Original Article

Study of the Association of Serum Cystatin C and Plasma Fibronectin with Diabetic Nephropathy in Patients with Type 2 Diabetes Mellitus.

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Abstract:

Background: Diabetic nephropathy is the most frequent cause of end-stage renal disease (ESRD), thus many markers was studied for early diagnosis of diabetic nephropathy. This study was done to evaluate clinical usefulness of serum cystatin C levels and plasma fibronectin for early detection of renal impairment in patients with type 2 diabetes and to evaluate the association between serum cystatin C and plasma fibronectin with albuminuria. **Methods:** A prospective, randomized, double-blinded study was performed on 50 type2 diabetic patients divided into two groups, groub1 included 25 type2 diabetic patients with nephropathy and group2 include 25 type2 diabetic patients without nephropathy as a control group. Serum cystatin C, plasma fibronectin, Serum creatinine, serum lipids, glycosylated hemoglobin and urine analysis included albumin creatinine ratio were measured. GFR was estimated by Cockcroft and Gault formula. **Results:** The study showed a statistically significant difference between the two studied groups according to serum cystatin C for early detection of diabetic nephropathy (P<0.001). On the other hand according to plasma fibronectin there was no statistically significant difference between the two studied groups (P=0.960). Regarding albuminuria there was a significant correlation between cystatin C and albumin creatinin ratio in diabetic nephropathy group (P<0.001) but there was no significant correlation with plasma fibronectin (P=0.334). **Conclusion:** Serum cystatin C was significantly higher in albuminuric type2 diabetic patients compared to normoalbuminuric patients and can be also used as an early marker of of diabetic nephropathy. More studies needed to be applied on plasma fibronectin as our study results that it could be a weak marker for early diabetic nephropathy.

Keywords: Diabetic nephropathy, Cystatin C, Fibronectin, Albuminuria.

Introduction:

Diabetic nephropathy (DN) is one of the microvascular complications of diabetes, it is characterized by a progressive increase in proteinuria and decline in GFR with a high risk of cardiovascular morbidity and mortality.[1-3] Diabetic nephropathy occurs in 20–40% of patients with diabetes and is a leading cause of ESRD worldwide. Patients with persistent albuminuria (30–299 mg/24 h) who progress to more significant levels (≥300 mg/24 h) are likely to progress to ESRD[4-7]. The terms microalbuminuria (30 – 299 mg/24 h) and macroalbuminuria (≥300 mg/24h) will no longer be used , but rather referred to as persistent albuminuria at levels (30 – 299 mg / 24 h) and levels ≥ 300 mg/24 h , with normal albumin excretion defined to be less than 30 mg / 24 h [8,9].Screening for increased urinary albumin excretion can be performed by measurement of the albumin to creatinine ratio in a random spot collection[10].Early diabetes is heralded by glomerular hyperfiltration and an increase in glomerular filtration rate (GFR). This is believed to be related to increased cell growth and expansion in the kidneys, possibly me diated by hyperglycemia itself. Increased albumin

excretion typically occurs after 5 years in type 1 diabetes. Overt nephropathy, with urinary protein excretion higher than 300 mg/day, often develops after 10 to 15 years. End stage renal disease (ESRD) develops in 50% of type 1 diabetic with overt nephropathy within 10 years[11]. Type 2 diabetes has a more variable course. Patients often present at diagnosis with increased albumin excretion because of delays in diagnosis and other factors affecting protein excretion. Without intervention, approximately 30% progress to overt nephropathy and, after 20 years of nephropathy, approximately 20% develop ESRD. Because of the high prevalence of type 2 compared with type 1 diabetes, however, most diabetics on dialysis are type 2 diabetics [12].

Cystatin C, a cysteine protease inhibitor, is freely filtered by the renal glomeruli, metabolized by the proximal tubule and identified as a promising marker of chronic kidney disease. Cystatin C is produced at a constant rate by nucleated cells and released into blood stream with a half-life of 2 hr. Its concentration is almost totally dependent on GFR.[13] Some studies have demonstrated that serum cystatin C is an early renal marker in diabetic patients, but not all studies have done so[14-16].

Fibronectins are large glycoproteins found in plasma, in extracellular matrix, and on cell surfaces. They promote cell-cell and cell-matrix interactions and thus play a role in tissue construction and reconstruction, the intravascular accumulation of cellular fibronectin reflects injury to blood vessels. Vessel wall damage with characteristic endothelial extracellular matrix changes has a role in development of microvascular complication and nephropathy in diabetic patient[17, 18].

Aim of the Study:

The aim of the study is to compare the level of serum Cystatin C and plasma fibronectin in patients with and without diabetic nephropathy and to Study the relationships between Cystatin C, fibronectin and other markers of diabetic nephropathy.

Patients and Methods:

After approval of Local Ethics Committee and with written informed consent, the present study was carried out in Alexandria University Hospital on 50 type2 diabetic patients divided into two subgroups: Subgroup I: 25 diabetic patients with diabetic nephropathy (Stage I to stage V). Subgroup II: 25 diabetic patients without diabetic nephropathy (i.e. normal GFR and urinary albumin creatinine ratio ‹ 30 mg / 24) as a control group. Evaluation of patients was carried out through proper history taking, clinical examination and routine laboratory investigations. Regarding cystatin C analysis, after 30 minutes incubation and washing, polyclonal anti-human cystatin C antibody, conjugated with horseradish peroxidase (HRP) is added to the wells and incubated for 30 minutes with captured cystatin C. Following another washing step, the remaining HRP conjugate is allowed to react with the substrate solution (TMB). The reaction is stopped by addition of acidic solution and absorbance of the resulting yellow product is measured. The absorbance is proportional to the concentration of cystatin C. A standard curve is constructed by plotting absorbance values against concentrations of cystatin C standards, and concentrations of unknown samples are determined using this standard curve. Regarding fibronectin analysis, fasting overnight venous blood sample (about 5 ml) was drawn from each control and diabetic nephropathy individuals. The blood was collected into precooled plastic or siliconized tubes containing 3.2% buffered citrate in a ratio of 1:10 (blood: anticoagulant). Centrifuge within 30 minutes after the puncture at 2000g for 10min. at 4°C. Pipette off the plasma. Store as aliquots at temperature below -30°C.

Statistical Analysis:

Data were fed to the computer and analyzed using IBM SPSS software package version 20.0.[19]

Qualitative data were described using number and percent. Quantitative data were described using range (minimum and maximum), mean, standard deviation and median. Comparison between different groups regarding categorical variables was tested using Chi-square test. The distributions of quantitative variables were tested for normality using Kolmogorov-Smirnov test, Shapiro-Wilk test and D'Agstino test, also Histogram and QQ plot were used for vision test. If it reveals normal data distribution, parametric tests was applied. If the data were abnormally distributed, non-parametric tests were used. For normally distributed data, comparison between two independent populations were done using independent t-test. Correlations between two quantitative variables were assessed using Pearson coefficient. For abnormally distributed data, comparison between two independent populations were done using Mann Whitney. Correlations between two quantitative variables were assessed using Spearman coefficient. Significance of the obtained results was judged at the 5% level.

Results:

Fifty type2 diabetic patients were enrolled in the study. All patients completed the study. Group I included 25 type2 diabetic patient with nephropathy and Group II 25 type2 diabetic patient without nephropathy. There was no significant difference in anthropometric measures (weight, height and BMI) between the two groups). There was significant difference in renal functions (creatinin, urea and albumin creatinin ratio) between the two groups. There was significant difference in GFR between the two groups. There was significant difference in cystatin between the two groups. There was no significant difference in fibronectin between the two groups. There was significant difference between Cystatin C and Albumin creatinine ratio in diabetic nephropathy group ($p<0.001$). With no significant difference between fibronectin and albumin creatinine ratio in diabetic nephropathy group ($p=0.334$).

Table (I): Comparison between the two studied groups according to anthropometrics

	Diabetic Nephropathy (n = 25)	**Control (Diabetic only) (n = 25)**	**T**	**P**
Weight (kg)				
Min. – Max.	69.0 – 97.0	70.0 – 90.0		
Mean ± SD.	80.64 ± 8.24	78.52 ± 5.74	1.056	0.296
Median	80.0	79.0		
Height (cm)				
Min. – Max.	165.0 – 184.0	160.0 – 132.0		
Mean ± SD.	174.28 ± 5.37	95.20 ± 17.61	1.045	0.301
Median	172.0	95.0		
BMI (kg/m2)				
Min. – Max.	24.16 - 29.41	21.13 - 30.10		
Mean ± SD.	26.49 ± 1.58	26.42 ± 1.91	0.140	0.890
Median	25.95	26.23		

t: Student t-tes.

Table (II): Comparison between the two studied groups according to renal function

	Diabetic Nephropathy (n = 25)	Control (Diabetic only) (n = 25)	Test of sig.	P
Creatinin (mg/dl)				
Min. – Max.	1.20 – 12.0	0.10 – 1.40		
Mean ± SD.	4.35 ± 2.82	0.88 ± 0.29	Z=6.001*	<0.001*
Median	3.0	0.90		
Urea (mg/dl)				
Min. – Max.	36.0 – 214.0	20.0 – 50.0		
Mean ± SD.	110.16 ± 43.70	38.60 ± 8.23	t=8.046*	<0.001*
Median	105.0	40.0		
ALB Creatinin(mg/dl)				
Min. – Max.	60.0 – 2597.0	7.60 – 133.0		
Mean ± SD.	417.46 ± 510.97	19.85 ± 23.88	Z=5.968*	<0.001*
Median	265.0	16.50		

t: Student t-test

Z: Z for Mann Whitney test

*: Statistically significant at p ≤ 0.05

Table (III): Comparison between the two studied groups according to eGFR

	Diabetic Nephropathy (n = 25)	Control (Diabetic only) (n = 25)	Z	P
GFR (ml/ min)				
Min. – Max.	5.83 – 62.20	52.0 – 129.20		
Mean ± SD.	27.25 ± 17.23	93.43 ± 20.19	6.005*	<0.001*
Median	27.20	89.50		

Z: Z for Mann Whitney test

*: Statistically significant at p ≤ 0.05

Table (IV): Comparison between the two studied groups according to cystatin C

	Diabetic Nephropathy (n = 25)	Control (Diabetic only) (n = 25)	T	P
Cystatin C (0.62-1.11ng/L)				
Min. – Max.	0.95 – 2.58	0.66 – 1.27		
Mean ± SD.	1.55 ± 0.41	0.95 ± 0.18	6.812*	<0.001*
Median	1.47	0.96		

t: Student t-test

*: Statistically significant at p ≤ 0.05

Table (V): Comparison between the two studied groups according to fibronectin

	Diabetic Nephropathy (n = 25)	Control (Diabetic only) (n = 25)	T	P
Fibronectin (25 - 40µg/ml)				
Min. – Max.	71.0 – 129.0	72.0 – 132.0		
Mean ± SD.	94.96 ± 15.66	95.20 ± 17.61	0.051	0.960
Median	94.0	95.0		

t: Student t-test

*: Statistically significant at p ≤ 0.05

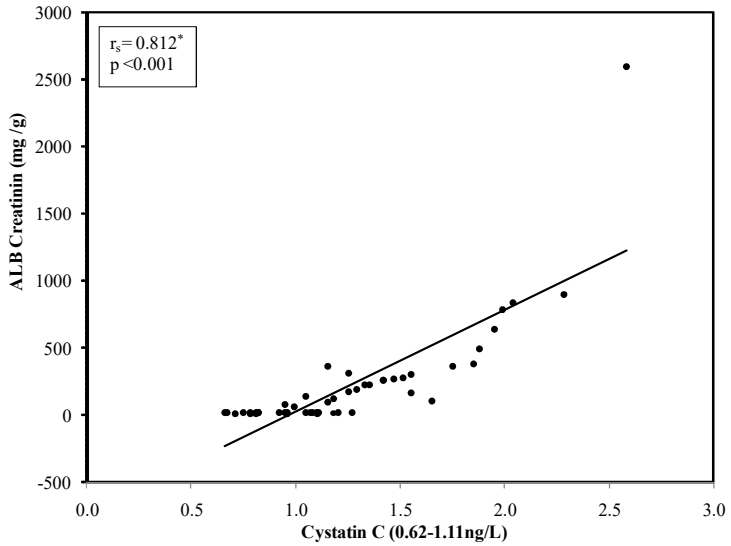

Figure (1): Correlation between cystatin C and ALB creatinine

Discussion:

Diabetic nephropathy (DN) is one of the most common microvascular complications of DM, greatly affecting the life quality and survival of the patients. As global prevalence of diabetes is steadily increasing, the number of patients with DN is expanding day by day. DN is now the leading cause of ESRD, a disease that I s described as a medical catastrophic of worldwide dimensions.[20] Morphological changes are known to start earlier DN than laboratory abnormality. Also some patients with albuminuria have normal renal structure, while some normoalbuminuric diabetic have well established nephropathic lesions. Also albumin excretion rate is a predictor of renal disease in hypertension and cardiovascular diseases, so it is not a sensitive marker for DN.[21] Measuring GFR is the best functional parameter in renal disease using creatinine clearance. This requires 24h urine collection and blood sample and measure creatinine level in blood and urine and volume of urine. There are several factors that may interfere with the accuracy of the test like incomplete collection of urine. Other methods of assessment of GFR are Cockcroft-Gault formula or MDRD but also there were not accurate methods.[22] Thus, we are still in need of identifying earlier marker early markers of DN needed to be identified. It has been reported that a decline in the renal function of patients with diabetes was not always

accompanied by an increased AER. To overcome these limitations, many clinicians additionally used creatinine in evaluating such patients. However, serum creatinine also depends on creatinine production, extrarenal elimination and tubular handling. Therefore, other biomarkers for estimation of renal function have been searched for, and one of them can be cystatin C. In our study, we measured the serum level of cystatin C and plasma level of fibronectin in different stages of DN and comparing them with ACR and eGFR to detect the usefulness of using those markers to diagnose DN and detect the progression of the disease in T2DM patients.

Data presented in our study dealt with 25type 2 diabetic patients with nephropathy (stage I to stage V). as cases and 25 diabetic patients without nephropathy (i.e. normal GFR and urinary albumin creatinine ratio (30 mg / 24) as a control group.

In our study, there was no significant correlation between serum cystatin C and the age in the control group and the patients group (P=0. 0.791).This may reflected the important of cystatin C to use as renal function test in elderly or even in very young patients. In contrast to serum creatinine is highly affected by the age. This data matched with Filler G et al[23] who found that measuring of cystatin C not affected by the age.

In contrast, Christensson AG et al[24] reported that there was significant positive correlation noticed between serum cystatin C and age.

In our study, there was no significant correlation between serum cystatin C and the sex in the control group and the patients groups (P=0.499).

This data matched with Erlandsen JE et al[25] and Uhlmann EJ et al[26] whom reported that there was no significant difference as regarding measuring cystatin C in both gender.

In contrast, some authors like Croda-Todd et al[27] and Finney H et al[28] found that there were statistically significant differences as regarding measuring cystatin C in adults' males and females and was higher in male gender than female.

From the previous data, cystatin C is another marker of renal function that has been proposed as potentially superior to serum creatinine level for estimating renal function, because it is thought to be produced at a constant rate by most nucleated cells. Moreover, cystatin C production has been reported to be not affected by age, gender, or muscle mass. In contrast serum creatinine level is commonly used to estimate renal function. Serum creatinine is not only determined by its renal excretion, but also by its production in muscular tissue, which is dependent on age, weight, and gender. Therefore, when using serum creatinine level to estimate renal function, one needs to adjust for these factors.[29]

In our study, there was a statistically significance difference between patients group and control group regarding blood urea (P<0.001). Blood urea is not an accurate marker for measuring renal function. It is filtered by glomerulus and reabsorbed by the renal tubule. Concentration of urea in serum could vary with diet, also can increased in dehydration associated with poor glycemic control that cause polyuria.

Regarding serum creatinine in diabetic nephropathy group,it ranges from 1.20 – 12.0 with a mean 4.35 ± 2.82 and in control group without nephropathy it ranges from 0.10 – 1.40 with a mean 0.88 ± 0.29.So in our study there was a statistically significant difference between patients groups and control group as P value <0.001 .

In contrast to our results, Vervoot G et al[30] showed significant decreases in serum creatinine concentrations among patient group compared to control group. These

results may be explained based on glomerular hyperfiltration that may develop at initial stages of the DN.

In our study, either serum creatinine or blood urea have no significance correlation with serum cystatin C.

The same result was reported by Plebani M, Dall'Amico R, Mussap M, et al whom reported that Cystatin C and creatinine are probably two independent markers of GFR.

In contrast to our result, Tian et al[31] reported that there was significant positive correlation between serum cystatin C and serum creatinine suggesting that serum cystatin C increased similar to serum creatinine for measuring renal function in diabetic patients.

In our study, there was a statistically significance difference between patients group and control group regarding albumin creatinine ratio (P<0.001). As classical evaluation of DN includes appearance of albuminuria, decreased creatinine clearance and increased serum creatinine.

Albuminuria expresses underlying endothelial dysfunction as presence of chronic hyperglycemia cause disruption of the endothelial permeability through production and activation of mediators such as ROS, VEGF and proinflammatory cytokines. This disturbance of endothelial cell podocyte communication contributes to and amplifies the endothelial lesions, leading to albuminuria.

With more progression, there is increase in urinary albumin excretion due to underlying inflammatory process with excess production of growth factor, deposition of ECM with subsequent interstitial fibrosis, and glomerulosclerosis leading to renal function deterioration. In contrast to control group with subsequent less or insignificant inflammatory process in the kidney, the level of ACR did not correlate significant with renal function.

In our study, there was strong positive correlation between serum cystatin C and ACR in patients group (P<0.001) and there was no correlation in the normoalbuminuric control group (P=0.517).

In agreement with these results, Mojiminiyi et al[32] found that cystatin C was significantly higher in patients with DN than in normoalbuminuric diabetic patients, which can be explained in that there is already impaired renal function in DN patients.

In addition, Yang et al[33] reported that serum cystatin C concentration increased significantly in patients from normoalbuminuric to patients with increase albumin excretion rate.

In our study, we used Cockcroft-Gault formula for measuring eGFR. There was significant difference between the patients groups and control group (P<0.001).

The current Kidney Disease Outcomes Quality Initiative (K/DOQI) guidelines advocate creatinine based equations for estimating GFR to identify patients with potential kidney disease and to classify them into different stages on the basis of these results. These stages also include individuals with normal or near-normal GFR. Such stratification requires an accurate and precise measurement of GFR that is inexpensive, reliable, and widely available.[34] In our study, there was a significant negative correlation between eGFR with serum cystatin C (P<0.001). As cystatin C is produced at a constant rate by nucleated cells and released into the blood stream with a half-life of ~2 h and is freely filtered and almost completely taken up and degraded, but not secreted, by proximal tubular cells, so with the occurrence of pathological changes in diabetic kidney the filtration capacity is decreased with subsequent retention of cystatin C and increased serum level.

The result of previous studies of the role of cystatin C in detecting early renal failure in

diabetic patients was contradictory. Some authors showed that cystatin C was more effective than creatinine in detecting initial reduction of GFR in T2DM as well as in T1DM as Mussap M et al[34], Xia LH et al[35], and Harmoinen et al[36] showed that serum cystatin C was more sensitive than serum creatinine for estimation of GFR in T2DM patients and Tan et al[37] showed the same in T1DM patients.

In contrast to these result, Oddoze et al[38] found that serum creatinine was better than serum cystatin C for the estimation of GFR in albuminuric and proteinuric diabetic patients. Oddoze et al selected a heterogeneous group of type 1 and type 2 diabetic patients.

In general, unlike healthy subjects, diabetic patients are continuously exposed to the various metabolic and hemodynamic risks associated with this disease. Recent studies have mainly focused on tubular damage, which is known to correlate with acute kidney injury in patients with DN. (39) Some cross-sectional studies have reported that several tubular markers increase more in diabetic patients than in healthy controls, and this correlated with the severity of albuminuria.[40]

In our study, there was statistically significant relationship of serum cystatin C in patients groups in comparison to control group (P<0.001).

In my opinion the strong correlation between serum cystatin C in the patients group was due to decrease eGFR in this group in comparing to normoalbuminuric control group. From our study, we can consider that serum cystatin C can be used as albuminuria for detection of DN.

In contrast to our study, Jeon YK[41] found that in normoalbuminuric patients, the cystatin C levels of serum was significantly increased in patients with GFR ≤ 60 mL/min/1.73 m2 than those with GFR > 60 mL/min/1.73 m2. It was thought that this increment was probably due to the tubular phase before glomerular manifestation. He suggested that the cystatin C levels of serum was related to subclinical tubular impairment and can be an earlier measurable marker of renal involvement before onset of albuminuria.

Another marker which can be increase in type 2 diabetic patients is fibronectin, and in this study we show the relation between plasma fibronectin and other markers of diabetic nephropathy. Plasma levels of circulating cellular fibronectin could reflect matrix changes and hence vessel wall damage in patients with diabetes, high levels of plasma fibronectin may be the effect of a widespread endothelial injury of small vessels, which early involves the renal glomerular microvasculature and may affect urinary albumin excretion and development of nephropathy.

In our study, There was statistically significant correlation between plasma fibronectin and (age and HbA1c), (P=0.016, <0.001 respectively).

There was no statistically significant correlation between plasma fibronectin and ACR, eGFR and s.Cr (P=0.334, 0.736, 0.152 respectively).

In agreement with our study, Takahashi et al.[42] found no relation between plasma fibronectin in diabetic patients and albuminuria.

On the other hand, DeGiorgia et al.[43] found a significant correlation between the PF with non-insulin dependent diabetes mellitus. Although Skrha et al. did not evaluate the intact plasma fibronectin; they found a positive correlation between the plasma free N-terminal fibronectin 30-kDa domain and albuminuria.

More studies needed to be applied on plasma fibronectin as our study results that it could be a weak marker for early diabetic nephropathy.

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