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MEASUREMENT OF CYCLOPHILIN A AS A NEW BIOMARKER FOR DIABETIC NEPHROPATHY IN TYPE II DIABETES MELLITUS

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ABSTRACT

Diabetic nephropathy (DN) is one of the most important microvascular complications associated with type II diabetic patients. Albuminuria is the most commonly used marker to predict onset and progression of DN clinically but it lacks both sensitivity and specificity to detect its early stages. So, it is critical to find earlier and reliable markers for DN. Cyclophilin A (CyPA) is a cytosolic and extremely abundant protein, has various intracellular functions. Keywords: Diabetic nephropathy (DN), Cyclophilin A (CyPA)

KEYWORDS: *Diabetic nephropathy, patients, Albuminuria, diagnose, kidney disease*

1-INTRODUCTION

Diabetic nephropathy (DN) is a serious and progressive complication related to diabetes. It can increase the risk and progression of end-stage renal disease. Diabetic nephropathy is clinically defined as a rise in urinary albumin excretion [microalbuminuria (30-300mg albumin/gm creatinine) then macroalbuminuria (>300mg albumin/gm creatinine)], decreased glomerular filtration rate (GFR) and elevated blood pressure.[1]Albuminuria have some limitations to detect early stages of DN such as it can be elevated in some cases such as exercise, acute illness, heart failure and there are some diabetic patients develop DN with normal albuminuria[2]so, new biomarkers are required. CyPA is an intracellular protein has various intracellular functions such as intracellular signaling, protein trafficking, and regulating the activity of other proteins.[3]CyPA was revealed to be secreted by monocytes in response to hyperglycemia in diabetic patients indicating that secreted CyPA could be a potential secretary marker in type II diabetes mellitus.[4] Furthermore, a relatively high expression of CyPA in normal kidneys may be associated with kidney damage.[5] Serum CyPA can be used as a potential biomarker of DN and may be raised earlier than albuminuria.

2- OBJECTIVES

This is a case-control study to detect the validity of using cyclophilin A as an earlier and reliable biomarker for diabetic nephropathy. In this study 112 subjects were enrolled and divided into three groups;

- **The first group** was healthy control group included 16 subjects (14.3% of the study population).

-**The second group** was diabetic patients without nephropathy (stage0) with no evidence of renal

disease included 16 subjects (14.3% of the study population).

-**The third group** was diabetic nephropathy patients included 80 subjects represented 71.4% of the study population divided into five stages;

• **Stage1** DN patients having normoalbuminuria (ACR<30mg/gm) and GFR more than120 mL/min/1.73m^2), included 16 subjects (14.3% of the study population).

• **Stage2** DN patients having normoalbuminuria (ACR<30mg/gm) and normal GFR (90-120 mL/min/1.73m^2), included 16 subjects (14.3% of the study population). *Differentiation between stage1 and stage2 diabetic nephropathy patients depending on the estimation of GFR.

• **Stage3** DN patients having microalbuminuria (ACR30-300mg/gm) included 16 subjects (14.3% of the study population).

• **Stage4** DN patients having macroalbuminuria (ACR>300mg/gm) included 16 subjects (14.3% of the study population).

• **Stage5** DN patients having GFR <15 mL/min/1.73 m^2 included 16 subjects (14.3% from study population). Stages of DN were classified according to [6, 7].

- Inclusion criteria: All males and females aged more than 40 years and diagnosed with type II diabetes were included.

- Exclusion criteria: Patients having autoimmune diseases causing secondary diabetes [e.g. systemic lupus erythematosus (SLE)], Patients with other chronic diseases such as chronic liver disease, Patients suffering from chronic kidney disease other than diabetic nephropathy (e.g. congenital kidney diseases, renal artery stenosis and hydro-nephrosis) and Patients with history of cardiovascular diseases

3-METHODOLOGY

- Clinical Examination

• Including blood pressure measurement after resting for 5 min in sitting position using mercury sphygmomanometer.

•Calculation of body mass index (BMI) using the following equation:

BMI = (Weight/kg) / (Height/m) ^{2}

-Laboratory Investigations

•Including sampling blood sample for measurement of fasting blood glucose, serum creatinine, serum total cholesterol, high density lipoproteins (HDL), low density lipoproteins (LDL), triglycerides and serum cyclophilin A, hemoglobin (HbA1C) and Spot urine sample from each patient to measure urinary albumin /creatinine ratio (ACR).

• Calculation of estimated glomerular filtration rate (eGFR) by using

MDRDEquation

$$=175 \times \left[plasma\ creatinine\ \left(\frac{mg}{dl}\right)\right]^{-1.154} \times [\text{Age}]^{-0.203} \times [\ 0.742\ if\ female\ ]$$

•All of the above laboratory investigations were performed at Clinical Pathology Department, Suez Canal University Hospital. Ismailia. Serum cyclophilin A concentrations were measured by using ELISA kit provided by **(Biotech Co., LTD)**.

4- STATISTICAL DESIGN

Statistical analysis was conducted by using SPSS software version 22.0. Quantitative data were calculated as mean ± standard deviation (SD). Chi-square and Kruskal-Wallis test were used for comparison between study groups. Pearson correlation coefficient test used to associate between study variables. Statistical significant difference if P-value less than 0.05.

5- RESULTS

The mean age for all study groups was from 42.6± 1.4 years to 58.9±2.7 years with statistical significant differences (p-value =0.001) .The gender distribution was (50%) female and (50%) male without statistical significant differences (p-value =0.9).

The duration of type II diabetes mellitus ranged from 3.1±0.9 to 15.4±2.9 years in all diabetic patients in this study with (p-value=0.009).

The mean values of body mass index (BMI) were higher in the diabetic nephropathy group in comparison to control group and stage 0 with (p-value <0.004). Systolic and diastolic blood pressure showed higher values in diabetic nephropathy groups than control group and stage0 due to progression of the disease with (p-value <0.001)

Study groups	**Healthy Control group**	**Diabetes without nephropathy**	**Stage 1 DN**	**Stage 2 DN**	**Stage 3 DN**	**Stage 4 DN**	**Stage 5 DN**	**P-value**
Variables	**Mean± SD**	**Mean± SD**	**Mean ± SD**	**Mean ± SD**	**Mean ± SD**	**Mean ± SD**	**Mean ± SD**	
Age (years)	42.6± 1.4	45.8±3	48.9± 2.6	52.9± 7.8	52.9± 5.1	50.1± 2.7	58.9± 2.7	*0.001
Gender male (%)	50%	50%	50%	50%	50%	56.3%	43.8%	0.9
Duration of DM (years)	0	3.1±0.9	5.3± 0.9	6.9± 1.4	9.4± 1.8	9.7± 1.5	15.4± 2.9	*0.009
BMI ($kg/m^{2)}$	24.7± 2.6	25.8±2.7	26.2± 3.1	27.1± 3.8	32.3± 4.7	29.5± 5.7	28.1± 3.8	*0.004
SBP (mmHg)	117.4± 5.4	119.8±6	118.1± 4.8	120.9± 4.6	135± 10.3	140± 4.8	145±7.7	<0.001
DBP (mmHg)	75±4.8	75.6± 4.7	75.6± 4.1	75± 4.1	81.9± 7.3	89.7± 4.6	94.4± 6.8	<0.001
Kruskal-Wallis test between DN stages for quantitative variables and chi-square test used for qualitative variables								

Table (1); Demographic characteristics of the study population

Statistical significant difference when P-value<0.05
BMI = Body Mass Index, DM = Diabetes mellitus,
SBP= systolic blood pressure, DBP=diastolic blood pressure

Study groups	Healthy Control group	Diabetes without nephropathy	Stage 1 DN	Stage 2 DN	Stage 3 DN	Stage 4 DN	Stage 5 DN	P-value
Variables	**Mean± SD**	**Mean± SD**	**Mean ± SD**	**Mean ± SD**	**Mean ± SD**	**Mean ± SD**	**Mean ± SD**	
FBG (mg/dl)	86.8±6.8	157±9.1	223.2±31.5	288.8±15.5	193.1±19.9	225.4±43.2	216.6±20.4	*0.001
Total Cholesterol(mg/dl)	114±10.7	159±15.4	136.9±26.6	174.4±14.3	224.9±40	223.8±48.9	240.3±47.8	*0.001
TG (mg/dl)	93.8±14.2	97.3±16.8	110.1±11.5	100.7±14.1	137.9±61.2	125.9±53.1	203.2±51.4	*0.001
HDL (mg/dl)	50.6±6.2	48.7±6.2	49±5.3	49.4±4.4	43.8±13.4	46.86±9.4	45.1±13.1	0.2
LDL (mg/dl)	45.4±8.7	91.6±14.7	103.9±12.5	104.8±16.1	153.6±41.2	151.6±45.3	115±50.8	*0.001
HBA1C%	4.8±0.3	6.9±0.2	10.1±0.9	8.6±1.0	9.1±1.2	8.8±1.2	9.2±1.1	*0.01
SCr (mg/dl)	0.7±0.3	0.8±0.1	0.59±0.12	0.8±0.2	1.1±0.3	2.5±1.3	6.8±2.5	*0.001
ACR (mg/g)	18.1±4.1	20.3±3.4	20.1±4.6	21.4±3.8	154.4±57.4	1500.5±606	-	*0.001
CypA (ng/ml)	0.34±0.06	0.32±0.07	2.8±0.4	4.8±0.8	8.6±0.8	17.2±2.1	28.8±4.9	*0.001
eGFR (ml/min)	103.9±9.1	104.6±6.2	142.3±16.3	95.9±11.4	75.5±24.5	32.4±16.3	8±2.5	*0.001

Kruskal-Wallis test between DN stages for quantitative variables and chi-square test used for qualitative variables.

Table (2); Showing laboratory investigations of the study population

Statistical significant difference when P-value<0.05

FBG= fasting blood glucose, HDL=high density lipoproteins, TG= triglyceride, LDL=low density lipoproteins SCr= serum creatinine, eGFR=estimated glomerular filtration rate

Variables	CypA		ACR	
	R	P value	R	P value
Age(years)	0.6	*<0.001	-0.02	0.8
Duration of DM (years)	0.8	*<0.001	0.2	0.06
BMI (kg/m2)	0.2	*0.01	0.2	*0.01
SBP (mmHg)	0.8	*<0.001	0.4	*<0.001
DBP (mmHg)	0.9	*<0.001	0.4	*<0.001
FBG (mg/dl)	0.3	*0.001	0.1	0.1
HBA1C%	0.4	*<0.001	0.2	*0.008
SCr (mg/dl)	0.9	*<0.001	0.06	0.6
Total Cholesterol (mg/dl)	0.6	*<0.001	0.3	*<0.001
TG (mg/dl)	0.6	*<0.001	0.05	0.6
HDL (mg/dl)	-0.1	0.2	-0.1	0.2
LDL (mg/dl)	0.5	*<0.001	0.3	*<0.001
eGFR (ml/min)	-0.9	*<0.001	-0.4	*<0.001
Pearson correlation coefficient test (R)				

Table (3); Correlation between CyPA and ACR and other study variables Statistical significant difference when P-value<0.05

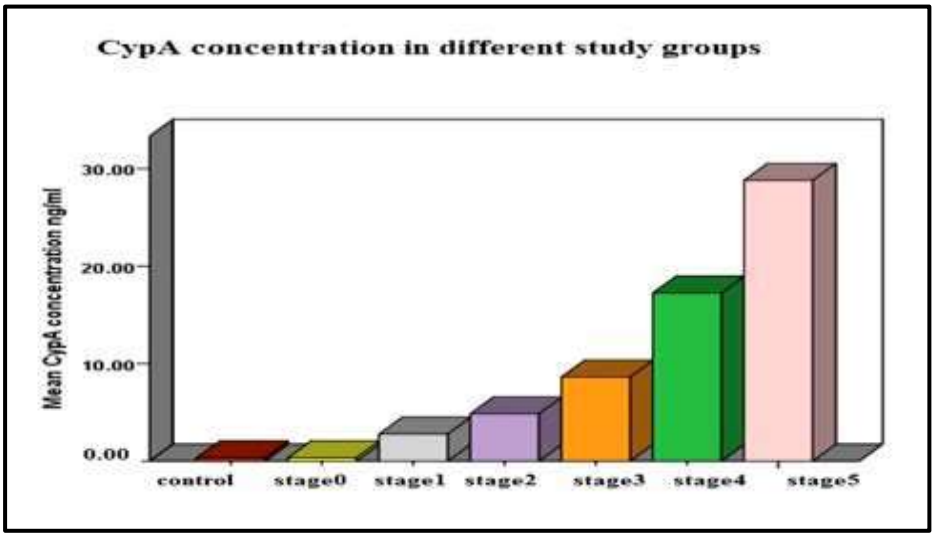

Figure (1); Showing CypA concentration in all study groups

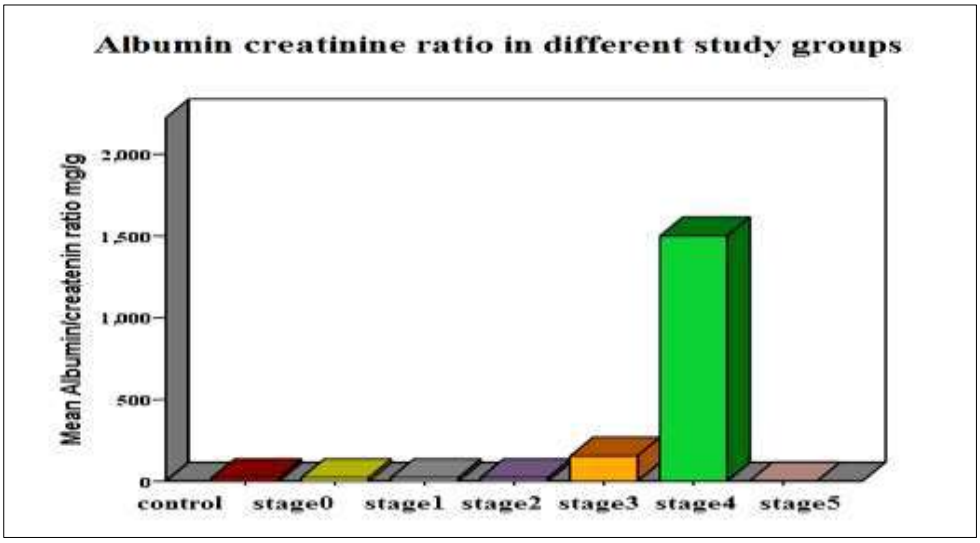

Figure (2); Showing ACR in all study groups

CKD stages	No CKD	Stage1	Stage2	Stage3	Stage4	Stage5	P-value
	Mean± SD	Mean± SD	Mean± SD	Mean± SD	Mean± SD	Mean± SD	
CypA (ng/ml)	2.1±1.9	8.4±0.6	10.6±2.8	12.3±4.4	17.8± 2.4	28.8±4.9	*<0.001
eGFR (ml/min)	111.7± 21.3	100.2± 9.6	78.5±11.5	49.1±7.4	19.3± 2.6	8±2.5	*<0.001
Kruskal-wallis test							

Table (4); Correlation between CypA and eGFR in chronic kidney disease (CKD) stages
*Statistical significant difference when P-value<0.05

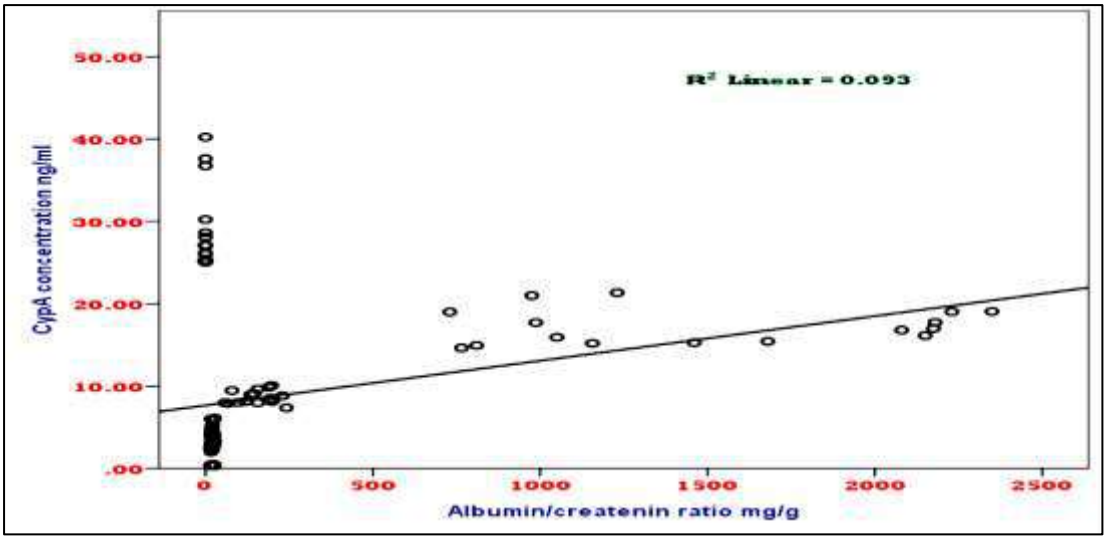

Figure (3); Showing linear regression between serum CypA and ACR among study groups
-By increasing ACR **1mg/g**, the concentration of serum CypA increased by **0.01ng/ml** and **R^2** linear was **0.093**

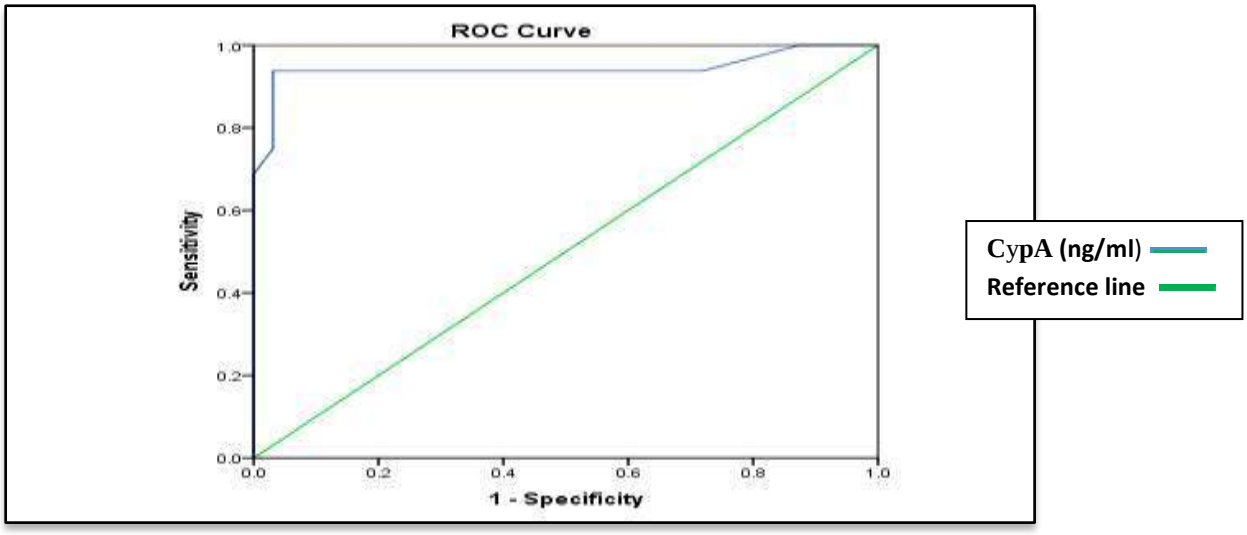

Figure (4) Receiver Operating Characteristic (ROC) curve analysis for cypA

Area under curve	0.943
Standard Error	0.030
95% Confidence Interval	0.911 – 1.000
P- value	**0.001***
Cut-off	0.3900ng/ml
Sensitivity (%)	93.8%
Specificity (%)	81.2%

Table (5): Illustrate the ROC curve of serum cyclophilin A

By determining the area under the curve and by comparing serum CypA concentration in the patients having diabetes without nephropathy (stage0) or control group with patients in stage1 DN, the concentration of serum cyclophilin A which can diagnose the first stage of diabetic nephropathy is 0.3900 ng/ml with a significant P-value =0.001 with sensitivity =93.8%, specificity =81.2% and with area under curve=0.943. As a result, serum cyclophilin A can serve an earlier diagnostic marker for diabetic nephropathy than albumin/creatinine ratio with high specificity and sensitivity.

6-DISCUSSION

The present study aimed to use serum cyclophilin A as a new biomarker for diabetic nephropathy during a case control study of 112 subjects [56males (50%) and 56 females(50%)] divided into three groups (16healthy control subjects, 16 diabetic patients with no evidence for nephropathy (stage0) and 80 diabetic nephropathy patients divided into five stages) as well as ***Tsai et al.***[8] study who used urinary cyclophilin A as a new biomarker for diabetic nephropathy through a Cross-Sectional study of 120 subjects (20 healthy control subjects and 100subjects with diabetic nephropathy divided into five stages).

Kidney function parameters such as serum creatinine, eGFR, ACR were measured in this study, serum CyPA was measured as a related kidney function parameter, other variables represented as risk factors for DN such as fasting blood glucose, lipid profiles (total cholesterol, triglyceride, HDL and LDL), hemglobinA1C, body mass index and blood pressure were measured.

In this study, there was a positive correlation between serum CyPA and age among study populations with (p-value **<0.001)** also, with the duration of diabetes mellitus with p-value **<0.001**. As a result, the greater age and the longer duration of diabetes mellitus can accelerate DN, supported by ***Viswanathan et al.***[9].

This study showed that there was statistical significant difference in serum CyPA among study groups (p-value=0.001**)** being higher in stage1 DN than the control group and stage0. The main values of serum CypA in control group was (0.34±0.06), in stage0 (0.32±0.07), in stage1 DN (2.8±0.4), in stage2DN (4.8±0.8), in stage3 DN (8.6±0.8), in stage4 DN (17.2±2.1) and in stage5 DN (28.8±4.9). Moreover, there was no statistical significant difference in serum CypA level between stage0 and control group. As a result serum CypA increased significantly early in stage1 DN which may possess more sensitive marker for DN. This is compatible with ***Tsai et al.***[8] study in which urinary CypA indeed increased

significantly in stage 2 DN and its increase persisted throughout the later stages.

There was statistical significant difference in HBA1C among study groups (P-value=0.01) that was compatible with ***Zeng et al.***[10], moreover, there was statistical significant difference between CyPA and the glycemic status (between CyPA and HBA1C with p-value<0.001 and between CyPA and FBG with p-value 0.001) that was in agreement with ***Ramachandran et al.*** [11] who found statistical significant difference between CypA with each of FBG (p-value< 0.01) and HBA1C (p-value= 0.019). In this respect, there was statistical significant difference between ACR and HBA1C with p-value =0.008, this finding supported by ***Idowu et al.*** [12], the increase in serum glucose levels and HBA1C in this study might be explained by increasing the risk of diabetic nephropathy progression which contributes to beta cell destruction in type II diabetes then increases diabetic complications. This finding not agreed with ***Wu et al.***[13] who not found statistical significant difference in HBA1C among study groups with (p-value=0.433).

In the current study there was statistical significant difference between CyPA and serum creatinine (p-value=**0.001**), this finding supported by ***Tsai et al.***[8] and there was no statistical significant difference between ACR and serum creatinine (p-value=**0.6**), as CyPA elevated before the obvious elevation in serum creatinine these results can highlight that CyPA may be more appropriate clinical marker in the monitoring of early stages of DN and CKD progression rather than ACR or serum creatinine.

The present study showed that eGFR having statistical significant difference among study groups (p-value=**0.001**) due to the pathologic mechanism of DN; the healthy control and stage0 have normal renal function (normal GFR), although stage 1DN subjects have the highest GFR (glomerular hyperfiltration) then reduced to normal range in stage 2 DN then progressive decline occurred in the other stages of DN reaching to ESRD at which GRF (**<15ml/min/1.73 m²)** due to progression of DN, that was in agreement with ***Doi et al.*** [14].

The present results showed that there was a positive correlation between serum CypA and severity of albuminuria When ACR increased by 1mg/g, the concentration of serum CypA increased by 0.01ng/ml that was compatible with ***Tsai et al.***[8] study which had statistical significant difference (p= 0.007) between urinary CypA with both albuminuric and non-albuminuric patients and proved that when ACR increased by 1 mg/g, the concentration of urinary CypA increased by 0.030 ng/ml.

From the current results, increasing albuminuria was actually relatively late in early-stage of DN because the first obvious increasing in ACR was in the stage 3DN. The main values of ACR in different study groups were; in control group **(18.1±4.1**), in stage0 (**20.1±3.4**), in stage1 (**20.1±4.6))**, in stage2 **(21.4±3.8)**, in stage3 **(154.4±57.4)** and in stage 4 **(1500.5±606)**, so ACR was not sensitive enough to detect early stages of DN, some patients have renal pathological changes without microalbuminuria and it can be detected in another non-DM related nephropathy, such as retinopathy and congestive heart failure so it also lacks specificity for DN ***Zachwieja et al.***[15] .

7-CONCLUSION

In the present study serum CypA concentration was higher in DN group rather than the control group and stage (0). The first obvious increase in serum CypA concentration was in stage 1 DN before the elevation of albuminuria (the trade marker which elevated in stage 3 DN) with high specificity and sensitivity so, serum CypA can be used as an earlier biomarker for DN than albuminuria.

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