Serum Cystatin C and Beta-2-microglobulin as Early Biomarkers of Diabetic Nephropathy in Patients with Type II Diabetes Mellitus

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Abstract: Changes in glomerular filtration rate (GFR) provide a valuable indicator of the progression of diabetic nephropathy (DN). This study was designed to demonstrate the clinical values of serum cystatin C (Cys C) and β_2 microglobulin in the assessment of renal function in type 2 diabetics by comparing with the GFR. **Patients and Methods:** 75 type 2 diabetic patients with (urinary albumin excretions (UAE) < 30 mg/24h) (n=39) and without (UAE 30-300 mg/24h) (n=29) microalbuminuria and 32 controls were enrolled in the study. Serum Cys C, β_2 microglobulin, creatinine, urinary microalbumin levels and eGFR values were determined in all groups. **Results:** Serum CysC, β2-microglobulin, glucose and HbA1c concentrations were significantly high in normoalbuminurics and in microalbuminurics compared to controls. In the patients with microalbuminuria, serum CysC and glucose concentrations increased significantly in comparison to patients with normoalbuminuria, while no differences were observed for β2-microglobulin levels. Serum creatinine concentrations and GFR values were not different between both diabetic group and controls. CysC was positively correlated with β2-microglobulin and creatinine and negatively with GFR values; β2-microglobulin was also positively correlated with serum creatinine in microalbuminurics. A significant inverse correlation was found between β2-microglobulin and GFR values in both microalbuminurics and normoalbuminurics. **Conclusions:** Increased CysC and β_2 microglobulin in the microalbuminurics may be early indicators of incipient DN. The significant elevation of CysC and β_2 microglobulin in the normoalbuminurics compared to controls may be indicative of an increased risk for DN.

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1. Introductıon

Kidney disease is a significant problem in the diabetic population. The proportion of patients with end-stage renal disease (ESRD) caused by diabetes has progressively increased during the last few decades, and diabetic nephropathy (DN) is now the single most common cause of ESRD in the Western world. In 2010, 44% of all new cases of ESRD in the U.S. were diagnosed in diabetic patients, >80% of whom have type 2 diabetes [1]. An increasing number of type 2 diabetic patients live long enough for nephropathy and end-stage renal disease to develop, due to the improvement in the treatment strategies. Therefore, prevention of DN or at least slowing down the disease process, has emerged as a key issue [2]. Determination of microalbuminuria has been suggested as an early predictor of diabetic glomerular disease [3, 4]. However, its excretion rates are altered by blood pressure variations and exercise [5]. In addition, there is a 40% day-to-day variability in microalbuminuria [6].

Glomerular filtration rate (GFR) is the most important clinical renal function to monitor in health and disease. The ideal marker for GFR should be produced endogenously at a constant rate and should be freely filtered in the renal glomeruli. Also it should not be reabsorbed, secreted or eliminated by other means. In clinical practice, serum creatinine is the most widely used index for the noninvasive assessment of GFR. Despite its specifity, serum creatinine demonstrates an inadequate sensitivity, particularly in the early stages of renal impairment. It has significant disadvantages such as inability to measure renal function impairments of 50% or less [7]. Moreover, because creatinine is not an inert substance and is secreted by the proximal tubules, elevating the true GFR by up to 30%, and because of inaccurately timed urine collections creatinine clearance measurements are of limited value [8]. Besides, both creatinine clearance and serum creatinine values are affected by dietary protein and muscle mass, as well as the documented analytical interferences for creatinine with the Jaffé and less

affected enzymatic methods [7, 9]. Thus, serum creatinine can only be used as a crude indicator of a significantly impaired renal function [7]. Taken together, although creatinine is a cheap and simple test for the assessment of GFR, it is hampered by many biological and technical problems.

The measurement of plasma concentration of various low molecular weight (LMW) proteins has been proposed as a useful tool to evaluate the impairment of GFR [10, 11]. Among these, cystatin C (CysC) (MW 13.3 kDa) and β2-microglobulin (MW 11.8 kDa) have been suggested as better markers of GFR than creatinine [12, 13]. CysC, is a non-glycosylated, basic protein (p*I*=9.2) that is a member of the cystatin superfamily of cysteine protease inhibitors [14]. It is constitutively produced by all nucleated cells and therefore exhibits a stable production rate even in the presence of an acute inflammatory response [15]. It does not seem to be significantly influenced by gender and age beyond the first year of life [16]. β2-microglobulin is associated with the histocompatibility antigen complex on the surface of nucleated cells and is shed from the cells during cellular turnover. It is filtered by the glomeruli and reabsorbed by the proximal tubular cells where it is metabolized. Its plasma concentration increases with decreasing renal function. Its production, however, is drammatically different in patients with lymphoproliferative syndromes, infections and autoimmune diseases [17].

This study was designed to show the clinical values of serum Cys-C and β_2-microglobulin in the assessment of renal function in type 2 diabetics with and without microalbuminuria by comparing with the eGFR.

2. Subjects and Methods

Subjects

Seventy five consecutive type 2 diabetic patients who fulfilled the American Diabetes Association (ADA) criteria [18] were studied (42 women and 26 men). The mean age (± SD) of these patients was 57,57 (± 9.7) years [range 28-75].

A questionnaire which included age, duration of diabetes, use of medication for diabetes was made. Subjects who had an infectious disease, or a non-diabetic renal disease were excluded from the study. None of the patients had a history of cerebrovascular or ischemic heart diseases, neuropathy and hypertension. Thirty nine patients were microalbuminuric and twenty nine were normoalbuminuric. In order to exclude the effect of antihypertensives on GFR and microalbuminuria, we examined normotensive patients and controls in this study. Forty patients were taking insulin and twenty-eight were taking oral antidiabetic agents. Healthy age-matched subjects (n=32, 19 women and 13 men) served as non-diabetic controls. The mean age (± SD) of these subjects was 48.1 (± 9.3) years [range 35-69]. All subjects gave written, informed consent, which was approved by the Ethical Committee of Medical Faculty.

Methods

The renal function was assessed by serum creatinine, urinary microalbumin, and the GFR estimated. They were selected from Internal Internal Medicine department and outpatient clinics in Al-Azhar university, New Damietta hospital and other hospitals, at the period from January 2017, to April 2018.

Urinary microalbumin measurements were made on a Hitachi 704 analyzer (Hitachi Tokyo, Japan) by using commercialy available immunoturbidimetric assay kits (Roche Diagnostics, GmbH, Germany) and were evaluated on the basis of at least three consecutive measurements in 24 h. collections and values between 30-300 mg/ day were accepted as microalbuminuric and <30 mg/day was accepted as normoalbuminuric.

Blood samples were taken after a 12-14 h overnight fast from the subjects for the analysis of clinical chemical parameters including serum Cys-C and β_2-microglobulin, creatinine, HbA1c and glucose. CysC and β2-microglobulin were measured using latex particle-enhanced turbidimetry (PET) kits (Dako cystatin C PET kit code no:0071 and Dako β2-microglobulin PET kit code no:0052) on the Hitachi 704 automatic analyzer (Boehringer Mannheim GmbH, Mannheim, Germany). Creatinine was measured by Jaffé method [19], performed on an automatic analyzer (Dax 48, Bayer Diagnostics, Toshiba, Japan). HbA1c levels were measured by commercialy available immunoturbidimetric assay kits (Roche Diagnostic, GmbH, Germany) on a Hitachi 704 analyzer (Hitachi, Tokyo, Japan). Serum glucose was determined on an automatic analyzer (Technicon Dax-48, Bayer Diagnostic, Toshiba, Tokyo, Japan).

For statistical analysis the results were subjected to parametric and nonparametric tests (t-test, One Way anova, Student Newman-Keuls test, Mann-Whitney U and Pearson correlation tests) using SPSS (Statistical Packages for Social Sciences) for Windows Version: 10.0, as appropriate. Data are expressed as mean ± SD.

3. Results

On the basis of the urinary albumin excretions, patients were divided in two groups; 39 with microalbuminuria and 29 with normoalbuminuria. Their main characteristics were summarized in Table

1. Serum CysC concentrations were significantly high in normoalbuminurics and in microalbuminurics compared to controls. Microalbuminuric patients have a higher serum CysC levels than normoalbuminuric patients (p=0.001, for all). Serum β2-microglobulin concentrations were significantly high in both microalbuminurics and normoalbuminurics compared to controls (p=0.0002). However, there was not any significant difference between microalbuminurics and normoalbuminurics. Serum creatinine concentrations and GFR values were not significantly different between the groups. Serum glucose concentrations were higher in microalbuminuric and normoalbuminuric patients than controls. Furthermore, patients with microalbuminuria have higher serum glucose concentrations than the normoalbuminuric patients (p=0.001, for all). HbA1c levels were also significantly high in microalbuminuric and normoalbuminuric patients than controls (p=0.001). However, there were not significant differences between microalbuminuric and normoalbuminuric patients.

Serum creatinine values significantly differed (p=0.004) between males and females (102.54 ± 20.33 vs. 86.63 ± 18.56 μmol/L, respectively), while no differences were observed for CysC (males, 1.43 ± 0.38; females, 1.45 ± 0.42 mg/L, respectively) (p=0.87) and for β2-microglobulin levels (males, 2.60 ± 0.71; females, 2.46 ± 0.83 mg/L, respectively) (p=0.49) in the overall patient group.

In microalbuminuric patients: Serum CysC levels were positively correlated with serum β2-microglobulin (p = 0.003, r = 0.47) and serum creatinine levels (p = 0.033, r = 0.34) and negatively with GFRvalues (p = 0.029, r = - 0.35). A significant inverse correlation was found between serum β2-microglobulin concentrations and GFR values (p = 0.002, r = -0.48). Serum β2-microglobulin levels were also positively correlated with serum creatinine (p = 0.005, r = 0.44). Serum glucose and urinary microalbumin levels were positively correlated (p = 0.047, r=0.32). Serum β2-microglobulin, creatinine levels were positively and GFR values were negatively correlated with age (p = 0.001, r = 0.59; p=0.013, r = 0.39; p = 0.013, r = -0.39, respectively).

In normoalbuminuric patients: A significant inverse correlation was found between serum β2-microglobulin concentrations and GFR values (p=0.012, r=-0.46). GFR values were negatively correlated with age (p=0.001, r = - 0.63).

Table 1. Diagnostic performance of single markers for discriminating between healthy individuals versus all patients:

Marker	Cystatin	Beta 2 microglobulin	CKD-EPI creatinine-cystatin equation	CKD-EPI cystatin C equation
Area under ROC curve				
Cut-off	1.1	2.2	86	73
Sensitivity (%)	80	95	84	84
Specificity (%)	96	92	92	92
Positive predictive value (%)	98	97	97	97
Negative predictive value (%)	62	85	66	66
Accuracy (%)	84	94	84	84

Table 2. Diagnostic performance of single markers for discriminating between Diabetes vs micro albuminuria:

Marker	Cystatin	Beta 2 microglobulin	CKD-EPI creatinine-cystatin equation	CKD-EPI cystatin C equation
Area under ROC curve				
Cut-off	1.3	2.8	66	50
Sensitivity (%)	72	60	72	72
Specificity (%)	68	64	88	88
Positive predictive value (%)	69	63	86	86
Negative predictive value (%)	71	62	76	76
Accuracy (%)	70	62	80	80

Table 3. Diagnostic performance of CKD markers for discriminating between micro albuminuria vs macro albuminuria:

Test Result Variable (s)	AUC	S.E	P value	95% CI	
				Lower Bound	Upper Bound
Beta 2 microglobulin	0.95	0.029	< 0.0001	0.889	1.002
EPI_Cr	0.97	0.021	< 0.0001	0.931	1.015
Cystatin	0.99	0.007	< 0.0001	0.981	1.008
MDRD_study	0.98	0.017	< 0.0001	0.945	1.013
EPI_Cr_cyst	0.99	0.007	< 0.0001	0.981	1.008
EPI_cyst	0.99	0.007	< 0.0001	0.981	1.008

Table 4. Diagnostic performance of combined markers:

Healthy vs all patients						
AUC	Cutoff	Sensitivity (%)	Specificity (%)	PPV	NPV	Accuracy (%)
0.98	1.9	91	96	99	77	92
Diabetes vs micro-albuminuria						
0.71	2.2	72	60	64	68	66
Macro-albuminuria vs micro-albuminuria						
0.98	2.4	96	92	92	96	94

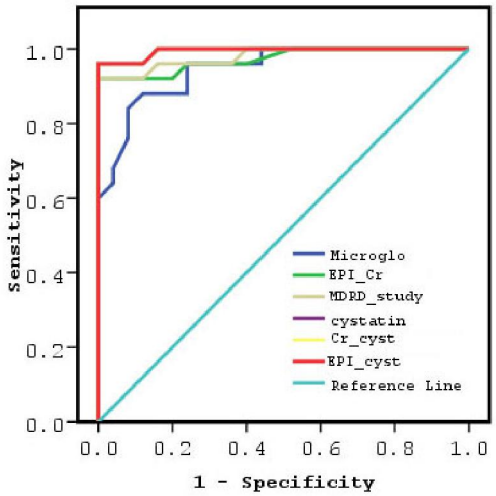

Figure 1. Area under ROC Curve of single markers for discriminating between micro albuminuria vs macro albuminuria.

4. Discussion

The decline in renal function in type 2 diabetic patients leads to a reduction in GFR and in a proportional increase in microalbuminuria. There has been broad acceptance of microalbuminuria as a marker of increased DN risk. However, assessment of microalbuminuria cannot replace the GFR estimation, because they may represent different aspects of renal damage. Besides, albumin excretion rates are altered by variations in blood pressure and exercise as well as blood glucose levels and there is an intra-individual variability during the evolution of albuminuria, and day-to-day variation is known to amount to up to 40-50% [20]. As noted by Mogensen, the presence of microalbuminuria is indicative of stage III DN [21]. This suggest that microalbuminuria is a late manifestation in the course of DN. Furthermore, microalbuminuria is not specific for diabetes or early nephropathy alone but is considered to reflect generalized vascular damage [22]. Thus, it would make sense to try to define normoalbuminuric patients at increased DN risk in order to apply aggressive preventive strategies.

The clinical methods utilised to measure renal function ideally should be accurate, reproducible, simple, safe, inexpensive and free of cumbersome features. Standard clearance techniques require timed urine collections, which are not only time-consuming but subject to erroneous and inaccurate collection. While single-shot clearance techniques may be more accurate and less cumbersome, the injection of agents is not practical if repeated measurements are required and involve radiation exposure [23]. The use of endogenous markers is thus of great advantage due to speed and simplicity. In clinical practice, serum creatinine and creatinine clearance are widely used as indirect markers of GFR. It was demonstrated that CysC and β2-microglobulin had diagnostic accuracies very similar to that of creatinine as markers of GFR [10, 24]. One of the most significant advantages of CysC in comparison with traditional markers of renal impairment is that very small reductions in GFR cause significant increases in CysC serum levels [25]. To be of use for monitoring renal function, any parameter is expected to have a low intraindividual variation. It has been reported that CysC possesses a higher intraindividual variance but a lower interindividual variance than serum creatinine, allowing earlier detection of impaired renal function [26]. Although CysC may be potentially better for detecting the onset of an abnormal GFR, it is said to be not as sensitive as serum creatinine for detecting changes within the same individual [26]. However, creatinine levels do not change until significant impairment of the renal function occurs.

Although CysC has been proposed as a reliable serum marker of GFR [16, 27, 28], the results in patients with diabetes mellitus are controversial. It has been reported that serum CysC is a better marker of GFR than creatinine and creatinine clearance in type 2 diabetic patients [29-32]. By contrast, Oddoze et al found that serum CysC was not better than serum creatinine or β2-microglobulin for the estimation of GFR in patients with steady-state diabetes [33]. In our study, while serum creatinine and GFR values were not different between both diabetic group and controls, the significant increase in serum CysC levels among microalbuminuric patients compared with the normoalbuminuric patients and control subjects suggests that the CysC levels were higher in patients with renal involvement. Similar to our results, Mojiminiyi et al. reported that serum CysC was significantly higher in patients with microalbuminuria than in normoalbuminuric patients [34]. Furthermore, Oberbauer has demonstrated that the renal clearance

of CysC in patients with proteinuric DN but a normal GFR is reduced due to its molecular size [35]. This could explain the increased levels of serum CysC and the statistically not different GFR values in our patients. Positive relations between serum CysC and serum β2-microglobulin, creatinine and negative with GFR values in the microalbuminuric patients further supports this observation. Furthermore, the significantly high CysC levels in normoalbuminuric patients compared with the control subjects indicate that increased serum CysC concentrations precedes microalbuminuria in DN.

Serum β2-microglobulin is also said to be a good endogenous marker of GFR, better than serum creatinine [13]. With declining renal function, increase in serum β2-microglobulin more and before than serum creatinine has been demonstrated [13, 36]. In this study, serum β2-microglobulin concentrations were also high in both microalbuminuric and normoalbuminuric patients compared to controls. However, there was not any significant difference between normoalbuminurics and microalbuminurics. Similar to our results, Piwowar et al found that serum β2-microglobulin concentrations were high in both normoalbuminuric and microalbuminuric type 2 diabetic patients and there were not differences between the two diabetic groups [31]. Thus, increased serrum β2-microglobulin levels were also early indicators of the renal involvement in type2 diabetes. Increased serum β2-microglobulin levels precedes microalbuminuria. However, the increase in serum β2-microglobulin levels do not discriminate the presence or absence of increased albumin excretion in these patients. Thus, serum CysC concentrations allow us to recognise the presence of microalbuminuria, in other words, to show the degree of renal involvement in type 2 diabetes mellitus while serum β2-microglobulin levels do not. In addition, while serum creatinine levels differed between males and females, serum β2-microglobulin and CysC levels did not differed between sexes. This known disadvantage of serum creatinine also makes these markers more preferable.

It has been well demonstrated that there is a positive relationship between hyperglycaemia, and microvascular complications. Meigs et al. reported that long-term hyperglycemia and subdiabetic glycemia increase risk for microalbuminuria [37]. We found positive relations between serum glucose and microalbumin levels in microalbuminuric diabetics. In addition, serum glucose levels were higher in microalbuminurics than in normoalbuminurics. Normoalbuminuric patients have a higher glucose level than controls. The development of DN closely correlates with the duration and the magnitude of the preceding hyperglycemia. Hyperglycemia has been causally linked to vascular and glomerular dysfunction by a variety of biochemical mechanisms, including increased polyol pathway flux, increased de novo diacylglycerol synthesis with resultant activation of protein kinase C isoforms, altered intracellular redox state and advanced glycation end product (AGE) formation [38, 39].

5. Conclusion

CysC and β2-microglobulin seems to be alternative markers to serum creatinine in the assessment of DN. The more prominent rise in serum CysC values allows a more rapid diagnosis of decline in GFR, with an earlier therapeutic treatment.

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