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ASSESSMENT OF SERUM CYSTATIN-C BASED EGFR EQUATIONS AND KIDNEY DYSFUNCTION IN PATIENTS WITH SICKLE CELL ANAEMIA: A SINGLE CENTRE EVALUATION OF CASES AND HEALTHY CONTROLS.
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ABSTRACT

Background: **Serum creatinine and its estimated glomerular filtration rate (eGFR) may be imprecise in assessing renal impairment of patients with sickle cell anaemia (SCA).**
Objective: **To evaluate serum Cystatin-C and Cystatin-C eGF Requationsin the assessment of kidney dysfunction in Nigerians with SCA and controls.**
Design**: A prospective, cross-sectional and case-control study**
Setting: **The study was conducted at the haematology units of the Obafemi Awolowo University Teaching hospital complex, Ile-Ife and the Ring Road State Hospital, Ibadan in the South Western Nigeria,**
Subject**s: The study participants included 46 sickle cell anaemia patients (HbSS) and 36 HbAA healthy controls in whiche GFR was calculated using the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI, 2009 and 2012) equations for Creatinine, cystatin-C and Creatine and cystatin-C combined.**
Results: **Mean serum cys-C was similar in patients and control group [0.29 (0.09) vs 0.26 (0.07) mg/L] while albuminuria occurred in 44.4% of patients only. Twice the number of patients with hyper filtration using CKD-EPI Cr equation was identified by CKD-EPI Cr-cys-C equation andnone by CKD-EPI cys-C. CKD-EPI cys-C identified more patients with eGFR 60 mL/min/1.73m2than the creatinine based only and/or Cr-cys-C equations. Patients with CKD stage 3 were identified by CKD-EPI Cr and CKD-EPI cys-C at 2.2% and 8.7% respectively. Age 19years, male sex, low eGFR and albuminuria were predictive of having high levels of serum cystatin C.**
Conclusion**: Serum Cystatin C values appear lower for the sickled cell anaemia patients and healthy controls in the Nigerian population. Of the CKD-EPI equations, CKD-EPI cystatin-C equation best identified SCA patients with low eGFR (eGFR < 60 mL/min/1.73m2).**

INTRODUCTION

Sickle cell nephropathy (SCN) range from functional abnormalities, to gross anatomic alterations of the kidney including hyperfiltration and glomerulopathy seen in individuals with sickled cell disease (SCD), which predispose them to chronic kidney disease

CKD) and/or end stage renal disease (ESRD) requiring dialysis and/or kidney transplantation [1- Chronic sickling underlies the several mechanisms of kidney injury but the exact mechanism of glomerular abnormalities in SCD remains undefined, overall, the production of renal vasodilating substances from ischaemic tubules, glomerular injury and ischaemic changes is often implicated [4-7].

Microalbuminuria and overt proteinuria are features and could precede renal abnormalities. As the disease progress, sickle nephropathy becomes irreversible with several associated factors including worsening anaemia, hypertension, increasing proteinuria, and microscopic haematuria [1].

Early assessment of kidney function using estimated glomerular filtration rate (eGFR) from creatinine is a standard clinical practice, but this is fraught with imprecision [8]. Cystatin-C is an endogenous marker of renal function whose serum concentration is known to correlate well with glomerular filtration rate (GFR) than serum creatinine [9], its serum concentration is exclusively determined by the glomerular filtration rate [10]. Cystatin C is considered to be a better alternative and a good marker than serum creatinine for eGFR determination [11-13], with the equation combining serum cystatin C and creatinine providing a more precise GFR estimate [14].

Previous but limited reports in sickle cell patients showed that serum cystatin-C detects renal deterioration early and correlates well with the levels of albuminuria, identify hyperfiltration and Compare well with GFR [15, 16].Bevc et al showed that among patients with CKD, cystatin-C prediction equation has a higher diagnostic accuracy in patients with low eGFR than creatinine based formulae [17].The cystatin-C based eGFR equations has not been previously evaluated in SCD patients from the sub Saharan Africa. In this report, we evaluated serum Cystatin-C and Cystatin-C based eGFR equations using the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation derived from serum creatinine (CKD-EPI, 2009), Cystatin C and Cystatin C with creatinine (CKD-EPI, 2012) in assessing kidney dysfunction in indigenous Nigerians with SCA.

PATIENTS AND METHODS.

Study Population

The study participants were sickle cell anaemia patients(SCA) attending routine clinic at the haematology units of the Obafemi Awolowo University Teaching hospital complex, Ile-Ife and the Ring Road State Hospital, Ibadan in the South Western Nigeria, they were patients previously and prospectively studied from February to July 2009. Those included were patients aged 15 years and above, with documented evidence of homozygous sickle cell gene phenotype (HbSS), and in steady state clinical condition at least two weeks prior to recruitment.

None of the patients was at this time on hydroxyurea or renal replacement therapy. Patients with established renal impairment were excluded. Institutional ethical approval was obtained from the Health and Research Ethics Committee of the Obafemi Awolowo University Teaching Hospitals' Complex, Ile-Ife, Nigeria. All the patients and controls gave their written informed consent. A total of 46 patients and 36 controls were studied. A designed proforma was used to obtain defined bio-data and clinical profile of the patients. Measurement and Assessment of Kidney Function abnormalities. Proteinuria was defined as at least 1+ protein following urinary dipstick examination.

All the subjects and controls had serum sample from venous blood obtained for Cystatin-C and creatinine following standard procedure. Urinary dipstick examination (Medi-Test 9®, MN, Germany), as well as quantitative rinary albumin estimation was performed. The estimated glomerular filtration rate (eGFR) in mL/min/1.73m2 was obtained for serum

creatinine using CKD-EPI Cr [14, 18], and cystatin C inependently (CKD-EPI cys-C) and in combination with creatinine (CKD-EPI Cr-cys-C) [14] using the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equations. The serum creatinine was measured by the modified Jaffe kinetic method using the reagent kits (Linear Chemicals, Montgat, Spain).

Urine sample was tested for the presence of blood, haemoglobin, protein and other All the study participants had quantitative urinary microal bumin determined in duplicates using Fortress® diagnostic kit by immunoturbidimetry assay method for urinary albumin. The kit content for analysis included: assay buffer (R1), antibody reagent (R2), and calibrators C1-C5 (R4) as standards.

The urinary creatinine was also determined using the Fortress® diagnostic kit for urinary creatinine estimation, following strictly the manufacturer's instructions. All absorbance (corresponding to the concentration of the measured substance) was measured using a UV spectrophotometer (DU S20, Beckman Coulter). The urine albumin/creatinine ratio was determined according to protocol previously described; value >30μg/mg (range = 30-300 μg/mg) indicate micro-albuminuria [19].

Serum cystatin-C kit (Cat. ALX-850-292-K101) was obtained from Alexis Biochemicals (Axxora, Germany) and the value of serum Cystatin-C was determined using ELISA microplate reader (Perlong DNM-9602) at 450nm In summary, standards and samples were incubated in the micro-titration wells coated with anti-human cystatin C antibody in duplicates. After a thorough wash, anti-human cystatin-C antibody labelled with horseradish peroxidase (HPR) was added to the wells and incubated with the immobilized antibody-cystatin-C complex. Following another wash, the remaining HPR conjugated antibody was allowed to react with the substrate. The reaction was stopped by the addition of an acidic solution (stop reagent).

The plate was read at 450nm using ELISA microplate reader. The absorbance was proportional to the concentration of Cystatin-C in the serum. A standard curve was plotted with mean absorbance at 450nm against tables, while Chi square was used to test the significance of difference between the selected non-continuous variables.

A multivariate analysis using binary logistic regression model was used to determine variables associated with high serum Cystatin-C concentration. All analyses were done at 95.0% significant level.The log of Cystatin-C concentration of known standards. The concentrations of unknown samples were determined using the standard curve. Kidney function was categorized according to K/DOQI guidelines (eGFR <15, 15–29, 30–59, 60–89, and >90 mL/min/1.73m2) for eGFRcr, eGFRcys and eGFRcr-cys [19]. SCA patients with eGFR values.>140 mL/min/1.73m2 were defined as having renal hyperfiltration [15] as previously described.

STATISTICAL ANALYSIS

The data entry was done using STATA IC software version 12.0 for Windows, descriptive analysis was performed and results expressed as mean and standard deviation or as frequencies presented in tables, while Chi square was used to test the significance of difference between the selected non-continuous variables. A multivariate analysis using binary logistic regression model was used to determine variables associated with high serum cystatin-C concentration. All analyses were done at 95.0% significant level.

RESULTS

Demographic and clinical characteristics of the patients and controls were similar with no statistically significant difference except for the urinary albumin/creatinine ratio. (Table 1); patients tended to have a higher mean (SD) of 48.9 (86.2) µg/mg (p = 0.059). Of the 46 SCA patients and 36HbAA controls studied, the mean (SD) value of the serum Cystatin-C of the patients was 0.29 (0.09) mg/L not statistically different from that of the control group, 0.26 (0.07) mg/L.

Table 1

Characteristics of the study population

Parameter	Patient group	Control group	P - Value
N	46 (100%)	36 (100%)	
Male	14 (30.4%)	24 (66.7%)	
Female	32 (69.6%)	12 (33.3%)	
Mean age (±SD)	25.3 (9.9) years	28.0 (10.6) years	0.16
BMI (±SD)	18.9 (3.3) kg/m^2	23.9 (5.3) kg/m^2	0.75
Serum cystatin C (±SD)	0.29 (0.09) mg/L	0.26 (0.07) mg/L	0.14
Serum creatinine (±SD)	76.9 (26.5) mmol/L	80.2 (19.4) mmol/L	0.64
Mean Urine alb/Cr (±SD)	48.9 (86.2) µg/mg (N = 36)	9.5 (4.4) µg/mg (N = 36)	0.059

Alb/Cr – albumin/creatinine ratio; N=36 for both patient and control group.

The serum Cystatin-C levels was not different among the male and female patients (X2 = 2.89; p = 0.52), neither was there difference across the weight (X2 = 2.89; p =0.68) or the BMI group (X2 = 2.68; p = 0.75). Of the patients that were studied formicro-albuminuria (N =36), 16 (44.4%) had micro-albuminuria while none of the controls had(Table2)

Table 2

Markers of kidney damage in the studied patients using eGFR equations (CKD-EPI) from serum creatinine and/or serum cystatin C only and with albuminuria.

Markers of kidney damage	Using CKD-EPI Cr n (%)	Using CKD EPI cys C n (%)	Using CKD EPI Cr-cys C n (%)
Hyperfiltration (eGFR ≥ 140 mL/min/1.73m^2)	18 (39.2%)	0 (0%)	36 (78.3%)
Reduced eGFR (eGFR < 60 mL/min/1.73m^2)	1 (2.2%)	4 (8.7%)	0 (0%)
Hyperfiltration and Microalbuminuria	9 (56.3%)	0 (0%)	12 (42.9%)
Reduced eGFR and Microalbuminuria	1 (100%)	1 (100%)	0 (0%)

Microalbuminuria only was found in 16/35 (44.4%) of the patients.

CKD-EPI-Chronic Kidney Disease Epidemiology Collaboration equation; eGFR – estimated glomerular filtration rate; Cr – Creatinine; cys C – serum cystatin C. Using the CKD-EPI equations with serum creatinine and Cystatin-C independently and in combination, twice the number of patients with hyperfiltration using CKD-EPI Cr equation was identified by the combination CKD-EPI Cr-cys-C equation while none was identified by CKD-EPI cys-C equation when used alone (Table 2). The Cystatin-C based only equation did not identify sickle cell patients with hyperfiltration in this study (Table 3). Similarly, CKD-EPI cys-C identified more patients with low eGFR(< 60 mL/min/1.73m2) than the creatinine based equation. No patient with low eGFR was identified by the CKD-EPI equation combining serum creatinine and cystatin C (Table 3).

Table 3

Chronic kidney disease (CKD) stage of 46 patients with SCA using the different CKD-EPI equations[14,17] for serum creatinine and cystatin C alone and in combination.

CKD stage Renal function	eGFR value in mL/min/1.73m^2	CKD-EPI Cr N (%)	CKD EPI cys C N (%)	CKD EPI Cr-cys C N (%)
Hyperfiltration	≥ 140	18 (39.1%)	0 (0%)	36 (78.3%)
1	90 - < 140	15 (32.6%)	30 (65.2%)	10 (21.7%)
2	60 - 89	12 (26.1%)	12 (26.1%)	0 (0%)
3	30 - 59	1 (2.2%)	4 (8.7%)	0 (0%)
4	15 - 29	0 (0%)	0 (0%)	0 (0%)
5	< 15	0 (0%)	0 (0%)	0 (0%)
	Total	46 (100%)	46 (100%)	46(100%)

CKD-EPI - Chronic Kidney Disease Epidemiology Collaboration equation; eGFR – estimated glomerular filtration rate; Cr – Creatinine; cys C – serum cystatin C.

The CKD-EPI equations using serum creatinine and cystatin C independently tend to identify patients with low eGFR with or without albuminuria better than the CKD-EPI Cr-cys-C equation (Tables 2 and 3). The severity of renal dysfunction was graded (Table 3) according to the Kidney Disease Quality Outcome Initiative (K/DOCQI) guidelines [20], while those with eGFR ≥ 140 mL/min/1.73m2 were defined as having hyperfiltration as previously published [15]. Those with stage 3 kidney damage were identified by CKD-EPI Cr and CKD-EPI cys-C at 2.2% and 8.7% respectively but none was identified by the combination equation.CKD-EPI cys-C equation was more sensitive in identifying patients with low eGFR (eGFR < 60 mL/min/1.73m2) with or without albuminuria. Remarkably, none of the patients studied was classified into CKD stages 4 and 5 by all the equations independently or in combination. Using the general logistic regression model; age ≤ 19 years, male sex, low eGFR(eGFR < 60 mL/min/1.73m2) and presence of micro-albuminuria were predictive of high serum levels of Cystatin-C in SCA patients (Table 4)

Table 4

Binary Logistic Regression Model showing the likelihood of SCD patients having high levels of serum cystatin C

Observed Parameters	Serum cystatin C value > 0.26 mg/L (mean value for the control group)		
	Odds-ratio *(standard error)*	p-value	[95% Confidence Interval]
Parameters			
Age in years			
15 – 19	19.91 (0.95)	0.034	1.43 –52.82
20 – 24	13.89 (2.97)	0.175	0.31 – 24.58
25 – 29	0.33 (0.41)	0.377	0.03 – 22.08
30 - 39	RG		
40+	8.86 (7.38)	0.996	0.00 – 1.00
Sex			
Male	RG		
Female	0.07 (0.09)	0.041	0.01 – 0.89
BMI in kg/m^2			
14.00 – 16.00	RG		
16.01 – 18.00	4.20 (5.60)	0.282	0.31 – 57.35
18.01 – 20.00	6.05 (12.66)	0.390	0.10 – 5.32
20.01 – 22.00	9.45 (43.62)	0.092	0.44 - 51.69
22.01 – 24.00	7.28 (8.05)	1.000	0.00 – 1.011
24.01 – 28.90	1.86 (6.48)	0.858	0.00 – 1712.16
eGFR in $mL/min/1.73m^2$			
GFR < 60	6.44 (0.01)	0.000	0.00 – 1.00
eGFR = 60 - 89	5. 03 (6.13)	0.185	0.46 – 54.86
eGFR = 90 <140	RC		
eGFR ≥ 140	0.18 (0.28)	0.268	0.01 – 3.67
Alb/Cr values			
Alb/Cr ≤ 30µg/mg	RG		
Alb/Cr >30 µg/mg	1.00 (0.00)	0.000	0.00 – 1.00
eGFR and Alb/Cr values			
eGFR <90 &Alb/Cr >30	RG		
eGFR >90 &Alb/Cr <30	0.07 (0.09)	0.046	0.01 – 0.96
eGFR ≥ 140 & Alb/Cr >30	0.14 (0.21)	0.026	0.02 – 0.79

However, there was no significant difference in the serum Cystatin-C levels of those patients with or without hyperfiltration (0.278 ± 0.81 vs 0.299 ± 0.97 mg/L; p = 0.49) in this study.

DISCUSSION

This study evaluated the usefulness of serum cystatin C in indigenous Nigerians patients with homozygous (HbSS) disease using the estimated glomerular filtration rate (eGFR) calculated from CKD-EPI equations.

Of all eGFR equations, the CKD-EPI equations has assumed a more accurate method of assessing renal function; it has been shown to be a reliable marker of GFR in clinical practice [21], and found to be useful and more applicable compared to other commonly used equations in patients with sickle cell disease [23]. Asnani et al recently observed that the cystatin-C based CKD-EPI equation has the greatest accuracy and least bias when compared to creatinine and/or cystatin-C-creatinine based equations [23].

In this study, and compared to other populations, serum cystatin C levels appears lower in our population, the mean serum cystatin C levels (SD) of 0.29 (0.09) mg/L in the patients was similar to that of the control group 0.26 (0.07) mg/L, these levels is lower than the reference value of 0.49-0.98mg/l reported for the Caucasians [24] and the mean value (0.95 – 1.32 mg/l) observed by Marouf et al [15] among the Kuwaiti or Asmani et al [23] among the Jamaican patients with SCA. The reasons for these differences are unclear but may not be unrelated to the ethno-racial differences or other non renal factors [25-27].

Groesbeck et al found a lower value in serum cystatin-C for non Hispanic black American and Mexican American compared to white American [25]. In addition, different assay methods of serum cystatin-C affect reported range of values [27]. In evaluating the markers of renal dysfunction (reduced eGFR, hyperfiltration, both with or without albuminuria); our study showed that greater number of patients with low eGFR (< 60 mL/min/1.73m2) were identified by CKD-EPI cys C compared to CKD-EPI Cr and cys C combining equations, this is consistent with the findings of high sensitivity of cystatin c in reduced renal function by Alvarez et al [16]. The robustness of cystatin C equation in assessing renal function is well established in children [16, 28], and also found useful in adult with SCD [23], our study reinforce the superiority of cystatin c equation over and above combined (creatinine + cystatin C) equation even in indigenous black population with SCA. Expectedly, all patients with reduced eGFR had albuminuria, cystatin C had been shown to correlate well with albuminuria [16].

Studies using creatinine based equations have shown over-estimation of eGFR values in SCD even in children [22, 29]; No patient with hyperfiltration was identified by CKD-EPI cys C in this study, while 39.2% and 78.3% were identified by CKD-EPI Cr and CKD-EPI Cr + cys C respectively. Serum creatinine levels tend to be lower in patients with SCD due to reduced muscle mass and increased renal tubular excretion of creatinine well documented in SCD patients [30], the outcome is a lower serum creatinine values and over-estimation of eGFR from creatinine based equations.

Compared with earlier study in the same group of patients [31], using the Cockcroft-Gault equation; similar kidney disease (CKD) staging was found with CKD-EPI Cr equation in the present study. About two percent of patients in the two groups had reduced eGFR while hyperfiltration was observed in 30.6% and 39.1% respectively; however, the CKD-EPI cystatin-C based equations re- classified the CKD staging of the patients. Many more patients were shown to have reduced eGFR and none with hyperfiltration using the CKD-EPI cystatin C equation. This study has again confirmed previous findings [23, 25] that young age, gender, albuminuria and low eGFR (< 60 mL/min/1.73m2) are predictive of increase serum cystatin C level across all population.

CONCLUSION

Serum Cystatin C values appears lower in the cases and control population in this study. While the CKD-EPI creatinine and Cystatin-C based equations classified kidney dysfunction differently in sickle cell anaemia patients; the CKD-EPI Cystatin-C equation identified those with low eGFR (eGFR < 60 mL/min/1) better than other equations. Albuminuria and eGFR < 60 mL/min/1.73m2 are predictive of high serum Cystatin-C level in these cohort.

COMPETING INTEREST

No competing interest declared by all the authors

AUTHORS CONTRIBUTION

RAB and KSA conceived the study and designed the protocol. RAB and NOA were involved in laboratory work while RAB and TKO participated in the data analysis. RAB, KSA, and NOA drafted the manuscript, while NOA performed expert review prior to submission. All the authors read and approved the final manuscript.

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