URINARY SE-CADHERIN AND PLASMA CYSTATIN C AS NOVEL BIOMARKERS OF DIABETIC NEPHROPATHY

Mohammad H. Aly, Neveen G. El-Antouny, Ghada E. Amr*, George E. Shaker
Internal Medicine& Clinical Pathology Departments, Faculy of Medicine, Zagazig University*

ABSTRACT

Background and aim : Diabetes is a major cause of chronic kidney disease (CKD). Urine albumin and estimated glomerular filtration rate (eGFR) are the two key markers for chronic kidney disease (CKD). The aim of our work was to explore the possibility of plasma cystatin C and urinary sE.cadherin as useful biomarkers for early detection of diabetic nephropathy (DN).

Methods: - A total number of 80 subjects were included and were classified into three main groups. Group I (20) normal subjects with no history of DM, hypertension or other diseases and with albumin / creatinine (Alb / Cr) ratio below 30 mg/ g. Group II (30) type 2 diabetic patients with Alb / Cr ratio below 30 mg/ g. Group III (30) type 2 diabetic patients with Alb / Cr ratio between 30 and 300 mg/ g. The latter two groups were divided in two sub groups A and B according to GFR by MDRD: normal (≥ 90 ml /min / 1.73 m2) and < 90 ml /min / 1.73 m2. All subjects underwent urine analysis, complete blood picture, liver function tests, kidney function tests, INR, fasting plasma glucose level, HbA1c, lipid profile, abumin/creatinine ratio, pelvi-abdominal ultrasound, plasma Cystatin C and urinary human sE-Cadherin.

Results: - Plasma cystatin C and urinary sE.cadherin/ cr levels were increased with micro-albuminuria. Also, plasma cystatin C and urinary sE.cadherin/ cr levels were significant between normoalbuminuric subjects with GFR ≥ 90 mL/min/1.73 m2 calculated by the MDRD equation and those below 90 mL/min/1.73 m2 being higher in the later. In multivariate logistic analysis, plasma cystatin C level was the only independent factor associated with eGFR < 90 mL/min/1.73m2 estimated by MDRD equation in patients with normoalbuminuria. There are high significant positive correlations of plasma cystatin C with age, total cholesterol unlike urinary sE.cadherin/ cr, but both had positive correlations with serum Cr, blood urea and Alb / Cr ratio and negative correlations with GFR.

Conclusion and Recommendations:- Plasma cystatin C and urinary sE.cadherin levels could be useful markers for detection of microalbuminuria and renal impairment in type 2 diabetic patients with normoalbuminuria.

Keywords: diabetic nephropathy, glomerular filtration rate, cystatin C, sE.cadherin.

INTRODUCTION

Diabetes is one of the most common causes of end-stage renal disease (ESRD). The classical definition of diabetic nephropathy is a progressive rise in urine albumin excretion, coupled with increasing blood pressure, leading to declining glomerular filtration and eventually end stage kidney failure **(1).**

There is an increased urinary microalbumin levels with increased duration of diabetes **(2).**

The definition of microalbuminuria is a urine albumin/ creatinine ratio (UACR) on a random urine sample of more than 30 mg (but less than 300 mg) of albumin per gram of creatinine. **(3)**.

Diabetic nephropathy occurs as a result of an interaction between hemodynamic and metabolic factors **(4).** Hemodynamic factors that contribute to the development of diabetic nephropathy include increased systemic and intraglomerular pressure, as well as activation of vasoactive hormone pathways including the renin angiotensin system and endothelin **(5)**. These hemodynamic pathways activate intracellular second messengers such as protein kinase C (PKC), Mitogen-activated protein (MAP kinase) **(6)**, nuclear transcription factors such as NF-kB and various growth factors such as the prosclerotic cytokine, TGF-β and the permeability enhancing growth factor, vascular endothelial growth factor, VEGF. Glucose dependent pathways are also activated within the diabetic kidney and result in enhanced oxidative stress and renal polyol formation **(7).**

Progressive renal function decline in diabetes is an early event that occurs in a proportion of patients without increased albumin excretion rate **(8).** The slope of glomerular filtration rate changes over time, which suggests that it is a more proximal marker than microalbuminuria of a person's trajectory toward impaired renal function and ESRD **(9).** Therefore, early renal function decline, rather than microalbuminuria, may be considered as an early marker of the committed process underlying progressive diabetic nephropathy **(10)**.

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 **(11).** Moreover, tubular involvement may precede glomerular involvement because several tubular proteins and

enzymes are detectable even before the appearance of microalbuminuria and a rise in serum creatinine **(12).** Moreover, microalbuminuria is not merely a predictor of diabetic nephropathy but also constitutes an evidence of renal damage. Therefore, other biomarkers for estimation of renal function have been searched for.

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 renal failure **(13).** Cystatin C is produced at a constant rate by nucleated cells and released into bloodstream with a half-life of 2 hr. Its concentration is almost totally dependent on GFR. Other studies have demonstrated that serum cystatin C is an early renal marker in diabetic patients **(14, 15),** but not all studies have done so **(16).**

E-cadherin is a member of the cadherin family of transmembrane adhesion proteins that form adherens junctions (**17).** E-cadherin is a 124-kDa, epithelial specific glycoprotein involved in many cellular processes including adhesion, recognition, signalling, communication, morphogenesis and oncogenesis **(18).** The seldom study that concluded that urinary sE.cadherin could be a novel biomarker for diabetic nephropathy was done by **Jiang et al. (19).**

Therefore, the aim of our study was to explore the possibility of plasma cystatin C and urinary sE.cadherin as useful biomarkers for early detection of diabetic nephropathy.

Patients and Methods:-

This work has been carried out in collaboration between the Internal Medicine, and Clinical pathology departments, Faculty of Medicine, Zagazig University, during the period from May 2012 to May 2013.

*** Subjects:**

A total number of 80 subjects were included and were classified into three main groups:

1) Group I:

Which included 20 volunteers with no history of DM, hypertension or other diseases and with normal Alb / Cr ratio (11 males and 9 females) (7 smokers and 13 non-smokers).Their age ranged from 40 years to 76 years with a mean values + SD of 59.8 ± 11.26 years. Their body mass index ranged from 26.20 to 35.30 with a mean values + SD of 29.99 ± 2.78 Kg/ m².

2) Group II:

Which included 30 type 2 diabetic patients with albumin / creatinine ratio (Alb / Cr ratio) below 30 mg/ g (12 males and 18 females) (10 smokers and 20 non-smokers). Their age ranged from 40 years to 77 years with a mean values + SD of 61.63 ± 11.11 years. Their body mass index ranged from 22.6 to 34.9 Kg/ m² with a mean values + SD of 30.06 ± 2.87 Kg/m². Their GFR according to MDRD formula ranged from 57 to 119 ml /min / 1.73 m^2 with a mean values + SD of 87.6 ± 12.54 ml /min / 1.73 m^2.

This group is further subdivided into 2 groups according to GFR:

(A) Normal GFR (17 subjects) according to MDRD formula ranged from 90 to 119 ml /min / m^2 with a mean values + SD of 99.94± 25.90 ml /min / 1.73 m^2.

(B) Decreased GFR < 90 (13 subjects) according to MDRD formula ranged from 57 to 87 ml /min / m^2 with a mean values + SD of 70.69 ± 8.08 ml /min / 1.73 m^2.

3) Group III:

Which included 30 type 2 diabetic patients with microalbuminuria as Alb / Cr ratio between 30 to 300 mg/ g (15 males and 15 females) (12 smokers and 18 non-smokers). Their age ranged from 45 years to 75 years with a mean values + SD of 61.93 ± 8.91 years. Their body mass index ranged from 23.3 to 39.5 Kg/ m² with a mean values + SD of 31.68 ± 3.84 Kg/ m². Their GFR according to MDRD formula ranged from 58 to 114 ml /min / m^2 with a mean values + SD of 79.73 ± 11.38.

This group is further subdivided into 2 groups according to GFR:

(A) Normal GFR (11 subjects) according to MDRD formula ranged from 91 to 114 ml /min / m^2 with a mean values + SD of 96.54 ± 7.62 ml /min / 1.73 m^2.

(B) Decreased GFR (19 subjects) according to MDRD formula ranged from 58 to 86 ml /min / m^2 with a mean values + SD of 70 ± 8.72 ml /min / 1.73 m^2.

Exclusion criteria were hepatic diseases, renal diseases, heart failure, thyroid diseases, autoimmune diseases, sepsis, inflammatory conditions, and malignancy.

A written consent was taken from all patients and control subjects according to Helsinki guidelines.

*** Methods:**

All subjects of the study were subjected to the following:-

A)Thorough history and full clinical examination.

B) Routine investigations:

They were all done according to the methods applied in the laboratories of zagazig university hospitals and included:

1- Complete blood picture.

2- Liver function tests: serum bilirubin (total and direct), serum albumin, serum ALT and AST.

3- Renal function tests: serum creatinine, blood urea.

4- Coagulation profile: PT, PTT and INR.

5- Urine analysis (for glucose, acetone, protein, pH, bilirubin and leukocytes.

6- Fasting plasma glucose level

7- HbA1c

8- Lipid profile: included serum total cholesterol level, serum triglycerides, HDL- cholesterol and LDL- cholesterol performed.

9-**Calcuation of glomerular filtration rate using MDRD equation (20):**

eGFR (mL/min/1.73 m2) = 175 x $(Scr)^{-1.154}$ x $(Age)^{-0.203}$ x (0.742 if female).

10- **Calcuation of glomerular filtration rate using cockcroft-gault equation (21):**

$$GFR_{Cockcroft} = \frac{(140 - age)x\ weight\ (kg)[\ x\ 0.85\ if\ female]}{72\ x\ serum\ creatinine\ (mg/dl)}$$

11-Calcuation of glomerular filtration rate using cystatin c based equation (22): GFR Dade (Epi) Assay = 76.7 X $cys^{-1.19}$

12-Albumin/Creatinine ratio

The urine creatinine value was divided by 100 to convert mg/dL to g/L and then divide the urine albumin value by the urine creatinine value to express ACR as (mg albumin/g creatinine) **(23).**

ACR (mg/g) = Urine albumin (mg/L) x100 /Creatinine in urine (mg/dl)

13- Abdominal ultrasound

C- Special investigations include:

1- Meaurment of urinary human sE-Cadherin by ELISA:

Specimen collection and preparation:-

First mid-stream urine of the day was aseptically collected, voided directly into a sterile container, centrifuged to remove particulate matter then stored at ≤ -20° C.

The concentration of soluble E-cadherin in urine samples was measured with an ELISA kit.

2- Meaurment of plasma Cystatin C by ELISA:

Six ml of heparinized peripheral venous blood was taken from each subject under complete aseptic conditions and then centrifuged at 3000 rpm for 5 minutes. Samples were separated and stored at -80℃, for measurement of plasma Cystatin C.

Statistical analysis:-

Statistical Package for Social Science (SPSS) version 9.0 and Grafpade program were used for analysis of data. Data were summarized as mean, SD and percentage. Non-parametric (Mann–Whitney U) test was used for analysis of quantitative data, as data were not symmetrically distributed. While the chi-square test was used for analysis of qualitative data, the Kruskal–Wallis H test was done for analysis of more than two quantitative data. Correlation analysis was done by simple Pearson's test. P-value less than 0.05 was considered significant.

RESULTS

Table (1a): Shows Characteristics of metabolic and laboratory parameters in patients with type 2 diabetes

	control	Normo-albuminuria	Micro-albuminuria	F or χ2	P
Number	**20**	**30**	**30**		
Age (years)	**59.8± 11.26**	**61.63± 11.11**	**61.93± 8.91**	**F=0.28**	**NS**
Sex (F/M)	**9/ 11**	**18/ 12**	**15/ 15**	**χ2=1.2**	**NS**
Smokers (% Yes)	**35.0%**	**33.3 %**	**40.0 %**	**χ2=0.3**	**NS**
History of HTN (%Yes)	**0.0%**	**33.3%†**	**50%†**	**χ2=14.06**	**HS**
Duration of DM (years)		**4.06 ± 1.7**	**8.36 ± 1.86‡**	**F=3.7**	**HS**
SBP (mmHg)	**117.75± 6.97**	**132 ± 9.96†**	**136.16 ± 8.37†**	**F=45.46**	**HS**
DBS (mmHg)	**77.5 ± 4.72**	**79.66 ± 6.93**	**81.5 ± 6.03**	**F=2.58**	**NS**
BMI (Kg/ m^2)	**29.99 ± 2.78**	**30.06 ± 2.87**	**31.68 ± 3.84**	**F=2.45**	**NS**
Hb (gm/ dl)	**12.51 ± 0.57**	**11.97 ± 0.89**	**12.25 ± 0.71**	**F=2.08**	**NS**

TLCs (x10³)	**7.05 ± 1.83**	**6.92 ± 1.53**	**6.53 ± 1.47**	**F=0.75**	**NS**
Pletelets (x10³)	**241.5 ± 64.66**	**242.46 ± 55.51**	**241.66 ± 59.29**	**F=0.002**	**NS**
Serum albumin (gm/ dl)	**4.14 ± 0.43**	**4.27 ± 0.29**	**4.27 ± 0.29**	**F=1.89**	**NS**
Total protein (gm/ dl)	**6.47 ± .28**	**6.59 ± 0.42**	**6.33 ± 0.31**	**F=1.86**	**NS**
ALT (I.U/ L)	**28.65 ± 7.7**	**27.4 ± 5.07**	**31.56 ± 6.5**	**F=1.56**	**NS**
AST (I.U/ L)	**31.35 ± 7.46**	**28.7 ± 5.93**	**30.53 ± 6.24**	**F=1.31**	**NS**
FBS (mg/ dl)	**84.35 ± 8.48**	**153.6 ± 9.69†**	**180.36 ± 9.78‡**	**F=634.21**	**HS**
HBA1c	**4.55 ± 0.32**	**7.12 ± 0.3†**	**8.28 ± 0.40‡**	**F=682.4**	**HS**
Serum TG (mg/ dl)	**104.45 ± 25.11**	**159.83 ± 19.36†**	**171 ± 13.54‡**	**F=78.7**	**HS**
Serum cholesterol (mg/ dl)	**163.5 ± 16.31**	**194.33 ± 16.33†**	**210.83 ± 13.83‡**	**F=56.6**	**HS**
HDL (mg/ dl)	**53 ± 7.14**	**45 ± 8.64†**	**37.33 ± 4.86‡**	**F=30.3**	**HS**
LDL (mg/ dl)	**91.5 ± 19.8**	**118 ± 22.9†**	**140.46 ± 16.5‡**	**F=36.4**	**HS**
Serum Cr (mg/ dl)	**0.81 ± 0.1**	**0.84 ± 0.14**	**0.92 ± 0.16‡**	**F=3.56**	**HS**
Blood urea (mg/ dl)	**21.8 ± 2.98**	**21.5 ± 3.57**	**31.66 ± 3.7‡**	**F=77.29**	**HS**
GFR by Cockroft &Gault (ml /min / 1.73 m2)	**104.15 ± 11.26**	**98.16 ± 18.1**	**96.6 ± 17.32**	**F=1.35**	**NS**
GFR by MDRD (ml /min / 1.73 m2)	**90.89 ± 8.81**	**87.6 ± 12.54**	**79.73 ± 11.38‡**	**F=3.98**	**HS**
GFR by cystatin C (ml /min / 1.73 m2)	**93.27 ± 10.68**	**90.51 ± 11.53**	**68.11 ± 7.41‡**	**F=52.72**	**HS**
Albumin/ cr ratio (mg/ g)	**15.1 ± 2.88**	**21.26 ± 4.77**	**198 ± 51.33‡**	**F=300.31**	**HS**
Plasma cystatin C (ng/ ml)	**855.75 ± 76.81**	**879.93 ± 90.46**	**1113.66 ± 99.27‡**	**F=67.75**	**HS**
Urine sE-Cadherin/ cr (ug/ g)	**798.5 ± 89.81**	**880.66 ± 136.24**	**2700.66 ± 282.33‡**	**F=826.23**	**HS**

† Means there's significant difference between control and other group
‡ Means there's significant difference between microalbuminurea and other groups

Table (1b): Shows predictors for microalbuminuria by linear regression

Score	Sig.	
Duration of DM	**47.07**	**<0.001**
HbA1c	**23.53**	**<0.001**
Plasma Cystatin C	**53.12**	**<0.001**
Urinary sE-cadherin/cr	**80**	**<0.001**

Among all factors associated with microalbuminuria, duration of DM, HbA1c, plasma cystatin C and urinary sE-cadherin/cr are the only predictors by linear regression.

Table (1c): t test with adjusted duration of disease (≤ 7 years) between normoalbuminria and microalbuminuria

	Normo-albuminuria	Micro- albuminuria	t	P
Number of subjects	**30**	**10**		
duration of disease	**4.06 ± 1.7**	**4.7 ± 1.06**	**0.61**	**NS**
Plasma Cystatin C	**879.93 ± 90.46**	**1009 ± 56.26**	**3.62**	**HS**
Urinary sE-cadherin/cr	**880.66 ± 136.24**	**2413 ± 200.72**	**4.22**	**HS**

This table shows that with adjusted duration of DM, plasma cystatin C and urinary sE.cadherin/ cr levels were significant between normoalbuminuria and microalbuminuria being higher in the later.

Table (1d): t test with adjusted HbA1c (≤ 7.6) between normoalbuminria and microalbuminuria

	Normo-albuminuria	Micro- albuminuria	t	P
Number of subjects	**30**	**8**		
HbA1c	**7.12 ± 0.3**	**7.17 ± 0.28**	**0.42**	**NS**
Plasma Cystatin C	**879.93 ± 90.46**	**1112.5 ± 124.49**	**3.84**	**HS**
Urinary sE-cadherin/cr	**880.66 ± 136.24**	**2746.25 ± 275.62**	**4.47**	**HS**

This table shows that with adjusted HbA1c, plasma cystatin C and urinary sE.cadherin/ cr levels were significant between normoalbuminuria and microalbuminuria being higher in the later.

Table (2a): Baseline characteristics of 30 diabetic patients with normoalbuminuria defined by using estimated eGFR (mL/min/1.73 m2) calculated by the MDRD equation

	eGFR ≥ 90	eGFR < 90	t	p
Number	**17**	**13**		
Sex (male/ female)	**7/ 10**	**5/ 8**	**X2 = 2.1**	**NS**
Age (years)	**53.7 ± 7.47**	**72 ± 4.33†**	**-7.85**	**<0.001**
SBP (mmHg)	**130.58 ± 11.97**	**133.84 ± 6.5**	**-0.88**	**NS**
DBP (mmHg)	**81.17 ± 6.96**	**77.69 ± 6.65**	**1.38**	**NS**
Duration of DM (years)	**3.05 ± 1.39**	**5.38 ± 1.04†**	**-3.2**	**<0.001**
BMI (Kg/ m²)	**28.8 ± 3.24**	**31.7 ± 2.86**	**-1.14**	**NS**
FBS (mg/ dl)	**153.82 ± 9.2**	**153.3 ± 10.67**	**0.14**	**NS**
Serum creatinine (mg/ dl)	**0.77 ± .097**	**0.93 ± 0.15†**	**-3.5**	**<0.001**
Blood urea (mg/ dl)	**19.64 ± 3.33**	**24 ± 2.12†**	**-4.1**	**<0.001**
GFR by Cockcroft (ml /min / 1.73 m2)	**111.94 ± 7.23**	**80.15 ± 10.12†**	**10**	**<0.001**
GFR by MDRD (ml /min / 1.73 m2)	**99.94 ± 25.9**	**70.69 ± 8.08†**	**10.85**	**<0.001**
GFR by cystatin c (ml /min / 1.73 m2)	**97.46 ± 10.24**	**81.43 ± 4.92†**	**5.18**	**<0.001**

Serum albumin (gm/ dl)	**4.27 ± 0.28**	**4.28 ± 0.3**	**0.12**	**NS**
Total protein (gm/ dl)	**6.76 ± 0.45**	**6.68 ± 0.37**	**0.51**	**NS**
ALT (I.U/ L)	**23.05 ± 4.69**	**26.84 ± 5.65**	**-2**	**NS**
AST (I.U/ L)	**25.17 ± 6.65**	**25.69 ± 6.82**	**-0.2**	**NS**
Hb (gm/ dl)	**12.33 ± 0.48**	**11.5 ± 0.33†**	**2.9**	**<0.05**
TLC ($x10^3$)	**6.94 ± 1.5**	**6.9 ± 1.62**	**0.05**	**NS**
Platelets ($x10^3$)	**240.58 ± 53.32**	**244.92 ± 60.36**	**-0.2**	**NS**
HBA1c	**6.96 ± 0.28**	**7.33 ± 0.17†**	**-4**	**<0.001**
Serum TG (mg/ dl)	**152.7 ± 14.34**	**169.53 ± 10.37†**	**-3.7**	**<0.05**
Serum cholesterol (mg/ dl)	**185.29 ± 15.04**	**206.15 ± 8.69†**	**-4.45**	**<0.001**
HDL (mg/ dl)	**50 ± 7.28**	**39.6154 ± 6.6†**	**4.03**	**<0.001**
LDL (mg/ dl)	**105.64 ± 21.44**	**134.23 ± 12.39†**	**-4.28**	**<0.001**
Albumin/ cr ratio (mg/ g)	**21.05 ± 5.03**	**21.53 ± 4.61**	**-0.26**	**NS**
Plasma cystatin C (ng/ ml)	**824 ± 73.88**	**953.07 ± 47.67†**	**-5.47**	**<0.001**
Urinary sE-Cadherin/ cr (ug/ g)	**822.94 ± 56**	**956.15 ± 76†**	**-4.15**	**<0.001**

† means there's significant difference between both groups (P <0.05: significant; P <0.001: high significant).

Table (2b): t test with adjusted age (below 60 years) between normoalbuminuric subjects with GFR ≥ 90 mL/min/1.73 m2 calculated by the MDRD equation and that below 90 mL/min/1.73 m2

	GFR	Mean	Std. Deviation	t	P
Age (years)	**≥ 90**	**51.23**	**6.69**	**-1.79**	**NS**
	<90	**58**	**2**		
Duration of diabetes (years)	**≥ 90**	**3.21**	**1.28**	**-1.69**	**NS**
	<90	**5.12**	**2.03**		
SBP (mmHg)	**≥ 90**	**130**	**12.91**	**0.002**	**NS**
	<90	**130**	**14.14**		
DBP (mmHg)	**≥ 90**	**81.92**	**7.78**	**0.34**	**NS**
	<90	**80**	**0.8**		
BMI (Kg/ m^2)	**≥ 90**	**28.31**	**2.88**	**-0.54**	**NS**
	<90	**29.45**	**0.07**		
FBS (mg/ dl)	**≥ 90**	**154.23**	**9.66**	**-0.46**	**NS**
	<90	**157.5**	**3.53**		
Serum cr (mg/ dl)	**≥ 90**	**0.74**	**0.076**	**-2.88**	**<0.05**
	<90	**0.9**	**0.00**		
Blood urea (mg/ dl)	**≥ 90**	**18.54**	**2.93**	**-2.27**	**<0.05**
	<90	**23.5**	**2.12**		
Albumin (gm/ dl)	**≥ 90**	**4.26**	**0.29**	**-0.63**	**NS**
	<90	**4.4**	**0.28**		
Total protein (gm/ dl)	**≥ 90**	**6.74**	**0.46**	**-0.69**	**NS**
	<90	**7**	**0.71**		
ALT (I.U/ L)	**≥ 90**	**22.23**	**4.19**	**-0.63**	**NS**
	<90	**30.5**	**3.54**		

AST (I.U/ L)	**≥ 90**	**26.15**	**6.83**	**0.41**	**NS**
	<90	**24**	**8.49**		
Hb (gm/ dl)	**≥ 90**	**12.36**	**0.53**	**0.28**	**NS**
	<90	**12.25**	**0.35**		
TLC (x10³)	**≥ 90**	**6.65**	**1.53**	**-1.39**	**NS**
	<90	**8.25**	**1.06**		
Pletelet (x10³)	**≥ 90**	**240**	**58.45**	**-0.82**	**NS**
	<90	**275**	**7.07**		
Serum TG (mg/ dl)	**≥ 90**	**153.46**	**22.2**	**0.21**	**NS**
	<90	**150**	**14.14**		
T. cholesterol (mg/ dl)	**≥ 90**	**180.38**	**13.76**	**-1.95**	**NS**
	<90	**190**	**20.9**		
ALB/Cr ratio (mg/ g)	**≥ 90**	**22.15**	**5.047**	**1.52**	**NS**
	<90	**16.5**	**2.12**		
HBA1c	**≥ 90**	**6.85**	**0.62**	**-1.313-**	**NS**
	<90	**7.4**	**0.24**		
Urine cr (mg/ dl)	**≥ 90**	**113.46**	**11.79**	**0.983**	**NS**
	<90	**105**	**0.00**		
Plasma Cystatin c (ng/ ml)	**≥ 90**	**792.53**	**51.78**	**-3.3**	**<0.05**
	<90	**917.5**	**10.61**		
Urinary e-cadherin/ cr (ug/ g)	**≥ 90**	**783.08**	**68.78**	**-2.797-**	**<0.05**
	<90	**925**	**35.36**		

This table shows that plasma cystatin C and urinary sE.cadherin/ cr levels were significant between normoalbuminuric subjects with GFR ≥ 90 mL/min/1.73 m2 calculated by the MDRD equation and those below 90 mL/min/1.73 m2 being higher in the later

Table (2c): Independent factors associated with eGFR < 90 mL/min/1.73 m2 calculated by the MDRD equation in normoalbuminuric patients by multivariate logistic analysis

	B	**Wald**	**Exp(B) Adjusted Odds**	**95% C.I.for EXP(B)**		**Sig.**
				Lower	**Upper**	
Plasma Cystatin C	**-2.012**	**5.9**	**18**	**2.5**	**23.5**	**0.04**

CI, confidence interval.

This table shows that plasma cystatin C level is the only independent factor associated with eGFR < 90 mL/min/1.73m2 estimated by MDRD equation in patients with normoalbuminuria.

Table (3a): Simple Pearson's Correlation between plasma cystatin C and each of the studied parameters.

	r	**P**
Age (years)	**0.56****	**< 0.001 (HS)**
Duration (years)	**0.88****	**< 0.001 (HS)**
SBP (mmHg)	**0.13**	**> 0.05(NS)**
DBP (mmHg)	**0.11**	**> 0.05(NS)**
BMI (Kg/ m²)	**0.17**	**> 0.05(NS)**
HBA1c	**0.71****	**< 0.001 (HS)**
Serum creatinine (mg/ dl)	**0.73****	**< 0.001 (HS)**

Blood urea (mg/ dl)	**0.94****	**< 0.001 (HS)**
Serum albumin (gm/ dl)	**-0.19**	**> 0.05(NS)**
Total Protein (gm/ dl)	**-0.18**	**> 0.05(NS)**
ALT (I.U/ L)	**0.1**	**> 0.05 (NS)**
AST (I.U/ L)	**0.19**	**> 0.05(NS)**
TLC (x10^3)	**-0.09**	**> 0.05 (NS)**
Hb (gm/ dl)	**-0.2**	**> 0.05 (NS)**
Platelets (x10^3)	**0.04**	**> 0.05(NS)**
Serum TG (mg/ dl)	**0.19**	**> 0.05 (NS)**
Serum cholesterol (mg/ dl)	**0.85****	**< 0.001 (HS)**
HDL (mg/ dl)	**-0.88****	**< 0.001 (HS)**
LDL (mg/ dl)	**0.88****	**< 0.001 (HS)**
GFR by Cockcroft (ml /min / 1.73 m2)	**-0.56****	**< 0.001 (HS)**
GFR by MDRD (ml /min / 1.73 m2)	**-0.65****	**< 0.001 (HS)**
GFR by cystatin c (ml /min / 1.73 m2)	**-0.98****	**< 0.001 (HS)**
Albumin/ cr ratio (mg/ g)	**0.83****	**< 0.001 (HS)**
Urine creatinine (mg/dl)	**-0.75****	**< 0.001 (HS)**
Urine sE-Cadherin/ cr (ug/ g)	**0.88****	**< 0.001 (HS)**

***:** significant; **: high significant.

Table (3b): Simple Pearson's Correlation between urinary soluble e-cadherin/ cr and each of the studied parameters.

	r	**P**
Age (years)	**0.168**	**> 0.05 (NS)**
Duration (years)	**0.862****	**< 0.001 (HS)**
SBP (mmHg)	**0.191**	**> 0.05 (NS)**
DBP (mmHg)	**0.179**	**> 0.05 (NS)**
BMI (Kg/ m^2)	**0.123**	**> 0.05 (NS)**
HBA1c	**0.751****	**< 0.001 (NS)**
Serum creatinine (mg/ dl)	**0.453****	**< 0.001 (HS)**
Blood urea (mg/ dl)	**0.876****	**< 0.001 (HS)**
Serum albumin (gm/ dl)	**-0.190**	**> 0.05 (NS)**
Total Protein (gm/ dl)	**-0.181**	**> 0.05 (NS)**
ALT (I.U/ L)	**0.187**	**> 0.05 (NS)**
AST(I.U/ L)	**0.197**	**> 0.05 (NS)**
TLC (x10^3)	**-0.175**	**> 0.05 (NS)**
Hb (gm/ dl)	**-0.003**	**> 0.05 (NS)**
Platelets (x10^3)	**0.008**	**> 0.05 (NS)**
Serum TG (mg/ dl)	**0.137**	**> 0.05 (NS)**
Serum cholesterol (mg/ dl)	**0.176**	**> 0.05 (NS)**
HDL (mg/ dl)	**-0.183**	**> 0.05 (NS)**
LDL (mg/ dl)	**0.185**	**> 0.05 (NS)**
GFR by Cockcroft (ml /min / 1.73 m2)	**-0.259***	**< 0.05 (S)**
GFR by MDRD (ml /min / 1.73 m2)	**-0.371****	**< 0.001 (HS)**
GFR by cystatin c (ml /min / 1.73 m2)	**-0.833****	**< 0.001 (HS)**
Albumin/ cr ratio (mg/ g)	**0.953****	**< 0.001 (HS)**
Urine creatinine (mg/dl)	**-0.574****	**< 0.001 (HS)**

Plasma cystatin C (ng/ ml)	**0.881****	**< 0.001 (HS)**

*: significant; **: high significant

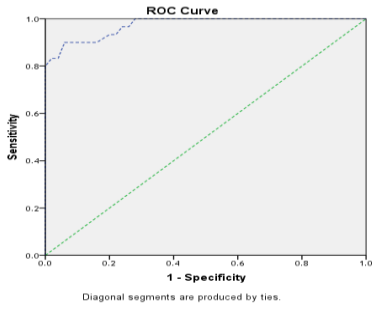
Table (4a): Sensitivity and Specificity of both biomarkers for detection of microalbuminuria.

	CUT OFF	Sensitivity	Specificity	+VE Predictive	-VE Predictive	Accuracy
plasma Cystatin C	**982.5**	**90%**	**92%**	**87%**	**93.8%**	**91.2%**
urinary sECadherin/cr	**1170**	**90%**	**70%**	**64.2%**	**92.1%**	**77.5%**
Both plasma cystatin C and Urinary e-cadherin/ cr		**90%**	**96%**	**93.1%**	**94.1%**	**93.7%**

Table (4b): Sensitivity and Specificity of both biomarkers for detection of renal impairment (GFR < 90 mL/min/1.73 m2 calculated by the MDRD equation)

	CUT OFF	Sensitivity	Specificity	+VE predictive	-VE predictive	Accuracy
plasma Cystatin C	**882.5**	**94.7%**	**82.6%**	**93.1%**	**86.3%**	**91.2%**
urinary sECadherin/cr	**910**	**89.5%**	**82.5%**	**92.7%**	**76%**	**87.5%**
Both plasma cystatin C and Urinary e-cadherin/ cr		**94.7%**	**87.2%**	**94.3%**	**89.4%**	**94.7%**

Fig (1) ROC Curve for plasma Cystatin C and urinary sECadherin/cr for detection of microalbuminuria



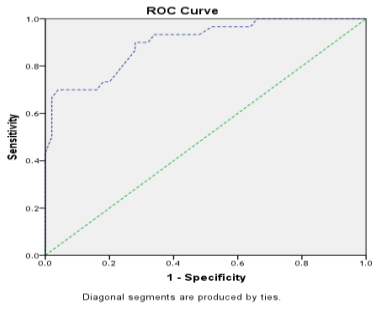
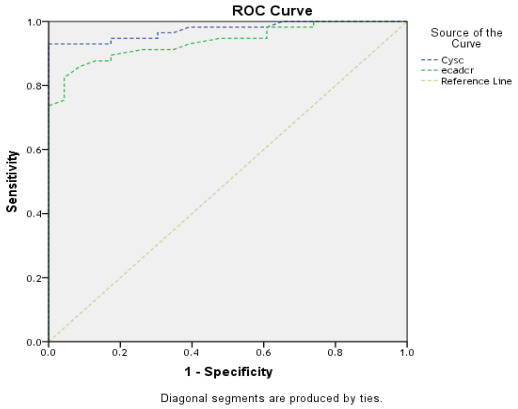


Fig (2) ROC Curve for plasma Cystatin C and urinary sECadherin/cr for detection of renal impairment (GFR < 90 mL/min/1.73 m2 calculated by the MDRD equation)



DISCUSSION

Diabetes is a major cause of chronic kidney disease (CKD) and is recognized as the most common cause of end-stage renal disease (ESRD) in the United States **(24).** Approximately 40% of US adults with diagnosed or undiagnosed diabetes had some degree of CKD in the 1999-2006 National Health and Nutrition Examination Survey **(25).** Even among adults with undiagnosed diabetes or prediabetes, the prevalence of kidney damage or dysfunction was substantial (17.7%) **(26).** The presence of CKD also adds considerably to the cost of diabetes management **(27).**

Our study aimed at detection of plasma level of Cystatin C and urinary soluble E-cadherin at early stages of diabetic nephropathy and whether they can be used individually or in combination for early detection of diabetic nephropathy in type 2 diabetic patients.

In present work, there was no significant difference between studied groups regarding age, sex, smoking state and body mass index but there was significant difference regarding duration of diabetes which goes in harmoney with **Kundu et al.**, **(2) and Assal et al., (28)** who found a significant difference between normo and microalbuminuric group of type 2 diabetes regarding duration of diabetes. They stated that longer duration of diabetes is a risk factor for development and progression of microvascular complications.

We found no significant difference between both diabetic groups (normoalbuminemia and microalbuminemia) regarding systolic and diastolic blood pressure; therefore we can exclude the effect of hypertension on UAE.

In the current work, a highly significant difference between studied groups regarding fasting blood glucose and glycosylated haemoglobin was observed. This goes in harmony with that reported by **Kundu et al., (2) and Sheikh et al., (29)** where they found that impaired glycemic control is associated with significant elevations in urinary microalbumin levels**.**

Our study showed no significant difference between control group and normo-albuminuric one but there's a significant difference between both diabetic groups regarding serum creatinine, urea and urinary albumin excretion and this result was supported by **Chae et al., (30)** who documented a significant difference between both diabetic groups as regard all previous parameters.

Our study showed no significant difference between control and normo-albuminuric as regard glomerular filtration rate (ml/min/1.73m²) by MDRD, Cockcroft and cystatin C based formulas. These results are supported by a lot of studies as **Tidman et al., (31)** and **Gunzler et al., (32)** but against little studies **Lu et al., (33)** and **Dwyer et al., (34)** who found significant difference between both groups as regard GFR. Renal insufficiency in normoalbuminuric patients was seen less frequently, likely reflecting greater contributions from aging, hypertension, and arteriosclerosis **(35).**

On the other hand our study showed significant differences among different groups of the study in GRR by MDRD and cystatin C based formulas but not by Cockcroft between control and micro-albuminuric groups and between normo-albuminuric and micro-albuminuric groups. Our results were confirmed by **Lorenzo et al., (36).**

Surprisingly, the decline in GFR in micro-albuminuric group was not noticed by Cockroft formula but noticed by MDRD and confirmed by cystatin C formulas. The explanation of this finding is that our subjects were obese as the mean of BMI of microalbuminuric group was 31.68 kg/h². In line with our results, others also found the estimation of Cockcroft-Gault to be more dependent on body weight or BMI than MDRD **(37) and (38).**

In this study, we not compare between the three methods of calculation of GFR as it should compare each of them with respect to 99mTC-DTPA, the most accurate method unlike **Trimarchi et al., (39)** did but in general the results of MDRD and cystatin C based equations are to some extent similar for calculating GFR and clearly differ from Cockroft formula.

Michels et al., (40) concluded that the Cockcroft-Gault equation is influenced by body weight and BMI and CKD-EPI gives the best estimation of GFR, although the performance is close to that of MDRD.

Marwyne et al., (41) concluded that the cystatin C-based eGFR equation was more accurate, sensitive and specific in overweight and obese subjects compared to the creatinine-based eGFR equations.

The cause of accuracy of cystatin C is that Cystatin C is degraded in renal tubular cells and not secreted by the kidneys, which means that plasma/serum levels are dependent on GFR. As there is no other endogenous source of this protein,

there is no need to measure urine levels to get a true GFR estimation unlike creatinine which need urine estimation to calculate GFR **(42).**

Tidman et al., (31) concluded that estimating GFR using formulae based on s. creatinine or s-cystatin C alone was equally accurate according to the NKF K/DOQI guidelines. A formula that combines both provided a greater accuracy. If Cystatin C, which is clearly more expensive, is used, the choice of the cystatin C determination method and an adjusted prediction equation is essential. Use of the MDRD seems to yield the best cost–benefit ratio for routine practice.

The production of cystatin C has been extensively reported to be independent of and unaffected by sex, age, height, weight, and muscle mass **(43).** However, there is conflicting evidence regarding whether cystatin C levels vary by gender. Some investigators found statistically significant differences between genders in adults **(44) and (45),** with males having higher cystatin C levels than females, whereas others did not **(46).** In agreement with the majority of previous studies, the results of the present study showed non-significant difference between males and females in the mean values of cystatin C. In contrast, there was statistically significant increase in the mean serum creatinine of males compared to females which may be due to the difference in muscle mass of males and females. Thus, we can confirm that, cystatin C, unlike creatinine was independent of gender.

In the present study, the significant positive correlation noticed between serum cystatin C and age indicating that serum cystatin C increased with age. Earlier studies have also shown positive correlation of serum cystatin C with age of the patients **(47) and (48).** Several authors noted an age-related rise in cystatin C levels after the 50 years **(49) (50),** which presumably reflects a decline of kidney function with age. In addition, cystatin C also had a significant positive correlation with duration of diabetes. These results are consistent with **Hosokawa et al., (48) and Assal et al., (28).** In disagreement with these results, **Shafey et al., (51)** reported that no correlation was found between cystatin C and duration of diabetes. On the other hand, there was non-significant correlation between cystatin C and BMI, and this is generally consistent with **Galteau et al., (52)** who have reported a moderate but biologically insignificant correlation between BMI and cystatin C. In contrast, **Al Wakeel et al., (53)** and **Muntner et al., (54)** have reported a significant correlation between serum cystatin C

The results of the current study revealed significant positive correlation between serum cystatin C and each of serum urea and creatinine suggesting that serum cystatin C increased similar to serum urea and creatinine. Similar findings were observed in the previous study done by **Tian et al., (55).**

The results of the current study revealed significant positive correlation between serum cystatin C and HbA1c. The results were confirmed by **Senghor et al., (56)**

The results of our study showed that plasma cystatin C was significant between normoalbuminuria and microalbuminuria being higher in the later by t test after adjustment of both duration of disease and HbA1c. This means that the rise in cystatin C is due to microalbuminuria and not due to other correlated factors like duration of disease and HbA1c.

In the present study, cystatin C levels showed positive significant correlation with cholesterol, LDL, and inversely correlated with HDL levels. These results are in accordance with the study done by **Krishna et al., (57)**.

The results of the current study demonstrated a strong positive statistical correlation between cystatin C and ACR. In contrast, cystatin C showed a strong inverse correlation with urine creatinine and eGFR. The results are previously confirmed by **Chae et al., (30) and Jeon et al., (19).**

The mechanism that explains positive correlation of plasma cystatin C with albuminuria is that proteinuria triggers chemokine expression of tubular epithelial cells and activates complements, which result in interstitial inflammation and fibrosis; proteinuria also induces the apoptosis of tubular epithelial cells **(58)**, also **Li et al., (59)** noted that in a rat kidney proximal tubular cell line (RPTC), albumin induced apoptosis in a time- and dose-dependent manner. Many authors suggested that plasma cystatin C is a predictor for tubular damage **(12), (28), (60) and (61).**

Another explanation is that filtered CysC is reabsorbed by megalin-facilitated endocytosis in proximal tubules and catabolized **(62).** Consequently, proximal tubular injury will reduce reabsorption and produce a diagnostic increase in urinary CysC **(62).** However, filtered albumin is

also reabsorbed by megalin–cubulin receptor-mediated endocytosis **(63).** Increased urinary CysC has been observed in the presence of proteinuria in children with nephrotic syndrome **(64).** Independently of tubular injury, competition for receptor-mediated transport between albumin and other LMW proteins could account for or make a significant contribution to increased urinary CysC in the presence of proteinuria **(65).**

In normoalbuminuric patients, the cystatin C levels of plasma were significantly increased in patients with GFR < 90 mL/min/1.73 m2 than those with GFR ≥ 90 mL/min/1.73 m2. t test with adjusted age was done, only plasma cystatin C and urinary soluble e-cadherin/ cr were found to be significant between both groups. In multivariate logistic analysis, plasma cystatin C level was the only independent factor associated with eGFR < 90 mL/min/1.73m2 estimated by MDRD equation in patients with normoalbuminuria.

It was thought that this increment was probably due to the tubular phase before glomerular manifestation. This suggests that plasma cystatin C and urinary soluble e-cadherin/ cr levela are related to subclinical tubular impairment and can be earlier measurable markers of renal impairment before onset of microalbuminuria. These data was supported before by **Jeon et al., (19)** that suggested that the cystatin C levels of serum and urine are related to subclinical tubular impairment and can be an earlier measurable marker of renal involvement before onset of microalbuminuria**.** cystain C could be one of the additional tubular factors which represent kidney state of diabetic patients.

Cho et al., (66) reported that E-cadherin (one of the proteomes) is present in the proximal and distal tubules of newborn mice.

To explore the changes of urinary level of sE-cadherin in DN patients, we performed ELISA analysis on 60 urine samples from DM and 20 urine samples from control subjects. To avoid the effect of urine volume, urinary levels of sE-cadherin were presented after correction for urinary creatinine concentration (sEcadherin/ Cr).

The results of the current study demonstrated no statistical correlation between urinary sEcadherin/ Cr and age. These results are in accordance with the study done by **Trnka et al., (67)** and in also this site **(68),** this exclude the effect of aging on the value of this biomarker unlike plasma cystatin C and serum Cr.

The results of our study showed that urinary sEcadherin/ Cr was significant between normoalbuminuria and microalbuminuria being higher in the later by t test after adjustment of both duration of disease and HbA1c. This means that the rise in urinary sEcadherin/ Cr is due to microalbuminuria and not due to other correlated factors like duration of disease and HbA1c.

In the present study, there's high positive correlation of urinary sEcadherin/ Cr with UACR and serum creatinine and high negative correlation with GFR. The results were matched with that of **Jiang et al., (19)**, **Trnka et al., (67) and Riaz et al., (69).**

In the present study, there's statistically high significant differences in urinary soluble e-cadherin/ cr between subjects of normal GFR and subjects of mild CKD (GFR< 90 mL/min/1.73 m2) in normoalbuminuric subjects, these results were matched with that of **Merchant et al., (70),** this mean that urinary soluble e-cadherin/ cr is a novel biomarker detecting the decline of kidney function in normoalbuminuric patients

The mechanism that explains positive correlation of urinary sEcadherin/ Cr with albuminuria and serum creatinine and high negative correlation with GFR is renal ischemia and apoptosis **(19).**

As we all know, with the development of renal damage of DN, ischemia and apoptosis of renal tubular epithelial cells will increase **(71) and (72).** Previous studies had demonstrated that ischemia and apoptosis could lead to degradation and cleavage of E-cadherin by proteolytic enzyme activation **(73) and (74).** Thus, the degradation and cleavage induced by renal damage may be a major reason for the increase of urinary sE-cadherin.

Another explanation is epithelial-to-mesenchymal transition (EMT). During this process, epithelial cells acquire features of mesenchymal cells such as myofibroblasts, resulting in loss of E-cadherin expression, the acquisition of mesenchymal markers such as αSMA, and the increased deposition of extracellular matrix (ECM) **(75)** leading to tubulointerstitial fibrosis.

Many studies have shown EMT induced by advanced glycation end (AGE) products or tissue growth factor (TGF)-β could suppress the expression of E-cadherin **(76)** and **(77).**

The validity of plasma cystatin C and urinary sE-cadherin/ cr in detection of microalbuminuria was assessed we found out that

plasma cystatin C is more specific and the same sensitivity of urinary sE-cadherin/ cr. The specificity of both was increased if used together and the accuracy of both is more than the accuracy of every one alone.

Also, the validity of plasma cystatin C and urinary sE-cadherin/ cr in detection of renal impairment was assessed we found out that plasma cystatin C is more sensitive and the same specificity of urinary sE-cadherin/ cr. The specificity of both was increased if used together and the accuracy of both is more than the accuracy of every one alone.

To gather definitive support that plasma cystatin C and urinary sE-cadherin/ cr are appropriate biomarkers for early prediction of diabetic nephropathy, further research on diabetic subjects with hyperfilteration as this is the earliest stage of DN comparing with both control and microalbuminurea.

In conclusion, plasma cystatin C and urinary sE.cadherin levels could be useful biomarkers for detection of microalbuminuria and detection of renal impairment in normoalbuminuric type 2 diabetic patients.

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