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RELIABILITY OF SOME SELECTED NOVEL MARKERS IN DETECTING EARLY RENAL DYSFUNCTION IN HIV POSITIVE PATIENTS ON TDF REGIMEN: A NIGERIAN STUDY

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ABSTRACT: *This study evaluated the predictability of novel biomarkers (Urine Cystatin C, NGAL and Albumin) in detecting early renal dysfunction. About 140 patients (53 (37.9 %) male and 87 (62.1%) female) who attends ARV clinic at the University's Teaching Hospital, Port Harcourt were included in the study. The study was designed in 3 phase to include Visit 0, 1 and 2 which lasted for about 12weeks (3months). Visit 1 was 4weeks from visit 0 and Visit 2 was 8 weeks from Visit 1. Laboratory assessment was carried out on samples collected from the patients, Albumin was 0.90±0.56, 1.36±0.89, and 1.36±0.94; Urine Creatinine was 479±1.90, 489.06±445.09 and 514.85±595.55; Urine Total Protein was 15.04±26.73 9.50±5.07 and 6.53±3.84; while NGAL was, 1902.51±902.59, 1941.48±743.60 and 4881.60±2792.01and Cystatin C was 889.70±1201.04, 1062.38±1165.38 and 1577.92±506.61 for Visit 0, 1 and 2 respectively. Significant difference was observed in the measured parameters across all Visits from 1st visit to end of the study. The differences observed between the markers across all visits were significant. Using differential reliability test, NGAL has 241.67% better chance of predicting renal dysfunction compared to Cystatin C, while Cystatin C has 166.67% better chance of predicting renal dysfunction compared to albumin, while NGAL also has 811.1% better chance of predicting renal impairment as compared to albumin. However, creatinine clearance did not pick up renal dysfunction. This study is therefore recommended to Physicians in other to help in diagnosing early renal dysfunction in HIV patients, especially those on tenofovir (TDF) based ARV regime which has been proven to cause renal dysfunction.*

KEYWORDS: HIV, Biomarkers, Tenofovir, NGAL, Cystatin C and Albumin

INTRODUCTION

Human immune-deficiency syndrome (HIV) is the number one health challenge in sub-Saharan Africa. Nearly three quarters of the people living with HIV reside in sub-Saharan Africa with poverty, ignorance and illiteracy driving the scourge (GARPR, 2015). Nigeria along with Uganda and South Africa accounts for almost half of the HIV load in Africa. While Nigeria closely follows South Africa with the 2nd heaviest burden of HIV in Africa (PEPFAR Nigeria, 2015); with an estimated 3.4 million people living with the virus (GARPR, 2015) and a national prevalence of 3.4% as well as 9% worldwide (Avert, 2013).

With the commendable success of ART in reducing morbidity and mortality, due to HIV, chronic non communicable diseases such as renal failure, have become an important cause of morbidity and mortality within the infected population (Michelle *et al.*, 2010; Aidsmap, 2011; FIC, 2014). Patients infected HIV have variously been reported as having a higher incidence of developing renal abnormalities than the uninfected population (Michelle *et al*, 2010; UNAID, 2011; Aidsmap, 2011; FIC 2014). There are increasing evidences that HIV infection

of the renal system is involved with HIV associated nephropathy (HIVAN) while coinfection with diabetes and hypertension has led to other forms of renal abnormalities (Crowley *et al.*, 1996).

Unlike many diseases, kidney disease often may not any symptoms in early stages (usually detectable through rigorous laboratory assessments), until it becomes very advanced (Andrew, 2008).

However advances in Biomarker application and technology could enable the clinician to make early or more definitive diagnosis, stratify risk and gather evidence for prognosis. This will make for treatment selection and prevent further complications.

Biomarkers investigated for various stages of (early and late) renal dysfunctions include Cystatin C (Low Molecular Weight Protein), Neutrophil Gelatinase Associated Lipocalin [(NGAL) (Up regulated Protein)], as well as albumin (Functional Marker) (de Geus *et al*., 2012).

The coveted drug or therapy to permanently eliminate HIV from the body cells has proven difficult despite numerous breakthrough and ground breaking discoveries in medical science and technology. This is as a result of the inherent ability of the virus to replicate and mutate rapidly once in a host cell, hence making a cure or vaccine difficult to compound. Perhaps it can only be managed with various antiretroviral drugs especially the HAARTs. One of such drugs preferably used to manage HIV infection, is Tenofovir (TDF); a nucleoside reverse transcriptase inhibitor (NRTI), considered safe and potent. Following its approval by the United State Food and Drug Administration in 2001, there have been a dramatic upward movements on the drug charts for its use. However, renal toxicity has been linked to its use (with incidence ranging from 0.3 to 2%) (karras *et al.,* 2003; Choi *et al*., 2003; Rollot *et al.*,2003; Peyriere *et al.*,2004; Labarga *et al*., 2009; Ketan *et al.*, 2010).

Unlike many diseases, kidney disease often may show no symptoms in early stages, until it becomes advanced (with about 80% of the kidney lost) (Andrew, 2008); hence biomarkers come in handy in early detection of renal disease.

A number of authors (Vishal *et al.*, 2009; de Geus *et al*., 2012 and Edgar *et al.*, 2014 have studied the use of biomarkers in detecting early renal dysfunction. Effective volume depletion occurs at the early (pre-renal stage). According to Samir *et al.*, 2005 AIDS-related kidney disease is now a fairly common cause of end-stage renal disease. Hence the study seek to estimate the predictability of some novel biomarkers (CYSTATIN C, NGAL and ALBUMIN) in predicting early renal dysfunction.

MATERIALS AND METHODS

It was a cohort study involving 140 (53 males and 87 females aged) volunteer HIV patients visiting the outpatient clinic of University of Port Harcourt Teaching Hospital ARV clinic. Patients' eligibility was assessed through Pre-randomization and their consent sought and obtained in accordance with the Helsinki Declaration. Ethical clearance was also obtained from the University of Port Harcourt (UPH) ethics Committee with the code number: UPH/R&D/REC/04.
The study was done in 3 scheduled visits from commencement; Visit 0, visit 1 and Visit 2.

Visit 0 (recruitment) where baseline samples were collected; visit 1 (4 months from recruitment); and Visit 2 (end of visit) 3 months from recruitment.
Sample collection for laboratory assessment was done on each visit. While Socio-demographic information was obtained using questionnaires.

Inclusion Criteria

i. Patient must have been on ART for at least 6 months.

ii. Patient must be 18 years old and above

iii. Patient should not be on any known medication capable of altering kidney and liver functions

Exclusion Criteria

Patients' exclusion criteria includes:

i. Inability to give consent (approval)

ii. Being bedridden

iii. Being on medications capable of altering kidney or liver function

Study Procedure

Patients who met the inclusion criteria were considered eligible for the study. Urine and blood samples were collected at 0 week, 4 weeks and 12 weeks for the laboratory assessment renal function markers (Serum albumin, NGAL and Cystatin-C). Blood for glucose, phosphate, creatinine, uric acid, Albumin, protein. Urine for urinary creatinine, uric acid, glucose, phosphate, albumin, protein.

Sample size was determined using Li *et al*. (2007) and Zhong (2009);

$$N = 2 \times \left(\frac{Z_{1-\frac{\alpha}{2}} + z_{1-\beta}}{\delta} \right)^2 \times S^2$$

Where;

Z_α which is 0.05 at 95% confidence desired (two tailed test) = 1.96

Z_β which is the power to detect such a difference (set at 80%) with a 20% withdrawal rate = 0.84

δ is the difference to be detected in the percentage change = 5

S^2 becomes the Polled standard deviation of both comparison = 15^2 = 225

Hence,

$$N = 2 \times \left(\frac{1.96 + 0.84}{5} \right)^2 \times 15^2$$

⇨ N = 140

Laboratory Sample Collection Methods

Morning spot urine samples were collected from patients using urine sample bottle. Urine was split into 2. One for NGAL and Cystatin-C analysis, the other for Urine analysis which was done within 4 hours of collection to avoid denaturing of protein parameters. WHO phlebotomy practices were followed in obtaining the blood samples using vacutena needle and syringe. Blood samples for haematological analyses were collected using the EDTA bottles, while those for clinical chemistry, were put in lithium heparin bottles. The blood samples were eventually taken to the laboratory for analyses.

Laboratory Analysis for Biomarkers

Biomarkers investigated for early and late renal dysfunctions include Cys C, NGAL, which were measured on stat fax® 4200, a compact standalone 8channel microplate reader, an ELISA (Enzyme Linked Immuno Sorbent assay) which uses a calorimetric principle. It read of colour intensity in the sample and measures at a wave length of 450nm. The more intense the colour, the higher the biomarker in the urine samples. These biomarkers (NGAL and Cystatin C) were made in the United Kingdom by R and D Systems® with expiry date in 2019 and Store ceiled products at 2 to 8 °C.

Samples were analyzed using good laboratory practice under standard operating procedures as directed by manufacturers.

Reference Range for NGAL is 7.81 - 500 pg/mL (manufacturer's values)

Reference Range for Cys C is 62.50 - 2,000 pg/mL (manufacturer's values)

Data Analysis

Descriptive statistics was used to determine mean values with post hoc multiple comparison test used to compare group mean. ANOVA and students t-test was used to differences in mean values between the visits (0, 1 and 2) and sex differences respectively. Dunnetts multiple comparison test was used to determine group differences. Significance level was set at 95%, hence $P< 0.05$.

Changes were compared in summary statistics. All these was carried out using Statistical Package for the Social sciences (SPSS) version 23.0 and XLSTAT (4.0.1, 2015).

RESULTS

Table 1: Descriptive statistics of urine biomarkers of HIV Patients on tenofovir regimen and test of significance at Visit 0, 1 and 2

			Descriptive statistics					*ANOVA*		
						95% C. I for Mean				
Urine parameters	***Visit***	***N***	***Mean***	***S.D***	***S.E***	***Lower Bound***	***Upper Bound***	***F-value***	***P-value***	***Remarks***
Urine Albumin	Visit 0	140	0.9	0.56	0.06	0.77	1.02	9.28	<0.01	S
	Visit 1	140	1.36	0.94	0.1	1.16	1.57			
	Visit 2	140	1.36	0.89	0.1	1.17	1.56			
	Total	140	1.21	0.84	0.05	1.1	1.31			
Urine Creatinine	Visit 0	140	514.85	595.55	64.98	385.61	644.1	37.64	<0.01	S
	Visit 1	140	489.06	445.09	48.56	392.47	585.65			
	Visit 2	140	479	419.7	0.21	4.38	521			
	Total	140	336.23	487.91	30.74	275.7	396.77			
Urine Total Protein	Visit 0	140	6.53	3.84	0.42	5.69	7.36	6.23	<0.01	S
	Visit 1	140	15.04	26.73	2.92	9.24	20.83			
	Visit 2	140	9.5	5.07	0.55	8.4	10.6			
	Total	140	10.35	16.19	1.02	8.34	12.36			
NGAL	Visit 0	140	1902.51	902.59	98.48	1706.64	2098.38	80.31	<0.01	S
	Visit 1	140	1941.48	743.6	81.13	1780.1	2102.85			
	Visit 2	140	4881.6	2792.01	304.63	4275.69	5487.5			
	Total	140	2908.53	2232.59	140.64	2631.54	3185.51			
CYS-C	Visit 0	140	889.7	1201.04	131.04	629.06	1150.34	10.57	<0.01	S
	Visit 1	140	1062.38	1165.38	127.15	809.48	1315.28			
	Visit 2	140	1577.92	506.61	55.28	1467.98	1687.86			
	Total	140	1176.67	1047.27	65.97	1046.74	1306.6			

***N**=distribution, **S.D**=Standard deviation, **S.E**=Standard error of mean, **F-value** = fischer's value, **p-value** = probability value, **S** = significant, **NS** = Not significant, P<0.05*

Table 2: Dunnett's multiple comparison test of urine biomarkers of HIV Patients on tenofovir regimen and test of significance at Visit 0, 1 and 2

Urine parameters	*Visit (I)*	*Visit (J)*	*Mean Difference (I-J)*	*S.E*	*95% Confidence Interval*		*P-value*	*Remarks*
					Lower Bound	*Upper Bound*		
Urine Albumin	Visit 0	Visit 1	0.00	0.14	-0.34	0.34	1.00	NS
		Visit 2	0.47	0.11	0.19	0.74	<0.01	S
	Visit 1	Visit 0	0.00	0.14	-0.34	0.34	1.00	NS
		Visit 2	0.47	0.12	0.18	0.76	<0.01	S
Urine Creatinine	Visit 0	Visit 1	-484.27	48.56	-602.53	-366.02	<0.01	S
		Visit 2	-510.07	64.98	-668.30	-351.84	<0.01	S
	Visit 1	Visit 0	484.27	48.56	366.02	602.53	<0.01	S
		Visit 2	-25.79	81.12	-221.55	169.97	0.98	NS
Urine Total Protein	Visit 0	Visit 1	-8.51	2.95	-15.68	-1.34	0.01	S
		Visit 2	-2.97	0.69	-4.65	-1.30	<0.01	S
	Visit 1	Visit 0	8.51	2.95	1.34	15.68	0.01	S
		Visit 2	5.54	2.97	-1.68	12.76	0.18	NS
NGAL	Visit 0	Visit 1	2979.08	320.16	2202.12	3756.05	<0.01	S
		Visit 2	2940.12	315.25	2174.33	3705.91	<0.01	S
	Visit 1	Visit 0	-2979.08	320.16	-3756.05	-2202.12	<0.01	S
		Visit 2	-38.96	127.60	-346.74	268.81	0.99	NS
CYS-C	Visit 0	Visit 1	515.54	138.65	179.69	851.38	<0.01	S
		Visit 2	688.21	142.22	343.63	1032.79	<0.01	S
	Visit 1	Visit 0	-515.54	138.65	-851.38	-179.69	<0.01	S
		Visit 2	172.68	182.59	-267.60	612.96	0.72	NS

N*=distribution,* ***S.E****=Standard error of mean,* ***p-value*** *= probability value,* ***S*** *= significant,* ***NS*** *= Not significant, P<0.05.*

Table 3: Descriptive statistics of urine biomarkers of HIV Patients on tenofovir regimen and test of significance at Visit 0 according to sex

Urine parameters	*Sex*	*Descriptive statistics* *N*	*Mean*	*S.D*	*S.E*	*T-test* *T-value*	*P-value*	*Remarks*
Urine Albumin (g/l)	F	87	1.95	1.41	0.24	-0.39	0.70	NS
	M	53	2.10	1.43	0.31			
Urine Creatinine (mmol/l)	F	87	5.78	1.96	0.33	0.52	0.61	NS
	M	53	5.50	2.13	0.47			
Urine T.Protein	F	87	7.65	5.70	0.96	0.07	0.94	NS
	M	53	7.55	3.90	0.85			
NGAL	F	87	2727.80	1403.87	237.30	0.50	0.62	NS
	M	53	2553.19	1003.32	218.94			
CYS-C	F	87	1052.31	915.94	154.82	-1.21	0.23	NS
	M	53	1492.62	1797.73	392.30			

***N**=distribution, **S.E**=Standard error of mean, **p-value** = probability value, **S** = significant, **NS** = Not significant, P<0.05.*

Table 4: Descriptive statistics of urine biomarkers of HIV Patients on tenofovir regimen and test of significance at Visit 1 according to sex

Urine parameters	**Sex**	**Descriptive statistics** **N**	**Mean**	**S.D**	**S.E**	**T-test** **T-value**	**P-value**	**Remarks**
Urine Albumin (g/l)	F	87	1.40	0.90	0.13	0.41	0.68	NS
	M	53	1.31	1.01	0.18			
Urine Creatinine (mmol/l)	F	87	509.33	543.43	75.36	0.53	0.60	NS
	M	53	456.12	206.78	36.55			
Urine T.Protein	F	87	14.35	25.36	3.52	-0.30	0.77	NS
	M	53	16.14	29.19	5.16			
NGAL	F	87	1932.56	966.23	133.99	0.39	0.70	NS
	M	53	1853.69	800.80	141.56			
CYS-C	F	87	1089.69	1118.97	155.17	0.27	0.79	NS
	M	53	1018.00	1254.23	221.72			

***N**=distribution, **S.E**=Standard error of mean, **p-value** = probability value, **S** = significant, **NS** = Not significant, P<0.05.*

Table 5: Descriptive statistics of urine biomarkers of HIV Patients on tenofovir regimen and test of significance at Visit 2 according to sex

Urine parameters	*Sex*	*Descriptive statistics* *N*	*Mean*	*S.D*	*S.E*	*T-test* *T-value*	*P-value*	*Remarks*
Urine Albumin (g/l)	F	87	0.92	0.57	0.08	0.60	0.55	NS
	M	53	0.85	0.56	0.10			
Urine Creatinine (mmol/l)	F	87	512.66	569.28	78.94	-0.04	0.97	NS
	M	53	518.43	645.33	114.08			
Urine T Protein	F	87	9.28	5.33	0.74	-0.49	0.62	NS
	M	53	9.85	4.68	0.83			
NGAL	F	87	1878.52	767.62	106.45	-0.99	0.33	NS
	M	53	2043.78	702.57	124.20			
CYS-C	F	87	910.79	1147.51	159.13	0.20	0.84	NS
	M	53	855.44	1301.49	230.07			

N*=distribution,* ***S.E****=Standard error of mean,* ***p-value*** *= probability value,* ***S*** *= significant,* ***NS*** *= Not significant, P<0.05.*

Table 6: Summary statistics showing the level (below, normal and above) of biomarkers in Patients at Visit 0, 1 and 2

Biomarkers		*Visit 0* *Below*	*Normal*	*Above*	*Total*	*Visit 1* *Below*	*Normal*	*Above*	*Total*	*Visit 2* *Below*	*Normal*	*Above*	*Total*
CYS C	**Count**	28	88	24	140	29	86	25	140	40	71	29	140
	% within CYS C	20.00%	62.86%	17.14%	100.00%	20.71%	61.43%	17.86%	100.00%	28.57%	50.71%	20.71%	100.00%
NGAL	**Count**	-	58	82	140	-	60	54	140	-	59	81	140
	% within NGAL	-	41.43%	58.57%	100.00%	-	42.86%	38.57%	100.00%	-	42.14%	57.86%	100.00%
ALBUMIN	**Count**	4	127	9	140	10	127	3	140	6	132	2	140
	% within Albumin	2.86%	90.71%	6.43%	100.00%	7.14%	90.71%	2.14%	100.00%	4.29%	94.29%	1.43%	100.00%

Following informed consent and ethical pass, laboratory samples (urine) was collected from Tenofovir dependent HIV patients who have been on the regimen for a minimum of 6 months.

Analysis was done and results were as presented hereunder. Descriptive statistics of the measured biomarkers were presented in Table 1, 3, 4 and 5, Analysis of variance [(ANOVA) (Table 1)] to determine significance between mean values at the 3 visits, while Dunnett's posthoc test (Table 2) was used to determine the actual pair of visits where mean values differ. Sexual dimorphism was determined using independent sample T-test (Table 3, 4 and 5). However, the summary statistics showing the level (below, normal and above) of these biomarkers in Patients was presented in Table 6.

There was a significant increase between the measured variables from visit 0 through visit 2 signifying renal dysfunction (Table 1). Sexual dimorphism was observed as significance difference was not observed between sex in Albumin, Cystatin C and NGAL at $P<0.05$.

Urine Cystatin C another renal marker was analyzed for in the urine samples of tenofovir dependent HIV patients during visit 0, 1 and 2, and it was observed that in visit 0, 20.00% have values below normal, 62.86% have normal values and 17.14% was above normal. In visit 1,

20.71% have below normal, 61.43% normal and 17.86% above normal.

While in visit 2, 28.57% of the patients were observed to have values below normal, 50.71% normal and 20.71% above normal (Table 4).

For NGAL, values below normal was not observed in all the visits, however 41.43% have normal values, while 58.57% was above normal in visit 1.

While in visit 1, 42.86% have normal values and 38.57% was above normal.

And in visit 2, 42.14% fell visit normal range, while 57.86% was above normal (Table 4).

Albumin values as observed in visit 0, were 2.86% (below normal), 90.71% (normal) and 6.43% (above normal).
In visit 1, values were 7.14% (below normal), 90.71% (normal) 2.14% (above normal).

While in visit 2, it was 4.29% (below normal), 94.29% (normal) 1.43% (above normal) (Table 4).

Cystatin-C and NGAL urine values above the reference range were suggestive of early renal disease. Values Suggestive of renal disease. Cystatin-C had more counts as compared to NGAL over a 12 weeks period (Table 4). On the contrary, low serum albumin is suggestive of renal disease.

The study therefore evaluated the reliability of novel biomarkers. The reliability was tested at visit 0 (baseline) where no intervention was given) and the markers; Cystatin C, NGAL and Albumin- where checked for their predictive ability in detecting renal dysfunction in HIV infected patients on TDF regimen for 6months and longer when compared to Creatinine clearance. This study found that Creatinine clearance showed no reliability when compared to NGAL.

However, NGAL was compared to Cystatin-C and it had 242% differential predictability; When Cystatin-C was compared to albumin, Cystatin-C was better predictor with 166.67% chance. When NGAL was compared to albumin, there was 811.1% predictability. Finding for NGAL better predictability are in keeping with Cowland and Borregaard (1997) who demonstrated NGAL gene expression to a certain extent in tissues of the body- kidney; and In

animal models, NGAL was demonstrated to have up-regulated more than 10 fold within the first couple of hours after ischemic kidney injury (Mishra *et al*., 2003). NGALs up-regulation can be picked up in the kidney (Matthaeus *et al.*,2001) and urine of lower animals such as mice after a 3hours administration of a chemotherapeutic agent and has been suggested as an early marker for detecting Kidney Injury (Mishra *et al.*,2004).

CONCLUSION

The study has effectively demonstrated that novel biomarkers such as Cystatin-C, NGAL and Albumin can be used, especially when creatinine clearance has failed in detecting early renal dysfunction. The study is therefore recommended to physicians especially those managing HIV patients in other to detect early renal dysfunction to enable proper intervention before complications sets in. As also observed by other authors, NGAL has better predictability compared to Cystatin-C and Albumin. The study therefore advise that preference be given to NGAL over other novel markers (Cystatin-C and Albumin).

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