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(RESEARCH ARTICLE)

Assessment of growth arrest-specific 6 protein as a biomarker of glomerular damage in sickle cell anaemia

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Abstract

Objective: To assess growth arrest-specific 6 (Gas6) protein as a biomarker of glomerular damage (glomerulopathy; nephropathy) in patients with sickle cell anaemia (SCA).

Methods: seventy SCA patients on routine clinic visit and seventy apparently healthy controls were recruited into the study and their blood and urine samples were collected while plasma Gas6 as well as urinary albumin and creatinine measured. Their socio-demographic and other anthropometric indices were documented. The study participants and controls were screened for urinary tract infection (UTI) using the urine dipstick combi-10 on their urine. The plasma Gas6 of all patients were compared with the albumin creatinine ratio (ACR) along with the ages. Albumin and creatinine levels were also compared with ages.

Results: Gas6 had an inverse though insignificant relationship with ACR and age in the patients; albumin, creatinine and albumin creatinine ratio varied directly with age in these patients.

Conclusion: Gas 6 was not found a useful marker of glomerulopathy in sickle cell anaemia.

Keywords: Albumin; Creatinine; Albumin creatinine ratio; Gas6; Glomerulopathy; Sickle cell anaemia

1. Introduction

The World Health Organization (WHO) has declared sickle cell disease, especially the homozygous inheritance of Haemoglobin S (HbSS), a public health priority, [1] with Nigeria accounting for about 50% of the over 300,000 children born annually with SCD globally.[2] Nigeria has the highest burden of sickle cell anaemia worldwide with a prevalence of about 20 per 1000 live births.[3-4] This monogenic disorder inheritable in an autosomal recessive Mendelian fashion is caused by ß-globin gene mutation in which the 17th nucleotide is changed from adenine to thymine, leading to the production of valine instead of glutamic acid at the position 6 of the ß-globin chain.[5] Following this mutation, a

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hydrophobic motif is produced in the deoxygenated HbS tetramer, resulting in the binding of β1 and β2 chains of two haemoglobin molecules. This crystallization produces a polymer nucleus, which grows and fills the erythrocyte, disrupting its architecture and flexibility and promoting cellular dehydration, with physical and oxidative cellular stress.[6] These processes lead to rigidity of RBCs as they traverse blood vessels with eventual occlusion and other related acute and chronic complications.

The burden of SCD is far reaching, some being related to the various associated complications, including renal related complications. Sickle cell nephropathy (SCN) is a recognized long-term complication of sickle cell anaemia (SCA) with associated high mortality and morbidity.[7] Studies have shown high prevalence of SCN (with varying degrees of chronic kidney diseases) among various age groups of SCA patients.[8-11]. Glomerular proteinuria, consisting predominantly of albumin,[12] is a common manifestation of SCN with a reported prevalence of 26% to 68% in adult SCA patients.[9]

Classically, urinary albumin excretion rate (AER) is categorized as normoalbuminuria (0-20μg/min or <30mg/day), microalbuminuria (20–200 μg/min or 30-300 mg/day) and overt albuminuria (>200 μg/min or >300 mg/day).[13] Patients usually progress from normoalbuminuria through microalbuminuria to overt proteinuria; efforts are therefore centered on detection at the stage of microalbuminuria to reduce morbidity and mortality in this group of patients. Several modalities developed for the estimation of urinary protein content are fraught with different limitations. The traditional use of 24-hr urine sample considered standard is cumbersome and associated with some technical challenges including inaccurate collection.[14] Albuminuria/proteinuria is more commonly diagnosed from elevated concentrations in a timed or spot urine sample and requires measurement on at least two or three occasions over a two- to three-month period.[15] The recommended use of spot urine for the measurement of albumin: creatinine ratio (ACR) is confounded by factors including muscle mass, metabolism, hydration status, age, gender, medications,[16] as well as the high creatinine excretion in SCA patients.[17] The standard urine dipstick primarily detects albumin semi-quantitatively; though specific, it lacks enough sensitivity to detect microalbuminuria. Renal biopsy is invasive and detects glomerulopathy when the damage is barely reversible.[18] There is therefore need for a biomarker that will detect glomerular damage with high sensitivity in a spot sample at an early stage of the disease so that any of the renal replacement therapy can be instituted.

Mesangial cell proliferation is noted to be a characteristic feature of many types of glomerular diseases and is usually associated with matrix expansion, leading to the development of kidney disease.[19] Growth arrest specific protein 6 (Gas6) is an autocrine growth factor for mesangial cells proliferation.[19] Studies have shown that Gas6 estimation aids detection of glomerular damage in conditions such as diabetes mellitus, systemic lupus erythematosis, etc,[20-21] and that Gas6 level correlated inversely with GFR.[22] Gas 6 could therefore be a real time marker of glomerular damage in SCA. The aim of this study is to analyze the utility of Gas6 protein as a biomarker for glomerular damage in SCA.

2. Material and methods

This is a hospital based descriptive cross-sectional study done at University of Ibadan/University College Hospital (UI/UCH), Ibadan over a nine-month period. The University College Hospital is a one thousand (1000) bedded hospital situated in Agodi, Ibadan Oyo State, Nigeria. Subjects for this study were recruited from the Haematology Day Care Unit and the Haematology clinic of the Medical Outpatient Department of the UCH, Ibadan. The clinics attend to patients with SCD for routine medical care and follow-up, with an average of 15 and 600 patients weekly and yearly respectively. The prevalence of SCA in the hospital is 3.1%.[23] The controls were normal subjects recruited from Ita Merin community in Ibadan Oyo state Nigeria. Exclusion criteria for both groups included diabetes mellitus (DM), urinary tract infection (UTI) and pregnancy, since their contribution to Gas6 concentration in urine is not known.

2.1. Sample Size

The minimum sample size for this study was determined from the formula below [55]

$$n = \frac{Z^2\,pq}{d^2}$$

Where:
n = minimum sample size
Z= constant at 95% confidence interval from Z table
p= prevalence of SCA in UCH Ibadan is 3.1%
q= 1-p
d= precision at 95% confidence interval = 0.05

$$\text{Calculation: } n = \frac{(1.962)(0.031)(0.969)}{(0.050)^2}$$

$$\frac{3.8416 \text{ x } 0.031 \text{ x } 0.969}{0.0025}$$

$$\frac{0.1153978224}{0.0025}$$

=46.2 + 10% attrition (4.6) + 50.8

~51

Seventy study participants and 70 controls were enrolled in this study

2.2. Sample collection

This descriptive comparative study made use of 2 groups- the patient and control groups. Five (5) milliliters of blood were drawn aseptically from the ante-cubital vein of each consenting patient and control participants, into an ethylenediaminetetraacetic acid Potassium 3 (EDTA-K3) bottle for Gas6 estimation. The samples were centrifuged at 3000rpm for 15 minutes and plasma decanted into plain bottles. Five to ten millilitres of urine sample were also collected from each patient into a sterile universal bottle. The urine samples were centrifuged at 1500rpm for 10 minutes and the supernatant decanted into plain sample bottles for both creatinine and albumin estimation. Urine and plasma samples were stored frozen at a temperature of -20^{0}C and were assayed within two months. The bio-data were also collected from both groups.

2.3. Sample Analysis

The following parameters were assessed in the collected samples.

2.3.1. Plasma Gas6

Plasma Gas6 protein was assayed using enzyme linked immunosorbent assay (ELISA) technique on the ELISA machine (Stat Fax 4200, Awareness technology). Model of the washer is Stat Fax 2600, Awareness technology.

2.3.2. Albumin

Urine albumin assay was done by immunoturbidimetric method as a measure of antigen-antibody insoluble immune-complexes formed. Automated chemistry analyzer platform (Landwind C100 plus) was used for this.

2.3.3. Creatinine

Urine samples were also analyzed for creatinine using creatininase enzymatic method and performed on the Landwind LW C100 *plus* autoanalyser.

2.4. Statistical Analysis

The obtained study data were analyzed using Statistical Package for Social Sciences (SPSS) version 20 (IBM Corp. Released 2011. IBM SPSS Statistics for Windows, Version 20.0. Armonk, NY: IBM Corp.). Data were presented as means and proportion. Results were presented with relevant charts, tables and statements.

2.5. Ethical Approval

This was obtained from the Institute for Advanced Medical Research and Training (IAMRAT) and the institutional ethics committee of University of Ibadan/University College Hospital (UI/UCH).

2.6. Informed Consent

Informed consent was obtained from all individual participants included in the study.

3. Results

3.1. Sociodemographic characteristics of the study subjects

Table 1 shows that majority of the study participants were between ages 18 and 27 years (45.71%) and largely females (54.29%). Similarly, the control group had males and females in a ratio of 1:1.4

Table 1 Demographic distribution of the study participants and Control group

	Study participants		**Control group**	
Gender				
Males	32 (45.7%)		29 (41.4%)	
Females	38 (54.3%)		41 (58.6%)	
Total	70 (100.0%)		70 (100.0%)	
Age range	Males (%)	Females (%)	Males (%)	Females (%)
18 – 27	17 (53.1)	15 (46.9)	4 (33.3)	8 (67.7)
28 – 37	8 (42.1)	11 (57.9)	8 (38.1)	13 (61.9)
38 – 47	5 (41.7)	7 (58.3)	6 (33.3)	12 (67.7)
48 – 77	2 (28.6)	5 (71.4)	11 (57.9)	8 (42.1)
Total	32	38	29	41
	70		70	

The median value and the interquartile range (IQR) for UACR in the study group was significantly higher than in the control group (p = 0.000). The median plasma Gas6 protein is lower in the study group. The control participants had higher urinary creatinine and plasma Gas6, but lower UACR than the study participants. (Table 2)

Table 2 Median Values and Interquartile Ranges of Biochemical Parameters in the Study Group and Control Participants

Variables	**Study group (n = 70)**		**Control group (n = 70)**		**p-value**
	Median	**IQR**	**Median**	**IQR**	
Microalbumin (mg/L)	30.0	18.5 – 66.3	26.7	17.6 – 39.6	0.126
Urinary Creatinine (g/L)	0.31	0.18 – 0.51	0.60	0.33 – 0.88	0.000
UACR (mg/g)	112.4	58.6 – 243.5	48.4	32.7 – 77.7	0.000
Plasma Gas6 . (ng/mL)	0.2	0.0 - 1.5	2.7	0.33 – 4.03	0.000

Table 3 showed that the median and the interquartile ranges of all the analytes increased with age.

The median and the interquartile ranges of Microalbumin, Urinary Creatinine and Urinary Albumin Creatinine Ratio showed no special trend. However, those of plasma Gas 6 were observed to decrease with increasing age (Tab. 4).

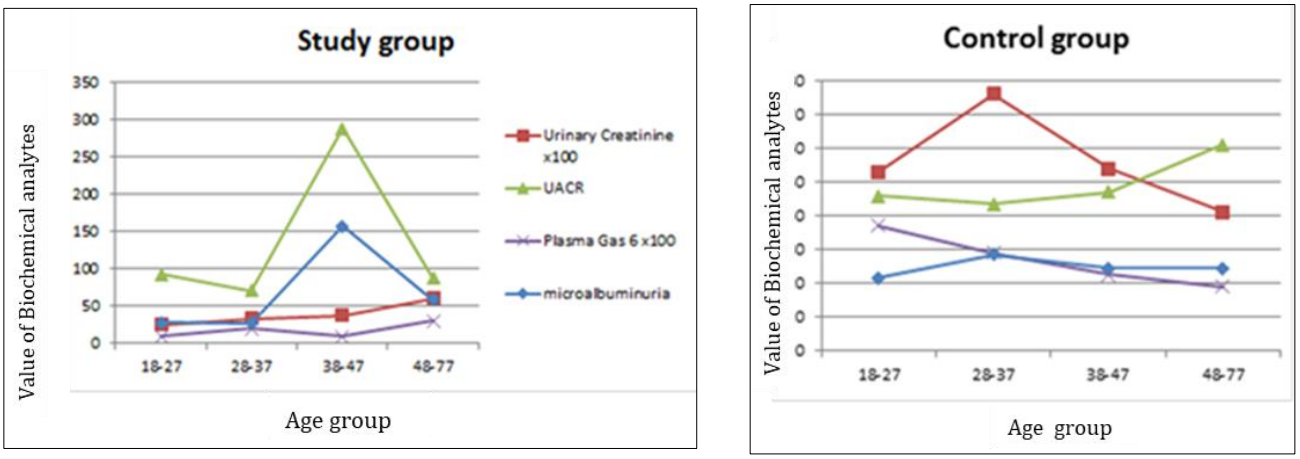
Table 3 Median and Interquartile Ranges of Biochemical Parameters of Various Age Groups in the Study Participants

Age Range in years (N)	Microalbumin (mg/L)		Urinary Creatinine (g/L)		Urinary Albumin Creatinine Ratio (mg/g)		Plasma Gas6 (ng/mL)	
	Median	IQR	Median	IQR	Median	IQR	Median	IQR
18 – 27 (31)	27.7	15.3 – 40.6	0.25	0.16 - 0.40	92.8	59.0 – 161.4	0.10	0.0 – 1.5
28 – 37 (19)	27.4	20.0 – 59.6	0.33	0.24 – 0.52	71.0	51.5 – 194.3	0.20	0.0 – 1.9
38 – 47 (13)	156.5	24.2 – 202.8	0.38	0.15 – 0.60	287.9	149.1-528.5	0.10	0.0 – 1.4
48 – 77 (7)	58.0	17.7 – 193.8	0.6	0.24 – 0.67	87.0	64.1 – 323.6	0.30	0.0 – 1.7

Table 4 Median and Interquartile Ranges of Biochemical Parameters of Various Age Groups in the Control group

Age Range in years (N)	Microalbumin (mg/L)		Urinary Creatinine (g/L)		Urinary ACR (mg/g)		Plasma Gas6 (ng/mL)	
	Median	IQR	Median	IQR	Median	IQR	Median	IQR
18 – 27 (12)	21.7	15.8 – 37.5	0.53	0.24 - 0.92	45.9	28.8 – 118.2	3.70	1.90 – 8.30
28 – 37 (21)	28.6	24.0 – 55.7	0.76	0.66 – 1.06	43.5	31.2 – 67.8	2.90	1.9 – 4.35
38 – 47 (18)	24.5	15.4 – 33.6	0.54	0.19 – 0.97	47.1	35.9-71.9	2.25	1.52 – 4.23
48 – 77 (19)	24.5	14.4 – 34.2	0.41	0.3 – 0.75	61.0	37.0 – 103.7	1.9	1.40 – 3.1

Figures 1a and 1b showed that the median and interquartile ranges of microalbumin and UACR in both the study and the control groups had similar patterns. However, plasma Gas6 decreased with age unlike that of the study group which showed no pattern with age.



a b

Figure 1 Comparing the age related trend of the median values of the analytes for the study and control groups

Correlating the age of the study group with Plasma microalbumin, urinary creatinine, and urinary albumin creatinine ratio gave p-values <0.05, but that for Gas 6 protein gave a p-value of 0. 382. Urinary creatinine and Gas6 correlated significantly and inversely with age in the control group. Table 5.

Table 5 Correlation Coefficient of Age with Biochemical Parameters Both Groups

Biochemical Parameters	Study Participants		Control Participants	
	Correlation Coefficient	P – Value	Correlation Coefficient	P - Value
Microalbumin	0.353	0.002*	-0.086	0.481
Creatinine	0.232	0.047*	-0.258	0.031*
UACR	0.230	0.05*	0.175	0.147
Gas6	-0.103	0.382	-0.317	0.007*

*Significant correlation

Gas6 showed no significant correlation with any of the biochemical parameters in both groups

Table 6 Correlation Coefficient of Gas6 with Biochemical Parameters in Both Groups

Biochemical Parameters	Study Participants		Control Participants	
	Correlation Coefficient	p - Value	Correlation Coefficient	p - Value
Microalbumin	-0.156	0.197	0.043	0.686
Creatinine	0.061	0.615	-0.084	0.491
UACR	-0.178	0.139	0.146	0.227

4. Discussion

Sickle cell disease (SCD), including SCA is a monogenic autosomal recessive haemoglobin disorder of public health importance associated with chronic complications including renal complications in 30-50% adults, with increased morbidity and mortality.[1] The modal age of the study participants was lower than that of the control group. This is probably because sickle cell anaemia patients have reduced life expectancy (14.3years),[24] compared with the Nigeria general population (54.5years) according to 2016 report.[25] It is estimated that 20% of HbSS patients die within the first two years of life, one third die before reaching age five while up to half would have been dead by the thirtieth year of life.[24] A study done amongst a group of 2,824 SCA patients reported that 73 (2.6%), who had genotype HbSS, died before age 3years.[26]

Improving the survival and life expectancy of SCA patients requires prevention and early detection of these complications.[27] Several studies have shown that Gas6 estimation aids early detection of glomerular damage in conditions such as diabetes mellitus, systemic lupus erythematosus, etc,[20-21] and correlated inversely with GFR.[22] Gas 6 could therefore be a real time marker of glomerular damage in SCA. This research evaluated the performance of Gas6 as a biomarker of nephropathy (glomerulopathy) compared with urinary albumin creatinine ratio in sickle cell anaemia patients.

Gas6 is a 75 kD multimodular vitamin K-dependent protein expressed in many tissues, including capillary endothelial cells, vascular smooth muscle cells, and bone marrow cells,[21] and serves as a ligand for TYRO-3, AXL and MER (TAM receptors).[28] GAS6/TAM signaling is involved in cell migration, survival and adhesion, as well as inflammatory cytokine release, hence promoting inflammation.[28] A study by Yanagita, et al., using mice models indicated that Gas6 induces glomerular cell proliferation in nephrotoxic nephritis and suggested that this may contribute to glomerular injury and the progression of chronic nephritis.[29]

In this study, we observed that Gas6 interquartile range was lower in SCA patients (0.0 - 1.5ng/mL) than in the healthy controls (0.33–4.03ng/mL). A study done amongst 69 diabetic patients showed that plasma Gas6 concentrations correlated well with albuminuria, being higher in patients with micro and macro-albuminuria compared to patients with normo-albuminuria and healthy controls.[20] Another study by Lee et al., similarly showed higher Gas6 IQR among

patients with renal disease than in the healthy group.[22] The disagreement could stem from the fact that none of these researchers worked on sickle cell anaemia patients.

It is noteworthy that SCA patients suffer repeated ischaemic injury to various organs of the body including the kidneys.[30] This could cause destruction and consequent depletion of the mesangial cells which are responsible for producing Gas6 (Gas6 production is usually in response to some growth factors and cytokines).[20] Hence, decreased Gas6 production and much lower Gas6 IQR in the sickle cell anaemia patients than in the healthy individuals. Gas6 levels in SCA represent a balance of the reduction in number of mesangial cells and the net production by the remaining mesangial cells undergoing hypertrophy and inflammation. Gas6 performance in this study shows that it was not a good biomarker for identification of renal complications in SCA, even though it is reported to be useful in detecting early renal complication in other chronic diseases such as diabetes mellitus.[31] The age of the study participants correlated insignificantly with Gas6 (p =0. 382). Thus, age has no effect on the concentration of plasma Gas6 and would not have been a confounding factor if Gas6 was found to be a reliable marker of glomerulopathy in SCA.

Even among healthy individuals, we observed lower plasma Gas6 median/interquartile range of 2.7μg/L/0.33 – 4.037μg/L compared to the findings of Jiang *et al*.,[32] and Sainaghi *et al*.,[33] with reported median/interquartile ranges of 16.9 μg/L/13–28 μg/L and 19.1ng/mL (17.2–21.4ng/mL) respectively. This may be largely due to genetically programmed racial differences. While our research was done in Nigeria, the studies quoted were done in Caucasians. There are evidences demonstrating the variability of the alleles in human GAS6, as well as its Mendelian pattern of inheritance.[34] At the least three genotypes of GAS6 already discovered and these include: GG, AG, and AA.[32]

Other parameters for assessing renal impairment performed better in this study. The urinary albumin and creatinine, and the albumin creatinine ratio correlated well with age in the SCA patients, indicating that glomerular damage increased with the age. The highest urinary albumin creatinine ratio was observed between age 38-47 years, agreeing with the report that SCA patients developed chronic kidney disease between ages 30 and 40 years.[35] Ischaemic depletion of mesengial cells leading to increased filtering of albumin through the glomeruli;[12] and reduced muscle mass with consequent reduced level of creatinine in SCA patient, account for higher urinary albumin and UACR than in the control group.[36]

We also observed higher levels of albuminuria in the control above the reference interval. Also, the interquartile range for the control group was higher than the reference interval for the general population. It has been reported that about 30% of apparently healthy individuals have microalbuminuria, likely due to mild or subclinical glomerulonephritis from exposure to infective processes and complex immune reactions.[37] Also antibiotics and other drugs abuse can cause kidney damage leading to proteinuria.[38]

5. Conclusion

Gas6 has not proven useful as a screening tool for glomerular damage in sickle cell anaemia probably due to loss of mesangial cells. Thus, UACR is still much more sensitive as a marker of glomerular damage in SCA than Gas6. There is therefore still the need for a biomarker that will perform better than UACR and detect glomerular damage much earlier.

Limitations of and recommendations from the study

- We did not measure vitamin K levels in this study therefore we would suggest that such study be carried out on SCA patients, both during crises and in steady state to ascertain its relationship with Gas6.
- In furtherance of a marker for early detection of kidney disease, another marker such as cystatin C may be worth exploring.
- A longitudinal study could help in assessing Gas 6 profile of SCA patients as they grow from childhood to adulthood.
- Specific Gas6 genotypes may be used for subsequent trials.
- There is need to review the acceptable reference interval of albumin creatinine ratio in the African population. This we hope to do soon.

Compliance with ethical standards

Acknowledgments

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Disclosure of conflict of interest

The authors declare that they have no conflict of interest.

Statement of ethical approval

Ethical approval was obtained from the Institute for Advanced Medical Research and Training (IAMRAT), the institutional ethics committee of University of Ibadan/University College Hospital (UI/UCH). The approval/registration number is: NHREC/05/01/2008a and the UI/UCH Ethics Committee assigned number is: UI/EC/15/0329 dated 18/12/2017. The study participants as well as the control group gave informed consent to the work.

Statement of informed consent

Informed consent was obtained from all individual participants included in the study.

Authors' contribution

ECOI: Conceptualization, methodology, software, investigation, resources, data curation, writing - original draft, project administration. **KSA:** Conceptualization, methodology, writing – review and editing, supervision, project administration; **TSA:** Methodology, writing – review and editing, supervision; **OAO:** Methodology, validation, writing – review and editing; **HCO:** writing – review and editing; **FEM:** software, writing – review and editing, visualization; **OOS** validation, formal analysis, investigation; **AOY** software, statistical analysis;

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