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A Study of Serum Cystatin C in Chronic Kidney Disease Patients Undergoing Hemodialysis

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Abstract
Introduction: *CKD has become a major public health problem [(1)]. According to Dash and Agarwal (2006) [(2)] the prevalence of CKD in India accounts to around 0.8% of the total population which would be around 7.85 million of 1 billion population. In recent years, a novel endogenous marker of renal function, cystatin C, is emerging as a more sensitive marker than serum creatinine in estimating GFR [(3,4,5,6)].*
Methods: *Serum cystatin C, creatinine and blood urea nitrogen were estimated in patients of end stage kidney disease both before and after dialysis and also in the normal control individuals and were statistically analysed.*
Observations & Results: *The pre-dialysis and post-dialysis cystatin C were both significantly ($p < 0.0001$) higher than the normal control. Significant Correlation ($p = 0.0214$) between cystatin C and creatinine of the pre-dialysis test group and post dialysis group ($p=0017$) was observed. However no significant ($p= 0.6359$) correlation was found in the normal control group.*
Conclusion: *Considering the unique geographical location, ethnicity and dietary habits of the people of N.E. India, this study would be useful to determine if there are any significant changes in the levels of serum cystatin C in patients with chronic renal disease on haemodialysis in this part of the country and will also help the clinician in formulating the treatment protocol in such patients.*
Keywords: *Cystatin C, Creatinine, ESRD.*

INTRODUCTION

CKD is a pathophysiologic process with multiple etiologies, resulting in reduction of nephron number and function and frequently leading to end stage renal disease (ESRD).

Under normal conditions, the glomerular filter clears molecules with molecular weights up to 58,000 Dalton (Da) but in renal failure (ESRD) these solutes are retained. These retention products are usually divided according to their molecular weight and physiological properties [(7,8)].

1. Low molecular weight compounds, that have molecular weight (MW) less than 500 Da (eg Urea-60, Creatinine-113),
2. Middle molecular weight compounds, which have MW between 500-12000 Da eg. Cystatin C, parathyroid hormone 9424 Da, microglobulin 11,818 Da.

3. High molecular weight compounds, that have molecular weight more than 12,000 Da eg. Leptin.

Cystatin C (HCC) is a cysteine protease inhibitor that belongs to the family 2 of the cystatin superfamily. It is a low molecular mass (13.4kDa) protein that was initially known as γ-trace, post γ-globulin, γ- CSF. [9]. It was described for the first time in chick egg white in 1968 [10]. Chicken Cystatin was the first inhibitor of the cysteine proteases of the papain superfamily to be isolated and characterized [11].

Butler and Flynn found the protein in 79% of urines of 31 patients with tubular disease [12,13] and advanced the hypothesis that the protein originated from plasma but could simply not be measured because of a lack of methodological sensitivity. This alkaline low-molecular-weight protein appears in an electrophoresis after the globulin band, hence the first names given to it, such as ‘‘post g protein’’ or ‘‘g trace’’. Slightly later, different authors confirmed that it was present in serum and other body fluids (colostrum, saliva, seminal fluid and ascites) [14; 15,16,13].

Human cystatin C is mainly catabolised by free filtration in the glomeruli followed by virtually complete tubular reabsorption. Direct studies of cystatin C in the rat have shown that the plasma renal clearance of cystatin C is 94% of that of the frequently used glomerular filtration rate (GFR) marker ^{51}Cr EDTA and that cystatin C thus is freely filtered in the glomeruli. These studies also indicate that at least 99% of the filtered cystatin C was found to be degraded in the tubular cells. The plasma disappearance of cystatin C in normal and nephrectomised rats indicated that the renal plasma clearance of cystatin C is about 85% of the total plasma clearance (renal + extra renal). Cystatin C was first suggested as a marker for GFR in 1979 , when it was observed that the plasma level of cystatin C was upto 13 times higher in patients on hemodialysis than in healthy patients. According to Sambasivan & Lepage & Filler 2005[17], cystatin C as a better tool for longitudinal monitoring patients with advanced CKD. Cystatin C could be used for longitudinal follow-up of patients with CKD, with improved intra patient variability compared with serum creatinine.

Through the present study, we intend to utilize the data for a new clinical approach for better management of patients of chronic kidney disease undergoing haemodialysis.

AIMS & OBJECTIVES

- Estimation of Serum cystatin C level, Serum Creatinine and Blood Urea Nitrogen (BUN) in groups 1) CKD patients before and after dialysis, 2) Normal healthy individuals.
- Statistical evaluation, interpretation, comparison, and correlation of findings in the study groups.
- Comparison of results with previous studies.

MATERIALS & METHODS

The present study was conducted in the Department of Biochemistry, the Department of Nephrology of Gauhati Medical College & Hospital, Guwahati, Assam. This work was sanctioned by the Institutional Ethics Committee, Gauhati Medical College, vide letter no. MC/90/2012/pt-11/17, dated the 21st April, 2012. The period of study was from June, 2012 to July, 2013. The target subjects were divided into 2 groups:

1. **Case group:** This group consisted of patients above 18 years diagnosed with chronic kidney disease undergoing hemodialysis. This group was further sub-divided into 2 sub-groups:
   Pre-dialysis sub-group &Post-dialysis sub-group
2. **Control group:** This group consisted of individuals without any kidney disease.

Exclusion Criteria

- Patients on glucocorticoids as intake of glucocorticoids increases serum cystatin C level.
- Patients with previous history of thyroid disorders, as cystatin C levels decreases in hypothyroid and higher in hyperthyroid patients.

Sample Collection

- The Pre-dialysis blood samples were collected from the arterial(dialyzer inflow) blood port by a 5 ml syringe before the commencement of dialysis.
- The steps followed while drawing the post-dialysis blood sample were

1. Turning off of ultrafiltration.
2. Slowing the blood pump to 100 mL/min for 10 s and then stopping the pump.
3. Drawing blood sample from the arterial (dialyzer inflow) blood port by a 5 ml syringe

Methods of Evaluation

Estimation of Serum cystatin C was done by double antibody sandwich ELISA method using ELISA Microplate Reader (Biorad 680). Serum Creatinine and Blood Urea were estimated using double beam UV Spectrophotometer (Spectra scan UV 2600). Blood Urea Nitrogen (BUN) was calculated from Blood Urea using the formula BUN = Blood Urea/2.14.

The estimated glomerular filtration rate of the patients were assessed by MDRD, equations from the pre-dialysis case group using standardized serum creatinine, age, race, gender.

After all the calculations and the biochemical estimations, the results obtained were statistically analyzed and compared between the the groups of the study. Baseline characteristics of the study participants were expressed in Mean ± SD. Serum cystatin C levels were compared with normal Control and Test groups (Pre-dialysis and Post-dialysis) with unpaired "t" test. The pre-dialysis and post-dialysis cystatin C values were compared with paired "t" test. The correlation of Serum cystatin C and Serum Creatinine levels and Serum cystatin C and Blood Urea Nitrogen levels of the pre-dialysis test group were assessed by Pearson correlation coefficient. The correlation of dialysis adequacy with cystatin C and between creatinine were assessed by Pearson correlation coefficient. The results were taken as significant when the probability (p) was less than 0.05 % of the observing values of "t" at a particular degree of freedom. Statistical analysis was performed using GraphPad InStat version 3.00. All the statistical graphs were prepared using Microsoft Excel 2007.

OBSERVATIONS & RESULTS

The normal control group consisted of 30 normal healthy individual with age ranging from 21 yrs to 68 yrs with mean value of 48.5. Most of the normal healthy control were in age group 51-60 yrs. The test group which consisted of 30 CKD patients undergoing hemodialysis with age ranging from 27-66 yrs, had mean value of 51.1yrs. Most of the CKD patients undergoing hemodialysis were in age group 51-60 yrs, with a relative frequency of 0.37. Out of total number of subjects in the control group and the case group, 5(17%) were females and 25(83%) were males.

BP	Study group	Mean ± SD	p Value	Median	95% CI
SBP	Control	121.5 ± 10.99	<0.0001	120	117.43-140.94
	Case	147 ± 16.22		150	125.64-153.06
DBP	Control	74.93 ± 7.22	<0.0001	74	72.24-80.99
	Case	83.47 ± 6.62		80	77.63-85.94

Table: showing means of blood pressure of the studied groups

BUN	MEAN	S.D.	S.E.M	MEDIAN	95% C.I.
NORMAL CONTROL	28.01	10.23	1.87	26.09	24.2-31.83
PRE DIALYSIS	149.49	59.38	10.84	145.14	127.3-171.66
POST DIALYSIS	78.26	42.01	7.67	68.49	62.58-93.95

Table: showing mean blood urea nitrogen concentration

CREATININE	MEAN	S.D.	S.E.M	MEDIAN	MIN	MAX	95% C.I.
NORMAL CONTROL	0.63	0.19	0.03	0.65	0.37	1.1	0.56-0.7
PRE DIALYSIS	11.63	4.53	0.83	10.18	5.58	25.4	9.94-13.33
POST DIALYSIS	8.54	2.7	0.49	8.17	4.09	14.02	7.53-9.55

Table: showing mean serum creatinine concentations

There was a very significant (p= 0.0068) correlation of creatinine with age in the normal controls (r= 0.4831, 95% CI 0.1487 to 0.7184). However, no significant (p=0.4138) correlation of the same was found in the pre-dialysis test sub-group (r=0.1549, 95% CI -0.2176 to 0.4880) and the post dialysis group (p=0.8825, r= -0.02818, 95% CI -0.3846 to 0.3356).

CYSTATIN C	MEAN	S.E.	S.E.M	MEDIAN	MIN	MAX	95% C.I.
NORMAL CONTROL	0.72	0.18	0.03	0.72	0.45	1	0.65-0.78
PRE-DIALYSIS	4.5	1.51	0.27	4.5	2.01	7.82	3.95-5.06
POST-DIALYSIS	4.89	1.5	0.27	4.89	2.11	7.85	4.35-5.44

Table: showing mean serum cystatin C concentration

Comparison 0F Cystatin C between groups	P Value
Normal control and pre-dialysis	<0.0001
Normal control and post-dialysis	<0.0001
Pre-dialysis and Post-dialysis	<0.0001

Table 4.14: comparision of cystatin c between the different study groups

There was no significant correlation (p = 0.1399) between cystatin C and age of the normal controls (r = - 0.2760, 95% C I – 0.5788 to 0.09367; r^2 = 0.07618), pre-dialysis (r= -0.1079, p= 0.5703) and post-dialysis (r= 0.0727, p=0.7028) sub-groups.

There was no significant correlation between cystatin C and body mass index (BMI) of the normal controls (r= -0.0299, p= 0.0.8753), the pre-dialysis (r = -0.1115, p = 0.5576) and post-dialysis (r= 0.1888, p= 0.3177) sub-group.

A significant Correlation (p = 0.0214) between cystatin C and creatinine of the pre-dialysis test group (r = 0.4185 ; 95% CI 0.06844 to 0.6768) was found. However no significant (p= 0.6359) correlation was found in the normal control group (r=0.09009, 95% CI -0.2793 to 0.4363, r^2 = 0.008116).

A significant correlation (p= 0.0021) between cystatin C and creatinine in the post-dialysis test group (r= 0.5397, 95% CI 0.2227 to 0.7535) was found.

There is no significant correlation of cystatin C with BUN with normal control (r=0.2091, p=0.2675), pre-dialysis (r= -0.05512, p=0.7723) and post-dialysis (r= -0.03818, p=0.8412). There was a significant (p= 0.0021) negative correlation of cystatin with the eGFR estimated from the MDRD formula the pre-dialysis group(r= -0.5390, 95%CI -0.7531 to -0.2217). However, no significant correlation was found between eGFR and cystatin c in the post dialysis group (r=-0.0305, p=0.872).

REDUCTION RATIOS								
	No.	**MEAN**	**S.D.**	**SE of Mean**	**MEDIAN**	**MIN**	**MAX**	**95% CI**
URR	30	45.97	20.49	3.741	42.75	1.3	90.8	38.32-53.62
CRR	30	24.77	14.86	2.712	24.5	1	74	19.22-31.31
CYRR	30	-10.55	13.01	2.382	-7	-66	-0.02	(-15.42)-(-5.68)

Table 4.19: showing the means of different reduction ratios for dialysis adequacy

The mean of urea reduction ratio (URR), Creatinine reduction ratio (CRR) and cystatin reduction ratio (CyRR) are 45.97 (SD $\pm$ 20.49), 24.77 (SD $\pm$ 14.86) and -10.55 (SD $\pm$ 13.01). There is highly significant ($p < 0.0001$) negative association of cystatin C reduction ratio with URR and CRR.

DISCUSSION

It was observed that maximum number of individuals in both the case and the control groups were in the age group 51-60 yrs. This signifies that the prevalence of CKD increases with increase in age[18]. These findings are in accordance with Chetri et al 2008[19].

In the present study, the majority of the patients were males, who constituted 83.33% of the total patients. This in accordance with Hoek et al. 2007 [20]. In 1998, Coggins et al [21] suggested that the low incidence of female patients might be attributed to the protective role of endogenous estrogen. They found that loss of renal function was lower in women than men especially in younger pre-menopausal women. However in the present study the increased incidence in male patients might be attributable to community, ethnic, genetics and environmental variations in the population of north east India.

In the present study the systolic and diastolic BP were significantly higher in the case in the test group ($p<0.001$ in both cases). This finding tallies to that of Ravera and colleagues 2006[22]. Kidney disease interferes with salt excretion, leading to volume overload and consequent hypertension. [23].

BUN was significantly raised in the test group (pre-dialysis) than the control group ($p<0.0001$), was significantly higher in the test group (post-dialysis) than the control group ($p<0.0001$) and was significantly lower in the post dialysis phase than the pre-dialysis phase in the test group ($p<0.0001$). This was in accordance with Ramprasad and Al-Gohnaim 2013[25].

Serum creatinine was significantly raised in the pre-dialysis group than the control group ($p<0.0001$), and was significantly higher in the post-dialysis in comparison to the control group with $p<0.0001$. The post dialysis serum creatinine level was significantly lower in comparison to the pre-dialysis group with $p<0.0001$. The results tally with that of Agraharkar & Nair & Patlovany, 2003 [26]. Creatinine excretion is also influenced by muscle mass, because creatinine formation occurs almost exclusively in the muscle; therefore, theoretically, the urinary excretion is the most specific index to define muscle mass [26,27]

The mean serum cystatin C concentrations in the control and test groups (pre- and post-dialysis) were 0.72 mg/L, 4.5 mg/L and 4.89 mg/L respectively. The serum cystatin C levels in both pre and post dialysis groups were significantly raised in comparison to the control group with a $p<0.0001$ in both the cases. Comparison of cystatin C levels between pre and post-dialysis groups also showed a significant difference with higher levels in the post dialysis group ($p<0.0001$). This is in accordance with Krishnamurthy et. al. 2010[28].

The rise in BUN, serum creatinine and cystatin C in the test group might be attributable to the fact that all the three parameters are excreted via glomerular filtration. So, with the decrease in glomerular filtration in CKD patients these parameters accumulates leading to increase in serum concentration. There is a significant fall in BUN and serum creatinine level following

hemodialysis in the test group because BUN and serum creatinine are low molecular weight molecules that is easily dialyzeable.

In the present study, there was rise in serum Cystatin C following hemodialysis in the post-dialysis phase in all the patients taken up for the study as compared to that of the pre-dialysis phase. This was inspite of the concomitant fall in serum creatinine and blood urea nitrogen, which are accepted indices for the adequacy of dialysis. The dialyzer used during our study was diacetate hollow fiber dialyzer, which is a low flux membrane dialyzer. The rise in the serum Cystatin C following dialysis could be attributed to several factors such as the nature of the dialyzing membrane and the composition of the dialyzing fluid. When dialysis is carried out using low flux membrane, the pore size is smaller than 1.5 nm which does not permit the removal of proteins such as cystatin C.

Lindstrom et al. 2008 [29] also observed that low flux hemodialysis did not reduce cystatin C. Their study also showed decrease in cystatin C levels with Hemodiafiltration and hemofiltration (to 28% and 44% respectively) ($p<0.001$). They found that cystatin C rebound post hemodialysis leading to a rise in cystatin C level by 12% in hemodiafiltration group. High flux hemodialysis decreases cystatin C post dialysis [30,31].

Another factor to be considered is the electrostatic interaction between micro-proteins and other plasma proteins adsorbed onto the dialyzer membranes. Kabanda et. al. in 1994 [32] observed that cystatin C being strongly cationic and the charged nature of the molecule might hinder its filtration.

The rise in serum Cystatin C could also be attributed to the effect of hemoconcentration that occurs during dialysis. The fall in serum urea and creatinine levels despite of haemoconcentration is because of the magnitude of reduction of these metabolites which occurs during hemodialysis [28].

There was no significant correlation of BUN with BMI in the normal control ($p= 0.8675$; $r= 0.03181$), pre-dialysis ($p=0.3212$; $r= 0.1875$) and post-dialysis ($p= 0.4439$; $r= 0.1452$) sub-group. There was a very significant correlation of creatinine with age in the normal controls ($r= 0.4831$, $p= 0.0068$). This was in accordance with Rowe et al 1976 [33]. However no significant ($p=0.4138$) correlation was found in the pre-dialysis phase ($r=0.1549$, $p=0.4138$) of the case group. No significant correlation was found with the post-dialysis phase ($r= -0.02818$, $p=0.8825$) of the case group. As shown in table 4.12, significant correlation between creatinine and BMI was found in the normal control ($r=0.4397$, $p=0.015$). This is in accordance with Banfi et. al. 2012 [34]. However, no significant correlation of the same was observed in pre-dialysis ($r= 0.01902$, $p= 0.9205$) and post-dialysis ($r= -0.02818$, $p= 0.8825$) phase of the case group.

Cystatin C is proposed to reflect GFR independent of age and body composition. As shown in table 4.15, no significant ($r= -0.2760$, $p= 0.1399$) correlation was found between cystatin C and age in the normal control group. Also no significant correlation was found in the pre-dialysis ($r= -0.1079$, $p= 0.5703$) and post-dialysis ($r= 0.0727$, $p= 0.7028$) phase of the case group. As depicted in table 4.16, no significant correlation was found between cystatin C and BMI in the normal control group ($r = -0.0299$, $p = 0.0.8753$), pre-dialysis ($r = -0.1115$, $p = 0.5576$) and post-dialysis ($r= 0.1888$, $p= 0.3177$) phase of the case group. This was in accordance with Grubb et al.1985 [35]; Kevil et al. 1998; Erlandsen and Randers 1999 [36]; Kumaresan and Giri 2011[37] ($r = -0.0358$, $p > 0.05$). But this study was contrary to the study of Knight et. al. 2004 [38]. On the other hand BUN is affected by age and serum creatinine is affected by both age and body mass. Thus cystatin C might prove to be a better marker of kidney function than BUN and creatinine in CKD patients undergoing hemodialysis.

As depicted in the table 4.17, a significant Correlation between cystatin C and creatinine of the pre-dialysis test group ($r = 0.4185$; $p = 0.0214$) and post-dialysis phase ($r=0.5397$, $p=0.0021$) was found. However no significant correlation was found with the normal control group ($r=0.09009$, $p=0.6359$). This indicates a significant positive

correlation between cystatin C and creatinine in both pre- and post-dialysis phase of the test group. There are evidences that cystatin C levels associate with clinical outcomes. Cystatin C levels have been shown to correlate positively with cardiac mortality in patients with coronary heart disease [(39)]. Urea and creatinine have been age old markers of CKD. But cystatin C is gradually paving its way and emerging as a new possible marker of CKD or more specifically as an important dialysis adequacy marker. However, a study in a bigger scale with more study subjects would have been more fruitful.

CONCLUSION

The findings of this study suggest that unlike creatinine, cystatin C is not dependent on age and body mass, and as such it would provide better accuracy in determining glomerular filtration rate than serum creatinine. However, cystatin C cannot be used to monitor dialysis adequacy. A more elaborate and comparative study with high flux dialyzer would have been desirable to precisely establish the role of serum cystatin C in ESRD patients. Another drawback of the study was that the gold standard for measuring GFR viz. Inulin Clearance, Urinary clearance of exogenous radioactive markers (125 I-iothalamate and 99m Tc-DTPA{Diethylene-triamine-pentaacetate}) were not used in this study due to practical limitations. Despite of such limitations we made a modest effort to establish the role of cystatin C as a new and better renal failure marker, and we are hopeful that this study will aid other studies in the near future. To conclude, serum cystatin C might be considered a marker of kidney function comparable to serum creatinine in patients with chronic kidney disease undergoing hemodialysis. Serum cystatin C is directly related with eGFR and not influenced by age and body mass giving a clear idea that cystatin C is a promising alternative marker to serum creatinine in these patients. Though, Serum cystatin C cannot be used to monitor dialysis adequacy, it might serve as a marker for dialysis inadequacy especially when low flux diacetate hollow fiber dialyzer is used during hemodialysis.

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