

Advanced Glycation End Products in Egyptian Type 2 Diabetic Patients with Nephropathy

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Abstract: In diabetes mellitus, hyperglycaemia accelerates non-enzymatic glycation and oxidative stress leading to damage of macromolecules, among others proteins. This manifests in the increased levels of advanced glycation end products (AGE). The aim of the present study was to assess the plasma levels of AGE in the patients with type 2 diabetes mellitus (T2DM) and to estimate its relation and connection with the degree of nephropathy. Plasma levels of AGE 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 AGE in comparison with the control group. AGE was increasing progressively and significantly from normoalbuminuria, through microalbuminuria to macroalbuminuria. In the current study, the diagnostic utility of AGE was determined by means of receiver operating characteristic (ROC) curve analysis which indicates that the elevation of AGE is a useful marker for the presence of nephropathy. There was a significant negative correlation between AGE levels and HDL-cholesterol. Highly significant correlation was observed between plasma AGE and ACR but no significant correlation was detected with serum creatinine concentration. In conclusion, there was a correlation between plasma levels of AGE and nephropathy severity in T2DM patients, measured by the degree of albuminuria. Therefore, plasma AGE could reflect the progression of kidney damage in the diabetic patients.

Key words: Type 2 diabetes mellitus, nephropathy, advanced glycation end products.

INTRODUCTION

Chronic hyperglycaemia in diabetes mellitus causes multiple biochemical events, such as accelerated glycation and oxidation of biologically important molecules. A relationship between glycation and oxidative processes has been observed and these mechanisms are commonly called "glycooxidation". It leads to structural damage of proteins, lipids and DNA as well as cell membranes and endothelium (Abou-seif and Youssef., 2004). These alterations of structural properties provoke functional abnormalities of macromolecules and/or its accumulation in biological systems. Glycooxidation products are believed to play an essential role in pathogenesis and progression of vascular complication in diabetes (Agnieszka *et al.*, 2008). Increased level of advanced glycation end products (AGE) and advanced oxidation protein products (AOPP) in serum of diabetic patients was reported (Kalousova *et al.*, 2002).

Advanced glycation end products are complex components formed from the non-enzymatic covalent binding of monosaccharides (reducing sugar e.g. glucose) to amino group of proteins to form reversible Schiff-base adducts (e.g. fructosamine or HbA_{1c} existing in blood). After diverse molecular rearrangements they are converted into the stable AGE, of which the best known are N-carboxymethyl-lysine (CML) and pentosidine (Tessier, 2010). Under physiological conditions, these processes undergo continually with low intensity. A mild rise of AGE level is associated with normal cellular aging. In some diseases such as diabetes, renal insufficiency, rheumatoid arthritis and osteoarthritis, AGE has been found to be elevated in body fluids such as plasma/serum, synovial fluid and urine (Nishizawa *et al.*, 2012). Circulating AGE are tissue-derived degradation products that may be produced either by extracellular proteolytic systems or during macrophage catabolism (Gugliucci and Bendayan, 1996). AGE can mediate their effects via specific receptors (RAGE), activating diverse signal transduction cascades and downstream pathways, including generation of reactive oxygen species (Tan *et al.*, 2007 and Yamagishi, 2011).

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The glycooxidation products are high in the condition of chronic hyperglycaemia and oxidative stress and have deleterious effects on the function of the kidneys, retina, nerves, and vascular tissues and, to a lesser extent, pancreatic islets in type 2 diabetes mellitus (T2DM) (Witko-Sarsat *et al.*, 1996, Piwowar *et al.*, 2009 and Shi *et al.*, 2008). 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 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).

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

MATERIALS AND METHODS

The study included sixty patients with type 2 diabetes mellitus from those attending the outpatient's clinics of the National Institute of Diabetes and Endocrinology, Egypt. 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. 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.

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, Inc. 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 glycation end products (AGE) (Brownlee, 1995) 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 ($\mu\text{g}/\text{mg}$) was calculated (Thomas, 1998).

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 ($X \pm SD$). 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:

Demographic and laboratory characterization of the studied population was given in Table (1). The mean levels of AGE were significantly higher in type 2 diabetic patients than those in the control group. Table (2) showed the comparison between the control group and the patients' groups regarding demographic and laboratory parameters. 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 AGE in the diabetic patients groups clustered in relation to albuminuria, described by albumin/creatinine ratio (Fig. 1). The mean levels of AGE were significantly higher in groups 1&2&3 of patients when compared to the control group. AGE increased progressively and significantly with the growth of albuminuria.

The receiver operating characteristic (ROC) curve analysis to calculate the best cut off point for AGE to discriminate between the control group and the patient's groups was 1245.833 ng/L. At this point the sensitivity was 75.0 % (45 out of 60 patients had AGE \geq 1245.833 ng/L) and the specificity was 73.3 % (22 out of 30 controls had AGE $<$ 1245.833 ng/L), Table (3). The area under ROC curve (AUC) that quantify the overall ability of AGE test to discriminate between the patient's groups and the control group was 0.801.

The best cut off point for AGE to discriminate between the control group and macroalbuminuria was 1494.222 ng/L. At this point the sensitivity was 100.0 % (17 out of 17 macroalbuminuria had AGE \geq 1494.222 ng/L) and the specificity was 93.3 % (28 out of 30 controls had AGE $<$ 1494.222 ng/L), Table (4). The area under ROC curve (AUC) that quantify the overall ability of AGE test to discriminate between the control group and macroalbuminuria was 0.915.

In the current study, no significant correlation was found between AGE and each of age, disease duration and anthropometric measurements except the significant positive correlation between AGE and age in all patients' group ($r=0.314$, $P<0.05$). AGE was positively and significantly correlated with FBG in all patients' group ($r=0.378$, $P<0.01$). On the other hand, there was no significant correlation between AGE and HbA1c. There was no significant correlation between AGE levels and any of the parameters of lipid profile such as total cholesterol, LDL-cholesterol or triglycerides except HDL-cholesterol in all patients' group ($r=-0.295$, $P<0.05$). A highly significant positive correlation was found between plasma AGE and ACR in all patients' group ($r=0.398$, $P<0.01$), Fig. (2) but no significant correlation was found between AGE and serum creatinine concentration.

Table 1: Demographic characters 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 \pm 8.42 | 50.98 \pm 8.15 |
| Disease duration (years) | - | 10.97 \pm 6.59 |
| BMI (kg/m ²) | 25.96 \pm 2.63 | 25.53 \pm 2.12 |
| FBG (mg/dL) | 87.73 \pm 13.78 | 261.48 \pm 94.59** |
| HbA1c (%) | 5.56 \pm 0.43 | 9.75 \pm 2.29** |
| Total cholesterol (mg/dL) | 178.90 \pm 32.64 | 201.75 \pm 40.42** |
| HDL-cholesterol (mg/dL) | 46.87 \pm 10.75 | 43.55 \pm 9.88 |
| LDL-cholesterol (mg/dL) | 138.97 \pm 41.1 | 144.28 \pm 54.59 |
| Triglycerides (mg/dL) | 112.83 \pm 26.78 | 179.68 \pm 76.64** |
| Sreum creatinine (mg/dL) | 0.77 \pm 0.18 | 1.03 \pm 0.53* |
| Urinary albumin/creatinine ratio (μ g/mg) | | |
| AGE (ng/L) | 16.00 \pm 8.99 | 473.20 \pm 872.88** |
| | 1105.45 \pm 348.19 | 1563.6 \pm 466.21* |

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

Table 2: Comparison between the control group and the patients' groups regarding demographic characters 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/m ²) | 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** |
| AGE (ng/L) | 1303.64±273.39* | 1502.02±404.37** | 1952.74±486.90** |

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

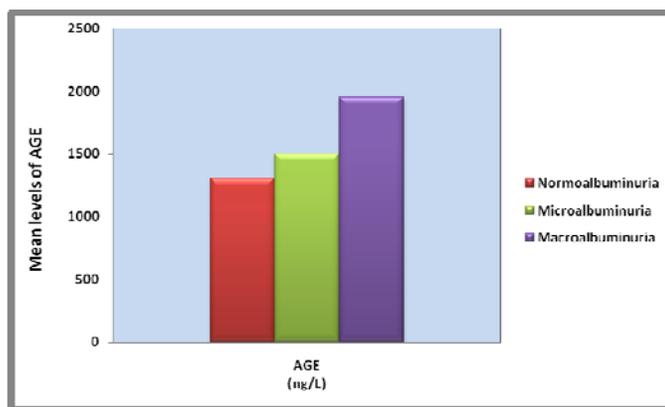


Fig. 1: Plasma levels of AGE in the diabetic patients groups with different stages of kidney diseases

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

| Parameter | Cut off | Sensitivity (%) | Specificity (%) | Area under curve (AUC) |
|------------|----------|-----------------|-----------------|------------------------|
| AGE (ng/L) | 1245.833 | 75.0 % | 73.3 % | 0.801 |

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

| Parameter | Cut off | Sensitivity (%) | Specificity (%) | Area under curve (AUC) |
|------------|----------|-----------------|-----------------|------------------------|
| AGE (ng/L) | 1494.222 | 100.0 % | 93.2 % | 0.915 |

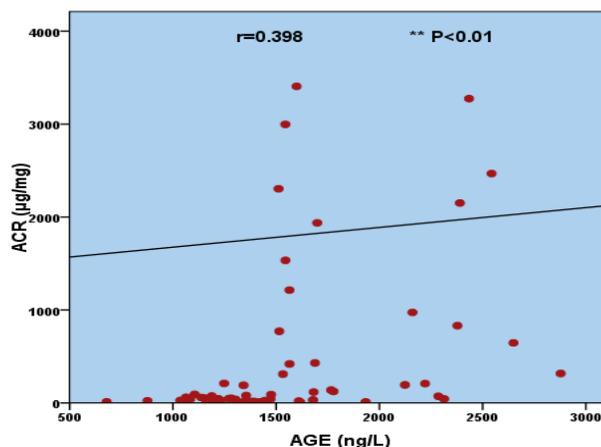


Fig. 2: Correlation between ACR and AGE in all patients' group

Discussion:

Diabetes mellitus is a complex metabolic disorder characterized by disturbance in glucose metabolism, insulin defect generation and prolonged exposure of the cells and tissues to hyperglycemia (Nishikawa *et al.*,

2000). 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). AGE 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 (Piwowar *et al.*, 2008).

The significance of AGE in development of diabetic nephropathy has been carefully studied for many years (Piarulli *et al.*, 2009 and Coughlan *et al.*, 2011). The observed increase in total AGE in plasma of T2DM patients in comparison to the control group in the current study is in agreement with previous reports obtained not only by measuring fluorescence of total AGE (Kalousova *et al.*, 2002) but also LMW-AGE (Sharp *et al.*, 2003) or N-carboxymethyl-lysine (Hirata and Kubo, 2004). In addition, AGE has been found to be about 31% higher ($p < 0.01$) in diabetic patients compared with the control group (Piwowar *et al.*, 2008). AGEs were elevated in non insulin dependent diabetes mellitus (NIDDM) in comparison to insulin dependent diabetes mellitus (IDDM) (Abou-seif and Youssef., 2004). Furthermore, other study observed significant elevation in serum AGEs (sAGEs) in the diabetic patients compared with controls, and significantly higher sAGEs was observed in the poor glycemic control (PGC) group than in the good glycemic control (GGC) group (Kostolanská *et al.*, 2009).

In this study, we also estimated plasma levels of AGE 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 AGE showed significant increase in each of normo-, micro- and macroalbuminuria groups compared to the control group.

The levels of AGE 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). In a more recent study, in comparison with healthy controls, AGE levels rose 2-fold in children and adolescents with chronic renal failure (CRF), 7-fold in end-stage renal disease (ESRD) patients, and 5-fold in the kidney transplanted children and adolescents, ($P < 0.01$). In patients with stabilized renal function, AGE levels did not change significantly during 12 months (Šebeková *et al.*, 2012). AGE has been shown to accumulate in kidney of T2DM patients with nephropathy (Van Dijk and Berl, 2004). AGEs are increased in situations with hyperglycemia and oxidative stress such as diabetes mellitus (Nishizawa *et al.*, 2012). The kidney plays an important role in clearance and metabolism of AGEs. Kidney podocytes and endothelial cells express specific receptors for AGEs. Their activation leads to multiple pathophysiological effects including hypertrophy with cell cycle arrest and apoptosis, altered migration, and generation of proinflammatory cytokines. AGEs have been primarily implicated in the pathophysiology of diabetic nephropathy and diabetic microvascular complications (Busch *et al.*, 2010). In chronic renal failure, reduced metabolism of AGE probably accounts for the rise of AGE and its precursors in serum, leading to uremic complication. Protein glycation, oxidation and nitration free adducts are formed by proteasomal turnover of tissue proteins and are also absorbed from digested food together with modified peptides (Henle, 2003). Low molecular AGE are filtered by renal glomeruli and then reabsorbed and metabolized by proximal tubule cells. In proteinuric states high molecular AGE are also delivered to proximal tubule cells (Saito *et al.*, 2005).

In the current study, the diagnostic utility of AGE was determined by means of receiver operating characteristic (ROC) curve analysis which demonstrated that AGE was good indicator for the presence of diabetic complications due to its high sensitivity (75%) and high specificity (73.3%) in discriminating between control group and T2DM patient's group. Our observations are in agreement with previous studies (Kostolanská *et al.*, 2009). On the other hand, AGE performance as marker for the presence of nephropathy was deeply unsatisfactory as it was worthless at identifying control group versus normoalbuminuria. Also, elevation of AGE was useful marker indicating the presence of nephropathy because 100% of microalbuminuria had values of AGE \geq the best cut off points (1056.056 ng/L). In addition, AGE was good indicator for the presence of nephropathy due to its high sensitivity (100%) and high specificity (93.2%) in discriminating between control group and macroalbuminuria.

In the present study, no significant correlation was found between AGE and each of age, disease duration and anthropometric measurements except the significant positive correlation between AGE and age in all patients' group. In contrast with our results, serum AGE in T2DM correlated significantly with diabetes duration (Aso *et al.*, 2000). In the current study, AGE was positively and significantly correlated with fasting blood glucose (FBG) in all patients' group. In agreement with our results, Aso *et al.*, (2000) reported that serum AGE in T2DM significantly correlated with fasting plasma glucose. The non enzymatic glycation of proteins is influenced by hyperglycemia, which bind to collagen and proteins that constitute glomerular basement

membrane and make glomerular barrier more permeable to proteins (Zelmanovitz *et al.*, 2009). The independent risk factor such as hypertension, together with intrinsic renal toxicity caused due to proteins filtered through glomerular vessels, may contribute to the progression of renal damage (Ruggenti *et al.*, 1998). There was no significant correlation between AGE and glycosylated hemoglobin (HbA1c). This is in contrast with Kostolanská *et al.*, (2009) who observed significant correlation between HbA1c and serum AGEs in poor glycemic control group of diabetic patients. 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 AGE levels and any of the parameters of lipid profile such as total cholesterol, LDL-cholesterol or triglycerides except HDL-cholesterol in all patients' group. In agreement with our result, Zhou *et al.*, (2008) found that in type 2 diabetic patients with incipient or overt nephropathy, the serum concentration of AGEs was significantly correlated with HDL-C. On the other hand, Kostolanská *et al.*, (2009) indicated a significant correlation between serum AGEs with triglycerides in poor glycemic control group of diabetic patients. Prolonged hyperglycemia, dyslipidemia and oxidative stress in diabetes result in the increased production and accumulation of AGEs in the kidney. Covalent AGE modifications significantly influence the structure and function of key protein targets. In addition, activation of AGE receptors, alone or in combination with other ligands, is able to promote renal damage, fibrosis and inflammation associated with diabetic nephropathy. The actions of AGEs synergize and potentiate the activity of other pathogenic mediators in the diabetic kidney, including oxidative stress, protein kinase C and renin-angiotensin system activation, which subsequently promote the development and progression of kidney disease in a vicious and progressive cycle. Furthermore, direct exposure to AGEs is able to generate lesions similar to those seen in diabetic nephropathy. The human body has a number of natural defenses against AGE accumulation, which are reduced in diabetic individuals, and in particular those with nephropathy, while the receptor for AGEs and its ligands are significantly increased (Thomas, 2011).

In our study, a highly significant positive correlation was found between plasma level of AGE and urinary albumin/creatinine ratio (ACR) in all patients' group but no significant correlation with serum creatinine concentration. Other studies showed significant correlation of LMW-AGE fluorescence with serum creatinine, 24-h urinary protein and urinary albumin excretion (Zilin *et al.*, 2001). In accordance with the current results Piwowar *et al.*, (2008) found that plasma fluorescence of AGE correlated significantly with urinary albumin/creatinine ratio ($p < 0.01$) but not correlated with plasma creatinine concentration.

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

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