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Research Article

The potential use of Endostatin and Angiopoietin-2 as valuable biomarkers for the prediction of Diabetic Nephropathy in Type 2 Diabetes Mellitus

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ABSTRACT

Diabetic Nephropathy (DN) is considered a serious capillary complication of diabetes mellitus (DM) which leads to end-stage renal disease. Endostatin (EST) is considered as collagen XVIII fragment formed during extracellular matrix remodeling (ECMR). EST acts as an anti-angiogenic factor. Angiopoietin-2 (Ang II) is a growth factor that increases in several conditions, such as hyperglycemia. The present study was to scrutinize the association of EST and Ang II serum levels with nephropathy in patients with type 2 diabetes mellitus (T2DM). A total of 30 healthy individuals (control) and 120 T2DM patients classified into 60 patients with microalbuminuria and 60 patients without microalbuminuria), aged 45-65 years were included. Fasting Plasma Glucose (FPG), HbA$_{1C}$%, lipid profile, urinary albumin/creatinine ratio of 30-300 mg/g (UACR), serum urea and creatinine levels were assessed. Both EST and Ang II were measured using the ELISA technique. Ang II and EST levels were elevated in patients with T2DM groups compared with the healthy control group (P<0.001). EST and Ang II were significantly correlated to UACR (r= 0.753, P<0.001) (r= 0.685, P<0.001) and therefore indicate progress to DN. Circulating EST and Ang II were significantly associated with T2DM and predict progression of DN and therefore can be used as biomarkers for the prediction of DN in such a group of patients.

Keywords: *T2DM; microalbuminuria; Diabetic nephropathy; Angiopoietin 2; Endostatin*

1. INTRODUCTION

DN is a serious capillaries complication of diabetes which leads to end-stage renal disease(ESRD) [1]. It is considered as the main cause of cases requiring dialysis or renal transplantation in the developing countries [2]. DN progression and pathogenesis are probable to be a result of metabolic and hemodynamic pathways interactions, which are disturbed in the case of diabetes [3]. One of the most important mechanisms involved in the pathophysiology and progression of DN is angiogenesis [4].

The process of angiogenesis is the formation of new blood vessels from existent vasculature. This process plays a significant role in both physiologic and pathologic events, including embryonic development, menstruation, wound healing, tumor growth, and diabetes [5].

Angiogenesis is a process needs several steps by endothelial cells, starting via basement membranes detachment, proliferation, migration, and maturation [6]. In the normal status, these events are strictly regulated by two main factors called pro-angiogenic and anti-angiogenic factors. Vascular endothelial growth factor (VEGF) and angiopoietins are the main characterized pro-angiogenic growth factors that play a role in angiogenesis [7], while EST is one of the main anti-angiogenic factor involved in the angiogenesis process [8].

The angiopoietins, the seven glycoproteins secreted ligands, including angiopoietin-1 to 7, play an important role in vasculature formation [9] where Ang I and II are the most famous members [10]. DM raises Ang II, which leads to vascular walls destabilization and promotes neovascularization when it is bound to VEGF [11]. Ang II is elevated in patients with DM and to be associated with endothelial damage and dysfunction [12]. However, the exact role of Ang II in the development of DN disease needs further exploration.

Breakdown of kidney endothelial, tubular and glomerular matrix collagen plays a crucial role in DN progression [13]. Collagen degradation releases EST an active derivate of collagen XVIII into the circulation [14]. EST level was considered a valuable marker for the patient with hypertension and end-stage organ damage especially in the heart, kidney, and vasculature [14]. Also, EST level was shown to be parallel with the renal function impairment, and the elevation of mortality risk in different settings [15]. The clinical importance of EST as a risk biomarker in diabetic patients with nephropathy still needs further investigations.

Therefore, the aim of this study is to investigate the association between endostatin and angiopoietin-2 with DN in T2DM patients with urinary albumin/creatinine ratio [UACR] of 30-300 mg/g and explore their suitability as risk biomarkers for the prediction of DN, which is considered one of the major complications of DM particularly in Egypt.

2. PATIENTS AND METHODS

2.1. Study Design

A total of 120 T2DM patients [66 men and 54 women] treated at the Diabetes and Endocrinology Clinic, Ain Shams Hospital, Ain Shams University, Cairo, Egypt, were recruited in the study for 4 months. Laboratory investigations were carried out at the Biochemistry Department laboratories, Faculty of Pharmacy, Ain Shams University, Cairo, Egypt. The average patient age was 45–65 years. They were enrolled in the study with at least 5 years' disease duration.

The patients were classified into three groups: group I consisted of 30 healthy control as 20 men and 10 women, and each of the eligible diabetic patients was randomly assigned by simple randomization to either Group II or Group III. Group II consisted of 60 T2DM patients without microalbuminuria and group III consisted of 60 T2DM patients with DN in the form of microalbuminuria (UACR: 30-300 mg/g) that were measured in two of three samples over 3 months before the study. All patients were on regular visits to the diabetes clinic. All diabetic patients were on Glimepiride as oral hypoglycemic drug and each one received his suitable dose according to his case. All participants signed their informed consent and the study was approved by the Human Ethical Review Committee, Faculty of Pharmacy, Ain Shams University, Cairo, Egypt (ENREC-ASU-240, date at 20/3/2019)

2.2. Exclusion Criteria

Patients were excluded if they have any of the following: patients with a history of liver disease or any disorder likely to impair liver functions or

elevated liver enzymes, hepatitis virus infection HBV or HCV or any evidence of infection, patients with any evidence of kidney impairment due to other causes than diabetes.

2.3. Blood Sampling and Biochemical Assays

Laboratory investigations: Venous blood samples were obtained from available participants on hospital admission after fasting overnight, plasma samples were collected on sodium fluoride-containing tubes to estimate fasting plasma glucose (FPG), while ethylene diamine tetra-acetic acid (K2-EDTA) in sterile vacutainer tubes were used for $HbA_{1C}\%$ assessment. Serum creatinine (S Cr) and lipid profile parameters were assessed using vacutainer serum collecting tubes that were centrifuged for 15 min at 1000x for serum separation. Serum samples were then stored at -80 °C for subsequent assessment of (EST and Ang II).

Morning urine samples were obtained from all participants for measurement of UACR using immuno-turbidimetric method (Cobas Integra 800; Roche Diagnostics, Mannheim, Germany). FPG and S Cr were measured by using commercial spectrophotometric kits (Bioscope Diagnostic, Cairo, Egypt). $HbA_{1C}\%$ was obtained with ion-exchange chromatography by Crystal Chem's HbA_{1C} kit. Lipid profile parameters were measured by commercial spectrophotometric kits (Spectrum, Cairo, Egypt). All spectrophotometric assays were performed by a UV∕visible 1650 spectrophotometer (Shimadzu, USA). Serum EST and Ang II were measured using kits from Sino Gene Clon Biotech Co., Ltd, Hangzhou, China using ELISA reader (Chromate®, USA).

2.4. Statistical Analysis

Data were collected, revised, coded, and entered into the Statistical Package for Social Science [IBM SPSS] version 20. The comparison between three groups with quantitative data was done using One Way ANOVA. The mean difference between the three groups is confirmed using post-hoc least significant difference (LSD) test. Spearman's rank correlation coefficient (r) was carried out between two measured parameters of the study groups. P values <0.05 were considered statistically significant and <0.01 was considered highly significant.

3. RESULTS

3.1. Baseline Clinical and Laboratory Characteristics of the Studied Population

Clinical and laboratory parameters: A total of 120 T2DM patients and 30 healthy controls were recruited. All these participants' age and sex ratios were compared. FPG, HbA1c, total cholesterol, triglyceride, and low-density lipoprotein cholesterol levels were significantly higher, and high-density lipoprotein cholesterol was significantly lower than their levels in diabetic patients. Moreover, UACR, serum Ang II, and EST were highly elevated in the DN group in comparison to their levels in the T2DM group and control group (P<0.01). In contrast, serum creatinine levels did not show a significant difference between the three groups (p= 0.67) (**Table 1**).

3.2. The relation between the Assayed Biomarkers.

The study was carried to investigate whether the levels of the assayed markers (UACR, EST and Ang II) are correlated with each other or not. The pooled data of the three markers UACR, EST and Ang II across the three groups were tested by using Spearman's rho (r) correlation. Ang II was positively correlated with UACR (r= 0.899, p<0.001), EST was positively correlated with UACR (r= 0.681, p<0.01), as well as EST and Ang II were positively correlated (r= 0.692, p<0.01) inpatient with DN (**Fig. 1**)

Table 1. The main anthropometric and biochemical parameters of the studied groups

	Group/numbers of Patients		
	Healthy Control/30	**T2DMGroup Without DN/60**	**T2DM Group With DN/60**
clinical data			
Age (years)	47.64 ± 3.71	50.00 ± 4.62	50.23 ± 4.61
Disease duration	-	5.44 ± 1.59	6.00 ± 1.60
BMI	24.92 ± 2.05	$27.77 \pm 2.87^*$	$28.70 \pm 1.18^*$
SBP	120.00 ± 8.26	$141.44 \pm 8.45^{**}$	$145.64 \pm 6.69^{**}$
DBP	78.50 ± 5.27	$88.56 \pm 5.23^{**}$	$89.00 \pm 4.34^{**}$
laboratory data			
FPG [mg/dL]	117.71 ± 17.55	$152.13 \pm 16.24^{**}$	$155.09 \pm 18.26^{**}$
HbA1c [%]	6.36 ± 0.47	$8.06 \pm 0.28^*$	$8.03 \pm 0.43^*$
TC [mg/dL]	164.93 ± 13.74	$191.50 \pm 24.66^*$	$185.73 \pm 25.35^*$
TGs [mg/dL]	137.29 ± 16.21	$148.88 \pm 18.06^*$	141.09 ± 25.30
LDL-ch [mg/dL]	99.93 ± 15.69	$132.81 \pm 29.57^*$	$104.24 \pm 32.11^{\partial}$
HDL-ch [mg/dL]	57.43 ± 7.27	$50.88 \pm 7.46^*$	53.27 ± 7.07
S.Cr. [mg/dL]	0.77 ± 0.14	0.82 ± 0.16	0.80 ± 0.15
UACR [mg/g]	17.11 ± 1.95	$27.66 \pm 4.48^{**}$	$167.29 \pm 36.86^{**,\S}$
Ang II [pg/mL]	10.83 ± 3.50	$21.17 \pm 5.44^{**}$	$34.04 \pm 6.53^{**,\S}$
EST [pg/mL]	1426.81 ± 601.59	$2329.97 \pm 483.94^{**}$	$3354.09 \pm 442.01^{**,\S}$

Data were expressed as mean ± SD. SBP:systolic blood pressure; DBP:diastolic blood pressure; FBG: fasting blood glucose; HbA1c:hemoglobinA1c; UACR: Urinary albumin creatinine ratio. HDL-cholesterol: High density lipoprotein-cholesterol; LDL-cholesterol: Low density lipoprotein-cholesterol; S Cr.: Serum Creatinine; Ang II: Angiopiotein-2; EST: Endostatin. P-value >0.05: Non significant

*,** Significant difference from Gp I at $p<0.05$ and $p<0.0$, respectively. π, § Significant difference from Gp IIat $p<0.01$ and at $p<0.01$ respectively. One Way ANOVA was used to compare anthropometric and biochemical parameters between 3 groups. The mean difference between the 3 groups are confirmed using post-hoc least significant difference (LSD) test.
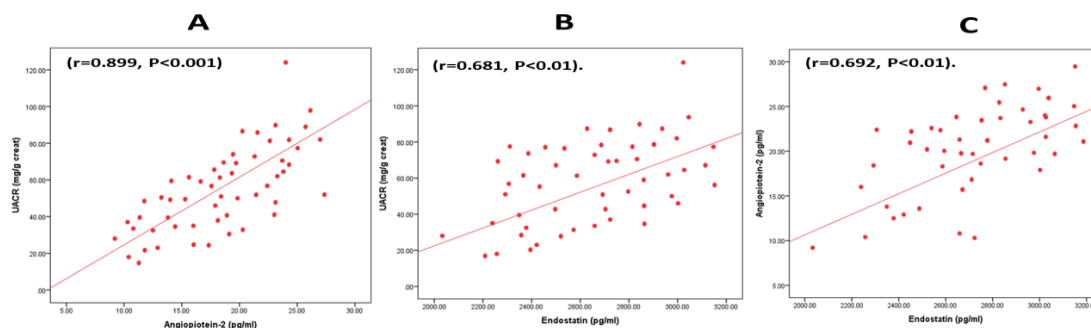

Fig. 1. Correlation between **[A]** UACR and angiopoietin 2 **[B]** UACR and Endostatin and **[C]** angiopoietin 2 and Endostatin in patients with diabetic nephropathy using Spearman correlation coefficient (P value< 0.05 was considered significant in all analyses).

3.3. Comparison between the studied angiogenic parameters variables among T2DM patients with and without nephropathy compared to control group

Compared to control group values, serum levels of EST in patients with T2DM with nephropathy more significantly elevated by 80.66%, while EST levels in patients with T2DM without nephropathy elevated by 48.09% (p<0.01). Also, Ang II more significantly elevated by 103.4% upon comparing with their levels in the control group, while Ang II levels in patients with T2DM without nephropathy changed by 63.59% (p<0.01). These results reflect that Ang II & EST levels may predict nephropathy progression.

4. DISCUSSION

DN is considered as the main causative factor of ESRD in the diabetic patient [6]. DN is associated with early changes in kidney structure including glomerular hyperfiltration, extracellular matrix expansion and glomerular basal membrane thickening [16]. Microalbuminuria is the main early sign of DN [1]. One of the main mechanisms that illustrate DN development is the renal micro blood vessel proliferation with increased circulating levels of angiogenic growth factors, including pro-angiogenic factors (e.g: angiopoietins) and anti-angiogenic factors (e.g: endostatin) [6]. The current study highlights the relationship between EST and Ang II with nephropathy in T2DM patients and whether or not both can be used as biomarkers for the prediction of DN in T2DM patients. EST was used as an anti-angiogenic drug and has been tested in patients with lung cancer, without serious side effects [17]. Also, EST had a protective action in diabetic nephropathy experimental animal models [18]. EST levels are elevated with physical stress [19], and its overexpression has been observed in profibrotic conditions in experimental studies [20].

Renal impairment is associated with peritubular capillaries loss and tissue hypoxia [21], also it is reported that up and downregulation of pro-angiogenic and anti-angiogenic factors can occur with the kidney diseases and renal hypoxia [22]. EST levels have been observed to be higher in renal impairment experimental studies [8]. In the current study, circulating EST levels were found to be higher in T2DM patients with nephropathy that might reflect the renal pathological angiogenic response that could lead to renal impairment progression. As EST is collagen XVIII cleavage derivative formed during ECMR, therefore elevated levels of circulating EST may have considered as a sign of pathological ECMR, as reported in an experimental study on mice with fibrosis [8]. Moreover, ECMR is observed in kidney diseases especially in renal cancers and fibrosis [23] and may be an important explanation for the association between endostatin and the present study outcomes. This is in line with the outcomes from longitudinal analyses where elevated EST levels predicted a more renal function impairment [15].

The process of angiogenesis, maturation, and remodeling can be stimulated through angiopoietins by using the N-terminus coiled-coil domain and a C-terminus fibrinogen-like domain. Also, angiopoietins may act as vital regulators in glucose and lipid metabolism [24]. Ang II overexpression inhibits Ang I binding to the Tie-2 receptor, which results in the development of DN[6]. Abnormal changes in Ang I/Ang II cause excess angiogenesis and inflammation, which play a vital role in the pathophysiologic mechanism of DN [12]. The accumulation of advanced glycation end products (AGE) due to chronic hyperglycemia stimulates VEGF formation through mesangial cells and hence, VEGF and Ang II mRNA expression can be up-regulated [25]. Lip et al., found in a cross-

sectional and interventional study, that VEGF plasma levels were significantly correlated with Ang II plasma levels among the T2DM group when compared with their levels in the healthy control [26]. These results are in contrast to the study conducted by Chen et al., who found that serum VEGF levels did not significantly change in the T2DM group compared to the healthy control group while VEGF urinary levels were significantly more elevated than their levels in the healthy control. Also, the study reported that serum Ang II levels were positively correlated with Ang II and VEGF urinary levels [27]. Besides, a previous study considering VEGF and angiopoietins in patients having T2DM with and without chronic vascular complications, reported the elevation of VEGF and Ang II levels in patients with T2DM compared to their levels in the healthy control regardless having cardiovascular disorders [28].

Conclusion

In conclusion, data obtained in this study showed that both EST and Ang II were significantly elevated in T2DM patients with albuminuria compared with healthy control subjects and could be used as a predictor of nephropathy progression.

Declarations

Ethics approval and consent to participate

The study was approved by the Human Ethical Review Committee, Faculty of Pharmacy, Ain Shams University, Cairo, Egypt (ENREC-ASU-240, the date on 20/3/2019).

Consent to publish

All participants signed their informed consent

Availability of data and materials

All data generated or analyzed during this study are included in this published article in the main manuscript.

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