

Comparison of serum levels of Cystatin-C and traditional renal biomarkers for the early detection of pre-hypertensive nephropathy

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Abstract

Objective: To compare serum Cystatin-C and serum creatinine levels along with estimated glomerular filtration rate of apparently healthy people of South Asian descent with pre-hypertension to determine which is better in detecting reversible renal dysfunction.

Methods: The comparative cross-sectional study was conducted at the Army Medical College, Rawalpindi, Pakistan, in 2013-14, and comprised apparently normal healthy male and female volunteers. The subjects were divided into normotensive group 1 and pre-hypertensive group 2. Serum Cystatin-C levels were measured by sandwich enzyme-linked immunosorbent assay technique whereas serum creatinine levels were measured by Jaffe's procedure. Glomerular filtration rate estimation was done by using standard equations. SPSS 20 was used for data analysis.

Results: Of the 78 subjects, 39(50%) were in normotensive group 1 and 39(50%) in the pre-hypertensive group 2. The mean age was 38.74 ± 5.71 years in group 1 and 38.07 ± 3.84 years in group 2. Serum Cystatin-C levels were higher in group 2 than in group 1 ($p=0.0001$), whereas serum creatinine levels manifested no statistical difference between the groups ($p=0.106$). Estimated glomerular filtration rate based on Cystatin-C significantly decreased in group 2 than in group 1 ($p=0.0001$). Serum Cystatin-C displayed a significant positive correlation and estimated glomerular filtration rate based on Cystatin-C negative correlation with the rising blood pressure values ($p=0.0001$). Serum Cystatin-C reflected a very high sensitivity and specificity at a cutoff value of 0.77 mg/l compared to serum creatinine.

Conclusion: Serum Cystatin-C and Estimated glomerular filtration rate based on Cystatin-C appeared to be better renal biomarkers in the detection of pre-hypertensive nephropathy.

Keywords: Cystatin-C, Creatinine, Glomerular filtration rate, Pre-hypertension, Hypertension, Nephropathy, Proteinuria, Cockcroft-Gault, Body mass index, Arteriosclerosis, Inulin clearance. (JPMA 69: 313; 2019)

Introduction

The seventh report of the joint National Committee for the detection, evaluation and treatment of high blood pressure (JNC-7) published in 2003 proposed new guidelines in which a person having systolic blood pressure (SBP) ranging from 120 mmHg to 139 mmHg and diastolic blood pressure (DBP) starting from 80 up to 89 mmHg will be considered pre-hypertensive.¹ Pre-hypertension is prevalent in Pakistani men and women with increasing body weight and waist-to-hip circumference ratio while following the World Health Organisation (WHO) cutoff values for Asians.²

The risk of end organ damage of kidneys and heart due to longstanding pre-hypertension is the current area of investigation by researchers. Arterial vasculature distortion

showed significant correlation with persistently higher than normal blood pressure termed pre-hypertension.³ Results of the Trial Of Preventing Hypertension (TROPHY) proved that pharmacological intervention with antihypertensive drugs that brought blood pressure (BP) below 120/80 mmHg had greatly helped in delaying the development of hypertension and its complications such as hypertensive nephropathy compared to the group receiving placebo over a four-year duration.⁴

Serum creatinine becomes a sensitive marker of renal damage with galloping rise in serum only when more than 50% of renal mass has been lost. Therefore, creatinine is relatively a poor index of nephropathy, especially during initial stages of renal disease, and poses a great challenge to physicians in its timely diagnosis.⁵

Despite accuracy and better renal handling of measured Glomerular filtration rate (mGFR) with inulin clearance, these are not commonly performed because they are labour-intensive, time-consuming and require special clinical settings and technical personnel.⁶

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Cystatin-C has been reported in New England Journal of Medicine as one of the most accurate renal markers to measure GFR.⁷ Cystatin-C, a gamma trace, is readily filtered by the renal glomeruli and metabolised by renal tubular epithelial cells which makes it a strong determinant of GFR, whereby reflecting its true levels in the serum.⁸ Northern blot analysis has demonstrated the presence of messenger ribonucleic acid (mRNA) of Cystatin-C in all nucleated cells. One of the notable reasons for the stable production of Cystatin-C in humans has been attributable to its gene which is of housekeeping type by virtue of studying the promoter part of gene.⁹

Cystatin-C not only detects the beginning of the deterioration of renal function, but also correlates well with structural changes such as renal glomerular endotheliosis.¹⁰ Cystatin-C, in terms of its diagnostic performance, has proven itself to be a better indicator of early decline in renal functions because of its independence from height, weight, age, gender, race and varying muscle mass. National health and nutrition examination survey (NHNES) manifested that serum Cystatin-C levels were found to be on the higher side from their normal baseline concentrations in subjects with hypertension without any overt renal pathology.¹¹

To our knowledge, no study has been documented till date in which Cystatin-C levels were checked exclusively in pre-hypertensive people to look for latent renal dysfunction when the damage is virtually reversible and is medically treatable. The current study was planned to measure serum Cystatin-C and serum creatinine levels along with equation-based GFR of apparently healthy people of South Asian descent with pre-hypertension.

Subjects and Methods

The comparative cross-sectional study was conducted at the Department of Physiology, Army Medical College, Rawalpindi, and the Centre for Research in Experimental and Applied Medicine (CREAM), Army Medical College, Rawalpindi, Pakistan in 2013-14. After approval was obtained from the institutional review board, sample size was calculated using the formula.¹²

$$N_{\text{Kelsey}} = \frac{(Z_{\alpha/2} + Z_{\beta})^2 \cdot p(1-p)(r+1)}{r(p_0 - p_1)^2}$$

Where, α = type I error probability = 0.05; β = type II error probability (1 - power of the test) = 80%; p_0 = the proportion of healthy normotensive population = 0.64%;¹³ p_1 = the proportion of disease free pre-hypertensive population = 0.31%;¹³ r = the ratio of population 2 to population 1 = 1; Z_{α} = value of Z at 95% confidence interval (CI) = 1.96; Z_{β} = value = 0.84 for the power of 80%.

Using non-probability convenience sampling, apparently normal healthy male and female volunteers were enrolled.¹² Those with slightly raised serum creatinine and those with blood sugar levels within pre-diabetes range on previous reports were excluded, and so were those who gave history of antihypertensive medication or use of steroid. Informed consent was taken from all study participants.

Five millilitres of blood was collected from antecubital vein of each subject under aseptic conditions and serum was subsequently obtained through ultracentrifugation which was subsequently stored at -80 degrees Celsius in Eppendorf tubes for the measurement of biochemical parameters i.e.; Cystatin-C and creatinine.

The subjects were divided into normotensive group 1 and pre-hypertensive group 2 after blood pressure of each subject was recorded according to the American Heart Association, JNC-7 and Clinical Practice Guidelines on Hypertension and Antihypertensive Agents in Chronic Kidney Disease (K/DOQI).^{14,15}

Anthropometric measurements such as height and weight were measured according to NHNES guidelines.¹¹ Protein remained undetectable in the urine samples of participants checked by urine dipstick method (Combur 10 Test[®]UX). Fasting and random blood glucose was normal in both groups. Serum Cystatin-C was measured by sandwich enzyme-linked immunosorbent assay (ELISA), Horse radish peroxidase (HRP) labelled antibody immunoassay kit (Cystatin-C, Human Biovendor). Serum creatinine was measured by Jaffe's reaction with ready-to-use assay kit (Pioneer Diagnostics, New York, USA). GFR was estimated by standardised formulae using serum creatinine-based equation, Cockcroft-Gault (eGFR_{CG}) written as (140-age) × (Wt in kg) × (0.85 if female) / (72 × Cr)¹⁶ and Cystatin-C-based Hoek formula (eGFR_{Cystatin-C}) written as 4.32 + 80.35 × 1/CysC¹⁷ in mg/l for all blood samples.

Data was entered into SPSS20. Mean ± Standard deviation (SD) values were calculated for variables. Normal distribution of data for all variables was checked with normal quantile-quantile (Q-Q) plot and by employing Kolmogorov-Smirnov test. $P > 0.05$ on test would indicate that the data was distributed normally. As data obtained for all variables was continuous, independent student t-test was applied in order to calculate statistical difference between the mean values provided that variables should have had normal data distribution. However, $p < 0.05$ was obtained for Kolmogorov-Smirnov test for the data distribution of serum Cystatin-C, so non-parametric Mann-Whitney U test was employed and its results were reported in median and interquartile range (IQR).

Degree of correlation between the selected variables were analysed with Spearman ranked correlation coefficient denoted by Greek small letter 'p' in case of departure of data from normal distribution and Pearson's product-momentum correlation coefficient in case of normally distributed data denoted by 'r'. Scatterplots or scatter graphs were drawn in order to illustrate relationship amongst study variables.

Logistic regression test was applied to check the effect of independant variable i.e. pre-hypertension over dependant variables taken as serum Cystatin-C and serum

creatinine. Predictor probabilities were derived after running the logistic regression and receiver operating characteristic (ROC) analysis was applied by using predictor probabilities as test variable and serum Cystatin-c and serum creatinine as state variables in order to find the area under curve (AUC), sensitivity and specificity of serum Cystatin-c and serum creatinine.

Results

Of the 90 individuals initially enrolled, 12(13.3%) were excluded, leaving the study sample to be 78(86.6%). Of them, 39(50%) were normotensive in group 1 and

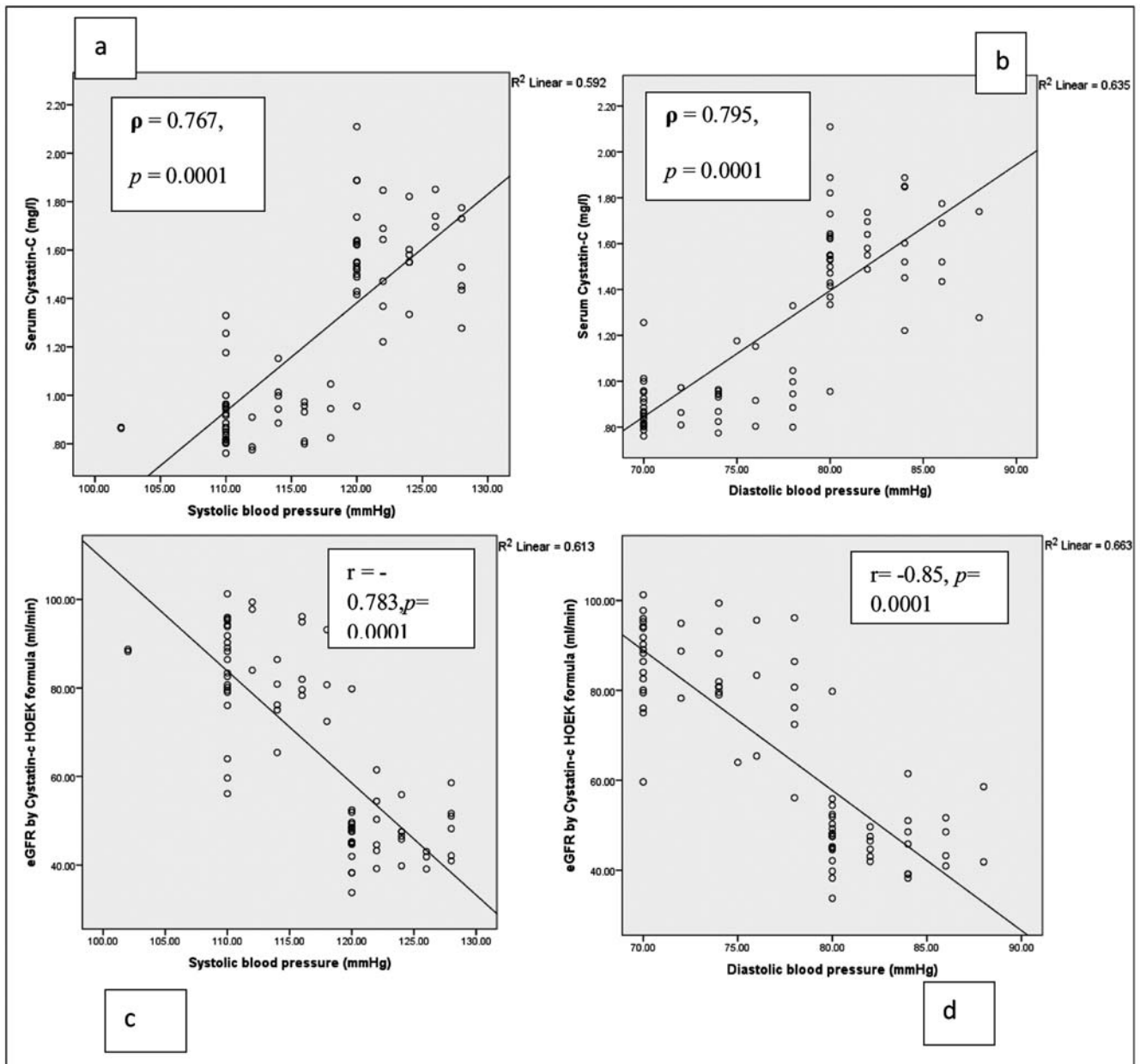


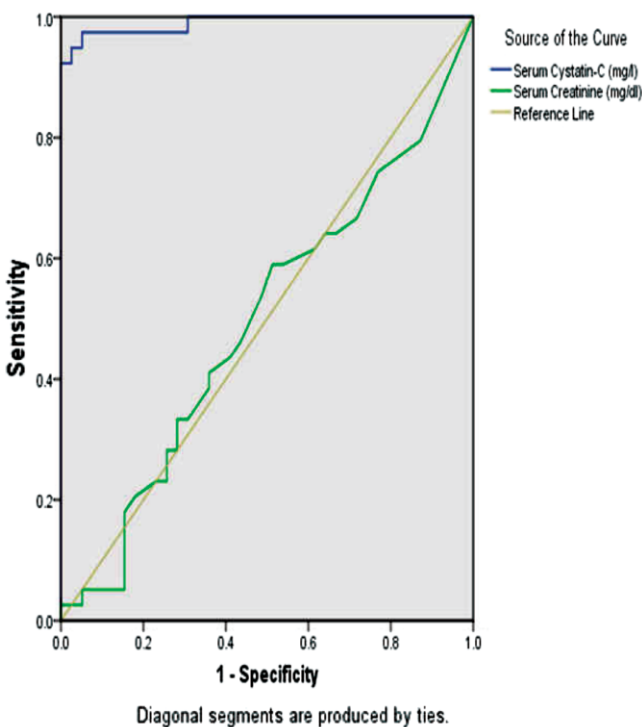
Figure-1: Scatter plots between study variables. Spearman ranked correlation coefficient 'p' Pearson's product-momentum correlation coefficient 'r'.

Table-1: Comparison of serum Cystatin-C levels with Mann-Whitney U test and serum creatinine levels with independent student t-test between normotensive and prehypertensive groups.

Variables	Normotensives Group=1 (n=39)	Prehypertensives Group=2 (n=39)	p-value
Serum Cystatin-C (mg/l)	*0.91 (0.818-0.964)	*1.55 (1.47-1.73)	0.0001
Serum Creatinine (mg/dl)	0.916 ± 0.163	0.917 ± 0.161	0.977
Cystatin-C-based HoekGFR (eGFR _{Cystatin-C})ml/min	*84.17 ± 10.86	*47.40 ± 7.85	0.0001
Creatinine based Cockcroft-GaultGFR (eGFR _{CG})ml/min	112.3 ± 24.8	117 ± 23.2	0.106

Table-2: Area Under Curve (AUC), sensitivity/specificity of serum Cystatin-C and serum creatinine.

Test Result Variables	Area under curve (AUC)	Sensitivity	Specificity	Cutoff level
Cystatin-C	0.990	100%	97%	0.77 mg/l
Creatinine	0.494	79%	87%	0.75 mg/dl

**Figure-2:** Receiver operating characteristics of serum Cystatin-C and serum creatinine.

39(50%) in pre-hypertensive group 2. There were 33(84.6%) males and 6 (15.4%) females in group 1, and 36(92.3%) males and 3(7.7%) females in group 2. Mean age of group 1 was 38.07 ± 3.84 years and that of group 2 was 38.74 ± 5.71 years. Mean height in group 1 was 169.53 ± 7.07 cms while it was 171.43 ± 6.84 cms in group 2. Mean weight in group 1 was 71.05 ± 8.66 kgs and 74.02 ± 7.20 kgs in group 2. Mean SBP in group 1 was 111.64 ± 3.58 mmHg and mean DBP was 72.79 ± 3.045 mmHg with no history of present or past illness. Mean SBP in group 2

was 122.61 ± 3.01 mmHg and mean DBP was 82.05 ± 2.53 mm Hg without any other comorbidity.

Serum Cystatin-C levels were 0.91 mg/l in group 1 and 1.55 mg/l in group 2 (p=0.0001) whereas serum creatinine difference in both groups remained statistically non-significant (p=0.977). Mean estimated GFR (eGFR) Cystatin-C was 84.17 ± 10.86 ml/min in group 1 and 47.40 ± 7.85 ml/min in group 2 (p=0.0001) whereas eGFR Cockcroft-Gault (CG) equation failed to exhibit any significant statistical difference between the groups (p=0.106) (Table-1).

Scatterplots a and b depicted a statistically significant positive correlation and a direct relationship amongst various variables. Serum Cystatin-C reflected a rise in SBP and DBP with ascending values (p=0.0001). Conversely, scatterplots b and c reflected statistically significant negative correlation and inverse relationship amongst variables, with eGFR_{Cystatin-C} tending to fall with rising SBP and DBP values (p=0.0001) (Figure-1).

The logistic regression model was statistically significant (p<0.0005) for serum Cystatin-C and correctly classified 93% of cases. The model explained 71% (NagelkerkeR²) of variance in terms of increase in serum Cystatin-C levels in participants. For every additional 1mmHg increase in BP, the odds of serum Cystatin-C to rise in the blood would increase by a factor of 0.516. Pre-hypertensive subjects had 70% greater odds of having a rise in serum Cystatin-C levels than normotensives. Statistically, no significant effect of rising BP values in blood in pre-hypertensive subjects was noted on serum creatinine (p>0.05).

ROC analysis exhibited a very high AUC of 0.990 for serum Cystatin-C than serum creatinine with a sensitivity of 100% and specificity of 97% which were markedly higher than

79% sensitivity and 87% specificity of serum creatinine (Figure-2, Table-2).

Discussion

All the subjects in the current study were of Pakistani origin. The pre-hypertensive Pakistani population has been documented to have higher circulating levels of various stress hormones like cortisol and aldosterone. Higher circulating levels of stress hormones have been attributable to stressful life at a younger age which will ultimately develop into full-blown hypertension and its associated complications like renal dysfunction.¹⁸

The earlier stages of reversible kidney disease have been coined as pre-chronic kidney disease that manifests itself with the excretion of low amount of albumin protein in the urine known as microalbuminuria.¹⁹ Hypertension leads to pathophysiological process of hyperfiltration through renal glomeruli. In our study, all normotensive and pre-hypertensive participants had undetectable protein in their urine samples. However, few studies showed protein levels in the urine gradually increased with rising values of BP from pre-hypertension stage 1 to stage 2 ($p < 0.001$).^{20,21}

Serum creatinine was essentially measured in all participants in our study because the current method of establishment of prevalence of the renal disease has been virtually based on serum creatinine cutoff values per million population.²² The current study revealed that serum creatinine remained within normal range of prehypertensive and normotensive groups (Table-1) and, as such, gave no indication of any degree of renal insult if there was any.²³ To address this problem, we chose to measure serum Cystatin-C concentrations, an efficient prognostic renal biomarker in the light of past studies in order to detect incipient and reversible renal pathology.⁷⁻¹⁰ Furthermore, studies have proven that cystatin-C, a novel non-invasive renal biomarker exhibited a high degree of correlation with mGFR based on iothalamate clearance.^{24,25}

Pre-hypertensive group in the study manifested increasing values of serum Cystatin-C (Table-1) and this trend was observed in a cohort study which reported a strong association between elevation in serum Cystatin-C levels and the development of full-blown hypertension leading to nephropathy.²⁶ Our study revealed raised serum Cystatin-C along with greater AUC than serum creatinine in pre-hypertensives (Figure-2, Table-2) and these results are compatible with another study with higher AUC (0.900) for Cystatin-C in hypertensives to serve as an early predictor of mild renal dysfunction compared to traditional renal biomarkers.²⁷ Moreover, a linear association between the rising levels of serum Cystatin-C, mild renal impairment,

high risk of cardiovascular disease and mortality has been established. Serum creatinine significantly rises mostly during late stages of renal dysfunction when patients mostly need either haemodialysis or renal transplantation.²⁸

Our study data is comparable to a study done in Japan which measured serum Cystatin-C in 60 subjects with essential hypertension and reported a strong correlation between serum Cystatin-C, a gradual rise in SBP and end-organ damage.²⁹ Significant positive correlation was found between serum Cystatin-C and SBP and DBP (Figure-1). Serum Cystatin-C levels could serve as an independent marker of hypertensive nephropathy and inflammation as proven by the positive correlation amongst increasing levels of serum Cystatin-C, higher SBP, pulse pressure, and a negative correlation with mild deterioration in equation-based eGFR.³⁰ Serum samples of 96 patients from a hospital with different grades of renal dysfunction based on guidelines of the Japanese Society of Nephrology established concrete association between mild to moderate renal dysfunction and soaring levels of serum Cystatin-C while having normal serum creatinine. These results proved that higher sensitivity and specificity of serum Cystatin-C can act as a good prognostic marker of reversible grades of nephropathy.³¹

Our study demonstrated higher sensitivity (100%) and specificity (97%) for serum Cystatin-C in Pakistani population (Table-2, Figure-2) and the results are comparable with another prospective study in which researchers found that serum Cystatin-C showed 94.7% sensitivity and 84.8% specificity at a cutoff value of 1.2 mg/l in Japanese patients with contrast-induced nephropathy after cardiac catheterisation owing to its ability to oscillate easily with mild to moderate acute renal dysfunction.³²

Our data showed that prehypertensive subjects had no evidence of proteinuria but serum Cystatin-C levels remained higher and GFR lower when measured with eGFR Cystatin-C (Table-1, Figure-1), depicting mild nephropathy which is in concordance with a study done among Korean diabetic patients where eGFR Cystatin-C served a better predictor in stage 2 diabetic nephropathy than creatinine-based GFR, especially during the stage of micro-albuminuria.³³

While monitoring decline in functional reserves of kidney, serum creatinine may or may not rise within days of the primary renal insult. This poses a great difficulty in continuous and early monitoring of damage induced by nephrotoxic toxins and drugs.³⁴ However, performance of serum Cystatin-C and serum creatinine in 72 cancer patients on combined chemotherapy remained in favour of serum Cystatin-C with 87% sensitivity and 100% specificity to detect mild to moderate renal deterioration.³⁵

Researchers demonstrated that serum Cystatin-C was not affected by confounding variables as no noticeable correlation was found between serum Cystatin-C and age, body surface area and BMI. However, creatinine correlated significantly with BMI and other variables with overall sensitivity of serum Cystatin-C remained 96.8%, whereas sensitivity of serum creatinine was upto only 61.3%.³⁶

Our second tool to detect renal damage was eGFR with the aid of equations based on serum Cystatin-C and creatinine. Creatinine-based Cockcroft-Gault (eGFR_{CG}) remained within normal range in all participants in the present study (Table-1). Researchers in another study demonstrated shortcomings of creatinine-based tests in which 29% subjects had an under estimation and 8% had an overestimation of creatinine-based GFR (Cohen's $\kappa = 0.47$). Creatinine-based eGFR_{CG} showed accuracy of nearly 80% in subjects who were underweight, whereas only 57% accuracy was reflected in overweight subjects ($p < 0.01$). Moreover, eGFR_{CG} was highly affected by other parameters such as age, BMI and gender when assessed by linear regression analysis.³⁷

On the contrary, serum Cystatin-C is independent of confounding variables unlike serum creatinine. Therefore, scientists proposed the estimation of GFR based on serum Cystatin-C in order to further improve the clinical outcome of Cystatin-C in mild to moderate fall in kidney functions and halting progression of nephropathy with medical management. Our results reflected a low eGFR Cystatin-C in pre-hypertensive group than normotensive control group, whereas serum creatinine-based eGFR_{CG} remained normal in both groups (Table-1). These results are compatible with another study in which scientists based on their findings regarded Cystatin-C as a highly sensitive and specific marker of renal function in patients with type 2 diabetes owing to its less biological variation and a good statistical correlation with actual renal function.¹⁷

However, 251 subjects in a study reported hardly any statistical difference in eGFR Cystatin-C when compared with eGFR_{CG} which is in contradiction of our data.³⁸

Nevertheless, more studies have conferred a consensus that because Cystatin-C performance is not influenced by the increasing BMI and other confounding variables, it could serve as an excellent early predictor of deteriorating kidney function than serum creatinine.^{39,40}

Besides, eGFR Cystatin-C overall performance excelled eGFR_{CG} to detect renal pathology whereas Cockcroft-Gault and other creatinine-based estimations proved to be less accurate in detecting renal dysfunction, especially in preclinical stages due to the main effect of varying

muscle mass and various other confounding variables.⁴¹

Cystatin-C has been an investigational renal biomarker that has not been included on the list of Joint Committee for Traceability in Laboratory Medicine database (JTCLM) and as a result lack of any official statement of approval by Food and Drug Administration (FDA), USA, for South Asian population.⁴²⁻⁴⁴ Results of our study are certainly very promising and have encouraged us to employ similar research design with addition of invasive renal markers such as renal biopsy and the measurement of GFR with inulin clearance to actually ascertain the extent of renal pathology and their degree of correlation with the efficiency of non-invasive novel renal biochemical marker of interest such as Cystatin-C or equation GFR based on Cystatin-C. We are hopeful that we will be able to save millions of lives with early diagnosis of pre-hypertensive nephropathy in Pakistan once serum Cystatin-C based tests become patent across the country.

Conclusion

Cystatin-C and Cystatin-based GFR can serve as better non-invasive prognostic endogenous biomarkers to detect reversible pre-hypertensive nephropathy than serum creatinine and other tests based on it.

Limitation

The study was carried out between Jan 2013- Dec 2014. "The delay of four years encompass the submission of the article to international medical journals which did not accept the manuscript. This gave the study authors an opportunity to bring further improvements in the presentation of the manuscript followed by the submission, processing and final acceptance of the manuscript by the 'Journal of Pakistan Medical Association'.

Disclaimer: The study is part of M.Phil-Ph.D thesis, and has been presented as Poster Presentation at an international competition of scientists held at the National University of Sciences and Technology (NUST).

Conflicts of Interest: None.

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