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Nephrin as a Biomarker of Sick Cell Glomerulopathy in Malawi¹

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Abstract

Background—Glomerulopathy is an increasingly identified complication in young patients with sickle cell disease (SCD). Hyperfiltration and albuminuria followed by declining glomerular filtration rates and eventual end-stage renal disease (ESRD) is assumed to be the typical progression of glomerular disease. There are only a few reported biomarkers to identify early-stage renal disease in SCD.

Procedures—We detail the renal profile of 101 children with SCD in Malawi and propose a novel urinary biomarker for the identification of early renal disease.

Results—Among children with sickle cell anemia, 24.8% had a urine albumin-creatinine ratio of 30 mg/g or above. In univariate analysis, only patients with higher urinary nephrin, a urinary marker of glomerular injury, had significantly greater odds of having albuminuria. In multivariable analysis, nephrin remained significantly associated with albuminuria. A nephrin-creatinine ratio (NCR) cut-point of 622 ng/mg, the 50th percentile, was associated with a 45.8 times greater odds of having albuminuria in children with nephropathy above this value. Further analysis revealed this urinary NCR cut-point to have 96% sensitivity, 64% specificity, 47% positive predictive value and 98% negative predictive value for the presence of albuminuria.

Conclusions—These data suggest that a substantial number of children with SCD in Malawi have renal disease and could be at risk for worsening nephropathy and ESRD as they age. Our data suggest that urinary nephrin could be utilized as an early marker of glomerular disease in SCD.

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CONFLICTS OF INTEREST

The authors have no relevant conflicts of interest to disclose.

Keywords

Sickle cell disease; Nephrin; Albuminuria; Glomerulopathy; D-Dimer; Sensitivity

INTRODUCTION

Sickle cell disease (SCD) is a monogenic disorder that affects millions of people worldwide. Renal disease, including glomerulopathy, is a prevalent complication among adult patients and is a prominent cause of morbidity and mortality ². The prevalence of albuminuria is up to 58% in adults with SCD, and a proportion of these patients will progress to end-stage renal disease (ESRD) ^{3,4}. The median time to development of ESRD is 23 years, with a 2-year life expectancy once ESRD is reached even with aggressive renal replacement therapy ⁵. Proteinuria appears to be an independent predictor of mortality among HbSS patients ⁶. Hyperfiltration and albuminuria have been observed as early as infancy, suggesting the presence of significant early glomerular disease in patients with SCD ^{7,8}.

Given the severe consequences of progressive disease, emphasis should be placed on the early identification of patients with renal disease. However, the surveillance of sickle glomerulopathy is limited by a dearth of clinically validated biomarkers. Current guidelines and best practices recommend the use of routine urinalysis as well as evaluation of microalbuminuria in the surveillance of renal disease. However, this strategy only identifies patients with clinically evident renal disease. A previous study of renal disease in patients with SCD found that the presence of measureable albuminuria is accompanied by a loss of glomerular permselectivity despite normal glomerular filtration rate (GFR), and suggests the presence of irreversible damage⁹. Early detection of renal disease with use of novel biomarkers could enable earlier intervention and subsequent slowing of disease progression, potentially limiting irreversible damage to the kidney.

The current treatment strategy of albuminuria is similar to that of other proteinuric renal diseases with the mainstay approach using angiotensin converting enzyme (ACE) inhibitors, and more recently with angiotensin receptor blockers (ARBs). There are no published studies of any long-term therapies for proteinuria in SCD. However, short-term treatment with ACE inhibitors significantly decreases both microalbuminuria and macroalbuminuria ^{10,11,12}. Hydroxyurea, approved by the US Food and Drug Administration to treat sickle cell anemia, may have renoprotective effects. Specifically, treatment of adult patients with hydroxyurea is associated with lower levels of albuminuria ¹³. Treatment of children with hydroxyurea also resulted in decreased glomerular hyperfiltration and glomerular hypertrophy ¹³. A recent prospective, open-label study reported improvement in albuminuria following 6 months of treatment with hydroxyurea ¹⁴.

Nephrin is a slit diaphragm protein that is critical for glomerular filtration by providing architectural support to podocytes. Nephrin shedding is used as a marker of glomerular-specific renal damage in a variety of settings and was observed prior to the development of albuminuria in a mouse model of proteinuric renal disease¹⁵. Increased levels of urinary nephrin are present in humanized sickle cell mice and correlate with eGFR in adults with

SCD^{16,17}. As nephrinuria develops before albuminuria, nephrin may be a suitable biomarker for early detection of glomerulopathy in SCD.

We have recently introduced hemoglobin electrophoresis testing and clinically characterized a cohort of children in Lilongwe, Malawi¹⁸. Malawi is a country of 18 million people in Southeastern Africa and has a substantial SCD burden, but only scarce resources for its diagnosis and treatment.^{19,20} In this study, we describe the renal profile of children with SCD followed at Kamuzu Central Hospital, a tertiary referral hospital, in Lilongwe, Malawi. We propose that nephrin, an early marker of glomerular injury, is associated with albuminuria and may represent a potential novel urinary biomarker for the identification of children with early renal disease.

METHODS

Consecutive patients seen at a pediatric chronic care clinic at Kamuzu Central Hospital in Lilongwe, Malawi, between January and May 2015 were recruited. The University of North Carolina at Chapel Hill (UNC) Institutional Review Board (IRB) and Malawi National Health Science Review Committee approved the study. Written informed consent was obtained from the parents of 119 enrolled children. One patient declined participation in the study. Children aged 7–17 years also provided informed assent prior to study participation.

Laboratory Analyses

Routine laboratory studies, including complete blood counts, chemistries, hemoglobin electrophoresis and urinalysis were performed by the UNC Project laboratory in Lilongwe, Malawi. The normal ranges for clinical laboratory variables are provided in supplemental table S1. Plasma and urine samples were aliquoted and frozen immediately at -80°C for subsequent analysis. Urine albumin-creatinine ratio (UACR) measurements were performed by the McClendon Clinical Laboratory at UNC Hospitals. Plasma hemoglobin was quantified by using a laboratory developed test (LDT, Core Laboratory, McLendon Clinical Laboratories, UNC, Chapel Hill) adapted to the Vitros 5600 chemistry platform (Ortho Clinical Diagnostics, Raritan NJ). Plasma levels of vascular endothelial growth factor (VEGF) (R&D Systems, Minneapolis, MN), soluble fms-like tyrosine kinase-1 (sFLT-1, also referred to as vascular endothelial growth factor receptor-1 [VEGFR-1]) (R&D Systems, Minneapolis, MN), D-dimer (RayBiotech, Norcross, GA), and urinary nephrin (Exocell, Philadelphia, PA) were measured using commercially available ELISA kits. Nephrin concentration in the urine was corrected by urine creatinine concentration, and expressed as nephrin-creatinine ratio (NCR; ng/mg). Estimated GFR was derived using the Schwartz formula for children²¹.

Quality control at UNC Project Malawi was assured by storing the plasma and urine samples immediately after collection at -80°C in freezers with Sensaphone® temperature monitoring, with back-up generators in the event of power outage. The samples were shipped on dry ice to UNC, Chapel Hill, with replenishment of dry ice by the courier during transit. Measurements of albumin-creatinine ratio have been performed in urine samples stored at -80°C .^{22,23}

Statistical Analysis

Demographic and laboratory variables were summarized by counts and percentages, if categorical, or by median and interquartile ranges (IQR), if continuous. Each variable was examined individually using a logistic regression model for albuminuria (UACR ≥ 30 mg/g), with adjustment for age and sex. Those variables with p-values < 0.35 in the univariate analyses were evaluated as potential predictors of albuminuria via a multivariable logistic regression model that was also adjusted for age and sex. Variable selection was conducted at the 0.05 level. To further assess the predictive capacity of urinary NCR for albuminuria, a receiver operating characteristic (ROC) curve was obtained, and area under the curve was estimated, with associated confidence interval. Sensitivities, specificities, positive predictive values, and negative predictive values were calculated for selected cut-points of urinary NCR in predicting albuminuria. Logistic regression analysis using a urinary NCR cut-point of 622 ng/mg (the median value in the cohort) was performed and the odds ratio for albuminuria with urine NCR above and below 622 ng/mg was estimated. Finally, Spearman correlation coefficients evaluating the associations of urine NCR with other laboratory variables were calculated. Reported p-values are for individual tests, unadjusted for multiple comparisons because of the exploratory nature of this study. The ROC curve for urinary NCR was generated using R (version 3.3.2), and other analyses were conducted using SAS University Edition (SAS Institute, Cary, NC).

RESULTS

One hundred and nineteen children were evaluated, with 101 confirmed to have sickle cell anemia (HbSS) (median age: 9 years [IQR: 4–11], 51% male), seven with sickle cell trait (HbAS), and nine with HbAA as determined by hemoglobin electrophoresis. Two patients had indeterminate results. Baseline demographic and laboratory data are shown in Table 1. As expected, HbSS patients had lower median hemoglobin levels, and higher WBC counts, as well as levels of lactate dehydrogenase, total bilirubin and indirect bilirubin than individuals with HbAA (See reference values for children in Malawi in Supplemental Table S1). Median urinary NCR was also higher in HbSS patients compared with individuals with HbAA. Albuminuria, defined as UACR of 30 mg/g or higher, was present in 24.8% of HbSS patients. The median UACR in the albuminuria subgroup of HbSS patients was 58.8 mg/g (IQR: 52.7 – 125.4) vs. 0.0 mg/g (IQR: 0.0 – 9.9) in patients without albuminuria. In univariate analyses, patients with higher urinary NCR had significantly higher odds of albuminuria ($p=0.0003$). However, no other significant associations were seen between laboratory variables and albuminuria (Table 2).

In the multivariable logistic regression analysis, only urinary NCR was significantly associated with albuminuria (odds ratio estimate = 1.002, 95% confidence interval [CI]: {1.001, 1.003}, $p = 0.0003$). Additional analysis using an NCR cut-point of 622 ng/mg, the median value for the cohort, revealed a 45.9 times greater odds of having albuminuria in children with NCR above this value. The area under the ROC curve that illustrates the ability of urinary NCR in predicting albuminuria was 0.89 (95% CI: [0.83, 0.96]) (Figure 1). An NCR of 622 ng/mg or greater, representing the 50th percentile for the population, provided a sensitivity of 96%, a specificity of 64%, a positive predictive value (PPV) of 47% and a

negative predictive value (NPV) of 98% for the presence of albuminuria. An NCR of 1213 ng/mg or greater, representing the 75th percentile for the population, provided a sensitivity of 64%, a specificity of 87%, a PPV of 62% and an NPV of 88% for the presence of albuminuria. An NCR of 2107 ng/mg or greater, representing the 90th percentile for the population, provided a sensitivity of 28%, a specificity of 96%, a PPV of 70% and an NPV of 80% for the presence of albuminuria.

Urinary NCR was positively correlated with total and indirect bilirubin levels while negatively correlated with D-dimer levels ($p < 0.05$). However, no significant correlations were seen between urinary NCR and hemoglobin or serum creatinine (Table 3). In addition, a significant correlation was not observed between NCR and eGFR ($r = 0.03$, $p = 0.84$).

DISCUSSION

Albuminuria is a common complication in SCD, with an increasing recognition of its high prevalence in young patients⁴. ACE inhibitors have become the “standard of care” for the treatment of albuminuria in SCD but adequately powered controlled studies of their effect on the progression of sickle glomerulopathy and subsequent chronic kidney disease are lacking. Despite the recommendation by an NHLBI expert panel that adults with moderately increased albuminuria (previously called microalbuminuria), without other apparent cause, be started on ACE inhibitor therapy, the natural history of albuminuria in patients with sickle cell anemia remains inadequately defined²⁴. However, multiple studies suggest that kidney function declines over time in patients with SCD^{25–27}. Indeed, we have recently reported a rate of decline in eGFR of 2.15 mL/min per 1.73 m² per year, much greater than that expected for healthy individuals^{28,29}.

The high prevalence of albuminuria in our patient cohort in Malawi suggests early, severe glomerular disease in young patients with SCD. This is similar to the results from a large, multicenter study in West Africa that reported a prevalence of 27% in children who were less than 10 years old³⁰. A recent study of patients from Cameroon reported a prevalence of albuminuria of 60%, although this cohort was older than our patient cohort, with an average patient age of 15 years³¹.

In the multivariable logistic regression analysis, only NCR was significantly associated with albuminuria. Urinary nephrin has been reported to correlate with urine albumin concentration in a small cohort of adult patients with SCD¹⁷. However, our study is the first to report the association of NCR with albuminuria in young children with SCD. Although the specificity and positive predictive value of NCR (with a cut-point at the 50th percentile) for albuminuria in our study are modest, the sensitivity and negative predictive value are high. As nephrin is a glomerular-specific protein, monitoring of this biomarker might be more indicative of glomerular injury than other putative biomarkers in the current literature³². Furthermore, evaluation of a urine biomarker is more convenient in a pediatric population than a plasma-based biomarker. With its high sensitivity and negative predictive value, nephrin represents a plausible and easily accessible biomarker for the early diagnosis of glomerulopathy associated with SCD.

In our patient cohort, D-dimer was negatively correlated with NCR. In addition, we observed that D-dimer appeared to be inversely associated with albuminuria, although this was not statistically significant. D-dimer is an assayable product of fibrin degradation that is elevated in the plasma during clotting and thrombosis. There is increasing evidence that SCD is a hypercoagulable state³³. In addition to an increased risk of thrombotic complications, patients with SCD have evidence of coagulation activation, with increased levels of markers of thrombin generation, even in the non-crisis, “steady states.” High concentrations of thrombin may contribute to podocyte injury³⁴. It is suggested that thrombin may contribute to progression of proteinuria in animal models of nephrotic syndrome³⁵. The role of coagulation activation in SCD pathophysiology is highlighted by a recent study which showed that genetic reduction in prothrombin levels in sickle mice, not only resulted in decreased levels of thrombin-antithrombin complexes and D-dimer, but also produced significant reductions in renal complications, including albuminuria, compared with wild type sickle mice³⁶. In light of these previous reports, the negative association between D-dimer and both NCR and UACR was surprising and unexpected. Additional studies in patients with SCD are required to clarify the relationship between coagulation activation and glomerulopathy.

There were no associations between hemoglobin or plasma hemoglobin and other markers of hemolysis with albuminuria. The absence of significant associations between the various markers of hemolysis and albuminuria suggests that hemolysis may not play a role in the pathogenesis of glomerulopathy in this population. The contribution of hemolysis to the pathophysiology of SCD-related glomerulopathy remains uncertain. While multiple observational studies show an association of albuminuria with markers of hemolysis in patients with SCD^{6,30,37–40} others have not shown such associations^{41–44}. Additionally, despite the reported role for hyperfiltration in the pathogenesis of albuminuria in SCD^{45,46}, we found no association between the presence of albuminuria and estimated GFR in this study. In addition, we did not observe any association between albuminuria and either VEGF or VEGFR-1.

Our study has several limitations. All the study patients were recruited from a tertiary hospital clinic in Malawi. As with other populations in sub-Saharan Africa, the highest mortality is thought to occur in the less than 5-year age group⁴⁷. As such, there is a possible survivor bias in our population resulting in an overall healthier cohort than might otherwise exist. The gold standard for measurement of albuminuria is a 24-hour urine collection. However, 24-hour collections are particularly tedious in the setting of a developing country such as Malawi. Furthermore, we have previously reported very strong correlations between spot assessments for UACR and 24-hour urine protein collections providing support for the use of spot urine collections to evaluate albuminuria in patients with SCD⁴⁸. As with all cross-sectional studies, this analysis demonstrates associations, but cannot prove causation.

CONCLUSION

These data taken together suggests that a substantial proportion of children with SCD in Malawi exhibit renal involvement early in life and could be at risk for worsening glomerulopathy and ESRD as they grow older. Our data further indicate that urinary nephrin

is associated with the presence of albuminuria and could be utilized as an early biomarker of glomerular disease in SCD. Confirmation of the ability of urinary nephrin to predict the development of albuminuria will require the conduct of longitudinal studies. However, early detection of renal disease through novel biomarkers could enable earlier intervention and possibly slow disease progression, potentially limiting irreversible damage to the kidney. Increased surveillance of children with SCD for renal complications can ultimately inform management strategies to improve outcomes and increase life expectancy in patients with SCD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS KEY

SCD	Sickle Cell Disease
ESRD	End-Stage Renal Disease
NCR	Nephrin-Creatinine Ratio
eGFR	Estimated Glomerular Filtration Rate
ACE	Angiotensin Converting Enzyme
ARB	Angiotensin Receptor Blocker
IRB	Institutional Review Board
VEGF	Vascular Endothelial Growth Factor
VEGFR-1	Vascular Endothelial Growth Factor Receptor-1
UACR	Urine Albumin-Creatinine Ratio
IQR	Interquartile Range
ROC	Receiving Operating Characteristic
PPV	Positive Predictive Value
NPV	Negative Predictive Value
HbSS	Hemoglobin SS

HbAS	Hemoglobin AS (Sickle Cell Trait)
HbAA	Hemoglobin AA
LDT	Laboratory Developed TestsFLT-1
sFLT-1	Soluble FMS-like Tyrosine Kinase-1
UNC	University of North Carolina at Chapel Hill
NHLBI	National HeartLung and Blood Institute
US	United States of America

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Figure 1. Receiver Operating Characteristic Curve for Urinary Nephrin-Creatinine Ratio for the Prediction of Albuminuria in Children with Sickle Cell Anemia. The area under the receiver operating characteristic curve was 0.89 (95% CI: [0.83, 0.96])

Table 1

Demographics and Laboratory Variables

Variable	Number	Median/Number	InterQuartile Range/Percentage
Age (years)	119	9	5 – 11
Sex	119		
Female (%)		57	48
Male (%)		62	52
Weight (kg)	114	20.5	14.5 – 27
Systolic Blood Pressure (mm Hg)	97	103	95 – 109
Diastolic Blood Pressure (mm Hg)	97	60	55 – 64
Oxygen Saturation (%)	114	93	88 – 97
White Blood Cell Count ($\times 10^9/L$)	112	13.95	10.95 – 18.3
Hemoglobin (g/dL)	112	7.4	6.9 – 8.1
Platelet Count ($\times 10^9/L$)	112	438.5	349 – 571
Lactate Dehydrogenase (U/L)	113	606	489 – 752
Total Bilirubin (mg/dL)	114	1.6	0.9 – 2.5
Direct Bilirubin (mg/dL)	114	0.6	0.4 – 0.8
Indirect Bilirubin (mg/dL)	114	0.95	0.5 – 1.8
Creatinine (mg/dL)	113	0.3	0.2 – 0.4
Urine Albumin-Creatinine Ratio (mg/g)	119	0.0	0.0 – 23.6
Estimated Glomerular filtration rate (mL/min per 1.73 m ²)	76	199.6	113.7 – 240.2
Vascular Endothelial Growth Factor (pg/mL)	119	96.74	34.5 – 210.6
D-Dimer (ng/mL)	119	1264.4	1034.7 – 1569.2
Urinary Nephryn-Creatinine Ratio (ng/mg)	119	620	301 – 1186

Table 2

Univariate Predictors of Albuminuria as Determined by Logistic Regression Analysis

* Variable	Odds Ratio	95% Confidence Interval	P-value
Systolic Blood Pressure	1.009	(0.985,1.035)	0.46
Diastolic Blood Pressure	1.013	(0.974,1.053)	0.52
White Blood Cell Count	0.991	(0.893,1.100)	0.87
Hemoglobin	0.918	(0.522,1.614)	0.77
Estimated GFR	1.002	(0.996,1.007)	0.62
Lactate Dehydrogenase	1.002	(1.000,1.005)	0.077
Total Bilirubin	1.240	(0.927,1.657)	0.15
Direct Bilirubin	1.879	(0.405,8.728)	0.42
Indirect Bilirubin	1.269	(0.918,1.753)	0.15
Plasma Hemoglobin	1.017	(0.997,1.037)	0.10
VEGF	1.000	(0.999,1.001)	0.37
VEGF R1	1.002	(0.994,1.010)	0.67
D-dimer	0.999	(0.998,1.000)	0.11
Urinary Nephritin-Creatinine Ratio	1.002	(1.001,1.003)	0.0003

* Adjusted for Age and Sex

UACR – Urine Albumin-Creatinine Ratio; VEGF – Vascular Endothelial Growth Factor; VEGF R1 - Vascular Endothelial Growth Factor Receptor
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Table 3

Spearman Correlation Coefficients of Laboratory Variables with Urinary Nephryn-Creatinine Ratio

Variable	Spearman Correlation Coefficient	P-value
WBC count	−0.072	0.49
Hemoglobin	−0.023	0.82
Platelet count	−0.014	0.90
Lactate dehydrogenase	0.19	0.063
Total bilirubin	0.26	0.011
Direct bilirubin	0.17	0.10
Indirect bilirubin	0.27	0.0086
VEGF	−0.05	0.62
D-Dimer	−0.25	0.012
Estimated GFR	0.027	0.84
Creatinine	0.089	0.39

WBC – White Blood Cell; VEGF – Vascular Endothelial Growth factor; Estimated GFR – Estimated Glomerular Filtration Rate