the diagnostic utility of AOPP was determined by means of receiver operating characteristic (ROC) curve analysis which indicates that the elevation of AOPP is a useful marker for the presence of nephropathy. There was a high significant positive correlation between AOPP levels and triglycerides in microalbuminuria group. Plasma AOPP correlated significantly with both of serum creatinine and ACR. In conclusion, AOPP may be helpful clinical markers for estimating kidney dysfunction, as well as AOPP is better able to identify diabetic patients with nephropathy. We suggest that AOPP is almost ideal for discriminating between T2DM patients with micro- and macroalbuminuria.

Key words: Type 2 diabetes mellitus, nephropathy, advanced oxidation protein products.

INTRODUCTION

Hyperglycaemia and oxidative stress have been implicated in the accelerated vascular damage associated with diabetes, which eventually manifests microvascular complications such as retinopathy, neuropathy, nephropathy and macrovascular disease e.g. peripheral arteriosclerosis, coronary disease, myocardial infarction and stroke. Although the mechanisms leading to such complications are not fully understood, the vicious circle mechanism between glycation and oxidation ('glycooxidation') has been proposed as one of the most important pathways. This leads, via several mechanisms, to multiple biochemical sequels, which display a disturbance of oxidative–antioxidative balance, and creates glycooxidative molecular damage (Kalousova *et al.*, 2005 and Kostolanská *et al.*, 2009). The majority of the glycooxidation products has not only altered physical and chemical properties but also biological ones, and can accumulate in biological systems. It brings about tissue degeneration, particularly in areas of blood vessels and thus take part in the origin of the diabetic vascular late complications e.g. nephropathy (Bonnefont-Rousselot, 2002 and Piwowar *et al.*, 2008).

Advanced oxidation protein products (AOPP) are formed as the result of oxidative stress not only by the action of reactive oxygen species (ROS) but also by chloramines (produced by myeloperoxidase in activated neutrophils) on serum proteins. AOPP are structurally analogous to advanced glycation end products (AGE) and show similar biological activities i.e. induction of proinflammatory cytokines and adhesive molecules (Tsukahara *et al.*, 2003 and Witko-Sarsat *et al.*, 1996). A few years ago it was suggested that AOPP reflected proteins damaged by oxidative stress, especially albumin (Tan *et al.*, 2007) but other authors have reported that fibrinogen is also responsible for blood plasma AOPP levels (Selmeci *et al.*, 2006).

Corresponding Author: Shaimaa A. El Khouly**,** Chemistry Department, Faculty of Science, Helwan University, Helwan, Egypt.
E-mail: shimaakholi@yahoo.com

Nephropathy is the most important long-term complication in diabetes. This is one of the main causes of end-stage renal disease and mortality in these patients. The structural and functional changes take place in the kidney during the early phases of diabetes, prior to microalbuminuria, including increase in glomerular filtration rate, glomerular hypertrophy and hyperplasia, and changes in the extracellular matrix (O'Connon and Schelling, 2005, Newman *et al*., 2005 and Hakim and Pflueger, 2010). The measurement of urinary albumin excretion rate and plasma creatinine concentration are the most reliable indicators of glomerular injury in developing nephropathy (O'Connon and Schelling, 2005 and Shumway and Gambert, 2002).

The aims of the present study were to determine plasma levels of AOPP in T2DM; to compare their relationship with nephropathy development; to elucidate the connection with common markers of nephropathy, e.g. albumin / creatinine ratio in urine and creatinine concentration in serum.

MATERIALS AND METHODS

As many as sixty patients with type 2 diabetes mellitus from those attending the outpatient's clinics of the National Institute of Diabetes and Endocrinology, Egypt, were studied. They were informed about the aim of these investigations and they gave their permission to conduct this study. All ethical aspects were considered including taking full informed consent from the patients and control individuals before participating in this study, which was approved by the Ethics Committee of the National Institute of Diabetes and Endocrinology. Patients with any acute or chronic disease other than diabetes, urinary tract infection, chronic renal failure, obesity and insulin treatment were excluded from the study.

The control group consisted of thirty healthy adults without inflammatory states or any abnormalities in lipids and carbohydrates metabolism as well as kidney disorders. They were 13 males and 17 females within the same age range.

Full history was taken including age, sex, duration of disease, family history of diabetes, type of treatment and presence of any complication of diabetes as diabetic nephropathy, cardiac complications as atherosclerosis, ischemic heart disease or any other associated diseases. Anthropometric parameters including weight and height were measured. The body mass index (BMI) that correlates weight in Kg in relation to square power of height in meters was calculated.

Eight ml of venous blood samples was collected from both patients and control subjects in the fasting state (for 12 hours) by standard venipuncture techniques into four sterile plastic tubes. The first tube with sodium fluoride/ potassium oxalate anticoagulant was centrifuged at 4000 rpm for 15 min to obtain plasma for the measurement of fasting blood glucose level (FBG) by enzymatic colorimetric method according to Burtis and Ashwood, (1994) using kits supplied from Abbott Laboratories, United States. The second tube with EDTA as anticoagulant in which whole blood was used fresh to determine glycated hemoglobin (HbA1c) by ion-exchange high-performance liquid chromatography (HPLC) according to Mayer and Freedman, (1983) using kits supplied from Bio-Rad Laboratories, lnc. The third tube with no anticoagulant was left to clot at room temperature, then centrifuged at 4000 rpm for 15 min to separate serum for determination of total cholesterol (Allain *et al*., 1974), triglycerides (Fossati and Prencipe, 1982), HDL-Cholesterol (Tholen *et al*., 2003), LDL-Cholesterol (Desideri-Vaillant *et al*., 2004) and creatinine (Soldin *et al*., 1978) by enzymatic colorimetric method using kits supplied from Abbott Laboratories, United States. The fourth tube with EDTA as anticoagulant was centrifuged at 10000rpm for 30 sec to obtain plasma, which was frozen and stored at -80°C but no longer than 2 months until used to determine advanced oxidation protein products (AOPP) (Witko-Sarsat *et al*., 1996) with double-antibody sandwich enzyme-linked immunosorbent assay (ELISA). The Kit was supplied from Glory Science Co., Ltd. Add: 2400 Veterans Blvd. Suite 16-101, Del Rio. TX 78840, USA.

First morning, urine samples were collected from the same patients and control subjects in sterile plastic cups for determination of albumin (Tholen *et al*., 2003) and creatinine (Soldin *et al*., 1978), then albumin/creatinine ratio (µg/mg) was calculated (Thomas, 1998). Type 2 diabetic patients were divided into three groups according to the value of the urinary albumin / creatinine ratio:

- *Group 1:* Twenty patients with normoalbuminuria (the urinary albumin / creatinine ratio below 30). They were 5 males and 15 females, whose ages ranged from 30 to 60 years.
- *Group 2:* Twenty three patients with microalbuminuria (the urinary albumin / creatinine ratio between 30 and 300). They were 8 males and 15 females within the same age range.
- *Group 3:* Seventeen patients with macroalbuminuria (the urinary albumin / creatinine ratio over 300). They were 5 males and 12 females within the same age range.

Statistical Analysis:

All analysis was done using the statistical package for the social science (SPSS software version 12, Chicago, Illinois). All numeric variables were expressed as a mean value and a standard deviation (X ± SD). Statistical comparisons were performed by analysis of variance (ANOVA) test which was applied for multigroup comparisons. Comparison between two groups was performed by using Student's t test (t-test).

Pearson's correlation test was used for correlating parametric variables. For all tests a probability $p < 0.05$ was considered statistically significant.

Receiver operating characteristics (ROC) curve was used to discriminate positive from negative results. The area under the curve (AUC) can range from 0.5 to 1 and diagnostic test that approaches 1 indicates a perfect discriminator. ROC curves also determined the threshold value for optimal sensitivity and specificity, which was constructed by calculating the true positive fraction (sensitivity percent) and the false positive fraction (100-specificity) of markers at several cutoff points.

Results:

The results showed the comparison between the control group and the patients' groups regarding the clinical and laboratory parameters (Table 1). The mean levels of both fasting blood glucose (FBG) and glycated hemoglobin (HbA1c) were significantly higher in each of normo-, micro- and macroalbuminuria cases group than those in the control group. There was a significant increase in the mean levels of total cholesterol and triglycerides in both of normoalbuminuria and macroalbuminuria when compared to the control group. A significant increase in the mean levels of triglycerides and HDL-cholesterol was found in microalbuminuria compared to the control group. The mean levels of serum creatinine and urinary albumin/ creatinine ratio (ACR) showed a significant increase in both of microalbuminuria and macroalbuminuria compared to the control group. The results showed the plasma levels of AOPP in the diabetic patients groups clustered in relation to albuminuria, described by albumin/creatinine ratio (Fig. 1). The mean levels of AOPP were significantly higher in groups 1&2&3 of patients when compared to the control group. AOPP increased progressively and significantly with the growth of albuminuria ($p < 0.01$). The results showed the clinical and laboratory parameters in the plasma of healthy people and T2DM patients (Table 2). The mean levels of AOPP were significantly higher in type 2 diabetic patients than those in the control group.

In this study, no significant correlation was found between AOPP and each of age, disease duration and anthropometric measurements except the significant positive correlation between AOPP and age in normoalbuminuria ($r=0.476$, $P<0.05$). AOPP were positively and significantly correlated with FBG in all patients' group ($r=0.470$, $P<0.05$). On the other hand, there was no significant correlation between AOPP and HbA1c. There was no significant correlation between AOPP levels and any of the parameters of lipid profile except triglycerides in microalbuminuria ($r=0.619$, $P<0.01$). A significant positive correlation was found between plasma level of AOPP and both of serum creatinine ($r=0.270$, $P<0.05$), Fig. (2) and ACR ($r=0.401$, $P<0.05$), Fig. (3) in all patients' group.

The receiver operating characteristic (ROC) curve analysis to calculate the best cut off point for AOPP to discriminate between the control group and the patient's groups was 10.793 ng/mL with 91.7 % sensitivity (55 out of 60 patients had AOPP $\geq$10.793 ng/mL) and 70.0 % specificity (21 out of 30 controls had AOPP <10.793 ng/mL), Table (3). The area under ROC curve that determine the overall ability of AOPP test to correctly identify the control group (normal) versus the patient's groups (abnormal) was 0.869.

The best cut off point for AOPP to discriminate between the control group and macroalbuminuria was 15.381 ng/mL with 100.0 % sensitivity (17 out of 17 macroalbuminuria had AOPP $\geq$15.381 ng/mL) and 93.3 % specificity (28 out of 30 controls had AOPP <15.381 ng/mL), Table (4). The area under ROC curve that determine the overall ability of AOPP test to discriminate between the control group and macroalbuminuria was 0.907.

Table 1: Comparison between the control group and the patients' groups regarding the clinical and laboratory parameters.

Parameters	Normoalbuminuria (n=20)	Microalbuminuria (n=23)	Macroalbuminuria (n=17)
Age (years)	50.40±8.6	50.48±8.89	51.88±5.16
Disease duration (years)	11.72±6.29	8.61±4.26	12.88±8.68
BMI (kg/m²)	25.40±1.69	25.84±2.09	25.25±2.64
FBG (mg/dL)	203.60±67.06**	292.83±92.72**	287.18±97.65**
HbA1c (%)	8.90±1.99**	9.87±2.24**	10.28±2.44**
Total cholesterol (mg/dL)	204.70±39.11*	196.65±36.83	205.18±47.80*
HDL-cholesterol (mg/dL)	47.15±9.31	41.57±7.49*	42.00±12.47
LDL-cholesterol (mg/dL)	146.15±47.23	148.74±62.19	136.06±54.01
Triglycerides (mg/dL)	140.70±50.04*	213.83±68.14**	179.35±93.42**
Sreum creatinine (mg/dL)	0.89±0.39	0.91±0.31*	1.38±0.75**
Urinary albumin /creatinine ratio (µg/mg)	15.55±6.91	91.22±58.71**	1528.41±107.24**
AOPP (ng/mL)	13.26±4.28**	16.18 ±6.15*	21.69±7.49*

Comparison to control group: significant difference at *P<0.05; high significant difference at ** P<0.01.

Table 2: The clinical and laboratory parameters of control group and patients with type 2 diabetes mellitus.

Parameters	Control group	T2DM group
Number (n)	30	60
Sex (M/F)	13/17	18/42
Age (years)	49.50±8.42	50.98±8.15
Disease duration (years)	-	10.97±6.59
BMI (kg/m^2)	25.96 ± 2.63	25.53±2.12
FBG (mg/dL)	87.73±13.78	261.48±94.59[**]
HbA1c (%)	5.56 ±0.43	9.75±2.29[**]
Total cholesterol (mg/dL)	178.90±32.64	201.75±40.42[**]
HDL-cholesterol (mg/dL)	46.87±10.75	43.55±9.88
LDL-cholesterol (mg/dL)	138.97±41.1	144.28±54.59
Triglycerides (mg/dL)	112.83±26.78	179.68±76.64[**]
Sreum creatinine (mg/dL)	0.77 ±0.18	1.03±0.53[*]
Urinary albumin/creatinine ratio (µg/mg)		
AOPP (ng/ml)	16.00±8.99	473.20±872.88[**]
	8.96±3.76	16.77±6.82[**]

Comparison to control group: significant difference at [*]P<0.05; high significant difference at [**] P<0.01.
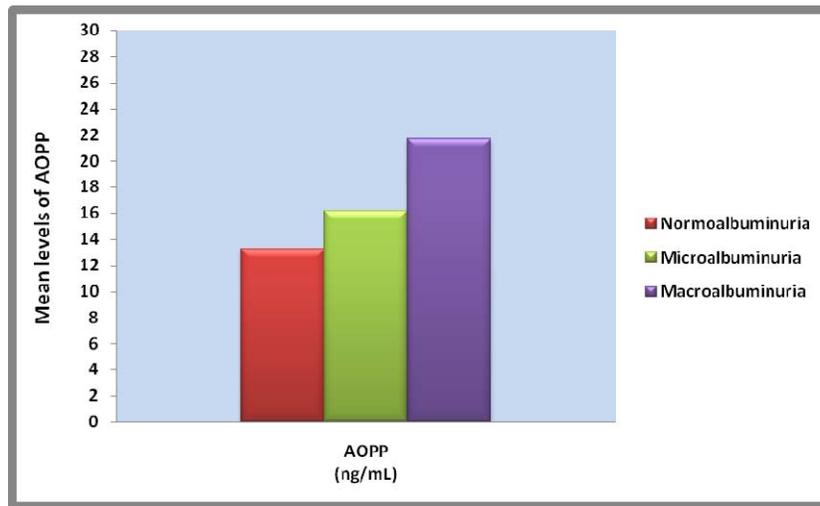

Fig. 1: Plasma levels of AOPP in the diabetic patients groups with different stages of kidney disease.
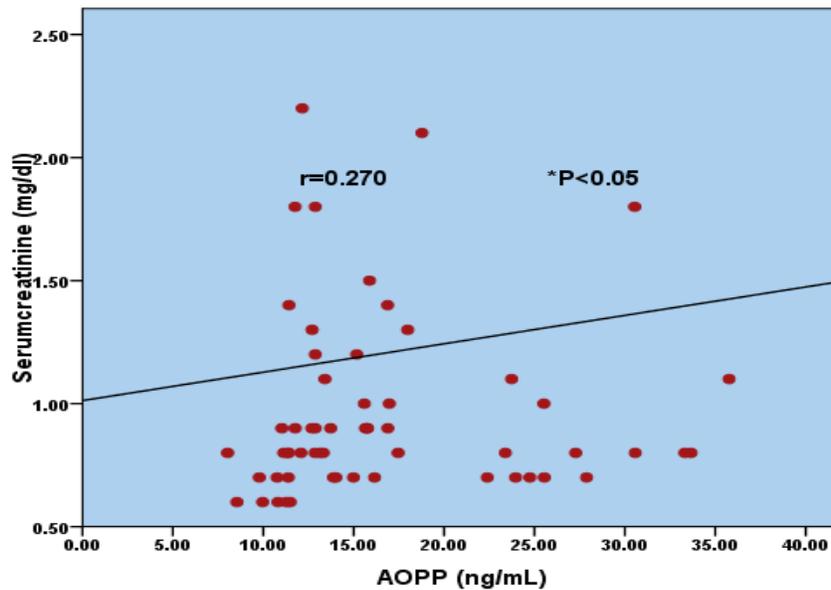

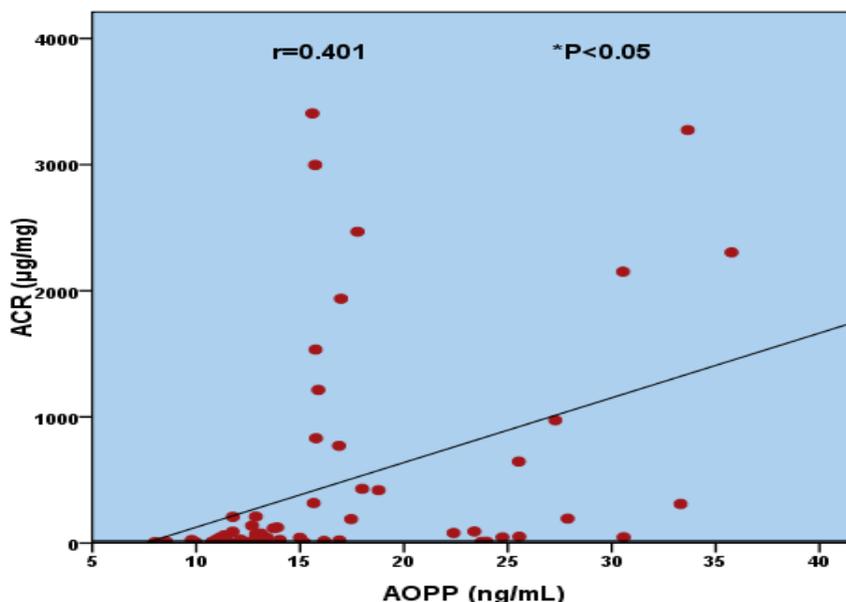**Fig. 2:** Correlation between serum creatinine and AOPP in all patients' group.

Fig. 3: Correlation between ACR and AOPP in all patients' group.

Table 3: ROC analysis for AOPP to discriminate between the control group and the diabetic patient's groups.

Parameter	Cut off	Sensitivity (%)	Specificity (%)	Area under curve (AUC)
AOPP (ng/ml)	10.793	91.7 %	70.0 %	0.869

Table 4: ROC analysis for AOPP to discriminate between the control group and macroalbuminuria.

Parameter	Cut off	Sensitivity (%)	Specificity (%)	Area under curve (AUC)
AOPP (ng/ml)	15.381	100.0 %	93.4 %	0.907

Discussion:

In diabetes mellitus, persistence of hyperglycemia was reported to cause increased production of oxidative parameters (Erciyas *et al.*, 2004). Oxidative stress may play an important intermediary role in the pathogenesis of diabetes complications. Thereby, advanced oxidation protein products (AOPP) are much higher in diabetic nephropathy than that of diabetic patients without vascular complications (Pan *et al.*, 2010). AOPP are elevated in patients with renal insufficiency and have the highest levels in patients on renal replacement therapy (Witko-Sarsat *et al.*, 1996). Nephropathy is the most important microvascular complication of diabetes mellitus and leads to end stage renal disease. It is characterized by progressive thickening of the glomerular basement membrane and by expansion of the mesangial matrix that correlates with glomerular filtration rate, damage of biological membranes and endothelium (O'Connon and Schelling, 2005 and Najafian *et al.*, 2011). In the nephropathy progression, two key mechanisms such as advanced glycation and oxidative stress, leading to formation of advanced glycation end products (AGE) and advanced oxidation protein products (AOPP), respectively, have been involved (Witko-Sarsat *et al.*, 1996 and Tan *et al.*, 2007).

There are less studies concerning AOPP (Tsukahara *et al.*, 2003, Piwowar *et al.*, 2009 and Pan *et al.*, 2010) and few of them is connected with the stages of diabetic nephropathy (Piwowar *et al.*, 2008), and hence it became the main aim of our present work. The observed increase in total AOPP in plasma of T2DM patients in comparison to the control group in the current study is in agreement with previous studies (Pan *et al.*, 2010 and Fathy *et al.*, 2009). In addition, AOPP has been found to be almost twice higher (p < 0.001) in diabetic patients compared with the control group (Piwowar *et al.*, 2008). In a previous work, there was a significant increase of AOPP concentration in the patients with long-term diabetes (more than 10 years) in comparison to patients of less than 5 years (Knapik-Kordecka *et al.*, 2007 and Piwowar *et al.*, 2007). AOPP were elevated in both non insulin dependent diabetes mellitus (NIDDM) and insulin dependent diabetes mellitus (IDDM) (Abou-seif and Youssef., 2004). Furthermore, other study observed significant elevation in AOPP levels in the diabetic patients compared with controls and higher AOPP levels was observed in the poor glycemic control (PGC) group than in the good glycemic control (GGC) group (Kostolanská *et al.*, 2009). AOPP was much higher in diabetic nephropathy than that of diabetic patients without vascular complications (Fathy *et al.*, 2009 and Pan *et al.*, 2010). They suggested that diabetes patients have more severe oxidative stress than normal persons and higher

oxidative stress in diabetic nephropathy than those in patients without complications. Oxidative stress may play an important intermediary role in the pathogenesis of diabetes complications.

In this study, we also estimated plasma levels AOPP in the course of diabetic nephropathy development, in the groups with normo-, micro- and macroalbuminuria, clustered according to urinary albumin/creatinine ratio. The data revealed that, plasma levels of AOPP showed significant increase in each of normo-, micro- and macroalbuminuria groups compared to the control group. The levels of AOPP were rising progressively and significantly with the degree of albuminuria. These findings were in agreement with previous studies (Piwowar *et al*., 2008 and Piarulli *et al*., 2009). On the other side, a progressive increase in AOPP (plasma and urine) was found in the course of albuminuria, but significant differences among the subgroups of patients (with normoalbuminuria, microalbuminuria and macroalbuminuria) were found only in plasma (Shi *et al*., 2008). In a more recent study, AOPP levels were 2.4-fold higher in children and adolescents with chronic renal failure (CRF) and end-stage renal disease (ESRD) patients, and 1.6-fold higher in kidney transplanted patients when compared with the controls (P < 0.01). In patients with stabilized renal function, AOPP levels did not change significantly during 12 months (Šebeková *et al*., 2012). AOPP have been shown to accumulate in kidney of T2DM patients with nephropathy (Van Dijk, and Berl, 2004). Several studies have reported that diabetic patients had increased susceptibility to oxidative stress with elevation of AOPP (Abou-seif and Youssef., 2004**,** Kalousova *et al*., 2002 and Gil-Del Valle *et al*., 2005) whether with (Piwowar *et al*., 2009 and Šebeková *et al*., 2012) or without nephropathy (Tsukahara *et al*., 2003 and Pan *et al*., 2010). AOPP promotes kidney inflammation in diabetic patients through the action of renal NADPH oxidase (Shi *et al*., 2008). Telci *et al*., (2000) explained that, damage to proteins may be more important than damage to lipids in oxidative stress *in vivo*.

In the current study, the diagnostic utility of glycooxidation parameters was determined by means of receiver operating characteristic (ROC) curve analysis which demonstrated that AOPP was good indicator for the presence of diabetic complications due to its high sensitivity (91.7%) and high specificity (70%) in discriminating between control group and T2DM patient's group. Our observations are in agreement with previous studies (Kostolanská *et al*., 2009 and Piwowar *et al*., 2008). On the other hand, AOPP performance as marker for the presence of nephropathy was deeply unsatisfactory as it was worthless at identifying control group versus normoalbuminuria. Also, elevation of AOPP was useful marker indicating the presence of nephropathy because 100% of microalbuminuria had values of AOPP ≥ the best cut off points (10.922 ng/ml). In addition, AOPP were good indicator for the presence of nephropathy due to their its sensitivity (100%) and high specificity (93.4%) in discriminating between control group and macroalbuminuria. Fathy *et al*., (2009) assessed the diagnostic utility of oxidative stress markers by means of ROC analysis and they demonstrated that AOPP and MDA were useful markers indicating the presence of complications. However, as markers of diabetes presence, they were not disease specific to be recommended in screening due to the involvement of oxidative stress in the pathogenesis of plenty of human disorders. Krzystek-Korpacka *et al*., (2008) in their study to validate the utility of oxidative stress markers in the evaluation of young type 1 diabetes, they concluded that, total antioxidant status (TAS) and protein oxidative damage represented by AOPP were good indicators of the disease presence.

In the present study, no significant correlation was found between AOPP and each of age, disease duration and anthropometric measurements except the significant positive correlation between AOPP and age in normoalbuminuria. In accordance with our results, no correlation between AOPP plasma levels and each of age, disease duration, weight, height and BMI in diabetic patient with or without complications (Fathy *et al*., 2009). In the current study, AOPP was positively and significantly correlated with fasting blood glucose (FBG) in all patients' group. There was no significant correlation between AOPP and glycosylated hemoglobin (HbA1c). This is in contrast with Kostolanská *et al*., (2009) who observed significant correlation between HbA1c and AOPP in poor glycemic control group of diabetic patient. Also, Gil-Del Valle *et al*., (2005) reported significant correlation between AOPP as an index of oxidant / antioxidant status and HbA1c as an indicator of glycemic control in diabetic patients. In addition, Fathy *et al*., (2009) reported that poor glycemic control was associated with enhanced oxidative stress as indicated by strong correlation between HbA1c and AOPP in diabetic patients with microvascular complications. Diabetics with higher HbA1c are at increased risk of overproduction of protein oxidation and lipid peroxidation products which may take part in the development of diabetes microvascular complications. This discrepancy may result from the differences in disease duration and / or the age of subjects under evaluation, since the levels of HbA1c have been age dependent and disease duration related (Levine *et al*., 2001).

In the current study, there was no significant correlation between AOPP levels and any of the parameters of lipid profile except triglycerides in microalbuminuria. This is in agreement with Kalousova *et al*., (2002) who observed a significant correlation between AOPP and triglycerides. On the other hand, Kostolanská *et al*., (2009) reported a significant correlation between AOPP with HDL-C in poor glycemic control group of diabetic patient. In addition, Fathy *et al*., (2009) indicated no correlation between AOPP plasma levels and lipid profile parameters in diabetic patients with or without complications.

In our study, a significant positive correlation was found between plasma level of AOPP and both of serum creatinine and albumin/creatinine ratio (ACR) in all patients' group. In accordance with the current results Piwowar *et al*., (2008) found that concentration of AOPP correlated significantly with the plasma creatinine (p <0.05) but not correlated with urinary albumin/creatinine ratio.

In conclusion, the current study supports essential relationship between the plasma levels of AOPP in T2DM patients and its utility in the monitoring of nephropathy development. Moreover, the measurement of AOPP in addition to routine laboratory assessments may be applied to detect and to determine the degree of diabetic nephropathy.

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