Evaluation of Serum Cystatin C in Type 1 Diabetic Children and Adolescents as an Early Indicator of Diabetic Nephropathy

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Abstract: Background: Diabetic nephropathy is a major cause of morbidity and mortality among young adults with type 1 diabetes. Clinical management and therapeutic intervention from early stage of DN is of major importance to prevent progression to end stage renal disease. **The aim of this study:** is to evaluate serum cystatin c and albuminuria in Type 1 Diabetic Children and Adolescents. **Methods**: In the present case control study, we evaluated the level of serum cystatin c in 85 patients with type 1 diabetes mellitus at Diabetes, Endocrinology and Metabolism clinic in pediatric hospital Cairo university, patients categorized into two groups (normoalbuminuric and microalbuminuric) according to A/C ratio. **Results:** Our study revealed increased level of serum cystatin c in microalbuminuric diabetic patients. Serum cystatin c negatively correlated with GFR. Also, it was found that serum cystatin c increased in parallel with the severity of renal disease, poor glycemic control and duration of diabetes. **Conclusion:** Serum cystatin c measurement might become a useful and accurate noninvasive tool for early detection of diabetic nephropathy.

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Key words: Type 1 diabetes mellitus - Diabetic nephropathy - Microalbuminuria – Serum cystatin c- Estimated glomerular filtration rate (eGFR).

Introduction

Diabetes is a metabolic disorder of multiple causes characterized by chronic hyperglycaemia and disorders of carbohydrate, fat and protein metabolism. It may be classified as autoimmune mediated type1 diabetes, or as insulin resistance associated type 2 diabetes, or a combination of these factors. Type1diabetes mellitus (T1DM) commonly occurs in childhood or adolescence, although the rising prevalence of type 2 diabetes mellitus (T2DM) in these age groups is now being seen worldwide **(ADA, 2010).**

T1DM may lead to many complications, either acute or chronic, chronic complications include nephropathy, retinopathy, neuropathy, stroke and cardiovascular diseases. Acute complications include diabetic ketoacidosis, hyperglycaemic hyperosmolar non ketotic coma which result from severe hyperglycaemia and profound hypoglycaemia may result from a relative excess of insulin **(Young, 2009)**.

Diabetic nephropathy (DN) is defined as persistent proteinuria greater than 500mg/ 24 hours or albuminuria greater than 300mg/24 hours and is usually associated with hypertension, and a diminishing glomerular filtration rate (GFR). End stage renal failure may occur many years later and requires dialysis or kidney transplantation. Early detection of diabetic nephropathy has an important value in the prevention of end stage renal disease in patients with type1 diabetes; the first clinical sign is microalbuminuria (**Donaghue *et al.*, 2009**).

Also, serum creatinine has been widely used as a marker of GFR, but it is not sensitive enough to detect decreased renal function. Therefore, various plasma low molecular weight proteins have been suggested as valuable markers of decreased renal function in place of serum creatinine. Among these markers, previous studies demonstrated that serum cystatin C (cysC) might be a superior marker for the evaluation of renal function than serum creatinine. However, the effectiveness of cysC for estimating GFR has not been sufficiently demonstrated in children with diabetes **(Aksun *et al.*, 2004).**

Cystatin C has a low molecular weight (approximately 13.3 kilodaltons), and it is removed from the bloodstream by glomerular filtration in the kidneys. If kidney function and glomerular filtration rate decline, the blood levels of cystatin C rise. Serum levels of cystatin C are a more precise test of kidney function (as represented by the glomerular filtration rate, GFR) than serum creatinine levels. This finding is based mainly on cross-sectional studies. Longitudinal studies are scarcer; some studies show promising results **(Stevens *et al.*, 2008).**

Aim of the work

The aim of this work is to evaluate the level of serum cystatin C as an early indicator of diabetic nephropathy in children and adolescents with type 1 diabetes and albumin creatinine ratio (ACR), to be correlated with renal function.

Subjects and methods

➢ **Subjects:**

The current case control study was conducted on 85 children and adolescents with type 1 DM, regularly attending for management and follow up at Pediatric Endocrinology and Diabetes unit at Cairo University at the period from February 2013 to June 2014.

Patients were collected according to the following criteria:

Inclusion criteria:

- Type 1 diabetes mellitus.
- Age of patients 2-18 years old.
- Minimum diabetes duration of 2 years at the time of enrolment.

Exclusion criteria:

- Duration of diabetes less than 2 years from date at diagnosis.
- Age of patients >18 years old.
- Patients with profound renal failure.
- Patients with other autoimmune endocrinal disorders e.g. (thyroiditis).

Patients were divided into two groups according to the albumin creatinine ratio:

- **Group (1) or microalbuminuric group (cases):**

Forty five patients with type1 DM and microalbuminuria (defined by morning spot ACR ≥ 30 mg/g creatinine in 2 samples within 3 months.

- **Group (2) or normoalbuminuric group (control):**

40 patients with type1 DM but without microalbuminuria (defined by morning spot ACR < 30mg/g creatinine).

➢ **Methods:**

All patient groups were subjected to the following:

○ **Full history taking laying stress on:** Age and sex of the patients, Duration of diabetes in years. Age of onset of diabetes. Family history of diabetes. Insulin therapy regimen: The daily total dose of insulin (calculated as IU/ Kg/day). The type of insulin.

- History and frequency of acute metabolic complications:

- History suggestive of DKA (any attacks of DKA at diagnosis or during follow up).History suggestive of severe hypoglycemic attacks (tremors, convulsions or coma).

- History suggestive of chronic Diabetic complications. Ophthalmic manifestations (persistent blurring of vision, flashes of light). Peripheral neuropathy manifestations (tingling, numbness and parathesia).

○ **Thorough clinical examination with special emphasis on:**

- Anthropometric measurements including: Standing height and height standard deviation score (SDS) for age and Sex. Weight and weight SDS for age and sex.

- Body mass index (BMI) and BMI standard deviation score **(CDC, 2009)**.

- Blood pressure measured by the conventional mercury sphygmo-manometer in the laying position then plotted on blood pressure curves according to the age and sex **(Bethesda, 1987).**

- Full neurological examination to detect evidence of peripheral neuropathy).

- Fundus examination to detect diabetic retinopathy.

○ **Laboratory investigations:**

a) Spot urinary albumin creatinine ratio. (Patient has persistent micro- albuminuria according to albumin-creatinine ratio (A/C) calculated in morning urine sample. An A/C ratio 30-300 µg/mg is considered positive for microabuminuria in absence of urinary tract infection. Two positive results for microalbuminuria within 3 months should.

b) Revising follow up records (HbA1C) last year.

c) Serum creatinine and calculating the glomerular filtration rate (GFR) from the **Schwartz formula**:

$$GFR = \frac{K \times \text{height (cm)}}{\text{S. creatinine (mg/dl)}}$$

Where k is 0.55 for children and adolescent females, and 0.70 for adolescent males (Vogt and Avner, 2008).

d) Serum cystatin C measurement using ELISA technique.

Quantitative assessment of serum cystatin c:

Serum cystatin c level was assayed using Human cystatin c Elisa by Catalog number RD191009100 manufactured by: Biovender- Laboratorni medicina, European Union.a.s. D-69120 Heidelberg Germany.

Collection of samples and storage: The blood samples was aseptically collected, centrifuged to get serum samples to be aliquoted and stored at ≤ - 20˚C till the time of assay.

Principle of assay:

This assay done using sandwich enzyme immunoassay technique. Standards, quality controls and samples are incubated in micro titrate plate wells pre-coated with polyclonal anti-human cystatin C antibody. 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. 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.

Statistical methods:

Quantitative variables with normal distribution were presented by mean and standard deviation (SD). They were compared by t-student test or ANOVA test if more than two groups.

For non parametric data, quantitative variables were presented by median and inter quartile range (IQR). They were compared by Mann-Whitney U test. Qualitative variables were presented by number and percent. They were compared by Chi-square or Fischer's exact test when appropriate. Receiver operator characteristic (ROC) curve was constructed to assess the reliability of cystatin c in early detection of microalbuminuria. Area under the curve (AUC) considered significant if >0.60 with significant p value. In all tests, p value considered significant if less than 0.05.

Results

The present case-control study included 85 Children with type 1 diabetes mellitus. Patients were divided into two groups. Group 1 included 45 patients with diabetic nephropathy (microalbuminuria) considered as (case group) and group 2 included 40 type 1 diabetic patients without nephropathy (normoalbuminuria) considered as (control group). Both groups are age and sex matched. The clinical, demographic and laboratory data of both groups were compared. All patients were 45 females (55.6%) and 40 males (44.4%).

There was no statistical significant difference between case and control groups as regard age and sex distribution. No statistical significant differences among case and control groups as regard standard deviation score (SDS) of weight, height and body mass index (BMI) were observed.

Table (1) Revealed statistical significant difference as regard systolic blood pressure and highly statistical significant difference as regard diastolic blood pressure among case and control groups.

Table (1) Comparison of blood pressure among case and control groups:

	Cases (45)	Controls (40)	*P*-value
Variable	Mean ± SD		
Systolic blood pressure (mmHg)	116.44 ± 14.48	106.00 ± 12.15	0.001 (S)
Diastolic blood pressure (mmHg)	74.89 ± 10.36	65.50 ± 9.32	0.0001 (HS)
Variable	N (%)		
Hypertension: No Yes	 40 (88.9) 5 (11.1)	 38 (95.0) 2 (5.0)	0.439 (NS)

P>0.05 non significant (NS), *p*<0.05 significant (S), *p*<0.01 highly significant (HS).

All patients in both groups were on multiple daily injection insulin regimens. The mean insulin dose was (1.23 ± 0.37 U/Kg/day) among micro-albuminuric group and (1.3 ±0.51 U/Kg/day) among the normoalbuminuric group, with no significant difference ($p = 0.342$). The types of insulin used were also similar in both groups with no statistical significant difference ($p = 0.587$).

Table(2) revealed statistical significant differences between case and control groups as regard HbA1c, A/C ratio and serum cystatin c but no statistical significant difference between case and control groups as regard serum creatinine and estimated GFR.

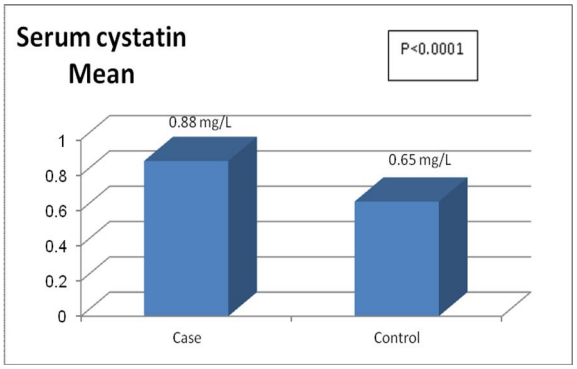


Figure (1): Comparison between case and control groups as regard serum cystatin c.

Table (2): Comparison between case and control groups as regard Laboratory parameters:

	Cases (45)	Controls (40)	*p*-value
Variable	Mean ± SD		
HbA1c (%)	10.16 ± 2.41	8.75 ± 2.46	0.009 (S)
A/C ratio (mg/gm)	88.59 ± 96.74	9.50 ± 4.67	< 0.0001 (HS)
Serum Creatinine (mg/dL)	0.68 ± 0.16	0.68 ± 0.12	0.993 (NS)
Serum cystatin c (mg/L)	0.88 ± 0.24	0.65 ± 0.13	< 0.0001 (HS)
Estimated GFR (mL/min/1.73 m2)	134.63 ± 31.85	128.26 ± 37.98	0.404 (NS)

P>0.05 non significant (NS), *p*<0.05 significant (S), *p*<0.01 highly significant (HS).

Figure 1 Showed highly significant difference between case and control groups as regard serum cystatin c.

Table (3) revealed significant positive correlation between duration of DM and each of A/C ratio and serum cystatin c. Also, there is significant positive correlation between serum cystatin c and each of A/C ratio, HbA1c and serum creatinine. Estimated GFR is significantly negatively correlated with serum creatinine. This table revealed also positive correlation between serum creatinine and A/C ratio. Similarly, positive correlation was found between HbA1c and A/C ratio.

Table (3) Correlations of some important parameters among all subjects:

		A/C ratio	Serum Creatinine	Serum Cystatin c
Age (years)	r	0.208	0.144	0.169
	P-value	0.056	0.188	0.122
Duration of DM (Years)	r	0.298	0.178	0.233
	P-value	0.006	0.104	0.032
HbA1c (%)	r	0.261	0.075	0.344
	P-value	0.016	0.496	0.001
A/C ratio (mg/gm)	r	-------	0.268	0.763
	P-value	-------	0.013	<0.0001
Serum (mg/dL) Creatinine	r	-------	--------	0.312
	P-value	-------	--------	0.004
Estimated GFR (mL/min/1.73 m2)	r	-0.046	-0.555	-0.180
	P-value	0.674	<0.0001	0.098

P>0.05 non significant (NS), *p*<0.05 significant (S), *p*<0.01 highly significant (HS).

Table 4 Revealed significant positive correlation between duration of DM and A/C ratio. Also, there is significant positive correlation between serum cystatin c and each of A/C ratio and serum creatinine.

Estimated GFR is significantly negatively correlated with serum creatinine and serum cystatin c. This table revealed also positive correlation between serum creatinine and A/c ratio.

Table (4) Correlations of some important parameters among cases:

		A/C ratio	Serum Creatinine	Serum cystatin c
Age (years)	r	0.098	0.121	0.017
	P-value	0.520	0.429	0.910
Duration of DM (Years)	r	0.334	0.247	0.252
	P-value	0.025	0.102	0.095
HbA1c (%)	r	0.200	-0.058	0.102
	P-value	0.188	0.703	0.507
A/C ratio (mg/gm)	r	-------	0.386	0.769
	P-value	-------	0.009	<0.0001
Serum (mg/dL) Creatinine	r	-------	-------	0.415
	P-value	------	-------	0.005
Estimated GFR (mL/min/1.7m2)	r	-0.160	-0.582	-0.296
	P-value	0.293	<0.0001	0.048

Table 5 revealed significant positive correlation between HbA1c and serum cystatin c. Also, there is significant negative correlation between estimated GFR and serum creatinine.

Table (5): Correlations of some important parameters among controls.

		A/C ratio	Serum Creatinine	Serum cystatin c
Age (years)	r	0.221	0.199	0.040
	P-value	0.170	0.219	0.808
Duration of DM (Years)	r	0.234	0.073	0.046
	P-value	0.147	0.656	0.777
HbA1c (%)	r	0.060	0.281	0.553
	P-value	0.713	0.079	<0.0001
A/C ratio (mg/gm)	r	-------	-0.241	0.101
	P-value	-------	0.133	0.534
Serum (mg/dL) Creatinine	r	-------	-------	0.241
	P-value	-------	-------	0.134
Estimated GFR (mL/min/1.73 m2)	r	0.025	-0.559	-0.266
	P-value	0.879	<0.0001	0.097

Serum cystatin c is significantly affected by HbA1c and A/C ratio. Also serum cystatin c is negatively affected by estimated GFR as shown in the following table.

Table (6) multiple regression showing parameters affecting serum cystatin c among all subjects:

Model	B	t	*P*-value
(Constant)	0.658	7.637	<0.0001
Age (years)	0.068	1.099	0.275
Duration of DM (years)	0.041	0.557	0.579
HbA1c (%)	0.014	2.193	0.041
A/C ratio (mg/gm)	0.002	10.160	<0.0001
Serum creatinine (mg/dL)	0.046	0.017	0.595
Estimated GFR (mL/min/1.73 m2)	-0.001	-2.122	0.037

Adjusted R^2 = 0.621. F ratio = 45.121, *p* value < 0.0001.

Serum cystatin c is significantly affected by A/C ratio as shown in the following table.

Table (7) multiple regression showing the parameters affecting serum cystatin c among cases:

Model	B	t	*P*-value
(Constant)	0.712	22.288	<0.0001
Age (years)	-0.059	-0.596	0.554
Duration of DM (years)	-0.005	-0.048	0.962
HbA1c (%)	-0.054	-0.541	0.591
A/C ratio (mg/gm)	0.002	7.879	<0.0001
Serum creatinine (mg/dL)	0.139	1.327	0.192
Estimated GFR (mL/min/1.73 m2)	-0.178	-1.846	0.072

P>0.05 non significant (NS), *p*<0.05 significant (S), *p*<0.01 highly significant (HS). Adjusted R^2 = 0.581. F ratio = 62.078. *p* value < 0.0001.

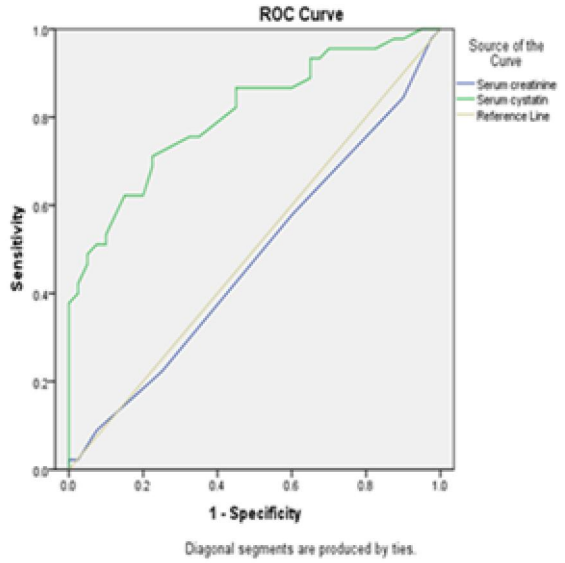
Table (8) multiple regression showing the parameters affecting serum cystatin c among controls:

Model	B	T	*P*-value
(Constant)	0.382	5.694	<0.0001
Age (years)	-0.020	-0.144	0.886
Duration of DM (years)	-0.016	-0.115	0.909
HbA1c (%)	0.030	4.088	<0.0001
A/C ratio (mg/gm)	0.068	0.500	0.620
Serum creatinine (mg/dL)	0.093	0.657	0.515
Estimated GFR (mL/min/1.73 m2)	-0.193	-1.431	0.161

P>0.05 non significant (NS), *p*<0.05 significant (S), *p*<0.01 highly significant (HS).

Serum cystatin c is significantly affected by HbA1c as shown in the table.

Figure 2 Receiver operator characteristic (ROC) curves Showed better diagnostic accuracy of serum cystatin c than serum creatinine in detecting microalbuminuria in diabetic patients. The sensitivity and specificity of serum cystatin c and serum creatinine were estimated via ROC curves. The area under the curves (AUC) was 0.806 for serum cystatin c and 0.477 for serum creatinine. The AUC was significantly higher for serum cystatin c P-value < 0.0001, but not for serum creatinine P-value = 0.715. The sensitivity and specificity of cystatin c were 86.7% and 55%, respectively with the upper reference limit as the cut-off.



For serum cystatin: Area under curve = 0.806. *P*-value < 0.0001 For serum Creatinine: Area under curve = 0.477. *P*-value = 0.715

Figure (2) Receiver operator characteristic (ROC) curves

Discussion

There is an unmet need for highly sensitive biomarkers for the detection of diabetic nephropathy. Currently this disease is not recognized early enough because of inadequate diagnostic methods, which increases the chances that early nephropathy and microalbuminuria will progress toward end-stage renal disease **(Novel biomarkers of diabetic nephropathy, 2011)**.

In the current study there was no significant difference between the microalbuminuric and normoalbuminuric group as regard anthropometric measurements (height, weight and BMI) the same finding was found by **Juretic *et al.* (2002).** But **Kordonouri *et al.* (2009)** found that short stature among patients with type 1 diabetes is associated with increased risk of developing nephropathy.

Our study revealed no statistical significant difference between both sexes as regard microalbuminuria. This finding was concordant to that found by **Soman and Soman (2009)** but **Lindsay *et al.* (2005)** found that A/c ratio was higher in females than males before puberty but thereafter the rate became equal.

Our study revealed that there was statistical significant difference between microalbuminuric and normoalbuminuric groups as regard systolic ($p<0.001$) and diastolic ($p<0.0001$) blood pressure and this is consistent with **Jeon, *et al.* (2011)** and **Assal *et al.* (2013) who** Revealed significant increase of systolic and diastolic blood pressure among microalbuminuric than normoalbuminuric patients.

Our study revealed no significant difference between microalbuminuric and normoalbuminuric groups as regard acute and chronic diabetes complications in contrast to **Kordonouri *et al.* (2009)** who found increased long term complications in microalbuminuric patients. This may be explained by that the two groups have almost the same mean duration of diabetes.

In the current study HbA1c at the time of the study was statistically higher among the microalbuminuric group and this was similar to **Nasser *et al.* (2011) and Jeon, *et al.* (2011)**, but disagrees with the finding of **Juretic et al. (2002)** Who found no significant association.

This study revealed significant increase of serum cystatin c among microalbuminuric group (0.88 ± 0.24 mg/l) in comparison to normoalbuminuric group (0.65 ± 0.13 mg/l). This result was concordant with **Chae, *et al.* (2012) and Assal *et al.* (2013)** this means that serum cystatin c may be an early marker of diabetic nephropathy and also **Ogawa, *et al.*,2008 who** found increased serum cystatin level was significant in overt nephropathy but not in early nephropathy**.**

In the current study there was significant positive correlation between serum cystatin c and Alb/Cr ratio ($p<0.0001$) (r=0.763) in all patients. The same was reported by **Jeon, et al (2011)** ($p<0.001$) (r=0.555), **Assal *et al.* (2013)** (p<0.001) (r=0.703). The same significant positive correlation was also found in our study between serum cystatin c and Alb/Cr ratio in microalbuminuric patients ($p<0.0001$) (r=0.769). The value of serum cystatin c was found to be directly proportional to the Alb/Cr ratio value, which mean that the more severe the kidney affection, the higher the values of serum cystatin c.

Regarding the relation between serum cystatin c and patients' age, no significant correlation was observed in our study (p=0.122) (r=0.169), this is not

consistent with **Jeon, *et al.* (2011)** who found that the serum level of cystatin C was related to age. On the other hand, we observed a positive correlation between serum cystatin c and duration of diabetes (p=0.032) (r=0.233), and this was consistent with **Assal *et al.* (2013)** who found significant positive correlation between serum cystatin and diabetes duration (p=0.002) (r=0.422).

Current study revealed positive correlation between serum cystatin c and glycemic control (HbA1c) (p=0.002) (r=0.344) among all subjects, and also among control group (normoalbuminuric) (p<0.0001) (r=0.553). Also multiple regression analysis shows that HbA1c affect serum cystatin c significantly (p<0.0001). This is suggesting that the incidence of diabetic nephropathy increases according to the progress of the disease and poor glycemic control. Our results disconcordant with **Jeon, *et al.* (2011)** who reported no positive correlation between serum cystatin c and HbA1c using Pearson's correlation analysis.

Our study revealed significant negative correlation between serum cystatin c and eGFR, (p=0.048) (r=-0.296) among microalbuminuric patients, this is consistent with results obtained by **Jeon, *et al.* (2011)** and **Assal *et al.* (2013)** this means that decrease in GFR leads to increase in serum cystatin c, and so the serum cystatin c could be a sensitive indicator of GFR. Serum creatinine was significantly positively correlated with serum cystatin c among all patients (p=0.004) (r=0.312), and among microalbuminuric patients (p=0.005) (r=0.415) and also this is consistent with **Jeon, *et al.* (2011)**, **Assal *et al.* (2013)** who reported also similar results.

This study revealed better diagnostic accuracy of serum cystatin c than serum creatinine in detecting microalbuminuria in diabetic patients using receiver operator characteristic (ROC) curves. The AUC was significantly higher (0.806) for serum cystatin c *P*-value < 0.0001, but not for serum creatinine (0.477) P-value = 0.715. The sensitivity and specificity of cystatin c were 86.7% and 55%, respectively. Similar results were observed by **Chae *et al.* (2012)** who found **the** AUC was 0.732 for serum cys C and 0.615 for serum creatinine. The AUC was significantly higher for serum cys C (P = 0.028), but not for serum creatinine (P = 0.069). The sensitivity and specificity of cys C were 87.3% and 66.2%, respectively. **Jeon, *et al.* (2011)** reported that the ROC curve analysis of serum cystatin for prediction of microalbuminuria in patients with diabetes showed that the (AUC) was 0.906 (Sensitivity, 81.0%; specificity, 87.1%). **Assal *et al.* (2013)** reported that the ROC curve analysis of serum cystatin for prediction of microalbuminuria in patients with diabetes showed that the (AUC) was 0.727. Sensitivity = 83.3% and specificity = 61.1%.

Conclusion and Recommendations

The present study has clearly demonstrated that microalbuminuric diabetic patients showed increased serum cystatin c, and the severity of renal damage caused by diabetic disease is well reflected by these levels. In addition, there was significant negative correlation between serum cystatin c and estimated glomerular filtration rate. Cerum cystatin c measurement might become a useful, practical and noninvasive accurate tool for early detection of microalbuminuria and renal insufficiency and also further researches are needed to detect the association between serum cystatin c and other factors that might affect its level.

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