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Full Length Research Paper

Serum of advanced glycation end products in Tunisian diabetic patients with chronic kidney disease

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Advanced glycation end products (AGEs) and their receptors are prominent contributors to diabetic renal disease and clinical importance of AGEs toxicity had been rarely reported. We measured serum AGE, sRAGE and pentosidine levels in diabetic patients with and without nephropathy and examined whether these biomarkers are related to renal function impairment. We included 30 healthy control subjects and 100 diabetic patients who were further divided into 2 subgroups: one with 30 patients who had normal eGFR, the other with 70 patients who had reduced eGFR. AGEs, sRAGE and pentosidine were measured in serum by ELISA. Serum levels of AGEs, sRAGE and pentosidine were significantly increased in diabetic patients compared to controls (579.78 ± 113.28 vs. 508.83 ± 119.68 pg/ml; 169.17 ± 30.41 vs. 148.72 ± 32.73 pg/ml; 247.84 ± 21.42 vs. 214.03 ± 55.05 pg/ml, P < 0.001, P < 0.01, P < 0.05 respectively). Diabetic patients with chronic kidney disease (CKD) showed an increased level of AGEs, sRAGE and pentosidine compared to diabetic patients without CKD (P < 0.01, P < 0.05, P < 0.05 respectively). In diabetic patients who had reduced eGFR, serum AGEs, sRAGE and pentosidine levels were significantly higher in patients with eGFR< 60, than in those with 60<eGFR<90 ml/min/1.73 m^2 (P < 0.001, P < 0.01, P < 0.001 respectively). Serum creatinine was positively associated with AGEs and sRAGE. In stepwise multivariate regression analysis, AGEs and sRAGE were independently associated with decreased renal function. Serum AGEs, sRAGE, and pentosidine levels are related with the presence and the severity of diabetic nephropathy in Tunisian population.

Key words: Diabetic patients, AGEs, reduced renal function.

INTRODUCTION

Diabetes mellitus (DM) is increasing at an alarming rate in Tunisian population; patients are prone to the development of macro- and microvascular diabetic complications that represent a major cause of morbidity and mortality. The microvascular complications, which affect the small vessels, involve neuropathy, nephropathy, and retinopathy. Chronic kidney disease (CKD) affects a significant portion of the world population with a prevalence of 7.2% in adults over age 30 and dramatically increasing to 23.4 - 35.8% over age 65 (Levey et al., 2005). Diabetic patients with CKD represent an important segment of Tunisian population, and mostly because of the high risk of cardiovascular disease (CVD) associated with renal insufficiency, detection and treatment of chronic renal disease is now a public health priority (Abderrahim et al., 2001; Ben Maïz et al., 2006). The causes of CKD are various and include glomerular kidney disease, tubular and interstitial kidney disease, pre renal and vascular disorders, diabetes mellitus (DM), and hypertension. DM is the most common cause of CKD in Tunisian patients. There are multiple etiologies involved in the pathogenesis of diabetic CKD, one recent hypothesis, has an important goal is the endogen production of advanced glycation end products (AGEs) and its exogenous accumulation by food nutrition in

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Abbreviations: AGEs; Advanced glycation end products, **CKD;** chronic kidney disease, CVD; cardiovascular disease, **DM;** diabetes mellitus, **eGFR;** estimated glomerular filtration rate, **sRAGE;** soluble form of receptor for advanced glycation end products.

Tunisian population. AGEs are formed by the Maillard process, a non-enzymatic reaction between ketones or aldehydes, the amino groups of proteins, lipids and nucleic acids that contributes to the aging of macromolecules (Stern et al., 2002; Takeuchi and Yamagishi, 2009). AGEs comprise several major molecular structures, such as pentosidine; it can be formed from glycoxidation of Amadori products or oxidation of arabinose. Pentosidine was preferentially located in interstitial collagen.

Previous studies have confirmed that AGEs and receptor for AGEs (RAGE) interaction elicits oxidative stress generation in various types of cells and subsequently evokes vascular inflammation, macrophage and platelet activation, and thrombosis, thereby playing an important role in the development and progression of vascular complications in diabetes (Hou et al., 2004). Several different receptors for AGEs have been identified, one of which is termed RAGE. Different cell types including human endothelial cells express RAGE (Kalousva et al., 2006). The functional role of these soluble forms of RAGE in the circulation remains unclear, but they may reflect the activity of the AGE-RAGE axis. In uraemia, in the absence of diabetes, a 2-to 3-fold increase in AGEs has been reported, which indicates that the kidney plays an important role in the accumulation of these compounds. Therefore, the first aim of this study was to examine whether serum AGEs, sRAGE and pentosidine are associated with the presence of diabetes, and with the decreased renal function in Tunisian CKD patients. The second aim of the study was therefore to investigate the relationship of AGEs, sRAGE and pentosidine to creatinine, an important determinant of kidney disease.

MATERIALS AND METHODS

Subjects

The local ethics committee approved this study. Written informed consent was obtained from all patients before the enrollment. In this prospective cohort study, we measured serum concentration of AGEs, sRAGE and pentosidine in 130 participants (age range, 50 to 75 years). The subjects were divided into 2 groups: Group I is composed of healthy volunteers (n=30) with no DM or renal function impairment. Group II is composed of type 2 diabetic patients (n=30) without renal function impairment. Group III is composed of type 2 diabetic patients (n=30) with renal function impairment who has 60 <eGFR< 90 mL/min/1.73m^2. Group IV composed of type 2 diabetic patients (n=40) with renal function impairment who has eGFR< 60 mL/min/1.73 m^2.

Etiologies of CKD in patients were chronic glomerular nephritis (46%), chronic tubulointerstitial nephropathy (30%) and vascular nephropathy (24%). The percentage of patients with CKD had DM as the primary cause of CKD is 38%. CKD is defined as decreased estimated glomerular filtration rate (eGFR). The eGFR was calculated using the 4-variable Modification of diet in renal disease (MDRD) study equation of (Levery et al., 2003). For each patient, a data sheet was completed with the patient's identification code, age, sex, and duration of diabetes. All diabetic patients received antidiabetic and traditional cardiovascular drugs.

In all subjects, venous blood was collected in the morning after an overnight fast. The samples were stored at -80°C until analysis. Random plasma glucose, hemoglobin A1C (HbA_{1C}) was measured using G7 HPLC Analyser (Tosoh Europe N.V), serum creatinine, uric acid, and lipid levels (HDL, LDL, cholesterol, and triglyceride), were measured using enzymatic methods by CX9 Auto-chemical analysis instrument (Beckman CX9, USA). A 24 h urine specimen was collected and analyzed for albumin by the turbidimetric method. AGEs, sRAGE, and pentosidine were quantitatively determined in serum by enzyme-linked immunosorbent assay (ELISA) kits provided by ABO Switzerland Company Limited, according to the manufacturer's instructions. Briefly, the microtiter plate has been pre-coated with an antibody specific to Human AGEs (or sRAGE or Pentosidine). Samples were added, after incubation and washing, plates were incubated with HRP, developed with TMB substrate, and OD_{450} was determined using an ELISA plate reader.

Statistical analysis

All values are expressed as mean ± SD, median and inter-quartile range. A P-value less than 0.05 was considered statistically significant. Significance between 2 groups was determined by independent sample student *t*-test for continuous variables. Continuous data from > 2 groups were compared with 1-way analysis of variance (ANOVA). All analyses were performed using the SPSS program (version 17). The relationship between two variables was tested by Pearson correlation. Multiple regressions were used to further explore the linear relationships between the variables. Regression variables were estimated as well as the correlation coefficient *r*. ANOVA was used to assess the significance of the regression with significance accepted at $P < 0.05$. A stepwise backward regression analysis was also calculated to detect independent associations of AGEs, sRAGE and pentosidine with decreased renal function (eGFR).

RESULTS

General clinical and biochemical parameters of the studied groups

The clinical characteristics and laboratory data of controls

Table 1. Clinical characteristics of control subjects and diabetic patients with and without CKD

	Control subjects	Diabetic patients without CKD	Diabetic patients with CKD
N (M/F)	30 (17/13)	30 (16/14)	70 (37/33)
Age (y)	52 ± 9	57 ± 12	63 ± 9
BMI (kg/m^2)	25.6 ± 1.2	30.4 ± 3.4	33.7 ± 5.4[b]
Duration of diabetes (y)	-	6 ± 3	8 ± 5
Glucose (mmol/L)	4.77 ± 0.91	10.54 ± 2.33	9.77 ±3.56
HbA_{1C} (%)	5.6 ± 0.2	8.9 ± 2.2	8.2 ± 3.1
Total cholesterol (mmol/L)	4.55 ± 0.74	5.22 ± 0.12	4.92 ± 0.65
Triglyceride (mmol/L)	1.42 ± 0.68	2.15 ± 0.98	2.32 ± 0.98
HDL (mmol/L)	1.52 ± 0.47	1.20 ± 0.31	0.91 ± 0.45
LDL (mmol/L)	2.25 ± 0.45	2.77 ± 0.33	2.97 ± 0.40
Creatinine (µmol/L)	88 (72-93)	96 (82-116)	317 (165-685)[a]
AGEs (pg/mL)	508.83 ± 119.68	579.78 ± 113.28	729.35 ± 90.58[a]
sRAGE (pg/mL)	148.72 ± 32.73	169.17 ± 30.41	187.57 ± 34.90[b]
Pentosidine (pg/mL)	214.03 ± 55.05	247.84 ± 21.42	297.84 ± 32.44[b]

Values are mean ± SD or median (inter-quartile range). BMI: body mass index; [a]*P*< 0.01 compared with diabetic patients without CKD and *P*< 0.001 with control subjects; [b]*P*< 0.05 compared with diabetic patients without CKD and *P*< 0.001 with control subjects.

Table 2. AGEs, sRAGE, and pentosidine levels in diabetic CKD patients with reduced eGFR.

	CKD patients with		
	60 <eGFR< 90(n= 40)	eGFR< 60(n= 30)	*P*
Duration of diabetes (y)	8 ± 2	13 ± 5	<0.01
HbA_{1C} (%)	7.6 ± 1.2	8.2 ± 1.1	NS
Creatinine (µmol/L)	175 (134-225)	270 (230-755)	<0.001
AGEs (pg/mL)	683.35 ± 204.55	729.96 ± 221	<0.001
sRAGE (pg/mL)	200.63 ± 48.83	214.79 ± 50	<0.01
Pentosidine (pg/mL)	287.58 ± 41.90	305.47 ± 36	<0.001

Values are mean ± SD or median (inter-quartile range).

and diabetic patients with and without CKD are shown in Table 1. In diabetic patients, we observed higher body mass index, HbA_{1C}, serum glucose, and lipids profiles in comparison to controls. Serum level of AGEs, sRAGE and pentosidine were significantly increased in diabetic patients than in controls ($P < 0.001$; $P < 0.01$; and $P < 0.05$ respectively). Patients with CKD showed an increased level of AGEs, RAGE and pentosidine compared with diabetic patients without CKD ($P < 0.001$ and $P < 0.01$, $P < 0.001$ respectively).

Relationship between AGEs, sRAGE, pentosidine levels and decreased renal function

Table 2 show results of serum AGEs, sRAGE, pentosidine levels in diabetic patients with decreased renal function. Patients with decreased renal function showed an increased level of serum AGEs, sRAGE, and pentosidine ($P < 0.001$, $P < 0.01$ and $P < 0.001$ respectively).

AGEs, sRAGE and pentosidine levels in diabetic patients with normal and decreased renal function

Figures 1, 2 and 3 show the distribution of serum AGEs, sRAGE and pentosidine levels in diabetic patients without renal function impairment (group II) and in diabetic patients with decreased renal function (group III and group IV). Serum level of AGEs, sRAGE and pentosidine were significantly increased ($P < 0.01$).

Results of Pearson correlation

Correlation analysis showed a close relationship between AGEs and sRAGE in all subjects ($r = 0.715$, $P < 0.001$).

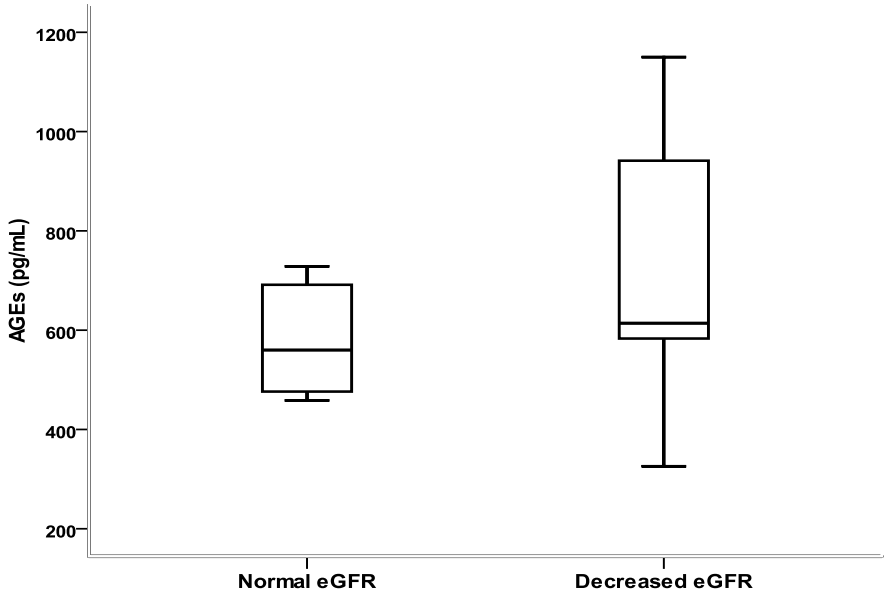

Figure 1. Box plots of serum AGEs levels in diabetic patients with normal and decreased renal function ($P < 0.01$).

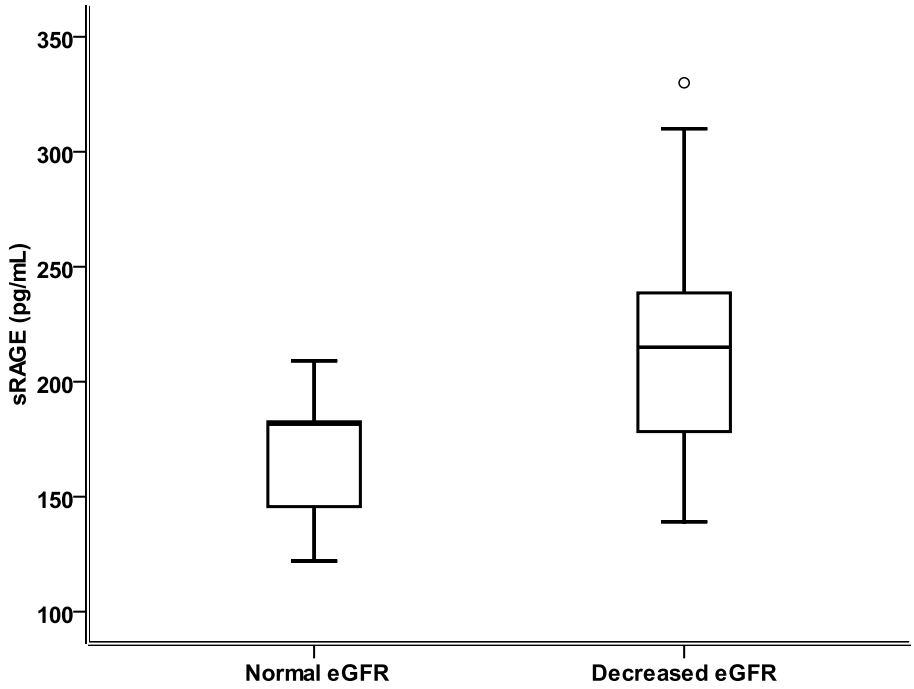

Figure 2. Box plots of serum sRAGE levels in diabetic patients with normal and decreased renal function ($P < 0.01$).

Serum creatinine was positively associated with AGEs and sRAGE but not with pentosidine. ($r = 0.792$, $P < 0.001$; $r = 0.749$, $P < 0.001$ respectively) (Figure 4). However, AGEs, RAGE and pentosidine were correlated with eGFR ($r = 0.745$, $P < 0.001$; $r = 0.677$, $P < 0.001$, $r = 0.540$, $P < 0.01$ respectively).

Results of multiple linear regression

Multiple linear regression was used in all diabetic patients to identify any related predictors of renal function impairment, evaluating the regression coefficients that represent the contributions of each independent variable

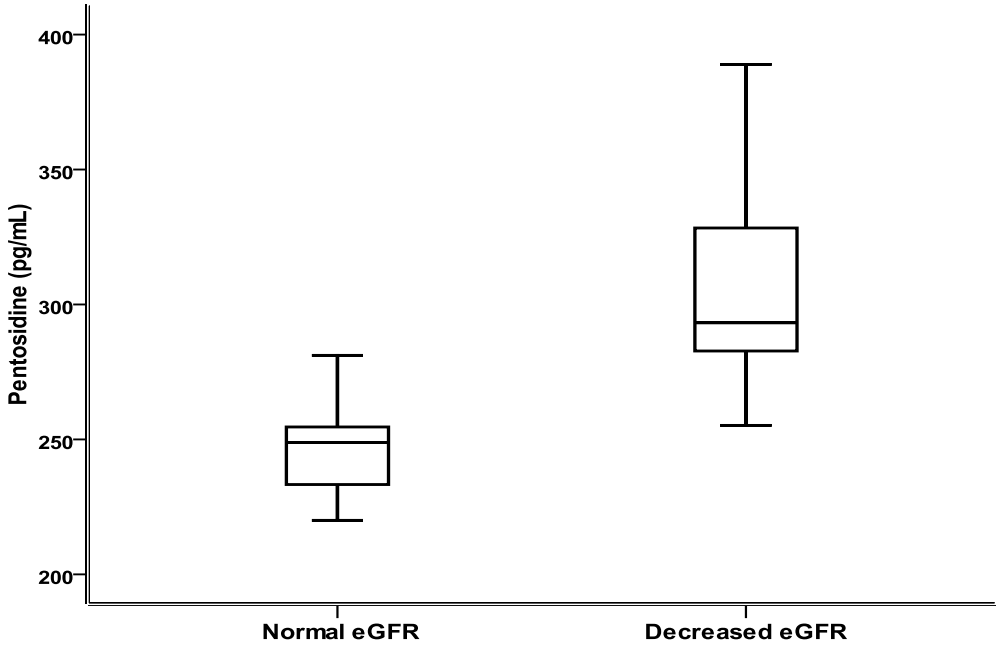

Figure 3. Box plots of serum Pentosidine levels in diabetic patients with normal and decreased renal function ($P < 0.01$).

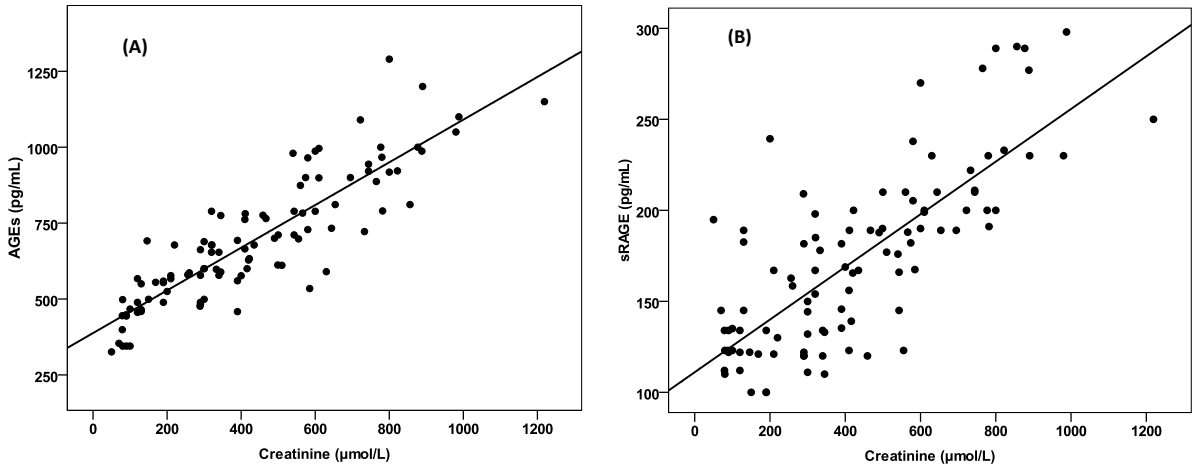

Figure 4. (A) Linear correlation between serum AGEs and serum creatinine ($r = 0.792$), and (B) linear correlation between serum sRAGE and serum creatinine ($r = 0.749$) in all diabetic patients with decreased renal function. The correlation was statistically significant ($P < 0.001$).

to the predictive value of the dependent variable (eGFR). This model, which was applied to AGEs, sRAGE and pentosidine variables (considered as independent variables), as well as eGFR as the dependent variable, showed that AGEs and sRAGE correlated negatively. Possible confounders (for example, age, sex, diabetes duration, HbA1c, BMI, lipids) were added to the model and were tested against eGFR and no significant correlation was found. Multiple regression revealed an independent correlation between eGFR and AGEs, sRAGE but not pentosidine (Table 3).

DISCUSSION

For our interesting goal, this study is the first in Tunisian CKD patients that showing a correlation between AGEs, RAGE, pentosidine and diabetes mellitus and its

Table 3. Variables estimates for multiple linear regression applied to AGEs, sRAGE, and pentosidine in diabetic patients with decreased renal function (eGFR).

Term	Estimate	Standard error	*T* ratio	*p*	Co-linearity statistics	
					Tolerance	VIF
Intercept	149.503	21.736	6.878	0.000		
AGEs	- 0.055	0.018	-2.880	0.008	0.654	1.528
sRAGE	-0.048	0.019	-2.722	0.009	0.739	1.354
Pentosidine	-0.180	0.082	-2.130	0.066	0.810	1.234

eGFR was the dependent variable; ANOVA revealed a statistically significant fit ($P < 0.001$); $r = 0.718$, $r^2 = 0.515$, r^2 adjusted= 0.457; VIF: variance inflation factor.

complications. Recently, we showed that AGEs, sRAGE and pentosidine were increased in Tunisian diabetic retinopathy (Kerkeni et al., 2012). Clinical studies for serum AGEs, sRAGE and pentosidine levels on CKD patients were not well known, and clinical importance of AGEs toxicity had been rarely reported in early and advance diabetic patients. Our study shows that serum AGEs, sRAGE and Pentosidine are elevated in Tunisian diabetic patients with normal renal function than the control subjects. CKD Patients with eGFR< 60 showed an increased level of serum AGEs, sRAGE, and pentosidine than CKD patients with 60 <eGFR< 90. RAGE also has roles in the pathogenesis of renal disorders that are not associated with diabetes, such as obesity-related glomerulopathy, hypertensive nephropathy and ischemic renal injuries (D'Agati and Schmidt, 2010). The present study shows that elevated serum sRAGE are associated with reduced eGFR in diabetic patients. Our findings also suggest that elevated serum AGEs and sRAGE were involved to the development of reduced eGFR. In fact, serum AGEs and sRAGE were positively associated with serum creatinine.

AGEs are metabolized and removed by the kidney but the kidney is also a site for accumulation of AGEs and AGE-related damage (Gugliucci and Bendayan, 1996). AGEs upregulate inflammation and the synthesis of fibronectin, laminin, and collagen IV in the kidney and promote glomerular sclerosis, fibrosis, and hypertrophy (Yang et al., 1994). The kidney is affected by AGEs, and declining renal function entails an increase in serum AGEs, thereby amplifying damage from AGEs. AGEs are not merely a marker of renal insufficiency, as treatment with AGE inhibitors improves renal function, suggesting a direct role of AGEs in the pathogenesis of reduced renal function (Williams et al., 2007). This is in contrast to what has been shown with hyperhomocysteinemia in kidney disease, where levels rise with declining renal function, but treatment has not been shown to be substantially beneficial (Jamison et al., 2007). In experimental study, Waanders et al. (2005) found that pentosidine accumulates in non-diabetic proteinuric kidney in damaged tubules. What could be the pathophysiological significance of renal pentosidine accumulation? Several studies support the *in vivo* and *in vitro* nephrotoxicity of AGEs (Vlassara et al., 1994; Yamagishi et al., 2003). As reviewed previously, AGE-modified proteins initiate a range of cellular responses including enhanced growth factor expression, cellular proliferation and apoptosis, angiogenesis and tissue remodelling by binding to AGE-specific receptors that might be involved in nephrotoxicity. Receptor for AGEs contributes to mesangial activation and transforming growth factor-β production; processes which converge to cause albuminuria and glomerulosclerosis (Wendt et al., 2003). These data suggest that renal AGEs and pentosidine accumulations that have resulted from a primary renal disorder, once present, can itself become a perpetuating factor in ongoing renal damage. Recently, Yamagishi et al. (2001) showed that the role of AGEs and oxidative stress in vascular complications in diabetes. Authors demonstrated that AGEs induce mesangial cell loss and dysfunction, glomeruloscerosis and tubulointertitial fibrosis. For more interesting of one product of AGE, pentosidine levels in CKD patients with tubulointertitial nephrophathy showed increased levels than CKD patients with chronic glomerular nephritis or CKD patients with vascular nephropathy (data not shown). We speculated that pentosidine, as a nephrotoxicity AGE, might accelerate the development and severity of diabetic nephropathy and increased pentosidine might be involved in tubulointertitial cell accumulation and causing dysfunction, as plausibly mediated by the AGE-RAGE axis activity. AGEs are nephrotoxic and renal accumulation may contribute progressive renal damage. Detection in early stage and intervention in AGEs formation (exogen or endogen synthesis) by drugs such as metformin or pyridoxamine may thus have renoprotective potential in CKD severity.

In conclusion, elevated serum AGEs, sRAGE and pentosidine are associated with reduced renal function and both AGEs and their receptor appear to be associated with the severity of diabetic nephropathy in Tunisian CKD patients. AGEs are potential target for interventions to prevent onset as well as progression of renal function impairment, as serum AGEs can be lowered by change in dietary pattern and

pharmacological treatment.

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