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The Role of Urinary Cyclophilin A as a New Marker for Diabetic Nephropathy

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ABSTRACT

Background: Type 2 diabetes mellitus is the most common single cause of end-stage renal disease (ESRD), where diabetic nephropathy (DN) is considered the cause in almost half of all patients with ESRD. Despite the availability of many modern therapies for glycemic control, there are no specific curative treatments yet for DN and many diabetic patients still progressed to severe renal damage. Currently, albuminuria is the most commonly used marker to predict onset and progression of DN clinically. However, this traditional marker for DN lacks both sensitivity and specificity to detect early stage of DN. Furthermore, there is a lack of a strong association between albuminuria and glomerular filtration rate (GFR). As such, it is crucial to find earlier and reliable markers for DN diagnosis and intervention providing an opportunity to stop the permanent damage caused by it.

Objective: This study focuses on Cyclophilin A (CypA) in urine. CypA is a protein with ubiquitous characteristics, mostly distributed in the cytoplasm and facilitates protein folding and protein trafficking. It has relatively high expression level in normal kidneys. Recently, CypA has been reported to be a reliable novel marker for early diagnosis of DN.

Subjects and Methods: Our study was conducted on 90 subjects of comparative age and sex. They were selected from Endocrinology Clinic after written consent at Ain Shams University Hospital and Railway Hospital. Participants were divided into: **Group I:** 30 healthy control subjects, **Group II:** 30 T2DM patients without albuminuria (normoalbuminuric), and **Group III:** 30 T2DM patients with albuminuric DN.

Results: Our study showed that regarding the level of urinary CypA there was a highly statistical significant difference between the three groups ($F= 221.730$, $p< 0.01$), being higher in GII (normoalbuminuric) (1.69±0.87 ng/ml) than in GI (control) (0.55±0.14 ng/ml) ($t= 7.04$, $p< 0.01$) and higher in GIII (albuminuric DN) (6.01±1.61 ng/ml) than GII ($t= 12.93$, $p< 0.001$) and GI ($t= 18.55$, $p< 0.0001$). In addition, we found that urinary CypA was significant higher in GIIIb (macroalbuminuria) (7.23±0.76 ng/ml) than in GIIIa (microalbuminuria) (4.79±1.25 ng/ml) ($t= 6.49$, $p< 0.01$). It worth mentioning that, the level of urinary CypA started to increase significantly in stage 2 DN (2.49±0.50 ng/ml) in spite of normal level of albuminuria (no albuminuria) comparing with each of stage 1 DN (1.03±0.15 ng/ml), diabetics with no renal affection (0.99±0.45 ng/ml) and GI (healthy control) (0.55±0.14 ng/ml). There was significant positive correlation between urinary CypA and each of: sCr in GII ($r= +0.39$, $p< 0.05$), GIIIa ($r= +0.89$, $p< 0.001$) and GIIIb ($r= +0.99$, $p< 0.001$) and ACR in GIIIa ($r= +0.93$, $p< 0.001$) and GIIIb ($r= +0.98$, $p< 0.001$).

Conclusion: Our study showed that there was a high significant difference in the level of urinary CypA between diabetic patients with any degree of renal affection and healthy subjects being higher in diabetics with renal affection even without the presence of albuminuria.

Keywords: Cyclophilin A – Diabetic nephropathy – Type 2 Diabetes – Albumin/ Creatinine ratio.

INTRODUCTION

Diabetes mellitus (DM) is a group of metabolic diseases in which there are hyperglycemia over prolonged period, it is due to either lack of endogenous insulin secretion by the beta cells of the pancreas or resistance to the action of insulin in insulin dependent cells (liver, muscles, and adipose tissues) [(1)]. Diabetic nephropathy is a progressive kidney disease caused by long standing hyperglycemia that contribute in non-enzymatic glycation reactions of proteins and peptides with production of advanced glycation end-products (AGEs) which are associated with inflammation and damage of renal glomeruli resulting in progressive proteinuria, hypoalbuminemia, edema, hypertension, and finally may lead to chronic renal failure [(2,3)].Type 2 diabetes mellitus (DM) is the most common single cause of end-stage renal disease (ESRD). ESRD in almost half of patients is due to diabetic nephropathy (DN), and these cases have the worst outcome compared to patients with other causes of ESRD. Reasons for poor outcome include inadequate markers for early detection of DN, the complicated mechanisms of DN and there are no specific curative treatments yet for DN [(4)].

Currently**,** the stage of severity of DN is determined according to the levels of albuminuria which lacks both sensitivity and specificity to detect early stage of DN. Furthermore, some DN patients with ESRD do not present with significant albuminuria [(5)]. The lack of strong association between glomerular filtration rate (GFR) and albuminuria, in addition to the need for early diagnosis of DN to

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prevent its permanent damage are presenting a strong motivation to find an earlier and reliable marker to diagnose DN as an alternative to albuminuria based staging technique. Cyclophilin A (CypA) represents a promising candidate as DN diagnosis marker [(6)]. CypA is an 18-kDa protein mostly distributed in the cytoplasm and facilitates protein folding, protein trafficking and T-cell activation. It has peptidyl prolyl isomerase activity which catalyzes the isomerization of peptide bonds from trans to cis form at proline residues, which allows it to regulate many biological processes, including intracellular signaling, transcription, inflammation, and apoptosis [(7)].

CypA is relatively high in the kidney, where proximal tubular epithelial cells (PTECs) are reported to contain considerably much more of CypA rather than other kidney tissues. As CypA is directly produced by normal kidney, so its level will increase in urine with any kidney damage. Therefore urinary CypA level would be the most suitable indicator for early diagnosis of DN as it is secreted by monocytes in response to hyperglycemia [(8)].

AIM OF STUDY

We aim to assess the relationship between the levels of urinary Cyclophilin A and type 2 diabetes with diabetic nephropathy.

SUBJECTS AND METHODS

Subjects: This study was conducted on 90 subjects. Patients were selected from Endocrinology Clinic at Ain Shams University Hospital and Railway Hospital in Cairo. A written consent was taken from all participants. Participants were divided as follow**:** **Group I:** 30 healthy control subjects. **Group II:** 30 T2DM patients without albuminuria (normoalbuminuric). **Group III:** 30 T2DM patients with albuminuric nephropathy which were subdivided according to Urinary Albumin/Creatinine ratio (ACR) into: 15 diabetic patients with microalbuminuria (ACR 30-300 mg/g), and 15 diabetic patients with macroalbuminuria (ACR >300 mg/g).

It is worth mentioning that, in our study although GII represents T2DM without DN diagnosed by absence of albuminuria according to ACR system, later on we subdivided them into: stage 1 DN consisted of three patients with increase in eGFR (hyperfiltration), stage 2 composed of fourteen patients with mild affection in eGFR and diabetics without DN who were thirteen patients with no any renal affection. The following exclusion criteria were taken in the consideration in the selection of our participants: Patients with other causes of renal diseases, liver diseases, malignancy, infectious and inflammatory diseases as well as patients with type 1 diabetes.

METHODS

All participants in the study were submitted to: Full medical history taking including duration of DM, thorough clinical examination, anthropometric evaluation including: weight, height, body mass index (weight in kilograms divided by square of height in meters), and laboratory investigations: Fasting plasma glucose after 8 hours overnight fasting (FPG) (mg/dl) was measured using an automated glucose oxidase method using Behring Diagnostics Reagents (SVR Glucose Test; Behring, La Jolla, CA). Glycated hemoglobin (HbA1c) (%) was measured by Quantitative colorimetric determination of Glycohemoglobin in blood.

Serum Creatinine (sCr) mg/dl was measured by colorimetric method using Standard, Picric Acid and sodium hydroxide reagents [(9)]. Lipid profile (total cholesterol, LDL, HDL, and triglycerides) (mg/dl). LDL-cholesterol was estimated from quantitative measurements of total and HDL-cholesterol and plasma triglycerides (TG) by using Friedewald equation: (LDL-chol) = (Total chol) – (HDL-chol) - (TG/5). The quotient (TG/5) is used as an estimate of VLDL-cholesterol concentration, as all of the plasma TG is carried on VLDL, and the TG:Cholesterol ratio of VLDL is constant at about 5:1[(10)].

Estimated glomerular filtration rate (eGFR) (ml/min/1.73m^2) was calculated using the modification of diet in renal disease (MDRD) equation: 186 x $(Creat)^{-1.154}$ x $(AGE)^{-0.203}$ x (0.742 if female) x (1.210 if African American). Urinary Albumin/Creatinine ratio (ACR) (mg/g) was calculated, where all DM patients were categorized by urinary Albumin/Creatinine ratio into normoalbuminuric (ACR <30 mg/g), microalbuminuric (ACR between 30 and 300 mg/g on a spot urine sample) and macroalbuminuric (ACR >300 mg/g on a spot urine sample) according to the Kidney Disease Outcomes Quality Initiative (KDOQI) guidelines for evaluation and stratification of chronic kidney disease. Urinary Cyclophilin A by ELISA (CypA) (ng/ml): spot urine test was used to measure the concentration of Cyclophilin A[(11)].

Statistical methods

Data were collected and entered into an excel sheet and was statistically analyzed using version 23 of IBM Statistical Package for the Social Sciences (SPSS). Data have been edited and revised before subjected to statistical analysis.

Qualitative data, e.g., gender, data were presented as count and proportion, while quantitative data, e.g., age, BMI, were presented as mean and

standard deviation. Chi square test was used to examine the relationship between two qualitative variables but when the expected count is less than 5 in more than 20% of the cells; Fisher's Exact Test was used. Comparison of quantitative variables across the four study groups was done using Analysis of variance (ANOVA) technique and due to inequality of the variance of the four groups Welch method is used to calculate the F Ratio. Student's t- distribution was applied to assess the statistical significance of the difference of a parametric variable between two independent means of two study groups. Regression and Pearson correlation coefficient (r) analyses were applied to illustrate the relationships between urinary CypA and the different studied variables.

The study was approved by the Ethics Board of Ain Shams University.

RESULTS

Table (1): Comparison of the four groups regarding selected quantitative variables

Parameter	Group I (n=30)	Group II (n=30)	Group III (n=30)		F ratio-Welch test	value
			Group IIIa (n=15)	Group IIIb (n=15)		
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD		
Age (years)	53.13 ± 9.05	53.03 ± 8.09	51.07 ± 8.43	52.20 ± 8.31	0.238	0.869
BMI (kg/m^2)	25.34 ± 3.02	26.34 ± 3.96	26.00 ± 2.63	23.96 ± 1.67	2.050	0.113
DDM (years)	-	7.29 ± 6.17	13.00 ± 4.14	16.40 ± 4.56	15.875	<0.01
SBP (mmHg)	113.67 ± 6.15	138.67 ± 12.79	151.00 ± 10.72	151.33 ± 11.09	68.337	<0.01
DBP (mmHg)	72.50 ± 6.92	90.33 ± 6.69	94.67 ± 11.25	95.67 ± 9.42	43.040	<0.01
FBG (mg/dl)	87.77 ± 6.88	176.33 ± 88.90	203.67 ± 86.67	227.87 ± 82.02	17.485	<0.01
HbA1c (%)	5.01 ± 0.46	7.57 ± 1.94	9.02 ± 1.75	10.05 ± 1.94	43.194	<0.01
Cr (mg/dl)	0.78 ± 0.09	1.06 ± 0.37	1.48 ± 0.31	2.06 ± 0.70	41.673	<0.01
ACR (mg/g)	9.29 ± 1.19	20.96 ± 4.25	226.83 ± 74.96	1267.53 ± 688.20	80.503	<0.01
eGFR (ml/min/1.73 m^2)	102.98 ± 8.09	96.59 ± 21.90	72.12 ± 22.48	41.26 ± 16.37	48.503	<0.01
CypA (ng/ml)	0.55 ± 0.14	1.69 ± 0.87	4.79 ± 1.25	7.23 ± 0.76	295.241	<0.01
T. Chol (mg/dl)	148.20 ± 35.76	209.03 ± 40.66	221.33 ± 37.81	226.20 ± 29.38	23.605	<0.01
LDL (mg/dl)	102.27 ± 34.29	145.43 ± 38.16	156.33 ± 32.26	170.93 ± 28.43	17.164	<0.01
HDL (mg/dl)	29.23 ± 6.31	35.43 ± 6.36	35.73 ± 15.05	40.67 ± 6.74	6.768	<0.01
TG (mg/dl)	98.57 ± 22.13	175.83 ± 75.93	198.33 ± 75.69	231.07 ± 80.95	17.662	<0.01

P< 0.05 is statistically significant, P< 0.01 is highly statistical significant, F ratio Welch test: ANOVA test
DDM: duration of diabetes, CypA: urinary cyclophilin A.

Table (2): Comparison between group I and group II regarding all variables

	Group I (n=30)	Group II (n=30)	t-test	P-value
Age (years)	53.13 ± 9.05	53.03 ± 8.09	0.045	0.964
BMI (kg/m2)	25.34 ± 3.02	26.34 ± 3.96	1.09	0.277
DDM (years)	-----	7.29 ± 6.17	6.48	<0.01
SBP (mmHg)	113.67 ± 6.15	138.67 ± 12.79	9.65	<0.001
DBP (mmHg)	72.50 ± 6.92	90.33 ± 6.69	10.15	<0.001
FPG (mg/dl)	87.77 ± 6.88	176.33 ± 88.90	5.44	<0.01
HbA1c (%)	5.01 ± 0.46	7.57 ± 1.94	7.06	<0.01
Cr (mg/dl)	0.78 ± 0.09	1.06 ± 0.37	3.98	<0.01
ACR (mg/g)	9.29 ± 1.19	20.96 ± 4.25	14.48	<0.001
eGFR (ml/min/1.73 m2)	102.98 ± 8.09	96.59 ± 21.90	1.49	0.139
CypA (ng/ml)	0.55 ± 0.14	1.69 ± 0.87	7.04	<0.01
T.Chol (mg/dl)	148.20 ± 35.76	209.03 ± 40.66	6.15	<0.01
LDL (mg/dl)	102.27 ± 34.29	145.43 ± 38.16	4.61	<0.01
HDL (mg/dl)	29.23 ± 6.31	35.43 ± 6.36	3.79	<0.01
TG (mg/dl)	98.57 ± 22.13	175.83 ± 75.93	5.35	<0.01

Student's t- distribution: t-test.

Table (3): Comparison between group II and group III regarding all variables

	Group II (n=30)	Group III (n=30)	t-test	P-value
ge (years)	53.03 ± 8.09	51.63 ± 8.24	0.66	0.509
MI (kg/m2)	26.34 ± 3.96	24.98 ± 2.40	1.61	0.113
DM (years)	7.29 ± 6.17	14.70 ± 4.62	5.27	<0.01
BP (mmHg)	138.67 ± 12.79	151.17± 10.72	4.10	<0.01
BP (mmHg)	90.33 ± 6.69	95.17 ± 10.21	2.17	0.034
PG (mg/dl)	176.33 ± 88.90	215.77 ± 83.82	1.77	0.082
bA1c (%)	7.57 ± 1.94	9.53 ± 1.89	3.97	<0.01
r (mg/dl)	1.06 ± 0.37	1.77 ± 0.61	5.45	<0.01
CR (mg/g)	20.96 ± 4.25	747.18 ± 715.17	5.56	<0.01
GFR (ml/min/1.73 2)	96.59 ± 21.90	56.69 ± 24.89	6.59	<0.01
ypA (ng/ml)	1.69 ± 0.87	6.01 ± 1.61	12.93	<0.001
Chol (mg/dl)	209.03 ± 40.66	223.77 ± 33.36	1.53	0.130
DL (mg/dl)	145.43 ± 38.16	163.63 ± 30.78	2.03	0.047
DL (mg/dl)	35.43 ± 6.36	38.20 ± 11.73	1.14	0.261
G (mg/dl)	175.83 ± 75.93	214.70 ± 78.78	1.95	0.057

Student's t- distribution: t-test.

Table (4): Comparison between microalbuminuric and macroalbuminuric groups regarding all variables

Parameter	Group IIIa (n=15)	Group IIIb (n=15)	t-test	P-value
Age (years)	51.07 ± 8.43	52.20 ± 8.31	0.37	0.714
BMI (kg/m2)	26.00 ± 2.63	23.96 ± 1.67	2.54	0.017
DDM (years)	13.00 ± 4.14	16.40 ± 4.56	2.14	0.041
SBP (mmHg)	151.00 ± 10.72	151.33 ± 11.09	0.08	0.934
DBP (mmHg)	94.67 ± 11.25	95.67 ± 9.42	0.26	0.794
FPG (mg/dl)	203.67 ± 86.67	227.87 ± 82.02	0.79	0.439
HbA1c (%)	9.02 ± 1.75	10.05 ± 1.94	1.53	0.138
Cr (mg/dl)	1.48 ± 0.31	2.06 ± 0.70	2.94	<0.01
ACR (mg/g)	226.83 ± 74.96	1267.53 ± 688.20	5.82	<0.01
eGFR (ml/min/1.73 m2)	72.12 ± 22.48	41.26 ± 16.37	4.30	<0.01
CypA (ng/ml)	4.79 ± 1.25	7.23 ± 0.76	6.49	<0.01
T.Chol (mg/dl)	221.33 ± 37.81	226.20 ± 29.38	0.39	0.697
LDL (mg/dl)	156.33 ± 32.26	170.93 ± 28.43	1.32	0.199
HDL (mg/dl)	35.73 ± 15.05	40.67 ± 6.74	1.16	0.256
TG (mg/dl)	198.33 ± 75.69	231.07 ± 80.95	1.14	0.262

Student's t- distribution: t-test.

Table (5): Comparison between the three subgroups of group II regarding all variables

Parameter	Group II (n=30)		
	No renal affection (n=13)	**Stage 1 (n=3)**	**Stage 2 (n=14)**
Age (years)	52.08± 9.53	51.67± 8.08	54.21± 7.02
BMI (kg/m2)	24.92± 2.09	27.38± 5.08	27.44± 4.83
DDM (years)	4.85± 4.46	2.90± 1.91	10.50± 6.67*
SBP (mmHg)	133.08± 9.90	156.67± 11.55*	140.00± 12.09□
DBP (mmHg)	86.92± 5.96	100.00±0.00*	91.43± 5.69□
FPG (mg/dl)	152.62± 85.22	171.33± 116.54	199.43± 87.72
HbA1c (%)	6.97± 1.64	7.09± 2.44	8.24± 2.02
Cr (mg/dl)	0.98± 0.34	0.64± 0.12	1.23± 0.34□
ACR (mg/g)	20.72± 3.74	20.37± 2.91	21.30± 5.08
eGFR (ml/min/1.73 m2)	104.32± 7.25	145.58± 13.88*	78.91±6.47*□
CypA (ng/ml)	0.99± 0.45	1.03± 0.15	2.49± 0.50*□
T.Chol (mg/dl)	188.15± 43.97	247.00± 37.24*	220.29± 28.03*
LDL (mg/dl)	125.62± 34.94	183.33± 42.36*	155.71± 31.75*
HDL (mg/dl)	36.08± 6.36	35.00± 2.00	34.93± 7.17
TG (mg/dl)	149.15± 76.12	198.67± 100.13	195.71± 68.65

- *,□: significant differences (P<0.05) as compared to No renal affection and Stage 1, respectively. Data is represented as mean ± standard deviation (SD).

Table (6): Comparison of urinary CypA level in different stages of renal affection

		No	**Urinary CypA (Mean ± SD)**	**F ratio-Welch test**	**P- value**
Stage of DN	**No renal affection**	43	0.69 ± 0.34	331.717	<0.01
	stage 1	3	1.03 ± 0.15		
	stage 2	14	2.49 ± 0.50		
	stage 3	15	4.79 ± 1.25		
	Stage 4	15	7.23 ± 0.76		

P< 0.05 is statistically significant, P< 0.01 is highly statistical significant, F ratio-Welch test: ANOVA test.

Table (7): Correlation of urinary Cyclophilin A level with quantitative variables

		Urinary CypA			
		Group I	**Group II**	**Group IIIa**	**Group IIIb**
No of cases		**30**	**30**	**15**	**15**
Age (years)	**r**	**+0.14**	**+0.14**	**+0.16**	**+0.11**
	P value	0.47	0.46	0.58	0.69
BMI (kg/m2)	**r**	**+0.05**	**+0.04**	**+0.08**	**+0.06**
	P value	0.81	0.84	0.77	0.83
DDM (years)	**r**	**--**	**+0.67**	**+0.90**	**+0.97**
	P value	--	<0.01	<0.001	<0.001
SBP (mmHg)	**r**	**+0.04**	**+0.24**	**+0.71**	**+0.83**
	P value	0.82	0.20	0.003	<0.001
DBP (mmHg)	**r**	**+0.04**	**+0.25**	**+0.65**	**+0.68**
	P value	0.86	0.18	0.008	0.005
FPG (mg/dl)	**r**	**+0.16**	**+0.12**	**+0.21**	**+0.27**
	P value	0.41	0.54	0.45	0.33
HbA1c (%)	**r**	**+0.22**	**+0.14**	**+0.28**	**+0.30**
	P value	0.22	0.46	0.31	0.28
Cr (mg/dl)	**r**	**+0.07**	**+0.39**	**+0.89**	**+0.99**
	P value	0.73	<0.05	<0.001	<0.001
ACR (mg/g)	**r**	**+0.24**	**+0.25**	**+0.93**	**+0.98**
	P value	0.21	0.18	<0.001	<0.001
eGFR ml/min/1.73 m2)	**r**	**-0.07**	**-0.76**	**-0.90**	**-0.98**
	P value	0.71	<0.01	<0.001	<0.001
T.Chol (mg/dl)	**r**	**+0.219**	**+0.220**	**+0.32**	**+0.29**
	P value	0.244	0.242	0.243	0.287
LDL (mg/dl)	**r**	**+0.19**	**+0.21**	**+0.23**	**+0.25**
	P value	0.30	0.28	0.41	0.37
HDL (mg/dl)	**r**	**+0.06**	**+0.02**	**+0.08**	**+0.08**
	P value	0.74	0.90	0.79	0.77
TG (mg/dl)	**r**	**+0.14**	**+0.22**	**+0.24**	**+0.25**
	P value	0.46	0.25	0.39	0.38

P< 0.05 is statistically significant.
P< 0.01 is highly statistical significant.
Pearson correlation coefficient test (r).

DISCUSSION

Diabetic nephropathy (DN) is one of the most common microvascular complications of diabetes and it is considered as a leading cause of end-stage renal disease since there are no specific treatments for it till now. Therefore earlier diagnosis and intervention may provide an opportunity to stop the permanent renal damage caused by DN [(4)].

In our study, we tried to find out the possibility of using the urinary cyclophilin A (CypA) as a new marker for diagnosis of diabetic nephropathy as early as possible.

Our study showed that there was a statistically significant difference in the level of urinary CypA between the three main groups (F= 221.730, P <0.01) being higher in GIII and GII than the control (GI). The urinary CypA in GIII (diabetics with albuminuric DN) (6.01±1.61 ng/ml) was statistically significant higher than in GII (diabetics without albuminuria) (1.69±0.87 ng/ml, t= 12.93, p <0.001) and in GI (control) (0.55±0.14 ng/ml, t= 18.55, p <0.0001). In GII the urinary CypA was statistically significant higher than in GI (t= 7.04, p <0.01). We also found that the level of urinary CypA was statistically significant higher in GIIIb (diabetics with macroalbuminuria) (7.23±0.76 ng/ml) than in GIIIa (diabetics with microalbuminuria) (4.79±1.25 ng/ml, t= 6.49, p <0.01).

This is in agreement with ***Tsai et al.*** [(8)] study which was the first study to use urinary CypA in early detection of DN. It was conducted on 120 subjects; 20 healthy control group and 20 diabetic patients in each stage of DN (5 stages). Urine

samples were collected to determine the expression of urinary CypA. They also treated mesangial (MES-13) and tubular (HK-2) cells with glucose or free radicals to observe the expression of secreted CypA in Western blot analysis. They found that the levels of urinary CypA was higher in groups of DN than in normal one. There was a highly statistical significant difference ($p< 0.001$) in levels of urinary CypA between all groups except between normal and stage 1 DN groups where there was no significant difference in level of urinary CypA between them. The lowest levels of urinary CypA was found in control and stage 1 DN, wherein the urinary CypA increased gradually with progression of DN till it reached the highest levels in stage 5 DN (ESRD).

Although these three subdivided groups of GII had no albuminuria (normal ACR), we noticed that the level of urinary CypA in patients with stage 2 DN (2.49±0.50 ng/ml) was statistically significantly higher ($p< 0.05$) than those in stage 1 (1.03±0.15 ng/ml) as well as those without DN (0.99±0.45 ng/ml). Whereas, there was not any statistically significant difference in the level of urinary CypA between diabetics without DN and those with stage 1 DN as well as control. So we can conclude that urinary CypA increased significantly early with renal affection even before the appearance of albuminuria (stage 2 DN) which may make it more sensitive marker for DN than ACR system.

This is compatible with ***Tsai et al.*** [8] study which was conducted on 100 type 2 diabetic patients in the different five stages of DN and clarified that by comparing with the control group, urinary CypA indeed increased significantly in stage 2 DN and its increase persisted throughout the later stages. The increment was more significant with worsening DN stage. They confirmed that there was no significant difference in concentration of urinary CypA between stage 1 DN and healthy control groups ($P= 0.117$). However, there were statistically significant differences between stages 1 and 2 ($P= 0.012$), stages 2 and 3 ($p= 0.003$), stages 3 and 4 ($p <0.001$), and stages 4 and 5 DN ($P= 0.005$).

In this study, there was a significant positive correlation between urinary CypA levels and the duration of diabetes in the three diabetic groups. We also noticed that the correlation was stronger in GIIIb which had the longest duration of diabetes (16.40±4.56 yrs, $r= +0.97$, $p <0.001$) than in GIIIa (13.00±4.14 yrs, $r= +0.90$, $p <0.001$) which in turn had a stronger correlation than in GII (7.29±6.17 yrs, $r= +0.67$, $p <0.01$). This emphasizes the role of CypA in producing micro and macro-vascular complications induced by prolonged duration of hyperglycemia.

This is in disagreement with ***Tsai et al.*** [8] study that showed no significant correlation ($p= 0.957$) between the level of urinary CypA and duration of diabetes. This discrepancy may be due to that in our research by chance the longest durations of diabetes were presented in GIIIb (the group with the most aggressive stage of renal affection). So the significant elevations in urinary CypA may be contributed by the severity of renal damage which was more aggressive in GIIIb and not by duration of diabetes itself. To confirm this correlation between urinary CypA level and duration of diabetes we need to do more studies where the duration of diabetes will be matched between three diabetic groups.

In the present study, we found that there was no significant positive correlation between urinary CypA and both of FPG and HbA1c in all studied groups. GI (FPG= 87.77±6.88 mg/dl, $r= +0.16$, $p= 0.41$) (HbA1c= 5.01±0.46 %, $r= +0.22$, $p= 0.22$), GII (FPG= 176.33±88.90 mg/dl, $r= +0.12$, $p= 0.54$) (HbA1c= 7.57±1.94 %, $r= +0.14$, $p= 0.46$), GIIIa (FPG= 203.67±86.67 mg/dl, $r= +0.21$, $p= 0.45$) (HbA1c= 9.02±1.75 %, $r= +0.28$, $p= 0.31$) and GIIIb (FPG= 227.87±82.02 mg/dl, $r= +0.27$, $p= 0.33$) (HbA1c= 10.05±1.94 %, $r= +0.30$, $p= 0.28$). Although these correlations between urinary CypA levels and FPG and HbA1c are non significant, they were much higher in GIIIa and GIIIb than GII. This illustrates that higher levels of hyperglycemia induced higher levels of Urinary CypA which may allow us to use CypA level also as a marker for diagnosis of DM.

This is in consistency with ***Tsai et al.*** [8] study that showed no significant correlation between urinary CypA and each of FPG ($p= 0.898$), and HbA1c ($p= 0.686$) as well as Ohtsuki et al., 2016 study which exhibited no significant correlation between CypA and HbA1c ($p= 0.232$).

In opposite to our study, the study was done by ***Ramachandran et al.*** [12]**.** It was conducted on 556 subjects had been divided into 5 groups: (i) patients with diabetes and diagnosed to have coronary artery disease (CAD) within 5 years of onset of diabetes, (ii) patients with DM and diagnosed to have CAD after 5 years and within 10 years of onset of diabetes, (iii) patients with only diabetes, (iv) patients with only CAD, and (v) controls. They found that there was a significant positive correlation between CypA and each of FPG and HbA1c ($p< 0.01$, $p= 0.019$, respectively).

The discrepancy between our results and these two studies may be returned to their studies

were conducted on larger number of diabetic patients than in our study. In addition, they assessed the plasma level of CypA whilst in our study we measured the urinary CypA level.

In our study, correlations between urinary CypA and other renal parameters (sCr, ACR, eGFR) were done in attempt to explain the presence of higher levels of urinary CypA with the more severe stages of DN.

We found that there was a significant positive correlation between urinary CypA and sCr in GII (r= +0.39, p< 0.05), while there was a highly significant positive correlations between urinary CypA and sCr in both GIIIa (r= +0.89, p< 0.001) and GIIIb (r= +0.99, p< 0.001). While there was no significant positive correlation between urinary CypA and normal sCr in GI (r= +0.07, p= 0.73).

This indicates that urinary CypA levels increase proportionally with the elevation in sCr. As the urinary CypA levels were low in GI who had normal sCr levels and it started to increase significantly in patients with stage 2 DN of (sCr 1.23±0.34 mg/dl). The increment of urinary CypA became more significant in GIIIa (sCr 1.48±0.31 mg/dl) whilst the highest significant increase in urinary CypA was in GIIIb who had the highest levels of sCr (2.06±0.70 mg/dl).

Our study is compatible with ***Tsai et al.*** [8] who studied the urinary CypA as a new marker of DN. They clarified that there was a significant positive correlation (p= 0.037) between urinary CypA and sCr. In addition, it demonstrated that the concentration of urinary CypA increased by 0.395 ng/ml for each 1 mg/dl increase in sCr. It put a constant equation illustrating the relation between urinary CypA and sCr (CypA= 2.241+Cr*0.395).

Our research established that there was a highly significant negative correlation between urinary CypA levels and eGFR in the diabetic groups (GII, GIIIa, and GIIIb) and this correlation became more significantly higher while the decrease in eGFR became more advanced. We noticed that this correlation was higher in GIIIb (eGFR 41.26±16.37 ml/min/1.73 m^2, r= -0.98, p< 0.001) than in GIIIa (eGFR 72.12±22.48 ml/min/1.73 m^2, r= -0.90, p< 0.001) which in turn higher than in GII (eGFR 96.59±21.90 ml/min/1.73 m^2, r= -0.76, p< 0.01). Whereas there was no significant negative correlation between urinary CypA and normal eGFR in GI (e GFR 102.98±8.09 ml/min/1.73 m^2, r= -0.07, p= 0.71).

So we concluded that the urinary CypA only significantly increased with renal affection and decrease of eGFR. Here we can explain the elevation of urinary CypA in GII whose their mean eGFR were within normal (96.59±21.90 ml/min/1.73 m^2) by the presence of 14 patients in this group who had stage 2 DN with mild decrease in their eGFR (78.91±6.47 ml/min/1.73 m^2) which most probably affected the urinary CypA levels in this group.

This is in agreement with ***Tsai et al.*** [8] study that proved the presence of significant negative association between urinary CypA and eGFR in DN patients (p= 0.013). Also, they found that the concentration of urinary CypA increased by 0.030 ng/ml with each 1 ml/min decrease in eGFR and they established an equation which illustrated the correlation between Urinary CypA and eGFR (CypA= 5.270+GFR*-0.030). Besides, the study showed that there was a trend of higher urinary CypA in the group with GFR less than 60 ml/min/1.73 m^2 as compared to the group with GFR more than 60 ml/min/1.73 m^2 (p< 0.060).

Our results showed that there was a highly significant positive correlation between urinary CypA and the severity of albuminuria (ACR) in both GIIIa and GIIIb. These positive associations were more significant in GIIIb (ACR 1267.53±688.20 mg/g, r= +0.98, p< 0.001) than in GIIIa (ACR 226.83±74.96 mg/g, r= +0.93, p< 0.001). While there was no significant positive correlation between urinary CypA and normal ACR as in GI (ACR 9.29±1.19 mg/g, r= +0.24, p= 0.21) and in GII (ACR 20.96±4.25 mg/g, r= +0.25, p= 0.18).

Our results are fit with that of ***Tsai et al.*** [8] study that illustrated that; there was a statistically significant difference (p= 0.007) in the levels of urinary CypA between both proteinuric and non-proteinuric patients where in non-proteinuric the concentration of urinary CypA decreased by 3.095 ng/ml. They also proved that when ACR increased by 1 mg/g, the concentration of urinary CypA increased by 0.030 ng/mL and they established an equation to link between them (CypA= 2.461+ACR*0.001).

From the previous results we can conclude that urinary CypA level is strongly correlated with the degree of renal affection and its level started to increase significantly in stage2 and continued to increase proportionally with the progression of DN. This confirms the presence of strong alternative relation between urinary CypA and DN.

To conclude, although the albuminuria-based system is the most common used marker for the diagnosis and follow up the progression of DN, it is far from ideal for a number of reasons. First, increased albuminuria is actually a relatively late manifestation of early-stage DN, so it is not

sensitive enough to detect early stages of DN. Second, some patients have renal pathological changes without microalbuminuria. Finally, albuminuria is not specific enough for DN because it can be detected in other non-DM related nephropathy, such as retinopathy and congestive heart failure [(13)].

In addition, due to that either GFR-based or albuminuria-based classifications of DN correlated significantly with urinary CypA. When comparing different stages of DN or Chronic kidney disease (CKD), there was only a trend of higher CypA in higher CKD stages, but truly statistically significant difference existed among the different DN stages. This finding supports the notion that urinary CypA is better correlated using the albuminuria-based classification, which is the better and earlier detection method for monitoring DN in clinical practice.

This will enable us to detect stage 2 DN early, so intensive blood sugar monitoring, timely diet restriction and exercise education would be useful to avoid further silent deterioration of DN.

CONCLUSION

Urinary CypA was higher in diabetics with macroalbuminuric DN than those with microalbuminuric DN who in turn had higher levels of urinary CypA than diabetics with normoalbuminuric DN. Urinary CypA had a positive correlation with serum creatinine, urinary albumin creatinine ratio and duration of diabetes, while it had a negative correlation with estimated glomerular filtration rate. Urinary CypA can be used as an early marker for DN as we found early significant high levels of urinary CypA in diabetic patients with stage 2 DN even before the appearance of albuminuria.

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