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Salivary creatinine – Potential biomarker only in late stage chronic kidney disease

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Abstract

Salivary creatinine estimation has been considered as an alternative for serum in chronic kidney disease (CKD). However, there is not enough data to validate its potential use as an early-stage biomarker. Hence, we aimed at stage-wise estimation of serum creatinine and correlation with salivary levels to validate saliva's diagnostic utility in renal dysfunction. Salivary and serum samples were collected from 30 healthy individuals and 150 chronic kidney disease patients with each stage comprising of 30 subjects. Serum and salivary creatinine levels were estimated, and data were subjected to statistical analysis. Students t-Test was done to compare serum and salivary levels between controls and individual kidney disease stage. To assess if there are any bias, subjects with CKD were grouped into one single group and analysed. Pearson's correlation was used to test the correlation between serum and salivary creatinine levels. Receiver operating characteristic analysis was done to assess the diagnostic performance of salivary creatinine. Cut-off values were established for salivary creatinine. Significant differences were found in serum creatinine between control and individual stages of CKD, however, differences in salivary creatinine were seen only in the late stages (stages 3-5) of CKD. When the CKD patients were considered as one group salivary creatinine showed significant differences between control and CKD patients. Correlation between serum and salivary creatinine was significant in all stages except stage 3 of CKD. Area under the curve for salivary creatinine was found to be 0.858. A cut-off value of 0.18 mg/dl gave a sensitivity of 85% and specificity of 70%. Salivary creatinine can be used as an alternative to serum only in late-stage chronic kidney disease.

Keywords: Chronic kidney disease, Creatinine, Saliva, Renal insufficiency

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1. Introduction

Chronic kidney disease (CKD) is a worldwide health problem and is one of the most common diseases of modern civilization, with adverse outcomes of kidney failure and premature death. CKD requires active therapy, monitoring and follow up to improve its outcome. However, in an Indian setting, it has become difficult for the common man to reach this goal because of the financial tag associated with therapy. Also, monitoring requires regular and periodic assessment of serum based renal function tests which require frequent blood sampling, that is invasive and has poor patient compliance [1, 2].

Although estimation of serum creatinine is considered ideal for screening renal function, several studies have suggested the role of saliva as an alternative, non-invasive diagnostic medium as the expression of these markers in saliva correlated well with serum levels [3, 4, 5]. However, the previous studies of salivary research associated with kidney disease evaluated the presence of these markers only in the late stages of kidney disease and have claimed the use of saliva as a diagnostic marker for kidney disease. As kidney disease is a progressive disorder evolving from a symptom-free early stage to advanced stage of loss of renal function, it is prudent to have a sensitive measure of a marker that should not only have good serum and salivary correlation, but also provide a dependable cut off point for each stage of renal disease. Hence, only a stratified stage-wise serum and salivary estimation and correlation of these markers will indicate its true potential as a diagnostic marker and to highlight the use of saliva as a non-invasive diagnostic marker in renal disease. With this background, our study aimed at estimation of serum creatinine

and correlation with their salivary levels in each stage of CKD for validation of its diagnostic utility in renal dysfunction.

2. Materials and methods

The study approval was obtained from Institutional Scientific and Ethical Committee **[IRB Approval number: SRMDC/IRB/2014/MDS/No.603]** and according to ethical principles, including the World Medical Association Declaration of Helsinki, a written and informed consent was obtained from all the study participants, and parents or legal guardians of minors or incapacitated adults prior to blood and saliva collection.

2.1. Study subject recruitment

The study subjects were recruited from Nephrology Department during the period June 2015 and August 2017. The demographic details and information on medical history were collected.

2.2. Inclusion and exclusion criteria for study subjects

2.2.1. Healthy control group (Group 1)

The subjects were recruited from student and staff volunteers. A total of 30 subjects were enrolled with the following eligibility criteria: age and sex matched (22-70 years), subjects with no kidney damage or loss of function and GFR over 90mls/min/1.73m^2, without any history of diabetes or hypertension and any other systemic illness, no known family history of chronic kidney disease, no pathological dry mouth syndrome, or inability to collect sufficient saliva samples on a reliable basis. Pregnant and lactating subjects and subjects under creatine supplements were excluded.

2.2.2. Chronic kidney disease (CKD) group (Group 2)

The sample collection was done using stratified random sampling method and a total of 150 patients were enrolled and sub-grouped into 5 stages with each group comprising of 30 cases based on Estimated GFR (eGFR) values calculated using Modification of Diet in Renal Disease (MDRD) [6] formula with the following criteria: Stage 1: Kidney damage with normal GFR. 90ml/min/1.73m^2 (Associated with risk factors); Stage 2: Kidney damage with mild decrease in GFR 60-89ml/min/1.73m^2; Stage 3: Moderate decrease in GFR. 30-59 ml/min/1.73m^2; Stage 4: Severe decrease in GFR. 15-29 ml/min/1.73m^2; Stage 5: Kidney failure. <15 ml/min/1.73m^2 or under dialysis. Subjects with history of altered GFR for less than 3 months, pregnant and lactating women, subjects under creatine supplements and subject with etiology of trauma in renal ailments were excluded.

2.3 Sample collection

Salivary samples were collected between 9 and 11 A.M under non stimulatory conditions. Participants were refrained from eating, chewing, and drinking at least one hour prior to sample collection [7]**.** Whole saliva samples were collected by spitting method. The study participants of both groups were asked to spit at least 5ml of saliva into the sterile centrifuge tubes. Each individual expectorated 5 ml of saliva by requesting subjects to swallow first, tilt their head forward, and expectorate all saliva into the centrifuge tubes for 10 min without swallowing. In patients under hemo-dialysis, salivary samples were collected before dialysis. Following collection, saliva was centrifuged immediately in a cooling centrifuge (-20ºC) at 3000 RPM for 15 minutes. Peripheral blood (2ml) was drawn from subjects using standardized phlebotomy procedures and transferred to centrifuge tubes without an anticoagulant and allowed to coagulate for one hour at room temperature. Sera were separated by centrifugation in a cooling centrifuge at 1500 RPM for 10 minutes.

2.4. Biochemical analysis (Estimation of creatinine)

The samples were assayed immediately in automatic biochemical analyser (Erba EM200) using creatinine estimation kit (Pathozyme Diagnostics) by Modified Jaffe's Kinetic Method [8, 9].

2.5. Statistical analysis

Statistical analyses were performed using statistical software program SPSS 22. Data were expressed as mean ± SD and a "p value <0.05" was considered statistically significant. Student t-Test was done to statistically analyse the level of significance in the serum and saliva of control and individual stages of CKD group. To assess if there are any bias in the relationship we also grouped all stages of CKD (considering all 150 study subjects) into one single group and analysis was done. Significance of mean serum and salivary levels within stages of CKD was done using ANOVA. Pearson's correlation coefficient was used to test the correlation between serum and salivary creatinine levels. Further, to validate the diagnostic utility of salivary creatinine ROC curve analysis was done and sensitivity and specificity based on cut-off values from ROC analysis was also determined.

3. Results and discussion

Demographic data of the study subjects are outlined in Table 1. Briefly, the study population included a total of 180 patients, out of which control group (Group 1) had a total of 30 subjects with 14 males and 16 females and CKD (Group 2) included a total of 150 patients which included 91 males and 59 females with an age range of 22 to 70 years. Further sub grouping was done based on the estimated GFR values: Stage

1-30 subjects with 18 males and 12 females, Stage 2-30 subjects with 15 males and 15 females, Stage 3-30 subjects with 19 males and 11 females, Stage 4-30 subjects with 15 males and 15 females, Stage 5-30 subjects with 24 males and 6 females.

Serum and salivary samples obtained from patients of both the group were analysed for concentration of Creatinine and the following observations were made. The mean values of serum and salivary levels of Creatinine in control and different stages of CKD are shown in Table 1, Chart 1 and 2. In control group, the mean values of serum creatinine is 0.703 mg/dl and saliva is 0.122 mg/dl. In CKD group, the mean values of serum and salivary creatinine for each stage is: Stage 1-0.786 mg/dl and 0.138 mg/dl; Stage 2-1.014 mg/dl and 0.276 mg/dl; Stage 3 - 1.770 mg/dl and 0.433 mg/dl; Stage 4-2.906 mg/dl and 0.680 mg/dl; Stage 5-6.663 mg/dl and 1.816 mg/dl respectively. When all the patients of CKD were grouped into one group we observed that the mean values were found to be 2.628 mg/dl in serum and 0.330 mg/dl in saliva. Significant differences in serum creatinine concentration were noted between control and individual stages of CKD group ($p<0.05$).

However, significant differences in salivary creatinine were seen only in the late stages (stages 3-5) of CKD (Table 2A). When the CKD patients were considered as one group salivary creatinine showed significant differences between control and CKD patients. Statistical comparison within stages of CKD group showed a significant difference between individual stages (Table 2B). Correlation between serum and salivary creatinine in control and individual stages of CKD was done and found to be significant in all stages, except stage 3 of CKD. Overall correlation was also found to significant (Table 2C, Figure 2).

Furthermore for validation using ROC analyis, only Stages 3, 4 and 5 were included since significant differences were found in the mean salivary levels of creatinine between control group and the stages 3, 4 and 5 of CKD. Area under the curve was found to be 1.000 for serum creatinine and 0.858 for salivary creatinine. We determined cut-off values for salivary creatinine and was 0.18 mg/dl at a sensitivity of 85% and specificity of 70% (Table 3,4 and Figure 1).

Saliva as a medium for diagnosis and monitoring of CKD could be of greater significance due to saliva's physiological role in excretion and its relative ease of collection and handling. Apart from diffusion of serum markers into saliva, it may also facilitate the body as an alternate route of excretion of substances when there is a compromise in renal function [9, 10]. This temporal association between stage of kidney disease and creatinine levels in both serum and saliva has made researchers to postulate the potential role of salivary creatinine as a valuable biomarker for predicting the severity of kidney disease.

This area of research pertaining to estimation of salivary markers in renal dysfunction has been open for discussion ever since 1922 where urea was assessed in saliva in nephritis by Philip et al [11]. Currently, even with almost a century gone by us, there is still a lacunae in proper justification and validation of the use of salivary biomarkers in renal dysfunction.

Although the current literature suggests that saliva can be used as a non-invasive diagnostic tool for estimating serum creatinine in chronic kidney disease patients, it is not without limitations. One of the important drawbacks of all reported data was that the study group either considered CKD as a single group or it included only stage 4 and stage 5 CKD patients. Extrapolation of the observed results from the late stage CKD (stage 4 and 5) to early stages of CKD (Stage 1, 2 and 3) would not be a true indicator to its application in a clinical scenario. Hence an attempt was made to throw more light on this grey area of research regarding the potential use of saliva as a diagnostic marker in CKD.

Our observation showed that the normal range of salivary levels was 0.05 to 0.2 mg/dl and found to be less than that of the serum levels with a range of 0.5 to 1 mg/dl; the data obtained were in accordance to Lloyd JE et al and Xia et al. Llyod et al 1996 and were one of the first few groups to describe the use of salivary creatinine as a potential screen for renal disease and salivary creatinine had not been measured in renal patients until then and were reported that salivary creatinine levels were 10-15% of the serum creatinine concentration [12]. Similar results were also observed by Venkatapathy et al and Reda et al who observed salivary mean concentrations to be generally less than serum creatinine values [5, 13]. In this study, salivary values by Jaffe's kinematic estimation was considered to be standard as the control group revealed a normal mean serum creatinine range and a corresponding 10-15% reduced concentration in their salivary values in accordance to other studies in literature.

When the CKD patients were grouped into a single category, we observed significantly high creatinine levels both in the serum and saliva of CKD patients when compared with controls as reported previously (Table 2A). Subsequently, to find out if there was any correlation between the serum and salivary creatinine levels and if changes in serum creatinine are accompanied by changes in salivary creatinine, correlation was performed and a strong correlation was observed in CKD group (Table 2C). This trend was similar to the observations by Venkatapathy et al and Davidovich et al [5, 14].

This is because when the kidneys are unable to excrete creatinine in CKD as a result in loss of function, it reflects as increased blood levels. Alternatively, it is also

suggested that when the concentration in blood increases, there is a corresponding increase in the salivary levels of these markers due to an increased concentration gradient of serum levels which result in the diffusion of the molecules into saliva [15]. It is also possible that when the normal excretory mechanisms are at fault there is an attempt by the body to eliminate it through an alternate route. Saliva, which also is known to play a role in excretion of substances may facilitate the body for an alternate route in such a compromised renal functional state.

However, our primary concern was to see if the same significance was present in a stage wise serum and salivary estimation and correlation to assess its clinical utility in the early stages of the disease. Comparison of mean serum creatinine levels between the control and the individual stages of CKD showed statistical significance in all stages. However, comparison of mean salivary creatinine levels were found to be statistically significant only between control and stages 3,4 and 5 of CKD group suggesting that changes in salivary creatinine levels in the early stages (stage 1 and 2) of CKD are only marginal and hence less significant than the control group (Table 2A). This may be due to the relative non-polar nature of creatinine and owing to the tight intercellular junctions of the salivary glands in a healthy or minimal pathology there is less permeability causing incomplete filtration of creatinine. This results in the minimal expression of creatinine in saliva which might be undetectable with greater sensitivity between normal and early stages of the disease. However, in advanced stages of the disease, possibly there is an alteration in the permeability of the salivary gland cells which causes increased diffusion resulting in significant amounts of creatinine in saliva [5, 16].

A stage-wise correlation analysis between serum and salivary creatinine was done revealed a strong positive correlation in the control group (Table 2-C). However, in the CKD group, a strong positive correlation was observed only in stages 1, 2, 4 and 5 and a negative correlation was seen in stage 3 disease. This might be due to the fact that clinical symptoms of the disease are expressed when there is reduction in the GFR by 50%, which happens in stage 3 CKD. It is possible that during this stage, there can be alteration either in the composition or rate of salivary secretions as a result of morphological, functional and DNA damage to the salivary gland apparatus [17].

Clarra Ersson et al 2012 showed that CKD patients had a significantly more DNA damage in salivary gland tissue compared with the controls. The observations in their study suggest that CKD might cause DNA induced damage to the peripheral tissues such as the salivary glands causing an altered functional state of the gland [18]. These changes could clinically translate to an altered relationship between serum and salivary creatinine in stage 3 disease as observed in this study. However, this hypothetical pathogenesis may not be similar among all patients and may be proved only by correlating the altered salivary levels with salivary glandular and renal nephrons damage. It is vital here to observe such specific changes by further subdividing the group into Stage 3A (45-59 mL/min/1.73m^2) and 3B (30-44 mL/min/1.73m^2) and validate the hypothesis. This will give further insight into salivary gland apparatus structural damage during specific stage of the kidney disease or damage.

Several factors may contribute to the expression of serum markers in saliva and their relationship. As a diagnostic marker, it is necessary to distinguish the healthy individual from disease. Hence, to validate the use of salivary creatinine as a diagnostic test, ROC analysis was done. ROC analysis of salivary creatinine revealed a higher area under curve of 0.858 at 95% confidence interval (statistically significant at $p < 0.05$, Table 3). Cut-off value was determined to be 0.18 mg/dl based on the best trade-off between sensitivity and specificity which was 85% and 70% respectively (Table 4). Xia et al in 2012 demonstrated an AUC of 0.897 (Sensitivity: 0.776 and Specificity: 0.989) and suggested the use of salivary as a diagnostic marker in CKD. Similarly *Venkatapathy et al 2014* demonstrated the total area under the curve as 0.967 for salivary creatinine. Sensitivity and specificity for different values of salivary creatinine were established and a cut-off value of 0.2 mg/dL was determined as this gave a best trade-off with sensitivity of 97.14% and specificity of 86.5%. In other study, total area under the curve was 0.79 and cut off value for salivary creatinine was determined to be 0.55 mg/dl [5, 19, 20]. Unlike our study in which we did ROC analysis following a stage wise estimation and correlation, other studies evaluated area under the curve for the study groups as a whole group without stage wise estimation.

Put together, the above observed findings suggest that creatinine may be expressed in significant amounts in saliva as an excretory mechanism in the late stages of renal dysfunction. Hence it is appropriate to use salivary creatinine as a late stage diagnostic marker and as an aid in monitoring renal function rather than an early stage diagnostic marker. However, the potential of saliva as an early diagnostic marker in CKD remains questionable because of the high variation rate of expression observed in the early stages of renal dysfunction.

Table 1: Descriptive statistics for creatinine in control and CKD stages

Group		Sample Size (n)	Age group (years)	Sex		Creatinine - mg/dl (Mean ± SD)	
				Males	Females	Serum	Saliva
Control		30	22-70	14	16	0.703± 0.145	0.122± 0.062
CKD group	**Stage I**	30	25-59	18	12	0.786 ± 0.118	0.138 ± 0.051
	Stage II	30	23-65	15	15	1.014 ± 0.152	0.276 ± 0.453
	Stage III	30	32-60	19	11	1.770 ± 0.448	0.433 ± 0.263
	Stage IV	30	22-69	15	15	2.906 ± 0.627	0.680 ± 0.405
	Stage V	30	23-70	24	6	6.663 ± 3.000	1.816 ± 4.984
CKD Group (all stages)		150	22-67	91	59	2.628±2.554	0.330 ± 0.360

The levels of serum and salivary creatinine were assessed by biochemical analysis in all the study subjects. The concentrations are expressed in milligram per deciliter

Table 2: Summary of statistical analysis for creatinine

2.A Comparison between groups T-test								
	Serum				**Saliva**			
	Mean and SD	**t-value**	**P value**	**Significance at p < 0.05**	**Mean and SD**	**t-value**	**P value**	**Significance at p < 0.05**
Control	0.703± 0.145	2.39984	0.019	Significant	0.122± 0.062	1.10558	0.273	Not Significant
Stage I	0.786 ± 0.118				0.138 ± 0.051			
Control	0.703± 0.145	8.07015	< 0.0001	Significant	0.122± 0.062	1.84819	0.069	Not Significant
Stage II	1.014 ± 0.152				0.276 ± 0.453			
Control	0.703± 0.145	12.39713	< 0.0001	Significant	0.122± 0.062	6.30076	< 0.0001	Significant
Stage III	1.770 ± 0.448				0.433 ± 0.263			
Control	0.703± 0.145	18.73263	0< 0.0001	Significant	0.122± 0.062	7.44531	< 0.0001	Significant
Stage IV	2.906 ± 0.627				0.680 ± 0.405			
Control	0.703± 0.145	10.86535	<	Significant	0.122± 0.062	1.86151	<	Significant

Stage V	6.663 ± 3.000		0.0001		1.816 ± 4.984		0.0001	
Control	0.703± 0.145	6.6408	< 0.0001	Significant	0.122± 0.062	5.54836	<0.001	Significant
CKD group	2.628±2.554				0.330 ± 0.360			

2.B Comparison within stages of CKD Anova test

	Serum			**Saliva**		
	Mean & SD (mg/dl)	**P Value**	**Significance**	**Mean ± SD (mg/dl)**	**P Value**	**Significance**
Stage I	0.786 ± 0.118	< 0.000	Significant at p <0.05	0.138 ± 0.051	0.025	Significant at p <0.05
Stage II	1.014 ± 0.152			0.276 ± 0.453		
Stage III	1.770 ± 0.448			0.433 ± 0.263		
Stage IV	2.906 ± 0.627			0.680 ± 0.405		
Stage V	6.663 ± 3.000			1.816± 4.984		

2.C

Correlation between serum and salivary creatinine in control and individual stages of CKD and CKD as a combined group.

(Pearson's Correlation)

	R score	**R Square**	**P value**	**Significance**
Control	0.756	0.571	<0.001	Significant at p <0.05
Stage I	0.499	0.249	0.005	Significant at p <0.05
Stage II	0.384	0.147	0.036	Significant at p <0.05
Stage III	- 0.301	0.090	0.106	Not Significant at p <0.05
Stage IV	0.568	0.323	<0.001	Significant at p <0.05
Stage V	0.823	0.676	<0.001	Significant at p <0.05
CKD group (All stages)	0.523	0.280	<0.001	Significant at p <0.05

Table 3: ROC analysis for serum and salivary creatinine

Area under the curve – ROC curve analysis			
Test Result Variable(s)	**Area**	**Significance**	**Level of significance**
Serum Creatinine	1.000	<0.001	Significant at p < 0.05
Salivary Creatinine	0.858	<0.001	Significant at p < 0.05

Table 4: ROC analysis - Cut off value for salivary creatinine

Parameter	**Cut off Value**	**Confidence Interval**	**Sensitivity**	**Specificity**
Salivary Creatinine	0.18 mg/dl	95 %	85%	70%

The diagnostic utility is assessed in terms of specificity and sensitivity

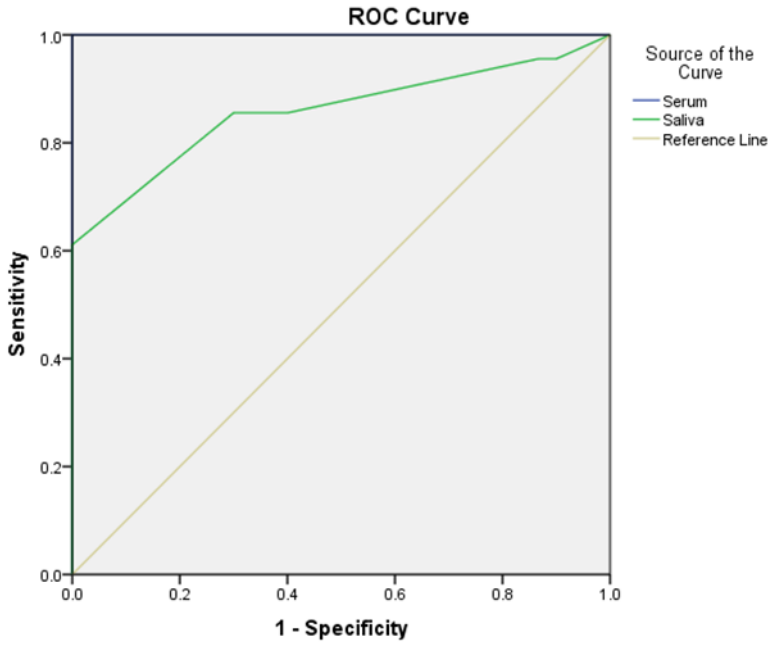

Fig. 1: Diagnostic utility of salivary creatinine assessed by ROC curve analysis

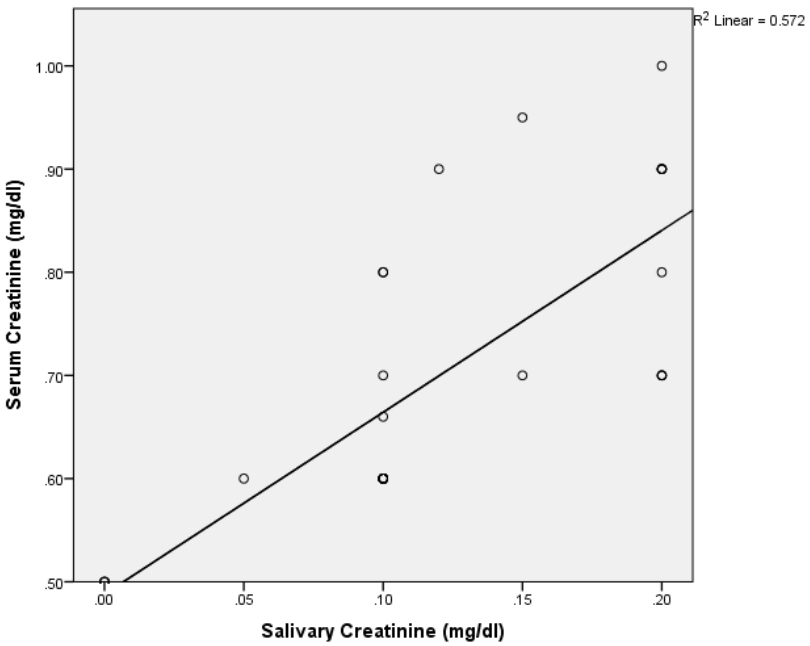

Figure (2a): Scatter diagram showing linear correlation between serum and salivary levels of creatinine among controls

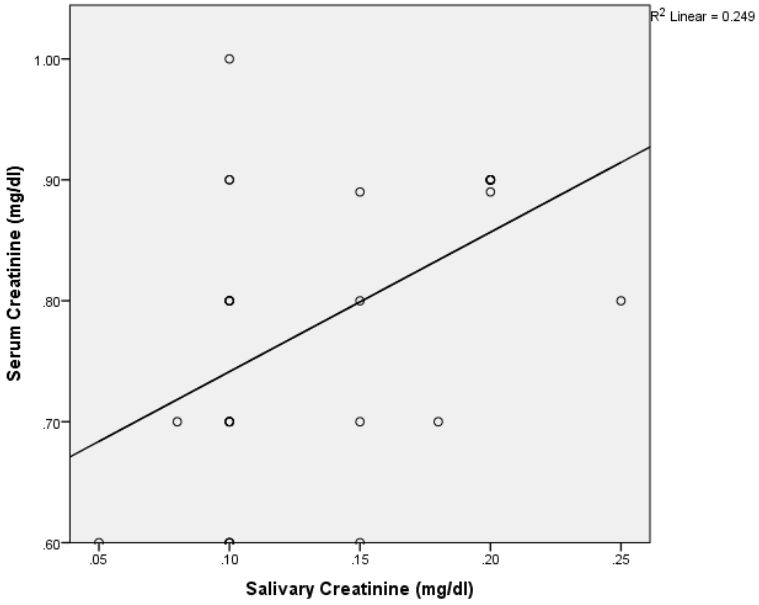

Figure (2b): Scatter diagram showing linear correlation between serum and salivary levels of creatinine among Stage I CKD

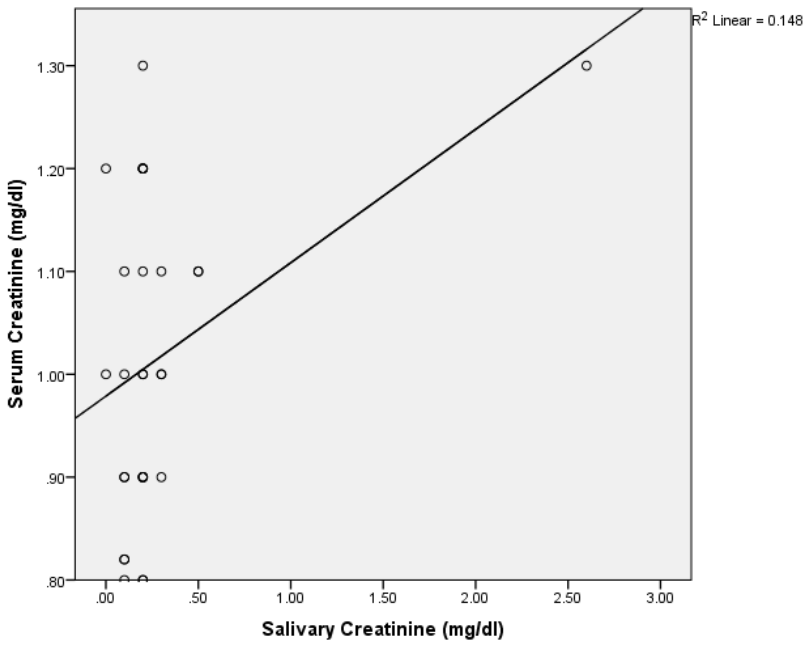

Figure (2c): Scatter diagram showing linear correlation between serum and salivary levels of creatinine among Stage II CKD

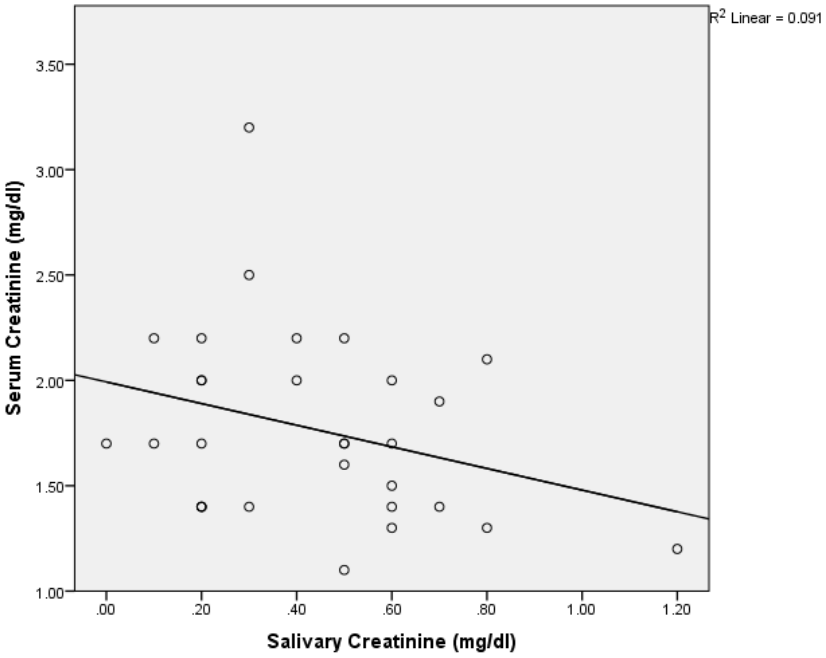

Figure (2d): Scatter diagram showing linear correlation between serum and salivary levels of creatinine among Stage III CKD

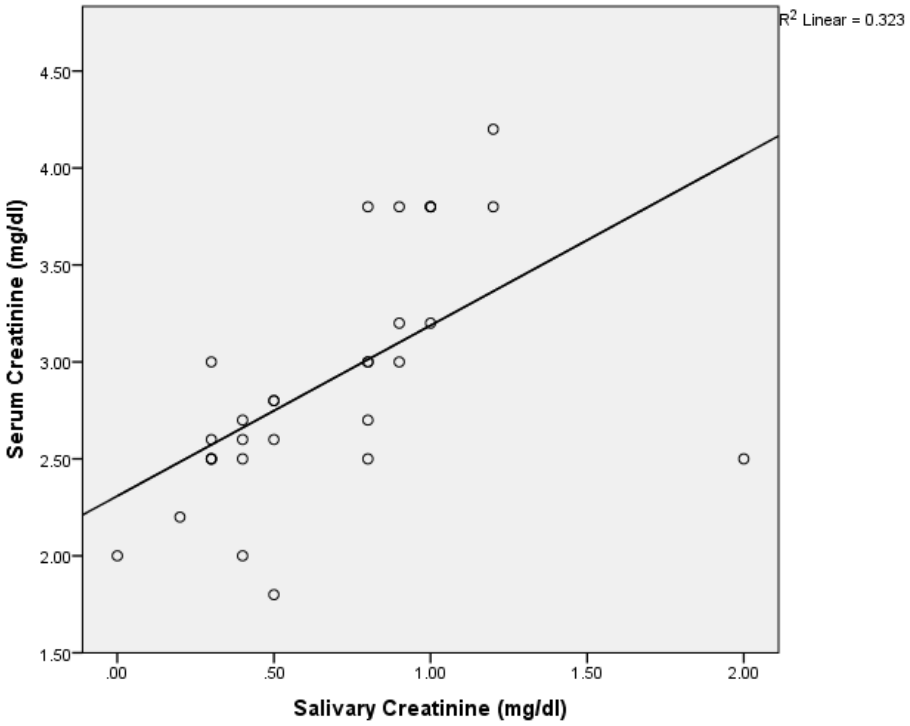

Figure (2e): Scatter diagram showing linear correlation between serum and salivary levels of creatinine among Stage IV CKD

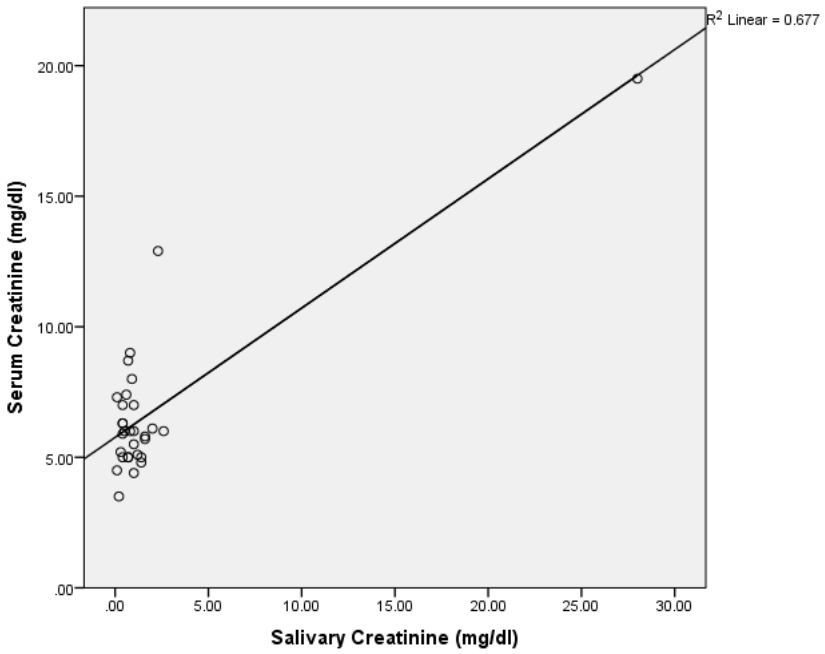

Figure (2f): Scatter diagram showing linear correlation between serum and salivary levels of creatinine among Stage V CKD

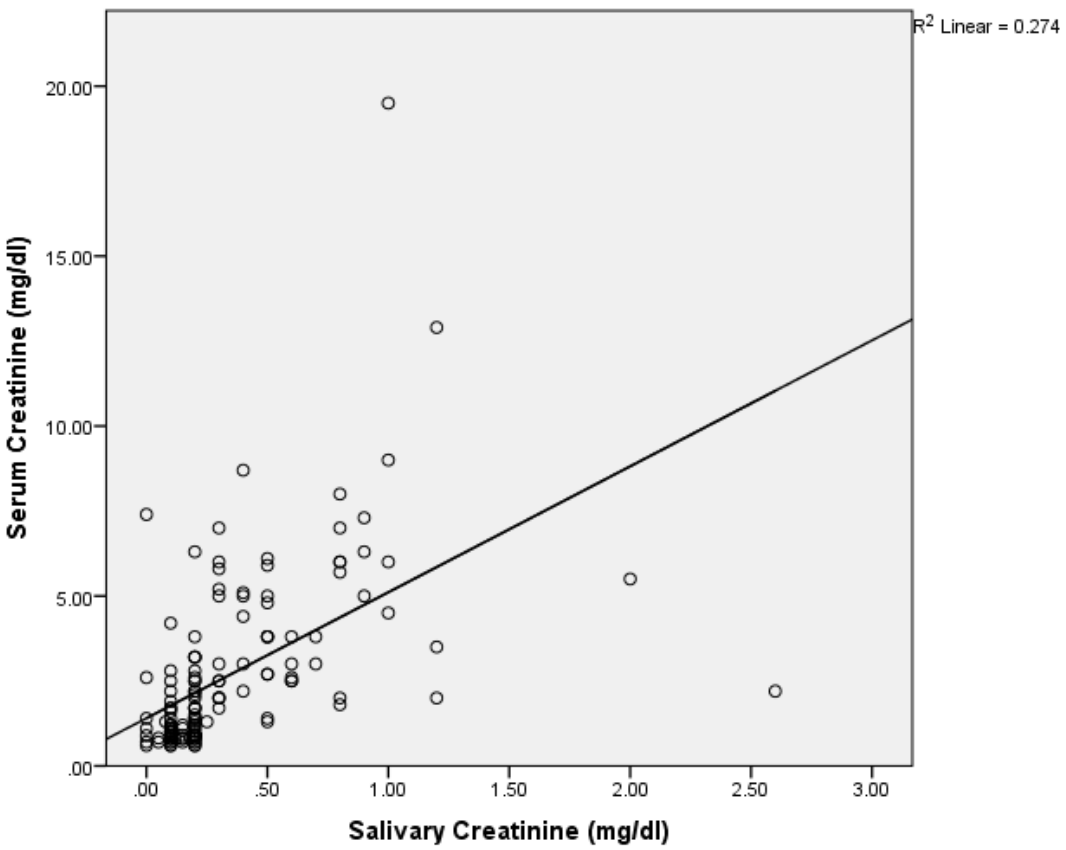

Figure (2g): Scatter diagram showing linear correlation between serum and salivary levels of creatinine among CKD group

Fig. 2: Scatter diagrams showing linear correlation between serum and salivary levels

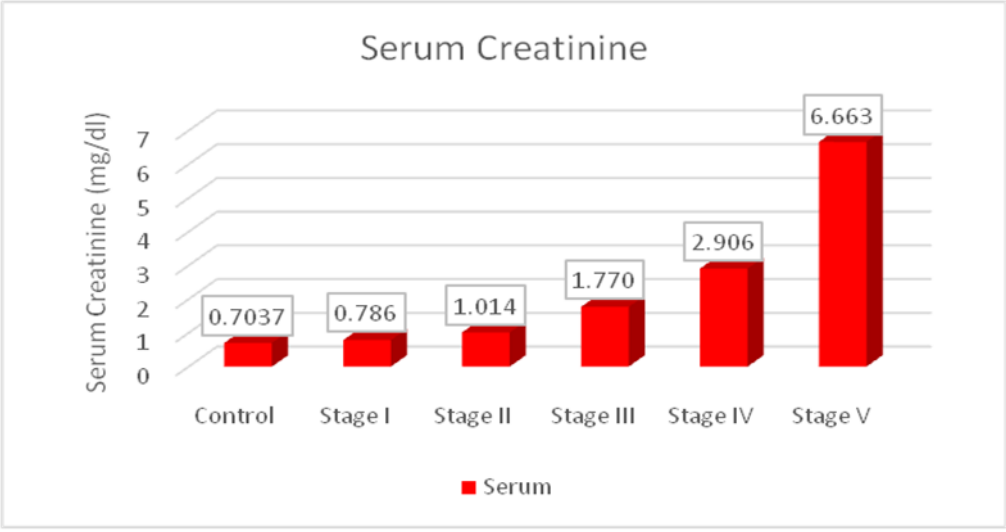

Chart 1: Mean serum creatinine concentration in control and stages of CKD

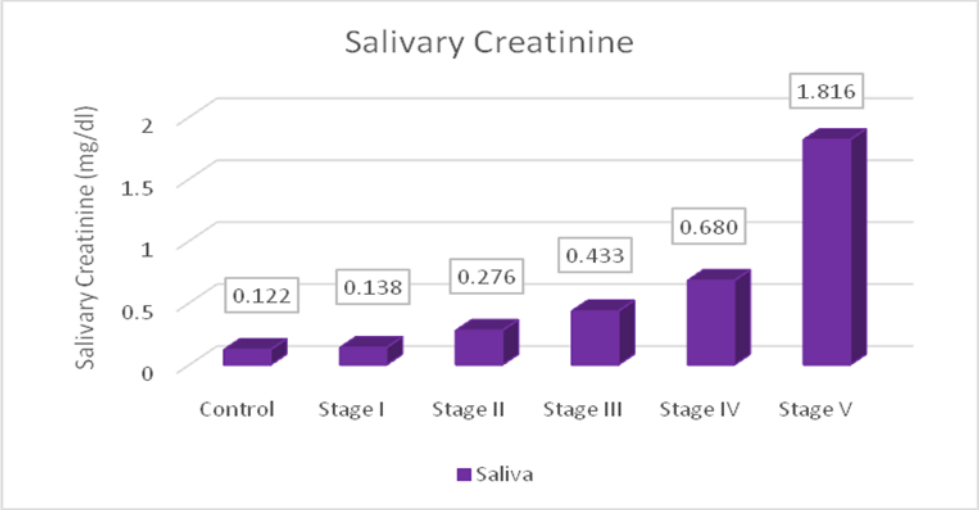

Chart 2: Mean salivary creatinine concentration in control and stages of CKD

Conclusion

Our study reinforces the fact that most studies are limited to late stages of disease which may not add further knowledge to the scientific literature. We would like to further emphasize and critically point that to consider saliva as a renal function marker, studies conducted henceforth should validate the diagnostic utility of saliva right from early to late stage of the disease with a larger sample size in a randomized controlled trial set-up. Highly sensitive estimation methodologies should be considered in analyzing these biomarkers, such an evaluation of the biomarkers in physiology (normal conditions) and as in a progressive manner of the disease will help to isolate the efficacy of saliva as a diagnostic marker and also will aid as a non-invasive tool for disease monitoring. With saliva having its distinct advantages as alternate medium to serum, it has a high potential to be used as a late stage marker which helps to differentiate patients with renal dysfunction and healthy subjects and for surveillance of the disease.

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