Hemoglobinuria is associated with chronic kidney disease and its progression in patients with sickle cell anemia

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Summary:

To evaluate the association between hemoglobinuria and chronic kidney disease (CKD) in sickle cell anemia (SCA), we analyzed 356 adult hemoglobin SS or Sβ+ thalassemia patients from the University of Illinois at Chicago (UIC) and 439 from the multi-center Walk-PHaSST cohort. CKD was classified according to National Kidney Foundation guidelines. Hemoglobinuria, defined as positive heme on urine dipstick with absent red blood cells on microscopy, was confirmed by ELISA in a subset of patients. The prevalence of CKD was 58% in the UIC cohort and 54% in the Walk-PHaSST cohort, and hemoglobinuria was observed in 36% and 20% of the patients, respectively. Pathway analysis in both cohorts indicated an independent association of LDH with hemoglobinuria and, in turn, independent associations of hemoglobinuria and age with CKD (P<0.0001). After a median of 32 months of follow-up in the UIC cohort, hemoglobinuria was associated with progression of CKD (halving of estimated glomerular filtration rate or requirement for dialysis; HR 13.9, 95%CI 1.7-113.2, P=0.0012) and increasing albuminuria (HR 3.1, 95%CI: 1.3-7.7; logrank P=0.0035). In conclusion hemoglobinuria is common in sickle cell anemia and is associated with CKD, consistent with a role for intravascular hemolysis in the pathogenesis of renal dysfunction in SCA.
Introduction:


In patients with SCA, the glomerular filtration rate (GFR) begins to decline as early as in the second decade of life. (Aygun, et al 2011) In the Cooperative Study of Sickle Cell Disease (CSSCD), creatinine clearance <100 mL/min was an independent predictor for early death. (Platt, et al 1994) In a large cohort study with over 25 years of follow-up, Powars et al found that a serum creatinine concentration of ≥1.5 mg/dL was observed in 4.2-11.6% of SCA patients and the average time to hemodialysis, renal transplantation, or death in these patients was 3.3 years. (Powars, et al 2005, Powars, et al 1991) Prevalences of abnormal levels of albuminuria range from 15-28% in adolescents to 40-68% in adults with SCA. (Abo-Zenah, et al 2009, Becton, et al 2010, Day, et al 2012, Dharnidharka, et al 1998, Guasch, et al 2006) Patients with SCA on hemodialysis have a poor prognosis with 40% mortality at 20 months of follow up. (Nissenson and Port 1989)

Single-institution studies have investigated correlations between increased markers of hemolysis and kidney disease in SCA patients with conflicting results. Several studies have not

Hemoglobinuria, which reflects intravascular hemolysis of a degree to exceed the binding capacity of haptoglobin, as estimated by urine dipstick analysis was reported in 15% of SCA patients from Saudi Arabia and 42% of SCA patients from Nigeria at steady-state.(Aleem 2008, Bolarinwa, et al 2012) Although acute hemoglobin-mediated damage to the kidneys has been observed in patients with paroxysmal nocturnal hemoglobinuria,(Forman, et al 1984) cardiac bypass surgery patients with acute hemolysis (Vermeulen Windsant, et al 2010), and pre-clinical animal models,(Baek, et al 2012, Minneci, et al 2005) the impact of chronic exposure of the kidneys to cell-free hemoglobin in SCA has not been previously explored.

In this study, the primary analyses were focused on laboratory and clinical factors associated with CKD in adult patients with SCA treated at the University of Illinois at Chicago (UIC), Comprehensive Sickle Cell Center. We repeated our analyses in the multi-center US and UK Walk-Treatment of Pulmonary Hypertension and Sickle Cell Disease with Sildenafil Therapy (Walk-PHaSST) cohort. The objective of this investigation was to test the hypothesis that hemolysis and hemoglobinuria are associated with CKD in SCA.
Methods:

Patients

We analyzed 356 adults with the diagnosis of SCA (Hb SS or Sβ0-thalassemia) receiving routine medical care at UIC between January 2003 and January 2012. The protocol was approved by the Institutional Review Board prior to undertaking the chart review. Laboratory and clinical data were extracted from the electronic medical record charting system, Cerner PowerChart. Laboratory data were collected from a steady-state clinic visit, defined as a visit without mention of the patient being in an acute vaso-occlusive pain episode and at least four weeks from a blood transfusion or an acute vaso-occlusive pain episode requiring medical attention in the emergency department, acute care center, or hospitalization. A total of 447 patients were screened and 356 had eGFR data at a steady-state clinic visit.

The validation cohort included 439 adults with SCA (Hb SS or Sβ0-thalassemia) from 8 US centers and 1 UK center were recruited from February 2008 to June 2009 with eGFR data. UIC was a participating site for the Walk-PHaSST study and patients from this site were excluded from the validation cohort. The local institutional review boards or ethics committees had approved the protocol prior to subject enrollment.

Laboratory and clinical data were obtained at steady state, defined as in usual state of health and at least three weeks from an acute pain event, hospitalization, or emergency room visit. Clinical variables included age, sex, type of SCA, medication history (hydroxyurea, anti-hypertensive medications, and NSAID therapy) and a history of SCA related complications (vaso-occlusive pain episode frequency, leg ulcer, priapism, avascular necrosis, stroke, and acute chest syndrome), blood pressure and oxygen saturation determined by pulse oximetry. A vaso-occlusive pain episode was defined as a pain episode severe enough to warrant an emergency
room, acute care center visit, or hospitalization. Laboratory variables included hemoglobin fractionation, hemoglobin, white blood cell count, platelet count, reticulocyte count, serum creatinine, bicarbonate, lactate dehydrogenase (LDH), total and indirect bilirubin, AST, urinalysis, urine albumin, and urine creatinine. Laboratory analyses at the University of Illinois Clinical Pathology Laboratories were performed using Clinical Laboratory Improvement Amendments (CLIA) approved methods.

**Chronic kidney disease (CKD)**

The eGFR was calculated using the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) formula. (Levey, et al 2009) We created eGFR categories of $\geq 90$, 60-89, and $<60$ mL/min/1.73m$^2$. Albuminuria was defined as microalbuminuria if the albumin to creatinine ratio was 30-300 mg/g creatinine and macroalbuminuria if the ratio was $>300$mg/g creatinine. CKD was defined according to the Kidney Disease Outcomes Quality Initiatives (KDOQI) guidelines to align the definition of CKD stage to the current National Kidney Foundation evidence-based guidelines: (2002) stage 0- eGFR $>60$ mL/min/1.73m$^2$ and albuminuria $<30$ mg/g creatinine; stage 1- eGFR $>90$ mL/min/1.73 m$^2$ and albuminuria $\geq 30$ mg/g creatinine; stage 2- eGFR 60-89 mL/min/1.73 m$^2$ and albuminuria $\geq 30$ mg/g creatinine; stage 3 and greater- eGFR $<60$ mL/min/1.73m$^2$.

**Hemoglobinuria**

In the UIC cohort, hemoglobinuria was defined by a reading of trace, small or moderate blood on automated urine dipstick analysis using the Aution 9EB Sticks on the IRIS 200Q machine and microscopy showing $<2$ red blood cells per high power field. In the Walk-PHaSST cohort,
hemoglobinuria was defined by a reading of trace, small, or moderate blood and microscopy showing 0–5 red blood cells per high power field.

Forty-three UIC SCA patients were prospectively evaluated from March to May 2013 to compare urine dipstick measurements of hemoglobinuria with the urine hemoglobin directly measured by the ELISA method (Human hemoglobin ELISA kit; Bethyl Laboratories, Montgomery, TX). They were selected based on consecutive appearances at routinely scheduled outpatient visits. Urine hemoglobin concentration (ng/mL) was prospectively and directly measured using an ELISA assay (Human hemoglobin ELISA kit; Bethyl Laboratories, Montgomery, TX) in 43 patients. Urine samples were diluted 1:1 with the assay’s diluents buffer and then corrected for the dilution factor.

**Longitudinal Follow-Up of UIC Patients**

139 UIC subjects with urinalysis on initial steady-state evaluation had a repeat urinalysis performed at steady-state after a median of 39 months (IQR 11-58 months). 164 study subjects with initial steady-state urinalysis and eGFR determination were followed for a median of 32 months (IQR 18-52 months). Over this period, CKD progression (defined by a reduction of eGFR by 50% or requiring hemodialysis/kidney transplant) was determined. 93 patients were followed for progression of albuminuria category (defined as progression from normoalbuminuria to micro or macroalbuminuria, or microalbuminuria to macroalbuminuria).

**Statistical Analysis**

Linear variables were compared according to eGFR, albuminuria, hemoglobinuria, and CKD stage with the Kruskall-Wallis test and categorical variables with Pearson’s chi square and
Cochran’s linear trend test. Multivariate analysis was performed using logistic regression in both cohorts. Variables with a $P \leq 0.1$ in univariate analysis were entered into the initial model and a stepwise approach was applied to select the final regression models. We also performed pathway analysis using structural equation modeling. The fitness of the final model to the observed data was determined by the comparative fit index (CFI), goodness-of-fit test $\chi^2$/degrees of freedom (df), and the root mean square error of approximation (RMSEA) with adjustment for site in the Walk-PhaSST cohort. A CFI $>0.9$, goodness-of-fit test $\chi^2$/df $<2$, or RMSEA $<0.05$ were considered to be an excellent fit of the data to the model. The Fisher’s exact test was used for comparing the initial urinalysis result to the most current steady-state urinalysis. The associations of hemoglobinuria status with CKD progression and worsening of albuminuria category were examined using the logrank method to compare Kaplan-Meier survival curves and Cox proportional hazards model to determine unadjusted hazard ratios. Systat 11 (Systat Software Corporation, Chicago, IL, USA) was used for most analyses and Lavaan (R-package, version 0.5-9) was used for pathway analysis.
Results:

Prospective Validation of Urine Dipstick to Screen for Hemoglobinuria

Forty-three consecutive steady-state patients were evaluated with both urine dipstick and hemoglobin concentration by ELISA. Seventeen patients with a positive urine dipstick for hemoglobin had significantly higher urine hemoglobin concentrations when directly measured by the ELISA method on the same urine sample compared to 26 patients with a negative dipstick for hemoglobin (medians of 23.1 vs. 11.5 ng/mL, respectively; \( P<0.0001 \)) (Figure 1).

Patient Characteristics

The UIC and Walk-PHaSST cohorts were similar with respect to age and gender distribution as well as proportions of patients on hydroxyurea or anti-hypertensive medications (Supplementary Table 1). Steady-state clinical and laboratory data were comparable between the SCA patients with baseline eGFR and albuminuria measurements available and those with eGFR but without albuminuria measurements.

Variables Associated with Decreased eGFR

Eleven percent of patients had eGFR 60–89 mL/min/1.73 m² and 6% had eGFR <60 mL/min/1.73 m² in the UIC cohort and 9% of patients had eGFR 60–89 mL/min/1.73 m² and 8% had eGFR<60 mL/min/1.73 m² in the Walk-PHaSST cohort. On univariate analysis in both cohorts, greater age and MAP, lower hemoglobin concentration, the presence of macroalbuminuria, and the presence of hemoglobinuria were associated with progressively lower eGFR category (\( P\leq0.004 \)). By logistic regression analysis in the UIC cohort, increasing age (\( P\leq0.0001 \)), the presence of hemoglobinuria (\( P\leq0.005 \)), and increasing MAP (\( P\leq0.15 \)) were
independently associated with progressively lower eGFR categories. In the Walk-PHaSST cohort, increasing age ($P<0.0001$), the presence of hemoglobinuria ($P\leq0.007$), and increasing MAP ($P\leq0.16$) were independently associated with progressively lower eGFR categories. In both the UIC and Walk-PHaSST cohort, MAP only achieved significance for the lowest eGFR category ($P\leq0.047$).

*Variables Associated with Albuminuria*

The prevalence of microalbuminuria in the UIC and Walk-PHaSST cohorts was 36% and 31%, respectively. Macroalbuminuria was present in 18% and 20% of patients, respectively. On univariate analysis in the UIC cohort, higher LDH, lower hemoglobin concentration and the presence of hemoglobinuria were associated with a progressive increase in albuminuria category ($P\leq0.004$). In the Walk-PHaSST cohort, higher LDH, AST, and MAP, lower hemoglobin concentration, and the presence of hemoglobinuria were associated with a progressive increase in albuminuria category ($P\leq0.004$). By logistic regression analysis in the UIC cohort, the presence of hemoglobinuria ($P<0.0001$) and increasing age ($P\leq0.008$) were independently associated with progressively higher albuminuria categories. In the Walk-PHaSST cohort, the presence of hemoglobinuria ($P\leq0.027$) was independently associated with progressively higher albuminuria categories.

*Variables Associated with Chronic Kidney Disease*

Chronic kidney disease was present in 58% of patients from the UIC cohort: 39% had stage 1 kidney disease, 8% had stage 2, and 11% had stage 3 or higher. Similarly, 54% of patients from the Walk-PHaSST cohort had CKD, 35% with stage 1, 6% with stage 2 and 13%
with stage 3 or higher. On univariate analysis in the UIC cohort, greater age and LDH, lower hemoglobin concentration, and the presence of hemoglobinuria were associated with CKD ($P \leq 0.004$) (Table 1A). In the Walk-PHaSST cohort, greater age, MAP, LDH and AST, lower hemoglobin concentration, and the presence of hemoglobinuria were associated with CKD ($P \leq 0.004$) (Table 1B). The variables remained significantly different according to CKD stage in each cohort after stratification by hydroxyurea status (data not shown). Hydroxyurea therapy was not associated with CKD stage in either the UIC or Walk-PHaSST cohort. By multivariate logistic regression, in the UIC and Walk-PHaSST cohorts the presence of hemoglobinuria, increasing age, and increasing MAP were independently associated with CKD (Table 2). Hemoglobinuria remained significantly associated with CKD stage on multivariate analysis when stratifying by hydroxyurea status (data not shown).

Variables Associated with Hemoglobinuria

Hemoglobinuria was observed in 36% of patients from the UIC cohort and 20% of patients from the Walk-PHaSST cohort. On univariate analysis in both the UIC and Walk-PHaSST cohorts the presence of hemoglobinuria was associated with albuminuria, higher LDH and AST, and lower hemoglobin concentration and eGFR (Figures 2A-F). Hemoglobinuria status did not differ by hydroxyurea use in either cohort and the variables remained significantly associated with hemoglobinuria in each cohort after stratification by hydroxyurea status (data not shown). By multivariate logistic regression in both the UIC and Walk-PHaSST cohorts, the natural log of LDH had the most significant independent association with hemoglobinuria (OR 18.9, 95% CI 6.4–55.6, $P < 0.0001$ and OR 2.6, 95% CI 1.6–4.3, $P = 0.0002$ respectively). In both cohorts LDH could be replaced by the other markers of hemolysis including AST and
hemoglobin concentration and association of LDH and hemoglobin concentration on multivariate analysis persisted after stratification by hydroxyurea status (data not shown).

Pathway Analysis of Chronic Kidney Disease

In pathway analysis, the UIC and Walk-PHaSST data were consistent with a model in which, consecutively, 1) higher LDH is associated with hemoglobinuria and 2) greater age and the presence of hemoglobinuria are associated the stage of CKD (Figure 3) (CFI=1.0, goodness-of-fit test $\chi^2/df \leq 0.8$, RMSEA<0.0001 for UIC and Walk-PHaSST). In contrast, the alternative pathway of 1) higher LDH and age associated with stage of CKD and 2) stage of CKD associated with hemoglobinuria had a poor fit of the data (CFI≤0.89, goodness-of-fit test $\chi^2/df \geq 3.7$, RMSEA≥0.11).

Clinical Correlates of Chronic Kidney Disease

Pulse pressure (medians of 46 vs. 50, 52 and 58 mmHg UIC and 50 vs. 50, 55 and 57 mmHg Walk-PHaSST; $P \leq 0.0056$) was higher in patients with increasing stage of CKD. There were no significant differences in oxygen saturation or histories of acute chest syndrome, avascular necrosis, stroke, or pain crisis frequency based on CKD in either the UIC nor Walk-PHaSST cohorts and histories of priapism and leg ulcers did not differ significantly after the Bonferroni correction.

Subset of UIC Patients Evaluated Longitudinally for Progression of Chronic Kidney Disease

One hundred and sixty-four SCA patients from UIC who had urine dipstick measurements were longitudinally followed to evaluate for progression of kidney disease. The
baseline characteristics of the patients in the longitudinal cohort were similar to those who were not evaluable for progression. In the patients followed longitudinally, age and MAP were similar between patients with versus without hemoglobinuria at the initial steady-state visit. Of the 139 SCA patients with repeat steady-state urinalyses, 61% (33/54) of patients who were initially hemoglobinuria positive remained positive on the most current urinalysis while 90% (8/85) of patients who were initially hemoglobinuria negative remained negative ($P<0.0001$) (Figure 4A).

The proportion of patients with CKD progression (defined by a reduction of eGFR by 50% or requiring hemodialysis/kidney transplant) at a median follow up of 32 months (IQR 18–52 months) was higher in patients with hemoglobinuria (13%, 7/56) versus without hemoglobinuria (1%, 1/108) (HR 13.9, 95% CI: 1.7–113.2; logrank $P=0.0012$) (Figure 4B). Progression of albuminuria category was observed in 42% (11/26) of patients with hemoglobinuria versus 13% (9/67) of patients without hemoglobinuria (HR 3.1, 95% CI: 1.3–7.7; logrank $P=0.0035$) (Figure 4C). Differences in rates of CKD or albuminuria progression were not observed based on baseline hydroxyurea status.
Discussion:

Chronic kidney disease is a common complication of SCA that is associated with other morbidities and early mortality. (Platt, et al 1994, Powars, et al 2005, Powars, et al 1991) We observed CKD in 58% of SCA patients from the UIC cohort and in 54% of SCA patients from the Walk-PHaSST cohort. The stage of CKD was independently associated with the presence of hemoglobinuria and increasing age in both the UIC and Walk-PHaSST cohorts. Hemoglobinuria, observed in 36% of adult patients from the UIC cohort and 20% of the adult Walk-PHaSST cohort, was associated with markers of hemolysis including increased LDH and AST and decreased serum hemoglobin concentration. These results and our pathway analysis are consistent with a scenario in which increasing age in combination with yet to be determined genetic and environmental factors lead to CKD in SCA. In parallel, chronic hemolysis in association with hemoglobinuria put patients at risk for CKD.

Our findings indicate that hemoglobinuria determined by dipstick urinalysis is a valid assessment of increased urine hemoglobin concentration and is fairly consistent on repeat testing at steady-state visits. In particular, we confirmed that significantly higher levels of directly measured urine hemoglobin concentrations were present in a subset of prospective patients with dipstick-defined hemoglobinuria.

age and MAP to be associated with CKD. Guasch and colleagues reported that patients with SCA and a creatinine clearance <90 mL/min/1.73 m² were significantly older and had significantly higher albuminuria levels with a median value in the macroalbuminuria range. (Guasch, et al 2006) Reduction in GFR with increasing age in SCA has also been observed in studies that directly measured GFR using radiolabeled tracers. (Aygun, et al 2011, Barros, et al 2006) Several studies have also consistently shown correlations between elevated blood pressure and albuminuria or reduced eGFR in patients with SCA (Abo-Zenah, et al 2009, Gordeuk, et al 2008, Guasch, et al 1996, Guasch, et al 2006) or sickle cell disease. (Becton, et al 2010, Powars, et al 1991) Hypertension is thought to directly damage the kidneys through direct transmission of increased systemic pressure resulting in focal and segmental glomerulosclerosis. (Raij, et al 1984) which is a common pathologic finding in patients with SCA. (Bhathena and Sondheimer 1991, Tejani, et al 1985)

New in our study is the finding that hemoglobinuria determined by urinalysis, reflecting intravascular hemolysis, is independently associated with CKD and its progression. Hemoglobinuria had highly significant associations with indirect markers of hemolysis and was a stronger predictor of CKD than the indirect hemolytic markers suggesting that cell-free hemoglobin may provide a mechanism for kidney damage in SCA. Furthermore, in the longitudinal cohort of patients, the presence of hemoglobinuria was significantly associated with a greater risk for CKD progression and progression of albuminuria category. These findings are consistent with reports of other investigators of a relationship between indirect markers of hemolysis and CKD in SCA. (Bartolucci, et al 2012, Day, et al 2012, Gurkan, et al 2010, Maier-Redelsperger, et al 2010)
The potential for cell-free hemoglobin to contribute to kidney disease in SCA is plausible based on prior laboratory investigations. In patients with SCA, plasma cell-free hemoglobin levels range from 0-20 μM heme at steady-state and increase during vaso-occlusive episodes to levels greater than 20-40 μM heme. (Naumann, et al 1971, Reiter, et al 2002) Circulating plasma cell-free hemoglobin rapidly dissociates into dimers that readily pass through the glomerulus; thus chronic hemolysis in SCA may result in continuous exposure of the kidneys to cell-free hemoglobin. (Szabo, et al 1965) Hemoglobin can exist in the kidney in the ferrous (Fe^{+2}), ferric (Fe^{+3}) and ferryl states (Fe^{+4}). (Gladwin, et al 2012) In the ferrous state iron will scavenge nitric oxide and participate in the Fenton reaction to generate hydroxyl radicals; both reduced nitric oxide and increased hydroxyl radicals can cause vasoconstriction, potentially impairing kidney perfusion. (Reiter, et al 2002) In the ferric state iron can form the oxo-ferryl radical and mediate lipid peroxidation, thought to be an important mechanism of injury in myoglobinuria. (Boutaud, et al 2010) Cell-free hemoglobin is rapidly converted to methemoglobin with liberation of the heme group, which may also elicit cytotoxic and inflammatory-mediated kidney damage. (Nath and Katusic 2012, Tracz, et al 2007) In chronic hemolytic disease states including SCA, the scavengers of free hemoglobin (haptoglobin) and free heme (hemopexin) are consumed resulting in increased circulation of cell-free hemoglobin and heme. (Muller-Eberhard, et al 1968)

Several animal models provide additional insights into potential pathophysiologic mechanisms linking cell-free hemoglobin to kidney damage in SCA. In a transgenic mouse model of SCA, increased heme, lipid peroxidation, and upregulation of heme oxygenase-1 were detected in whole kidney homogenates as well as cytosolic, mitochondrial, and microsomal fractions. (Nath, et al 2001) In a non-sickle canine model, free water-induced intravascular hemolysis triggered release of cell-free plasma hemoglobin predominantly in the ferrous state.
which resulted in increased nitric oxide consumption, elevated MAP, and decreased creatinine clearance. (Minneci, et al 2005) These effects on MAP and creatinine clearance could be attenuated by concomitant inhalation of NO suggesting that the ferrous form of cell-free hemoglobin and NO consumption play an important role in hemolysis-mediated kidney disease. In a guinea pig model, transfusion of longer-stored red blood cell (RBC) units was associated with sustained intravascular hemolysis and increased cell-free hemoglobin concentrations. (Baek, et al 2012) In this model, the cell-free hemoglobin initially predominated in the ferrous form with an increase in the ferric form over time. Transfusion with longer-stored RBC units was associated with elevated blood pressures, vascular wall injury, and kidney dysfunction. Proteomic analysis of the kidneys displayed increased markers of oxidative stress and tubular injury and histopathology revealed nephrosis and tubular degeneration. Co-infusion of haptoglobin with longer-stored RBC abated the increases in oxidative and tubular injury markers. These animal models suggest that intravascular hemolysis-mediated cell-free hemoglobin causes kidney injury by NO scavenging in the ferrous form in addition to oxidative injury in the ferric or free-heme form.

There are a number of limitations to this study. The large cohort analyses were cross sectional in nature, although a prospective cohort confirmed the validity of urine dipstick and microscopy to identify hemoglobinuria and a longitudinal cohort confirmed an association between hemoglobinuria and CKD. Although associations have been demonstrated between CKD and hemoglobinuria, determining a causal relationship will require future studies. Hemoglobinuria may be a consequence of CKD, although when testing this direction on our pathway analysis we observed a poor fit of the data to this model. Another limitation is that the urine dipstick and microscopy analysis to assess for hemoglobinuria may be positive in the
presence of myoglobinuria. This is less likely since plasma levels of myoglobin are not increased at steady-state in patients with SCD in comparison to healthy controls and only rise to 5.4 µg/dL during a vaso-occlusive pain episode which, is a concentration approximately 1000-fold less than plasma free hemoglobin levels. (Roth, et al 1981) Patients negative for urine dipstick defined hemoglobinuria had significantly lower albeit detectable levels of hemoglobinuria and future studies to directly measure cell-free hemoglobin in urine may help establish a threshold upon which renal damage is accelerated. Coinheritance of α-thalassemia, which is associated with lower rates of hemolysis, has been shown to be associated with decreased rates of development and progression of increased albuminuria and will also need to be assessed in future studies. (Day, et al 2012, Guasch, et al 1999, Nebor, et al 2010) The data on hydroxyurea use affecting nephropathy has been less clear. (Day, et al 2012, Steinberg, et al 2010) In this study, CKD stage and rates of CKD or albuminuria progression did not differ by hydroxyurea use. Finally, the differences in progression of renal dysfunction observed in this study according to hemoglobinuria need to be assessed in future studies including potential genetic differences that may modulate response to intravascular hemolysis through scavenger proteins or detoxifying enzymes.

In summary, in two independent large cohorts of adults with SCA, increasing age and intravascular hemolysis as reflected in hemoglobin-positive urine dipstick with negative microscopy were independent factors associated with CKD. The potential contribution of cell-free hemoglobin to kidney disease in SCA warrants further investigation including prospective studies with specific assays for plasma free and urine hemoglobin. Further research is also needed to determine if measures to decrease intravascular hemolysis and cell-free hemoglobin exposure can preserve renal function in patients with SCA. Investigations to identify genomic
and genetic markers that predispose patients for CKD might also assist in preventive and therapeutic interventions.

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**Authorship:**

S.L.S. collected and analyzed the data, performed statistical analysis, and wrote the paper. X.Z. analyzed the data, performed statistical analysis, and wrote the paper. T.K. contributed vital analytical tools, interpreted the data, and wrote the paper. B.O., C.L., J.R. collected and analyzed the data. J.P.L., R.F.M., M.G., J.H., J.D., R.F.M., M.T.G., J.A.L. wrote the paper. V.G.R. analyzed the data, performed statistical analysis, and wrote the paper.
Conflict-of-interest disclosure: The authors declare no competing financial interests.
References:


Figure Legends

**Figure 1:** Validation of hemoglobinuria defined by dipstick urinalysis. Patients with hemoglobinuria on dipstick urinalysis (n=17) had significantly higher concentrations of urine hemoglobin directly measured by ELISA that those without hemoglobinuria (n=26) (23.1 vs. 11.5 ng/mL, \( P < 0.0001 \)).

**Figure 2A-D:** Hemoglobinuria association with laboratory markers of hemolysis. In the UIC cohort lower hemoglobin concentration (8.0 vs. 9.0 g/dL, \( P < 0.0001 \)), higher LDH (426 vs. 279 u/L, \( P < 0.0001 \)), higher AST (49 vs. 37 u/L, \( P < 0.0001 \)), and higher reticulocyte percentages (16.1% vs. 11.7%, \( P < 0.0001 \)) were observed in patients with hemoglobinuria. Similarly, in the Walk-PHaSST cohort, lower hemoglobin concentration (8.1 vs. 8.9 g/dL, \( P < 0.0001 \)), higher LDH (540 vs. 428 u/L, \( P < 0.0001 \)), higher AST (49 vs. 42 u/L, \( P = 0.0052 \)), and a trend for higher reticulocyte percentage (9.6% vs. 8.5%, \( P = 0.07 \)) were observed in patients with hemoglobinuria.

**Figure 2E:** Hemoglobinuria association with increased albuminuria. In both the UIC and Walk-PHaSST cohorts, the presence of hemoglobinuria was associated with higher levels of albuminuria (UIC: 248 vs. 20 mg/g creatinine, \( P < 0.0001 \); Walk-PHaSST: 109 vs. 18 mg/g creatinine, \( P < 0.0001 \)).

**Figure 2F:** Hemoglobinuria association with decreased GFR. In both the UIC and Walk-PHaSST cohorts, with adjustment for age the presence of hemoglobinuria was associated with decreased eGFR (UIC: 120 vs. 131 mL/min/1.73 m\(^2\), \( P = 0.0014 \); Walk-PHaSST: 124 vs. 131 mL/min/1.73 m\(^2\), \( P = 0.035 \)).
**Figure 3:** UIC and Walk-PHaSST pathway analysis for CKD stage. Standardized betas are indicated in the model. Comparative fit index=1.0; goodness-of-fit test $\chi^2$/degrees of freedom≤0.8; Root Mean Square Error of Approximation of model<0.0001. According to this analysis 1) higher LDH is associated with hemoglobinuria, 2) higher age and presence of hemoglobinuria are associated with the stage of CKD.

**Figure 4A:** Reliability of hemoglobinuria based on repeat dipstick urinalysis. Sixty-one percent (33/54) of patients with hemoglobinuria on initial assessment remained positive for hemoglobinuria on repeat urinalysis while 90% (8/85) of patients without hemoglobinuria on initial assessment remained negative on repeat urinalysis.

**Figure 4B:** CKD progression based on the absence or presence of hemoglobinuria on initial urine dipstick analysis. CKD progression, defined by a reduction of eGFR by 50% or requiring hemodialysis/kidney transplant, was observed in 13% (7/56) of patients with hemoglobinuria versus 1% (1/108) of patients without hemoglobinuria (HR 13.9, 95% CI: 1.7–113.2; logrank $P=0.0012$).

**Figure 4C:** Albuminuria category progression rate based on the absence or presence of hemoglobinuria on initial urine dipstick analysis. Forty-two percent (11/26) of patients with hemoglobinuria had progression of albuminuria category versus 13% (9/67) of patients without hemoglobinuria (HR 3.1, 95% CI: 1.3–7.7; logrank $P=0.0035$)
Table 1A: UIC clinical and laboratory variables according to the presence or absence of chronic kidney disease (CKD) and its stage.

<table>
<thead>
<tr>
<th>Variable</th>
<th>CKD not present</th>
<th>Stage 1</th>
<th>Stage 2</th>
<th>Stage 3 or higher</th>
<th>p-value</th>
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<tbody>
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<td>Age (years)</td>
<td>88</td>
<td>81</td>
<td>17</td>
<td>22</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Gender (male : female)</td>
<td>88</td>
<td>81</td>
<td>17</td>
<td>22</td>
<td>0.7</td>
</tr>
<tr>
<td>MAP (mmHg)</td>
<td>87</td>
<td>81</td>
<td>17</td>
<td>22</td>
<td>0.1</td>
</tr>
<tr>
<td>HU therapy</td>
<td>87</td>
<td>81</td>
<td>17</td>
<td>22</td>
<td>0.5</td>
</tr>
<tr>
<td>Ferritin (ng/mL)</td>
<td>68</td>
<td>56</td>
<td>17</td>
<td>21</td>
<td>0.8</td>
</tr>
<tr>
<td>Hemoglobin (g/dL)</td>
<td>88</td>
<td>81</td>
<td>17</td>
<td>22</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>LDH (u/L)</td>
<td>79</td>
<td>69</td>
<td>17</td>
<td>19</td>
<td>0.00031</td>
</tr>
<tr>
<td>Indirect bilirubin (mg/dL)</td>
<td>86</td>
<td>79</td>
<td>17</td>
<td>19</td>
<td>0.1</td>
</tr>
<tr>
<td>AST (u/L)</td>
<td>69</td>
<td>72</td>
<td>17</td>
<td>16</td>
<td>0.034</td>
</tr>
<tr>
<td>Absolute reticulocyte count (x10^3/µL)</td>
<td>88</td>
<td>79</td>
<td>17</td>
<td>21</td>
<td>0.018</td>
</tr>
<tr>
<td>Reticulocyte percent (%)</td>
<td>85</td>
<td>78</td>
<td>15</td>
<td>21</td>
<td>0.038</td>
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<tr>
<td>Hemoglobin F (%)</td>
<td>84</td>
<td>80</td>
<td>16</td>
<td>19</td>
<td>0.4</td>
</tr>
<tr>
<td>Hemoglobinuria</td>
<td>83</td>
<td>74</td>
<td>16</td>
<td>9</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

Continuous variables are presented as median values (interquartile range). P values < 0.004 are significant after the Bonferonni Correction. MAP = mean arterial pressure; HU = hydroxyurea; LDH = lactate dehydrogenase; eGFR = estimated glomerular filtration rate.
Table 1B: Walk-PHaSST clinical and laboratory variables according to presence or absence of chronic kidney disease (CKD) and its stage.

<table>
<thead>
<tr>
<th>Variable</th>
<th>N</th>
<th>CKD not present</th>
<th>N</th>
<th>Stage 1</th>
<th>N</th>
<th>Stage 2</th>
<th>N</th>
<th>Stage 3 or higher</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>126</td>
<td>34 (25 – 45)</td>
<td>96</td>
<td>33 (24 – 43)</td>
<td>17</td>
<td>50 (33 – 55)</td>
<td>36</td>
<td>50 (45 – 55)</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Gender (male : female)</td>
<td>126</td>
<td>48% : 52%</td>
<td>96</td>
<td>47% : 53%</td>
<td>17</td>
<td>47% : 53%</td>
<td>36</td>
<td>50% : 50%</td>
<td>0.9</td>
</tr>
<tr>
<td>MAP (mmHg)</td>
<td>126</td>
<td>82 (78 – 88)</td>
<td>96</td>
<td>83 (77 – 89)</td>
<td>17</td>
<td>88 (83 – 92)</td>
<td>36</td>
<td>91 (85 – 102)</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>HU therapy</td>
<td>126</td>
<td>56 (44%)</td>
<td>96</td>
<td>43 (45%)</td>
<td>17</td>
<td>8 (47%)</td>
<td>36</td>
<td>14 (39%)</td>
<td>0.7</td>
</tr>
<tr>
<td>Ferritin (ng/mL)</td>
<td>121</td>
<td>265 (102 – 607)</td>
<td>96</td>
<td>321 (142 – 1185)</td>
<td>17</td>
<td>594 (149 – 1023)</td>
<td>34</td>
<td>487 (287 – 1801)</td>
<td>0.010</td>
</tr>
<tr>
<td>Hemoglobin (g/dL)</td>
<td>125</td>
<td>9.2 (8.1 – 10.1)</td>
<td>95</td>
<td>8.5 (7.5 – 9.4)</td>
<td>16</td>
<td>8.2 (6.9 – 8.5)</td>
<td>36</td>
<td>7.9 (6.2 – 8.9)</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>LDH (u/L)</td>
<td>117</td>
<td>372 (305 – 518)</td>
<td>89</td>
<td>476 (368 – 727)</td>
<td>16</td>
<td>601 (437 – 928)</td>
<td>34</td>
<td>469 (316 – 707)</td>
<td>0.00016</td>
</tr>
<tr>
<td>Indirect bilirubin (mg/dL)</td>
<td>-</td>
<td>Not available</td>
<td>-</td>
<td>Not Available</td>
<td>-</td>
<td>Not Available</td>
<td>-</td>
<td>Not Available</td>
<td>-</td>
</tr>
<tr>
<td>AST (u/L)</td>
<td>120</td>
<td>39 (29 – 53)</td>
<td>95</td>
<td>50 (37 – 69)</td>
<td>17</td>
<td>49 (42 – 65)</td>
<td>36</td>
<td>47 (33 – 60)</td>
<td>0.00027</td>
</tr>
<tr>
<td>Absolute reticulocyte count</td>
<td>111</td>
<td>246 (168 – 331)</td>
<td>92</td>
<td>269 (181 – 362)</td>
<td>16</td>
<td>266 (195 – 389)</td>
<td>36</td>
<td>216 (118 – 297)</td>
<td>0.058</td>
</tr>
<tr>
<td>Reticulocyte percent (%)</td>
<td>111</td>
<td>8.3 (5.9 – 11.2)</td>
<td>93</td>
<td>10.2 (6.9 – 15.2)</td>
<td>16</td>
<td>10.9 (6.4 – 17.2)</td>
<td>36</td>
<td>8.9 (5.2 – 14.3)</td>
<td>0.1</td>
</tr>
<tr>
<td>Hemoglobin F (%)</td>
<td>119</td>
<td>6.6 (2.9 – 14.7)</td>
<td>95</td>
<td>5.8 (2.9 – 11.8)</td>
<td>16</td>
<td>6.1 (2.5 – 11.9)</td>
<td>34</td>
<td>3.8 (1.4 – 10.6)</td>
<td>0.3</td>
</tr>
<tr>
<td>Hemoglobinuria</td>
<td>105</td>
<td>9 (9%)</td>
<td>72</td>
<td>21 (29%)</td>
<td>12</td>
<td>2 (17%)</td>
<td>22</td>
<td>12 (55%)</td>
<td>&lt; 0.0001</td>
</tr>
</tbody>
</table>

Continuous variables are presented as median values (interquartile range).

*P* values < 0.004 are significant after the Bonferonni Correction

MAP = mean arterial pressure; HU = hydroxyurea; LDH = lactate dehydrogenase; eGFR = estimated glomerular filtration rate
Table 2: Logistic regression model for chronic kidney disease (CKD) stage in the UIC and Walk-PHaSST cohorts.

<table>
<thead>
<tr>
<th></th>
<th>Variables</th>
<th>OR*</th>
<th>95% CI</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>UIC</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CKD Stage 1</td>
<td>74</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hemoglobinuria</td>
<td>12.8</td>
<td>5.3 – 31.1</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td></td>
<td>Age</td>
<td>1.5</td>
<td>1.1 – 2.0</td>
<td>0.029</td>
</tr>
<tr>
<td></td>
<td>MAP</td>
<td>1.0</td>
<td>0.6 – 1.4</td>
<td>0.8</td>
</tr>
<tr>
<td>CKD Stage 2</td>
<td>16</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hemoglobinuria</td>
<td>112.0</td>
<td>19.8 – 634.2</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td></td>
<td>Age</td>
<td>4.1</td>
<td>2.1 – 7.8</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td></td>
<td>MAP</td>
<td>1.4</td>
<td>0.7 – 2.9</td>
<td>0.3</td>
</tr>
<tr>
<td>CKD Stage 3 or higher</td>
<td>9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hemoglobinuria</td>
<td>110.4</td>
<td>13.7 – 891.1</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td></td>
<td>Age</td>
<td>11.7</td>
<td>3.6 – 37.9</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td></td>
<td>MAP</td>
<td>2.1</td>
<td>0.9 – 5.0</td>
<td>0.092</td>
</tr>
<tr>
<td><strong>Walk-PHaSST</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CKD Stage 1</td>
<td>72</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hemoglobinuria</td>
<td>4.5</td>
<td>1.9 – 10.7</td>
<td>0.00062</td>
</tr>
<tr>
<td></td>
<td>Age</td>
<td>0.9</td>
<td>0.7 – 1.2</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td>MAP</td>
<td>0.9</td>
<td>0.6 – 1.4</td>
<td>0.8</td>
</tr>
<tr>
<td>CKD Stage 2</td>
<td>12</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hemoglobinuria</td>
<td>2.2</td>
<td>0.4 – 12.4</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td>Age</td>
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<td>1.7 – 7.3</td>
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<tr>
<td></td>
<td>MAP</td>
<td>1.0</td>
<td>0.5 – 2.2</td>
<td>0.9</td>
</tr>
<tr>
<td>CKD Stage 3 or higher</td>
<td>22</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hemoglobinuria</td>
<td>11.3</td>
<td>3.2 – 40.4</td>
<td>0.00019</td>
</tr>
<tr>
<td></td>
<td>Age</td>
<td>3.3</td>
<td>1.8 – 6.1</td>
<td>0.00020</td>
</tr>
<tr>
<td></td>
<td>MAP</td>
<td>2.8</td>
<td>1.4 – 5.5</td>
<td>0.0032</td>
</tr>
</tbody>
</table>

* Odds ratios are based on the comparison to subjects without CKD.

MAP = mean arterial pressure.

Odds ratio for Age and MAP are based on 10-year and 10-mmHg intervals, respectively.
Figure 1:

- Urine Hemoglobin (ng/mL)
  - Absent
  - Present

- Urine Dipstick Hemoglobinuria

p < 0.0001
Figure 2A:

**Hemoglobin (g/dL)**

<table>
<thead>
<tr>
<th></th>
<th>Absent</th>
<th>Present</th>
</tr>
</thead>
<tbody>
<tr>
<td>UIC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Walk-PHaSST</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Hemoglobinuria**

p < 0.0001

p < 0.0001
Figure 2B: UIC Walk-PHaSST

<table>
<thead>
<tr>
<th>Hemoglobinuria</th>
<th>UIC</th>
<th>Walk-PHaSST</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absent</td>
<td>p &lt; 0.0001</td>
<td>p &lt; 0.0001</td>
</tr>
<tr>
<td>Present</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

LDH (u/L)

500 1000 1500 2000
Figure 2C:

![Graph showing AST (u/L) levels with labels for UIC and Walk-PHaSST, indicating statistical significance with p < 0.0001 and p = 0.005 respectively, and the presence or absence of Hemoglobinuria]
Figure 2D:

<table>
<thead>
<tr>
<th></th>
<th>UIC</th>
<th>Walk-PHaSST</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reticulocytes (%)</td>
<td>p &lt; 0.0001</td>
<td>p = 0.07</td>
</tr>
<tr>
<td></td>
<td>Absent</td>
<td>Present</td>
</tr>
<tr>
<td>Hemoglobinuria</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 2E:

<table>
<thead>
<tr>
<th>Albuminuria (mg/g creatinine)</th>
<th>UIC</th>
<th>Walk-PHaSST</th>
</tr>
</thead>
<tbody>
<tr>
<td>1000</td>
<td>p &lt; 0.0001</td>
<td>p &lt; 0.0001</td>
</tr>
<tr>
<td>100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absent</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Present</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hemoglobinuria</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 2F:

Hemoglobinuria Status:

- Absent
- Present

p = 0.0014

p = 0.035

eGFR (mL/min/1.73 m²)
**Figure 3: Pathway Analysis**

LDH

- Standardized βeta ≥ 0.31
- p < 0.0001

Age

- Standardized βeta ≥ 0.31
- p < 0.0001

Hemoglobinuria

- Standardized βeta ≥ 0.43
- p < 0.0001

Chronic Kidney Disease
Figure 4A:

The bar chart shows the repeat hemoglobinuria (%) by initial hemoglobinuria status.

- **Absent (n = 85):** Approximately 10% repeat hemoglobinuria.
- **Present (n = 54):** Approximately 60% repeat hemoglobinuria.

A statistically significant difference is observed, with a p-value of less than 0.0001.
Figure 4B:

Hemoglobinuria Status:
- Absent
- Present

CKD Progression-Free Survival

p = 0.0012

n = 108
n = 56

Months
Figure 4C:

- Hemoglobinuria Status:
  - Absent
  - Present

- Albuminuria Progression-Free Survival

- p = 0.0035

- n = 67

- n = 26