Original Article

Study of serum Cystatin C, beta-2 microglobulin and protein carbonyl in diabetic nephropathy

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Abstract

Aim: Diabetic nephropathy is the leading cause of mortality due to long term uncontrolled hyperglycaemia related complications.The aim of present study was to evaluate clinical values of serum Cys-C and β_2 microglobulin and protein carbonyl as a marker of renal dysfunction and accompanied oxidative stress in diabetes mellitus patients. **Material and Methods:** A total of 47 consecutive diabetic nephropathy patients (age > 40 years) attending the Diabetic OPD and dialysis unit at Sir J.J. Hospital were included in the study. Serum creatinine, Cystatin C, β-2 microglobulin, Total protein and Albumin, Urea, Uric acid and protein carbonyl, HbA1c, fasting and post prandial glucose were measured. Glomerular Filtration Rate (GFR) was calculated using formula by National Kidney Foundation calculator, for both creatinine and Cystatin C and the grade of kidney disease and risk of progression was graded as1 : Stage 1 : Mild; 2 : Stage 2: moderate; 3: Stage 3: high; 4: Stage 4: very high. P value of < 0.05 was considered statistically significant. Unpaired t test and Pearson correlation was used to data analysis using SPSS statistical software (version 19.0). **Results:** The GFR was significantly higher in male compared to the female ($p<0.01$). Protein carbonyl and β- 2 microglobulin were significantly higher in female compared to the male group ($p <0.08$ and 0.02).The correlation of serum uric acid and protein carbonyl were stronger when Chronic Kidney Disease (CKD) severity when calculated using serum Cystatin C ($r= 0.30$ and 0.37 respectively) as compared to serum creatinine value based GFR (0.48 and 0.64 respectively).Serum Cystatin C showed significant correlation ($r=0.35$) with β- 2 Microglobulin while serum creatinine was not found to be correlated with beta-2 Microglobulin. **Conclusions:** Cystatin C and β2 microglobulin can serve as an early and accurate marker of renal function as compared to serum creatinine in patients with diabetes mellitus induced nephropathy.

Key Words: Diabetes mellitus, Nephropathy, Oxidative stress, Cystatin C and protein carbonyl.

Abbreviations: Chronic Kidney Disease (CKD); Glomerular Filtration Rate (GFR).

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INTRODUCTION

Diabetic nephropathy is a severe complication occurring in diabetic patients and it is associated with many complications such as cardiovascular disease, progression of chronic kidney disease thus increased risk of all-cause mortality. Development of end stage renal disease needs costly renal replacement therapy in the form of dialysis or transplantation[1]. The proportion of patients with end-stage renal disease caused by diabetes has progressively increased during the last few decades, and diabetic nephropathy is now the single most common cause of ESRD in the Western world[2]. Indian CKD registry established under the aegis of Indian Society of Nephrology; has been collecting data and has reported on 38,193 patients collected from 154 centers; to include 38 centers from Indian Society of Pediatric Nephrology. It has made pertinent observations. It has reported that Diabetes mellitus as the cause of CKD was found in 31.2% of patients[3]. Prevention or slow down the process of renal dysfunction is the key to improve the life expectancy of diabetic patients. Diagnostic markers play very important role in the early diagnosis of kidney

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disease and thus deciding the treatment strategies to slowdown the loss of renal function and decrease adverse outcomes. Serum creatinine is the traditional marker used to measure renal function through assessment of GFR but for early detection of renal dysfunction, serum creatinine has shown low specificity and sensitiviy. Further its level in serum are affected by muscle mass and dietary proteins. It has significant disadvantages such as inability to measure renal function impairments of less than 50%[4]. Oxidative stress and associated oxidative damage play the significant role in the development and progression of diabetic complications. Exposure of proteins to ROS causes major physical change in protein structure thus affects its functions. Protein carbonyl is a measure of chemical and non enzymatic oxidative oxidation and serves as a marker of free radical intensity[5]. The measurement of low molecular weight proteins such as cystatin C, β2-microglobulin and oxidative stress markersuch as protein carbonyl have been suggested as better markers of renal impairments[2,6]. Cystatin C isis a non-glycosylated protein produced by all nucleated cells, freely filtered, reabsorbed and completely metabolized by the proximal tubule and therefore is not subjected to tubular secretion[7]. 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[8]. Protein carbonyls are the oxidation product of proteins and are reported as the potent biomarker of oxidative stress. They represent the stable end product generated upon formation of transient radical species, such as chloramines and nitrogen/carbon radicals, which are induced by oxidant stimuli[9]. The aim of present study was to evaluate clinical values of serum Cys-C and β_2 microglobulin and protein carbonyl as a marker of renal dysfunciton in diabetes mellitus patients.

MATERIAL AND METHOD

Present prospective cohort study was conducted at Department of Biochemistry, Sir J. J. Hospital, Mumbai. The study was approved by the institutional Ethical Committee of hospital.

Subjects

A total of 47 consecutive patients of either gender above 40 yrs of age with no history of any major inflammatory condition or malignancy in the past 6 months attending the Diabetic OPD and dialysis unit at Sir J.J. Hospital were included in the study. Subject with k/c/o Hypothyroidism/ Hyperthyroidism and developing post dialysis complications were excluded from the study. Out of the 47 patient, 35 were males and 12 were females. Mean age of the cohort was 73.13 (ranging from 61-87 years. Dialysis was carried out as per prescribed standardised procedure (haemodialysis HD / Peritoneal dialysis PD). Written, informed consent was obtained form all the study participants before their enrolment. Patient's clinical history and demographic data (name, age, gender, height, weight and past history of any chronic disease, dietary habits) was collected as per patient's records and history given by patient or accompanying relatives.

Sample Collection and Processing

Blood samples were collected in evacuated tubes by trained phlebotomists. Serum was separated and used for estimation of serum creatinine, Cystatin C, β-2 microglobulin,, Total protein and Albumin, Urea, Uric acid and protein carbonyl in plain tubes,HbA1c in EDTA tubes and fasting and post prandial glucose in fluoride tubes. The samples were analysed immediately or stored at -20degrees deep freeze. Stored samples were thawed and analysed for the said parameters and transported as per standard protocol mentioned by manufacturers. Serum Cystatin c was measured using Latex enhanced Immunoturbidimetry principle with ready to use reagents on AU-400 autoanalyser using standard kit by Agappe[10]. Serum creatinine was measured using modified Jaffe's method on AU-400. Protein carbonyl contentwas measured on semi-automated analyser using method of Levine *et al*, 1990[11]. HbA1c was estimated using direct quantitative determination in whole blood with ready to use reagents. Serum β- 2 microglobulin was measured using Enzyme Enhanced Chemiluminiscence method on Immulite 1000 analyser. GFR was calculated using standard formula by National Kidney Foundation calculator, for both creatinine and Cystatin C and the grade of kidney disease and risk of progression was graded as1 : Stage 1 : Mild; 2 : Stage 2 : moderate; 3: Stage 3 : high; 4: Stage 4 : very high.

Statistical Analysis

All quantitative data was presented as mean ± SD for normally distributed values and qualitative data was presented as number and percentages. Differences between the groups were analysed using unpaired t test. Pearson's correlation coefficient was employed to test the correlations between two quantitative variables. P value of < 0.05 was considered statistically significant. For all

statistical analysis, SPSS statistical software for Windows (version 19.0) was used.

RESULTS

Table 1 indicates the demographic data. Out of the 47 subjects with Diabetic nephropathy included in the study, 23.4% subjects were active and working as opposed to 76.6% who were leading a sedentary life. With respect to the dietary habits, 74.5% were non vegetarians and 25.5 % were vegetarians or had modified their dietary habits owing to nephropathy. According to the Kuppuswamy's scale (2016) for socioeconomic status, 55 % subjects belonged to the upper middle class, 17 % to the middle class and 27.4 % belonged to the lower socioeconomic status. With respect to habit of smoking, 54% were non-smokers, only 12% were smokers and 34% had previous H/o smoking. Out of the 47 subjects 57.4 % were alcoholic 42.6 % were non-alcoholic.

Table 1: Clinical and other characteristics of participants

Parameters	Number (%)
Lifestyle	
1-Sedentary	36 (76.6 %)
2-Working	11 (23.4 %)
Dietary Habits	
1-Non-veg (all 7 days)	14 (29.8 %)
2- Non-veg (thrice a week)	7 (14.9 %)
3- Non-veg (once a week)	14 (29.8 %)
4-Vegitarin	12 (25.5 %)
Socioeconomic Status	
1-Lower	13 (27.4 %)
2-Middle	8 (17.0 %)
3-Upper Middle	26 (55.0 %)
Smoking	
1-Smoker	6 (12.8 %)
2-Non-smoker	26 (55.3 %)
3-Previous H/o smoking	15 (31.9 %)
Alcohol	
1-All 7 days	3 (6.4 %)
2- Thrice a week	4 (8.5 %)
3- Once a week	4 (8.5 %)
4-Previous H/o Alcohol	16 (34.0 %)
5- Non-alcoholic	20 (42.6 %)
History of Diabetes	
1-0-5 years	0 (0 %)
2- 06-Oct	5 (10.6 %)
3- Nov-15	10 (21.3 %)
4-16-20	8 (17.0 %)
5- 21-25	9 (19.1 %)
6-26-30	12 (25.5 %)
7- more than 30	3 (6.4 %)
Previous Cardiovascular events	
1-Yes	29 (61.7 %)
2-No	18 (38.3 %)
Hypertension History	
1-0-5 years	1 (2.1 %)
2- 06-Oct	18 (38.3 %)
3- Nov-15	12 (25.5 %)
4-16-20	14 (29.8 %)
5- 21-25	1 (2.1 %)
6-26-30	1 (2.1 %)
7- more than 30	0 (0.0 %)
Dialysis History	
1- Peritoneal	12 (25.5 %)
2- Hemodialysis	35 (74.5 %)
3- No dialysis	0 (0.0 %)

Table 2 indicates the gender wise comparison of demographic and biochemical parameters of the study population. The mean height and weight of male group was significantly higher compared to the female group (p <0.0001 and 0.012 respectively). For biochemical parameters, the GFR levels based upon serum creatinine and Cystatin C were found to be significantly higher in male group compared to the female group (p<0.007 and 0.049 respectively). The value of oxidative stress marker protein carbonyl and β- 2 microglobulin were significantly higher in female compared to the male group (p <0.08 and 0.02). There was no significant difference in age, fasting glucose, Post prandial glucose, HbA1c, Blood Urea level, serum Total protein, Albumin n both the groups.

Table 2: Gender wise comparison of demographic and biochemical characteristics of study population

Parameters	Normal Ranges	Males (n=35) (Mean ± SD)	Females (n=12) (Mean ± SD)	p-value
Age (years)	NA	74.11 + 7.23	70.25 + 5.27	.097 NS
Height (cms)	NA	168.83 + 4.79	153.08 + 3.47	< 0.0001***
Weight (kgs)	NA	74.83 + 8.49	67.33 + 8.53	.012*
Serum Creatinine (mg/dl)	0.1-1.1	1.80 + 0.39	1.88 + 0.34	.52 NS
Serum Cystatin C (mg/L)	0.63-1.44	2.68 + 0.65	3.06 + 0.73	.10 NS
ACR(mg/g)	< 30	1.11 + 0.32	1.25 + 0.45	.26 NS
GFR (Creatinine) (ml/min/1.73m^2)	>75	45.46 + 19.38	28.67 + 10.62	.007**
GFR (Cystatin C) (ml/min/BSA)	> 60	25.74 + 11.33	18.25 + 10.31	.049*
Fasting Plasma Glucose (mg/dl)	90-110	136.69 + 24.68	130.25 + 20.67	.42 NS
PP Plasma Glucose (mg/dl)	<140	199.37 + 71.27	186.42 + 37.52	.55 NS
Blood Urea (mg/dl)	15 – 45	35.26 + 7.93	34.42 + 6.12	.74 NS
ProteinCarbonyl(µmol/g)	0.5	2.16 + 0.57	2.69 + 0.58	.008***
Total Protein (g/dl)	5.5 – 7.5	6.03 + 0.46	5.93 + 0.56	.53 NS
Albumin (g/dl)	3.5- 5.5	2.62 + 0.42	2.53 + 0.47	.53 NS
Uric Acid (mg/dl)	Males : 2-7 Females: 2 -6	7.41 + 0.77	7.54 + 0.75	.60 NS
Beta- 2 microglobulin (ng/ml)	670-2143	3407.09 + 920.83	4279.42 + 1602.14	.02*
HbA1c (%)	<6% Non-diabetic <7% known diabetic	7.14 + 1.39	7.16 + 0.92	.97 NS

Table 3 indicates correlation of uric acid and protein carbonyl content with severity of CKD due to Diabetic nephropathy. Serum uric acid and protein carbonyl showed significant correlation with CKD severity based on calculated GFR using serum creatinine and serum Cystatin C values. The correlation of Serum uric acid and protein carbonyl were stronger when CKD severity when calculated using serum Cystatin C (r= 0.30 and 0.37 respectively) as compared to serum creatinine value based GFR (0.48 and 0.64 respectively).

Table 3: Correlation of CKD severity with uric acid and protein carbonyl

Parameter	CKD staging based on serum Creatinine)		CKD staging based on serum Cystatin C)	
	r Value	P value	r Value	P value
Serum Uric Acid (mg/dl)	0.3054	0.03*	0.4832	0.0006***
protein carbonyl	0.376	0.0092**	0.6432	<0.0001***

Table 4 presents the correlation of serum creatinine, Cystatin C, beta-2 microglobulin and protein carbonyl with HbA1c and age. Serum creatinine and Cystatin C equally positively correlated with HbA1c and onlybeta-2 Microglobulin showed negative correlation (r=0.57 and 0.52 respectively) with age (r=-.41**).

Table 4: Correlation of serum creatinine Cystatin C, beta-2 microglobulin and protein carbonyl with HbA1c and age

Variables	Normal range	HbA1c (%)		Age (years)	
		R	P value	R	P value
Serum Creatinine (mg/dl)	0.1-1.1	.57**	0.0001**	-.08	0.6 ns
Serum Cystatin C (mg/L)	0.63-1.44	.52**	0.0001**	-.25	0.09ns
Serum beta-2 Microglobulin (ng/ml)	670- 2143	-.04	0.77NS	-.41**	0.004**
Serum Protein carbonyl (micromole/gram)	0.5	.24	0.10NS	-.22	0.14

Figure 1 shows the correlations of serum Cystatin C and serum creatinine with beta-2 Microglobulin. Serum Cystatin C showed significant correlation (r=0.35) with beta-2 Microglobulin while serum creatinine was not found to be correlated with beta-2 Microglobulin.

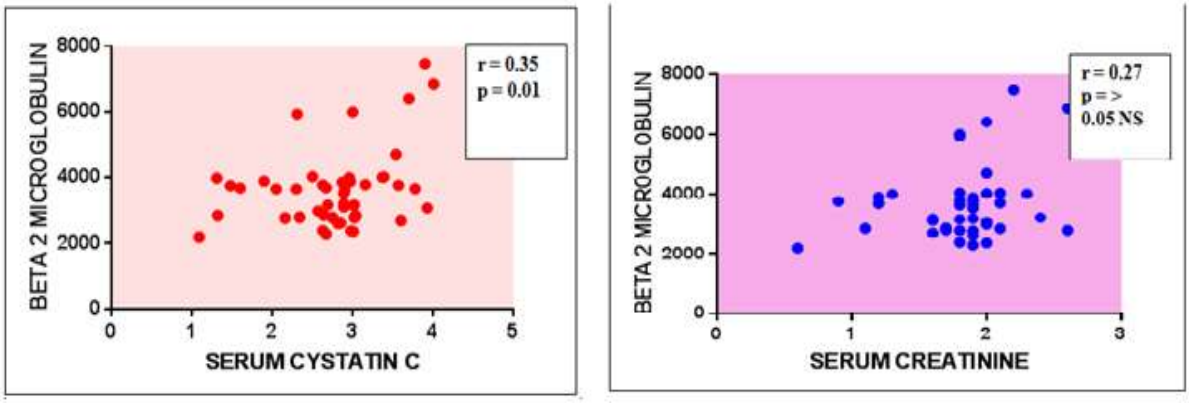

Figure 1: Correlation of beta-2 microglobulin with serum creatinine and serum Cystatin C

Table 5 shows the risk classification of Diabetic nephropathy subjects. As per CKD staging using GFR calculated with creatinine and Cystatin C. With GFR calculated using serum creatinine, 51.1% study participants were classified in Stage 2 CKD and 31.9 % in stage 4, however when GFR was calculated using Cystatin C 66% of study participants classified in Stage 4 with high degree of progression and 14.9 % in stage 5.

Table 5: CKD risk classification of study participants using serum creatinine and Cystatin C

CKD Stage	Based upon serum Creatinine levels		Based upon serum Cystatin C levels	
	Frequency	Percentage	Frequency	Percentage
1	7	14.9	2	4.3
2	24	51.1	7	14.9
3	1	2.1	--	----
4	15	31.9	31	66.0
5	0	0	7	14.9
Total	**47**	**100.0**	**47**	**100.0**

DISCUSSION

Diabetic nephropathy is a principal cause of end stage renal failure, which could account for disabilities and high mortality rates in patients with diabetes (12). Present study demonstrates the clinical value of serum Cystatin C, beta-2 microglobulin and oxidative stress marker protein carbonyl in the early diagnosis and management of chronic kidney disease due to diabetes mellitus. First we demonstrated the clinical, dietary; lifestyle and demographic history of study population (Table-1) which showed that our study cohort was an older population (mean age 73.13 years). Most of the individuals were non-vegetarians, following sedentary life style, had history of hypertension, cardiovascular events and were on dialysis. Gender wise comparison of biochemical parameters revealed that GFR, beta-2 microglobulin of male was significantly higher and protein carbonyl level was significantly lower in male compared to the female. High protein carbonyl level indicates high oxidative stress and lower GFR, beta-2 microglobulin levels shows loss of

renal function. Although in both male and female, the values of above mentioned parameters were higher when compared to the normal reference ranges but gender wise comparison shows that female were more affected by oxidative stress and had higher renal dysfunction as compared to male. Our result was in line with other studies. Vittorio Calabrese *et al*, 2007evaluated systemic oxidative stress in patients suffering from type 2 diabetes–induced nephropathy. They observed significantly high level of protein carbonyl (DNPH) ($P<$ 0.01 in all the samples from type 2 diabetic patients with nephropathy with respect to control group. They also observed significant positive correlations between protein carbonyl content and the degree of renal failure in diabetic uremic subjects[13]. Protein carbonyl is generated by oxidative modifications of proteins either by α-amidation pathway or by oxidation of glutamyl side chains, this leads to formation of a peptide in which the N-terminal amino acid is blocked by a α- ketoacyl derivative. However, direct oxidation of lysine, arginine, proline and threonine residues may also yield carbonyl derivatives. Oxidative damage causes decrease solubility, accumulation and impaired functions of proteins. Accumulation of plasma protein carbonyls, termed as "carbonyl stress," causes renal damage including diabetic nephropathy[14,15]. B- 2 microglobulin is a single chain, low molecular wt. non glycosylated protein produced by cells expressing MHC-I and associated with macro vascular complication in type-2 diabetes mellitus. Freely filtered at glomerulus are markers of tubular damage in various renal diseases. Because of its small size β2 microglobulin is filtered freely through the glomeruli. Changes in glomerular filtration rate provide a valuable indicator of the progression of diabetic nephropathy and B2MG in diabetics may be an early indicator of incipient diabetic nephropathy[16]. Cystatin C is produced in all nucleated cells, spontaneously filtered by the glomerulus without limits, and not secreted by the renal tubules makes it better and stable marker of renal function compared to serum creatinine because it is effected by gender, body muscle mass and diet. Same was observed in our study. In the present study, the levels of serum creatinine, β2 microglobulin and Cystatin C were measured in diabetic nephropathy subjects. When compared to normal ranges of these parameters, all were increased in diseased group and female had significantly higher β2 microglobulin compared to male. Although when serum creatinine and serum Cystatin C equally correlated with HbA1c but when both were correlated with β2 microglobulin, only Cystatin C showed significant positive correlation and serum creatinine did not show any significant correlation (Figure 1, Table-4). Further when subjects were classified according to CKD risk classification based on serum creatinine values, 51.1% subjects were in stage2 but upon using serum Cystatin C values for classification, 66.0% subjects reached to stage-4. When the oxidative stress markers were correlated with CKD severity, the correlation of serum uric acid and protein carbonyl with Cystatin C based CKD severity stage was better compared to serum creatinine based CKD severity (Table-3). All these data proves that serum Cystatin C is better marker of renal dysfunction compared to serum creatinine. Fathy *et al*, 2017 measured theclinical validity of serum Cystatin C for the early and accurate assessment of GFR compared to serum creatinine or β2 microglobulin in type 1 diabetes mellitus patients (n=80). They observed that the correlation of serum Cystatin C(r= 0.6) and β2 microglobulin(r=0.55) with 24 hour urine albumin compared to creatinine (r= o.5) and Cystatin C also had the highest negative correlation with GFR higher than that observed for serum creatinine or serum β2 microglobulin[17]. It can be concluded that Cystatin C and β2 microglobulincan serve as an early and accurate marker of renal function as compared to serum creatinine in patients with diabetes mellitus induced nephropathy. Monitoring of changes in protein carbonyl can be used to monitor oxidative stress induced progression and development of diabetic nephropathy.

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