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Biomarkers for early detection of sickle nephropathy

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Abstract

Renal complications affect nearly 30–50% of adults with sickle cell anemia (SCA), causing significant morbidity and mortality. Standard renal function tests like serum creatinine and glomerular filtration rate become abnormal in this disease only when renal damage has become extensive and largely irreversible. Moreover, not all patients develop sickle nephropathy (SN). Therefore, noninvasive biomarkers that predict early onset of SN are necessary. We performed a cross-sectional analysis for nephropathy in 116 patients with sickle cell disease, analyzing urinary kidney injury molecule-1 (KIM-1), liver-type fatty acid binding protein (L-FABP), *N*-acetyl-b-D-glucosaminidase (NAG), neutrophil gelatinase-associated lipocalin (NGAL) and transforming growth factor-β1 (TGF-β), together with conventional renal biomarkers (urine albumin and osmolality, and serum creatinine and cystatin C estimated GFR) during routine clinic visits when patients were at steady-state/baseline. We observed a distinct biomarker pattern: KIM-1 and NAG emerged as biomarkers with a strong association with albuminuria. Surprisingly, and in contrast to other acute/chronic renal disorders, NGAL, L-FABP, and TGF-β levels did not show any relationship with albuminuria in patients with SCA. Our study identifies potential biomarkers for SN, and suggests longitudinal validation of these biomarkers for early detection of SN, so that therapeutic interventions can be applied before renal damage becomes irreversible.

Introduction

The kidney is affected in several different ways in sickle cell anemia (SCA). Children with SCA develop hyposthenuria/urine concentrating defect (UCD), have supra-normal

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Author Contributions: PD and PM designed the study; GA, PM and JW did the regulatory paperwork and obtained clinical samples, NS and MB performed the experiments, NS and MK performed the data analysis and statistical analysis; PM conceptualized and executed the study, PD and PM interpreted the data. NS, GA, PD and PM wrote and edited the manuscript.

glomerular filtration rate (GFR) and proximal tubular function, and an impaired ability to acidify urine or excrete potassium, develop microscopic hematuria and even gross hematuria from renal papillary necrosis. [Reviewed in Refs. 1–4]. With increasing age, patients develop glomerulopathy, resulting in micro-albuminuria (MiA, defined as urine albumin of 30–300 mg/g urine creatinine), which progresses to macro-albuminuria (MaA, defined as urine albumin >300 mg/g urine creatinine), and subsequently to end stage renal disease (ESRD) [1,2,5,6].). With time, the most common glomerular lesion in SN is focal and segmental glomerulo-sclerosis, while membranous glomerulopathy has also been observed in some cases. Studies show that gross proteinuria and ESRD is observed in 15–30% of patients with SCA [7,8]. However not all patients with SCA develop SN, and it is not clear what factors predict or promote the progression of SN in susceptible patients.

Renal complications are not identifiable at early stages of SN, since the classic biomarkers of renal damage are not informative in this disease. Plasma creatinine levels are low due to supra-normal creatinine excretion in the urine, and therefore serum creatinine rises only in late stages of SN. SCA patients also have higher than normal GFR and renal blood flow; therefore, urinary creatinine clearance is high from the resulting hyperfiltration. Subnormal GFR and elevated serum creatinine levels develop only when significant proteinuria develops [9]. Besides, GFR estimated from creatinine clearance or serum creatinine levels [10] is fraught with errors due to high creatinine excretion in SCA, varying muscle mass and hydration status. Therefore, early interventions that would prevent the progression of renal damage cannot be applied, since SN is diagnosed when renal damage has become extensive and largely irreversible.

One alternative method of estimating GFR more accurately is plasma cystatin C, that is freely filtered at the glomerulus. Another commonly used biomarker of glomerulopathy is albuminuria, specifically MiA and MaA. Novel biomarkers are needed for early diagnosis of sickle glomerulopathy, before the damage to the kidney becomes irreversible, so that therapeutic interventions are effective at preventing progression of renal damage. Recently, several novel early biomarkers for detection of renal damage to specific regions of the nephron were studied in a rat renal injury model in a large prospective study by the Predictive Safety Testing Consortium, a collaborative consortium between industry, academia, the US Food and Drug Administration and the European EMEA [11–14]. Most promising of these biomarkers include urine kidney injury molecule-1 (KIM-1), liver-type fatty acid binding protein (L-FABP), *N*-acetyl-b-D-glucosaminidase (NAG) and neutrophil gelatinase-associated lipocalin (NGAL) [15]. Devarajan and coworkers have identified NGAL as a novel sensitive marker of renal tubular damage in acute and chronic nephropathy [16]. In this study, we explored the prevalence of SN using novel biomarkers KIM-1, NAG, L-FABP, NGAL, and TGF-β, in addition to plasma cystatin C, urine osmolality and urine albumin in patients with SCA and found a distinct pattern that characterizes SN.

Materials and Methods

Patient samples

The study was approved by the Institutional Review Board at Cincinnati Children's Hospital Medical Center and the University of Cincinnati. Concurrent urine and blood samples were collected from patients with sickle cell disease after obtaining informed consent (and assent, where applicable) during their routine clinical visit to the Cincinnati Sickle Cell Center by the Repository of blood, Bone Marrow, Urine, Buccal cells, Skin Tissue, and Data from Patients with Non-Malignant Blood Disorders. All patients were at baseline status (no acute sickle event at least three weeks prior to sample collection). Spot urine samples were obtained so to as to minimize the burden on the patients and simulate routine screening at clinic visits. Blood samples were centrifuged at 4°C at 500*g* for 15 min to separate plasma. Plasma and urine samples were stored at −80°C within 2 hr of collection until analysis.

Urine osmolality was measured using a Freezing point osmometer (Advanced Instruments 3250, Norwood, MA). Plasma Cystatin C was measured by nephelometry using a clinical laboratory platform (BN ProSpec; Siemens Healthcare Diagnostics, www.medical.siemens.com). Inter- and intra-assay coefficients of variation were <5% for batched samples analyzed on the same day. The GFR was estimated from the cystatin C measurements using the following formula: GFR = antilog [1.962 + 1.123*log(1/cystatin C)], as described by Alvarez et al. [9]. Urine albumin was measured by immuno-turbidometry using Dimension Xpand clinical chemistry system (Siemens Healthcare Diagnostics, www.medical.siemens.com).

Measurement of urinary biomarkers

Urinary levels of KIM-1 and NGAL (both from R&D systems, Minneapolis, MN), total TGF-β (Millipore, Billerica, MA) and L-FABP (CMIC Co., Tokyo, Japan) were measured by enzyme linked immunosorbent assay (ELISA), as per manufacturers' instructions. For KIM-1 and NGAL ELISA, 50 μl of sample was used per sample and each sample was analyzed in duplicates. For L-FABP assay 50 μl of samples were pretreated with the provided reagent. Totally, 20 μl of pretreated samples were then used in the assay in duplicates. Urine NAG activity was measured using a colorimetric assay (Roche Diagnostics, USA). Briefly, 5 μl of sample was incubated with 100 μl of substrate solution (3-Cresolsulfonphtha-leinyl-*N*-acetyl-b-D-glucosaminide) for 20 min at 37°C. The reaction was stopped with a stop solution containing sodium carbonate and the optical density (OD) measured at 580 nm. The OD values were subtracted from blank and NAG activity was calculated and the activity was expressed in units per liter (U/L). Urine active TFG-β was measured using an ELISA kit (R&D systems), as per the manufacturer's instructions. Briefly 100 μl of urine samples were treated with 20 μl 1N HCl for 20 min. The samples were then neutralized with 20 μl of 1N NaOH/0.5M HEPES (pH 7.5). All urinary biomarker levels were normalized with urine creatinine. Urine Creatinine was measured using an alkaline picrate assay (R&D systems). All urinary biomarkers were expressed as a ratio of urine creatinine.

Statistical analysis

We used summary statistics: mean, standard errors, frequencies and range and ANOVA using the GraphPad Prism software, version 5.0, to summarize the clinical and hematological/renal parameter characteristics of the current cohort of sickle cell disease patients in Tables I and II. Biomarker data were skewed, suggesting unequal variances among the different albuminuria groups (NoA, MiA, and MaA). We log-transformed the data to stabilize the variances and used general linear model (GLM) analysis to compare the albuminuria groups, while accounting for age effect, if it existed. The albuminuria groups were compared pair-wise by Tukey-Kramer test and p-values adjusted for the pair-wise multiple comparisons are reported in the Figures, whenever they were statistically significant ($P < 0.05$).

Results

Prevalence of albuminuria and hyposthenuria in sickle cell disease

We enrolled 116 patients with sickle cell disease (90 patients with Hb SS [hereafter referred to as SCA], 16 with Hb SC disease and 10 with Hb S/β+ thalassemia). Patients ranged from 6 to 56 years of age. The patient population was skewed towards a pediatric age group (median age 18 years): this allowed the cohort to contain a large subset of patients without gross evidence of renal pathology, allowing early biomarker evaluation.

The clinical characteristics and baseline laboratory data on hematological and renal parameters of the patients with SCA versus those with other forms of sickle cell disease are compared in Table I. The median age, blood pressure, blood urea nitrogen, serum creatinine and urine specific gravity were similar in both groups. However, patients with SCA had significantly lower hemoglobin, and higher reticulocyte counts, CRP and indirect bilirubin than those with other forms of sickle cell disease, showing the higher hemolysis, anemia, and inflammation present in patients with SCA. Also as expected, a significantly higher proportion of SCA patients were on hydroxyurea or chronic transfusions. While 40.5% of patients with SCA had albuminuria, only three patients in the “other” group had MiA while the rest had normal urine albumin levels. Notably, all patients, regardless of the sickle genotype, had a low specific gravity of 1.011.

Figure 1a shows the degree of albuminuria in all patients with sickle cell disease. MiA was defined as urine albumin of 30–300 mg/g creatinine, MaA was defined as urine albumin >300 mg/g creatinine. Urine albumin <30 mg/g creatinine was considered normoalbuminuria (NoA). Albuminuria was more commonly observed in patients with SCA (Hb SS disease) as compared with those with other forms of sickle cell disease: 34% of SCA patients had MiA (mean urine albumin 64 ± 9 mg/g creatinine) and 6% percent had MaA (mean urine albumin of 1521 ± 745 mg/g creatinine). The prevalence of albuminuria in patients with other forms of sickle cell disease was low, with 3 (12%) patients with MiA. Because of the low incidence and minimal MiA in patients with other forms of sickle cell disease, only SCA [Hb SS] patients were analyzed in detail for biomarker analysis.

Table II compares the characteristics of SCA patients with NoA, MiA, and MaA. There were no significant differences in hemoglobin, reticulocyte count and unconjugated bilirubin

(markers of hemolysis), leukocyte counts (marker of inflammation), or commonly used indicators of renal function (blood pressure, serum creatinine, BUN, specific gravity) between these groups, underscoring the fact that routine renal indicators such as BUN, creatinine, and blood pressure do not predict onset of SN. A similar proportion of patients with NoA or MiA groups were on hydroxyurea or chronic transfusions (Fig. 1b). The prevalence of albuminuria and its degree increased with age (Fig. 1c), with the median age of patients with NoA, MiA, and MaA being 12, 20, and 25 years, respectively. There were no significant differences in the urine albumin between males ($n = 40$) and females ($n = 50$) with SCA.

Hyposthenuria or a urine concentrating defect (UCD) was observed in nearly all patients with SCA, and appeared to gradually worsen with age (Fig. 1d). We found that the mean urine osmolality, even in the youngest age group of 6- to 12-year-old patients with SCA was 502 ± 22 mOsm/Kg H_2O, well below normal urine osmolality. It decreased further to 350–375 mOsm in adults with SCA. Only two patients in the entire Hb SS cohort had an osmolality >800 mOsm/Kg H_2O and 6 patients had an osmolality >600 mOsm/Kg H_2O. All the rest had a urine osmolality <600 mOsm/Kg H_2O. The urine osmolality of SCA patients on hydroxyurea (418 ± 14.7) was not significantly different from those that were not on hydroxyurea (461 ± 22), but SCA patients on chronic transfusions showed significantly higher urine osmolality at 527 ± 32 mOsm/Kg H_2O, ($P < 0.05$; Fig. 1e). While urine osmolality on first morning urine samples has been shown to be higher than 600 mOsm, it is pertinent to note that urine osmolality in this study was determined on a random spot sample and should be interpreted with caution, since lower osmolality may result from a high fluid intake.

Glomerular filtration rate in patients with sickle cell anemia

GFR, a measure of kidney function, normally ranges from 90 to 125 ml/min/1.73 m^2 body surface area [10]. The GFR values were estimated (eGFR) from plasma cystatin C levels [11]. After filtration, cystatin C is normally catabolized in the renal tubules and none appears in the urine, so urine collection methods cannot be used. However, equations have been developed linking estimated GFR (eGFR) to serum cystatin C levels; and GFR has been estimated using cystatin C in children with sickle cell disease [9]. GFR estimated from plasma cystatin C was on the high normal range in young individuals with SCA (Fig. 2a). It became subnormal only in patients >40 years of age. When eGFR was compared within the NoA, MiA and MaA groups, we observed that patients with MaA had reduced GFR values compared to patients with NoA and MiA, although this difference was not statistically significant due to few patients in the MaA group (Fig. 2b).

Next-generation urinary biomarkers in patients with sickle cell anemia

We measured urinary KIM-1, NAG activity, NGAL, TGF-β, and L-FABP levels, biomarkers which have been recently identified as useful in different forms of acute and/or chronic renal injury, and analyzed those across albuminuria levels, after adjusting for age. Since urine samples were spot samples, all biomarker data was normalized to urine creatinine.

Normal NAG activity in urine has been reported to be less than 2 U/l [17]. The urine NAG activity was elevated above 2U/l in most SCA patients (Fig. 3a). When compared in groups with different levels of albuminuria, NAG activity was increased above normal even in the NoA group, and levels increased significantly in patients with MiA ($P < 0.005$; Fig. 3b). It is therefore conceivable that elevations in NAG may precede MiA. It was also higher in patients with MaA as compared with NoA, although the differences were not statistically significant due to few patients in the MaA group. The potential of NAG as an early biomarker of SN needs to be confirmed in a longitudinal analysis.

Urine KIM-1 is detected in acute kidney injury. KIM-1 was detected in all SCA urine samples (Fig. 3c). When urinary KIM-1 levels were compared within the different albuminuria groups, they were detected at lowest levels in patients with NoA, significantly increased in patients with MiA ($P = 0.005$), and further increased in the MaA group ($P = 0.0015$; Fig. 3d), suggesting this may be another biomarker of relevance in SN that needs to be confirmed in longitudinal studies.

Surprisingly, urine NGAL levels were significantly subnormal (<50 ng/mL) in most patients with SCA. NGAL levels did not show any particular pattern with age or with albuminuria groups. The overall urinary NGAL in most patients were well below levels usually seen in patients with acute or chronic renal injury (Fig. 4a,b).

Urine L-FABP levels were the highest in the youngest group of 6–12 years old, a group that had minimal evidence of renal injury, as measured by urine albumin, NAG and KIM-1. Urine L-FABP also showed no trend with the degree of albuminuria (Fig. 4c,d).

Among the markers tested above, KIM-1, LFABP, and NAG showed no significant differences associated with gender of patients. However mean NGAL levels were significantly higher in the females (45 ± 10 ng/mL) than males (20 + 4.5 ng/mL; $P < 0.03$)

Recently, urine TGF-β levels have been reported to be elevated in patients with sickle cell disease [18]. We measured TGF-β in the urine of all SCA patients using the methodology described by Mohtat et al. [18] Except for one sample, TGF-β was undetectable in urine of all other patients with SCA. We then measured the total urinary TGF-β levels in 12 patients with NoA, 12 with MiA and all five patients with MaA using a second method. Many of the urine samples did not have detectable levels. The mean, levels of urine TGF-β were 9.6 ± 2.9, 5.8 ± 2.5, and 7.8 ± 5.1 ng/g creatinine in the NoA, MiA, and MaA groups, respectively (Fig. 5).

Discussion

In this study, we assessed the degree of SN in a relatively young cohort of patients so evaluations for novel urine biomarkers of renal damage could be done before gross nephropathy develops. Our clinical and biochemical data shows that standard renal tests are not indicative of nephropathy in this disease, and confirm previous reports that prevalence of albuminuria in SCA increases with age [19–23], and that albuminuria is more commonly seen in patients with SCA as compared with those with Hb SC or Hb S-β+ thalassemia [21]. Guasch et al. have reported that 70% of adults with SCA have albuminuria, while only 40%

of adults with other types of sickle cell disease have albuminuria [21]. Our data shows the same trend, although a lower percentage of SCA patients have albuminuria, due to inclusion of a younger patient population. A similar frequency of albuminuria has been reported in the younger patients with SCA [24–26]. Our studies indicate that albuminuria could be a useful, albeit delayed biomarker of SN. Studies in diabetic nephropathy suggest that glomerular damage has already commenced when MiA is detected, [27] and not all diabetic patients who have MiA go on to develop MaA [28]. Whether MiA is a predictable biomarker of progressive SN can only be determined by a longitudinal analysis. Recently, albuminuria in SCA has been associated with pulmonary hypertension, [29] suggesting that a common underlying mechanism that affects that cardio-pulmonary-renal axis may be implicated in sickle nephropathy and pulmonary hypertension.

Unlike albuminuria, UCD is reported to develop early and universally in patients with SCA [30–34]. Its onset is dependent on the sickle hemoglobin dosage [32,33]. Our studies confirm results reported nearly five to six decades back, showing presence of hyposthenuria in infants and children with SCA [31,33,34]. Hyposthenuria is also observed in older persons with sickle cell trait [9,30,33]. It is to be noted that urine osmolality was measured without water deprivation; despite this, patients on chronic transfusion in our cohort showed significantly improved urine osmolality. Two studies on small numbers of patients have showed improved osmolality on transfusions when patients were under 10 years or 15 years of age [33,34]. Since our observations were measured using spot urine samples in the clinic, the results have been normalized to urine creatinine for all biomarkers. They need to be interpreted with some caution for urine osmolality. We observed no significant difference in urine osmolality (or albuminuria) in patients that were on hydroxyurea versus those not on hydroxyurea, consistent with an earlier report on lack of effect of hydroxyurea on albuminuria in Hb SC patients [35]. However, it is likely that the hydroxyurea group of patients is a biased set of patients with more severe disease. Hence the data are not directly comparable, unless a prospective study is done. Perhaps the BABY-HUG cohort [36] will shed light on the role of hydroxyurea in reducing hyposthenuria.

The Predictive Safety Testing Consortium (PSTC), a collaborative consortium between industry, academia, the FDA and EMEA, performed studies on a rat renal injury model to identify the next generation renal biomarkers, and has recently published its detailed report [11–14]. We explored these new generation urinary biomarkers in SCA, simulating an outpatient setting with spot urine samples.

NAG is a lysosomal enzyme that is constitutively expressed in proximal tubular epithelial cells, and released in the urine when there is proximal tubular injury. It has been found to independently predict development of albuminuria in diabetes [11]. Voskaridou et al. showed that in Hb S-β thalassemia patients, NAG activity was a reliable predictor of proteinuria [17]. Our data shows that increased NAG is also observed in some patients with NoA and is associated with increasing albuminuria, corroborating its role as a potential biomarker for SN. Our data also suggest that despite supra-normal proximal tubular function in SN, concomitant proximal tubular damage must exist, leading to the increase in NAG release.

KIM-1 is a proximal tubular trans-membrane protein and has been shown to be expressed in fibrotic areas of damaged kidneys. KIM-1 is not expressed in normal kidneys but upregulated in de-differentiated proximal tubules after ischemic and nephrotoxic injury [11]. Ischemia-reperfusion is a well-recognized pathophysiology in sickle cell disease. Therefore we explored the role of KIM-1 as a potential urinary biomarker of SN. Several studies on acute kidney injury have indicated the usefulness of this marker in predicting adverse outcomes. However its usefulness in chronic kidney disease is still being debated. KIM-1 has been found to be elevated in various types of glomerulonephritis, chronic allograft nephropathy, and hypertension, but its levels have not been shown to correlate with proteinuria. Our data indicates that KIM-1 has a strong association with increasing albuminuria in SCA, and appears to be a promising biomarker of SN. This is perhaps reflective of patchy regeneration of proximal tubule cells that might accompany this form of chronic nephropathy. Lowering of urinary KIM-1 and NAG levels have been shown to be associated with the regression of MiA in type 1 diabetes mellitus [37].

However, it was surprising that urinary NGAL levels were subnormal in the majority of our SCA patient population, given that almost all of them show evidence of damage to the loop of Henle, manifest as hyposthenuria, and a proportion of them have albuminuria. NGAL is normally present in the systemic circulation, but only very small amounts are normally expressed in the kidney, and therefore very small quantities are normally detected in the urine. NGAL is one of the earliest molecules that is most upregulated in the loop of Henle and collecting duct segments after ischemic or nephrotoxic acute kidney injury, leading to markedly increased concentrations in the urine. While systemic NGAL is normally freely filtered across the glomerulus, it is largely and efficiently reabsorbed in proximal tubules [11,16]. Proximal tubular function is supra-normal in SCA, and it is likely that any filtered NGAL may be reabsorbed much more efficiently, resulting in subnormal urinary NGAL levels in SN. Alternately, other unidentified mechanisms peculiar to SCA may explain the low urinary NGAL levels. For example, the same pathology that leads to damaged distal nephron cells and hyposthenuria in SN may also preclude any ability of these cells to induce NGAL expression, even under conditions of renal stress. Thus, the characteristically low urine NGAL concentration in SN may play a more important role in excluding other forms of chronic kidney disease where increased urine NGAL is a reliable predictor of progression [11,16].

Elevated renal TGF-β signaling is well established in diabetic nephropathy and in hypertension. Urinary TGF-β levels have also been shown to be increased in diabetic patients and other forms of renal disease, with the highest levels seen in obstructive uropathy [38,39]. Tubular damage has been implicated with high urine TGF-β, more than glomerulopathies [38]. Increased TGF-β activity in the kidneys has not been shown in sickle cell disease, despite that fact that polymorphisms in genes in this pathway have been shown to be associated with stroke, priapism, GFR, and pulmonary hypertension [40–44]. Most recently, Mohtat et al. have reported elevated urine TGF-β levels in patients with sickle cell disease. Notably, they also showed an inverse correlation of urine TGF-β levels with increasing age, a finding incongruous to increasing SN with age [18]. In our study, urinary TGF-β was present at very low to undetectable levels in our patient population and showed no association with the degree of albuminuria. Perhaps, a larger study with determination of

TGF-β in a 24-hr urine collection would yield definitive results on the role of urinary TGF-β in sickle cell disease.

L-FABP seemed to show a negative correlation to age and albuminuria. L-FABP levels decreased with age, a finding that was unexpected and contrary to that predicted with increasing kidney damage. L-FABP is thought to be an endogenous antioxidant that suppresses tubulo-intestinal damage and is a marker of chronic kidney damage and its progression [11]. Therefore, decreasing levels of L-FABP with increasing albuminuria is unexpected in SCA, given the high oxidant state in this disease, and is a distinct finding ruling out its value as a biomarker for SN. L-FABP, like creatinine, may not be an important diagnostic tool for analyzing renal injury in SCA.

Currently there are no clinical tests readily available for L-FABP, KIM-1, and NGAL. However a chemiluminescent microparticle immunoassay has been developed for urine NGAL (ARCHITECT analyzer, Abbott Diagnostics, Abbott Park, IL), that is undergoing multicenter validation [45]. Use of KIM-1 as a biomarker for renal injury is also being undertaken in several preclinical and some clinical trials [46].

In conclusion, the molecular mechanisms behind renal damage in SCA are unknown. It is believed that renal injury is mainly from glomerular damage that manifests itself as proteinuria, [47] and UCD is mainly reflective of distal tubular damage, presumed to be secondary to the hypoxic hyperosmotic medullary environment that promotes sickling, causing progressive damage to the vasa recta and tubules. However, not all patients with SCA develop SN, and standard renal parameters become abnormal very late in the course of SN, necessitating screening tools/biomarkers to predict renal damage before it becomes irreversible. Our data shows a distinct biomarker pattern in SCA, identifying KIM-1 and NAG as two potential biomarkers of early SN. Our data also suggests cystatin C may be a good estimate of GFR in SCA and confirms existing data on SN using conventional renal parameters. It also emphasizes the need for longitudinal analysis of MiA, KIM-1, and NAG for their potential as screening tools for SN. A noninvasive urine biomarker analysis at routine clinic visits that can predict SN will allow early diagnosis and effective therapeutic interventions to prevent progression of renal damage and end stage renal disease.

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References

1. Pham PT, Pham PC, Wilkinson AH, et al. Renal abnormalities in sickle cell disease. Kidney Int. 2000; 57:1–8. [PubMed: 10620181]
2. de Santis Feltran L, de Abreu Carvalhaes JT, Sesso R. Renal complications of sickle cell disease: Managing for optimal outcomes. Paediatr Drugs. 2002; 4:29–36. [PubMed: 11817984]
3. Falk RJ, Scheinman J, Phillips G, et al. Prevalence and pathologic features of sickle cell nephropathy and response to inhibition of angiotensin-converting enzyme. N Engl J Med. 1992; 326:910–915. [PubMed: 1542341]

4. Powars DR, Elliott-Mills DD, Chan L, et al. Chronic renal failure in sickle cell disease: Risk factors, clinical course, and mortality. Ann Intern Med. 1991; 115:614–620. [PubMed: 1892333]
5. Hiti AL, Zeng L, Xiang Q, et al. Beta-globin haplotypes from blood spots for follow-up of newborn hemoglobinopathy screening. 1997; 54:76–78.
6. Bakir AA, Hathiwala SC, Ainis H, et al. Prognosis of the nephrotic syndrome in sickle glomerulopathy. A retrospective study. Am J Nephrol. 1987; 7:110–115. [PubMed: 3605231]
7. Ataga KI, Orringer EP. Renal abnormalities in sickle cell disease. Am J Hematol. 2000; 63:205–211. [PubMed: 10706765]
8. Wesson DE. The initiation and progression of sickle cell nephropathy. Kidney Int. 2002; 61:2277–2286. [PubMed: 12028473]
9. Alvarez O, Zilleruelo G, Wright D, et al. Serum cystatin C levels in children with sickle cell disease. Pediatr Nephrol. 2006; 21:533–537. [PubMed: 16491413]
10. Levey AS, Eckardt KU, Tsukamoto Y, et al. Definition and classification of chronic kidney disease: A position statement from Kidney Disease: Improving Global Outcomes (KDIGO). Kidney Int. 2005; 67:2089–2100. [PubMed: 15882252]
11. Koyner JL, Vaidya VS, Bennett MR, et al. Urinary biomarkers in the clinical prognosis and early detection of acute kidney injury. Clin J Am Soc Nephrol. 2010; 5:2154–2165. [PubMed: 20798258]
12. Vaidya VS, Ozer JS, Dieterle F, et al. Kidney injury molecule-1 outperforms traditional biomarkers of kidney injury in preclinical biomarker qualification studies. Nat Biotechnol. 2010; 28:478–485. [PubMed: 20458318]
13. Bonventre JV, Vaidya VS, Schmouder R, et al. Next-generation biomarkers for detecting kidney toxicity. Nat Biotechnol. 2010; 28:436–440. [PubMed: 20458311]
14. Hoffmann D, Adler M, Vaidya VS, et al. Performance of novel kidney biomarkers in preclinical toxicity studies. Toxicol Sci. 2010; 116:8–22. [PubMed: 20118187]
15. Devarajan P. Emerging urinary biomarkers in the diagnosis of acute kidney injury. Expert Opin Med Diagnostics. 2008; 2:387–398.
16. Devarajan P. Review: Neutrophil gelatinase-associated lipocalin: A troponin-like biomarker for human acute kidney injury. Nephrology (Carlton). 2010; 15:419–428. [PubMed: 20609093]
17. Voskaridou E, Terpos E, Michail S, et al. Early markers of renal dysfunction in patients with sickle cell/beta-thalassemia. Kidney Int. 2006; 69:2037–2042. [PubMed: 16501491]
18. Mohtat D, Thomas R, Du Z, et al. Urinary transforming growth factor beta-1 as a marker of renal dysfunction in sickle cell disease. Pediatr Nephrol. 2011; 26:275–280. [PubMed: 21107986]
19. Foucan L, Bourhis V, Bangou J, et al. A randomized trial of captopril for microalbuminuria in normotensive adults with sickle cell anemia. Am J Med. 1998; 104:339–342. [PubMed: 9576406]
20. Guasch A, Cua M, Mitch WE. Early detection and the course of glomerular injury in patients with sickle cell anemia. Kidney Int. 1996; 49:786–791. [PubMed: 8648921]
21. Guasch A, Navarrete J, Nass K, et al. Glomerular involvement in adults with sickle cell hemoglobinopathies: Prevalence and clinical correlates of progressive renal failure. J Am Soc Nephrol. 2006; 17:2228–2235. [PubMed: 16837635]
22. Guasch A, Zayas CF, Eckman JR, et al. Evidence that microdeletions in the alpha globin gene protect against the development of sickle cell glomerulopathy in humans. J Am Soc Nephrol. 1999; 10:1014–1019. [PubMed: 10232687]
23. Guasch A, Cua M, You W, et al. Sickle cell anemia causes a distinct pattern of glomerular dysfunction. Kidney Int. 1997; 51:826–833. [PubMed: 9067917]
24. Wigfall DR, Ware RE, Burchinal MR, et al. Prevalence and clinical correlates of glomerulopathy in children with sickle cell disease. J Pediatr. 2000; 136:749–753. [PubMed: 10839871]
25. Dharnidharka VR, Dabbagh S, Atiyeh B, et al. Prevalence of microalbuminuria in children with sickle cell disease. Pediatr Nephrol. 1998; 12:475–478. [PubMed: 9745872]
26. McBurney PG, Hanevold CD, Hernandez CM, et al. Risk factors for microalbuminuria in children with sickle cell anemia. J Pediatr Hematol Oncol. 2002; 24:473–477. [PubMed: 12218596]

27. Perkins BA, Ficociello LH, Ostrander BE, et al. Microalbuminuria and the risk for early progressive renal function decline in type 1 diabetes. J Am Soc Nephrol. 2007; 18:1353–1361. [PubMed: 17329575]
28. Perkins BA, Ficociello LH, Silva KH, et al. Regression of microalbuminuria in type 1 diabetes. New England J Med. 2003; 348:2285–2293. [PubMed: 12788992]
29. Ataga KI, Brittain JE, Moore D, et al. Urinary albumin excretion is associated with pulmonary hypertension in sickle cell disease: Potential role of soluble fms-like tyrosine kinase-1. Eur J Haematol. 2010; 85:257–263. [PubMed: 20491879]
30. Miller ST, Wang WC, Iyer R, et al. Urine concentrating ability in infants with sickle cell disease: Baseline data from the phase III trial of hydroxyurea (BABY HUG). Pediatric Blood Cancer. 2010; 54:265–268. [PubMed: 19621454]
31. Francis YF, Worthen HG. Hyposthenuria in sickle cell disease. J Natl Med Assoc. 1968; 60:266–270. [PubMed: 5661193]
32. Gupta AK, Kirchner KA, Nicholson R, et al. Effects of alpha-thalassemia and sickle polymerization tendency on the urine-concentrating defect of individuals with sickle cell trait. J Clin Invest. 1991; 88:1963–1968. [PubMed: 1752955]
33. Itano HA, Keitel HG, Thompson D. Hyposthenuria in sickle cell anemia: A reversible renal defect. J Clin Invest. 1956; 35:998–1007. [PubMed: 13367196]
34. Statius van Eps LW, Schouten H, La Porte-Wijsman LW, et al. The influence of red blood cell transfusions on the hyposthenuria and renal hemodynamics of sickle cell anemia. Clin Chim Acta. 1967; 17:449–461. [PubMed: 6051640]
35. Iyer R, Baliga R, Nagel RL, et al. Maximum urine concentrating ability in children with Hb SC disease: Effects of hydroxyurea. Am J Hematol. 2000; 64:47–52. [PubMed: 10815787]
36. Ware RE, Rees RC, Sarnaik SA, et al. Renal function in infants with sickle cell anemia: Baseline data from the BABY HUG trial. J Pediatr. 2010; 156:66–70. e61. [PubMed: 19880138]
37. Vaidya VS, Niewczas MA, Ficociello LH, et al. Regression of microalbuminuria in type 1 diabetes is associated with lower levels of urinary tubular injury biomarkers, kidney injury molecule-1, and N-acetyl-beta-D-glucosaminidase. Kidney Int. 2011; 79:464–470. [PubMed: 20980978]
38. Grenda R, Wuhl E, Litwin M, et al. Urinary excretion of endothelin-1 (ET-1), transforming growth factor- beta1 (TGF-beta1) and vascular endothelial growth factor (VEGF165) in paediatric chronic kidney diseases: Results of the ESCAPE trial. Nephrol Dial Transplant. 2007; 22:3487–3494. [PubMed: 17901069]
39. De Muro P, Faedda R, Fresu P, et al. Urinary transforming growth factor-beta 1 in various types of nephropathy. Pharmacol Res. 2004; 49:293–298. [PubMed: 14726227]
40. Sebastiani P, Ramoni MF, Nolan V, et al. Genetic dissection and prognostic modeling of overt stroke in sickle cell anemia. Nature Gene. 2005; 37:435–440.
41. Elliott L, Ashley-Koch AE, De Castro L, et al. Genetic polymorphisms associated with priapism in sickle cell disease. Br J Haematol. 2007; 137:262–267. [PubMed: 17408468]
42. Fertrin KY, Costa FF. Genomic polymorphisms in sickle cell disease: Implications for clinical diversity and treatment. Expert Rev Hematol. 2010; 3:443–458. [PubMed: 21083035]
43. Ashley-Koch AE, Elliott L, Kail ME, et al. Identification of genetic polymorphisms associated with risk for pulmonary hypertension in sickle cell disease. Blood. 2008; 111:5721–5726. [PubMed: 18187665]
44. Nolan VG, Ma Q, Cohen HT, et al. Estimated glomerular filtration rate in sickle cell anemia is associated with polymorphisms of bone morphogenetic protein receptor 1B. Am J Hematol. 2007; 82:179–184. [PubMed: 17034027]
45. Devarajan P. Biomarkers for the early detection of acute kidney injury. Curr Opin Pediatr. 2011; 23:194–200. [PubMed: 21252674]
46. Vaidya VS, Ford GM, Waikar, et al. A rapid urine test for early detection of kidney injury. Kidney Int. 2009; 76:8–10. [PubMed: 19528988]
47. Marsenic O, Couloures KG, Wiley JM. Proteinuria in children with sickle cell disease. Nephrol Dial Transplant. 2008; 23:715–720. [PubMed: 18065783]

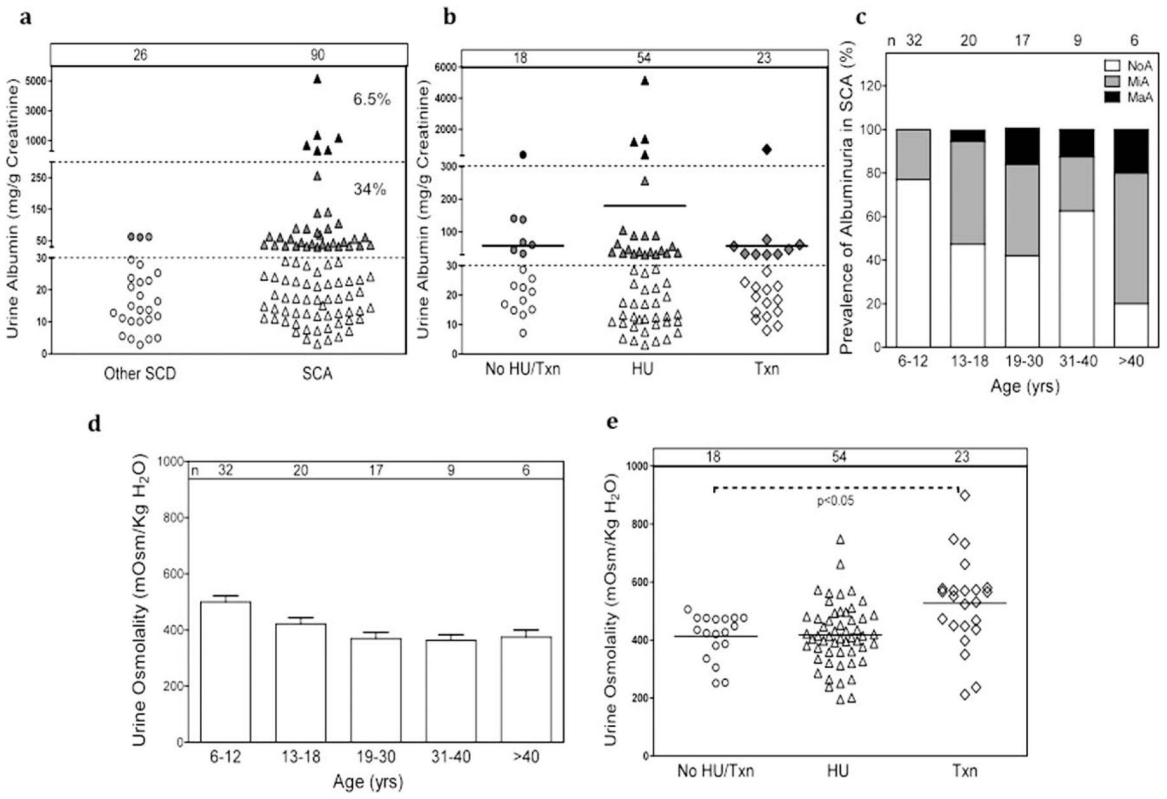

Figure 1.

Urine albumin and osmolality in patients with sickle cell anemia and other forms of sickle cell disease (a) Albuminuria in patients with SCA (Hb SS disease) and other forms of sickle cell disease (Hb SC and Hb S-β+ thalassemia; other SCD). The percentage of patients with NoA, MiA, and MaA is indicated on the right and the three albuminuria groups are separated by dotted lines. (b) Prevalence of albuminuria in SCA patients on hydroxyurea (HU), on chronic transfusions (Txn) or on neither HU nor Txn (No HU/Txn). The three albuminuria groups are separated by dotted lines. Each symbol in panels (a) and (b) represents an individual patient. Open symbols = NoA, gray symbols = MiA, black symbols = MaA. (**c**) The degree and prevalence of albuminuria at different ages in patients with SCA. Column bars represent a specified age group with open bar = NoA, gray bar = MiA and black bar = MaA. (d) Urine osmolality in patients with SCA across different age groups. (e) Urine osmolality in SCA patients on HU, Txn or No HU/Txn. Each symbol represents an individual patient. Numbers listed above each group or bar represent the number of patients in each group in all panels.

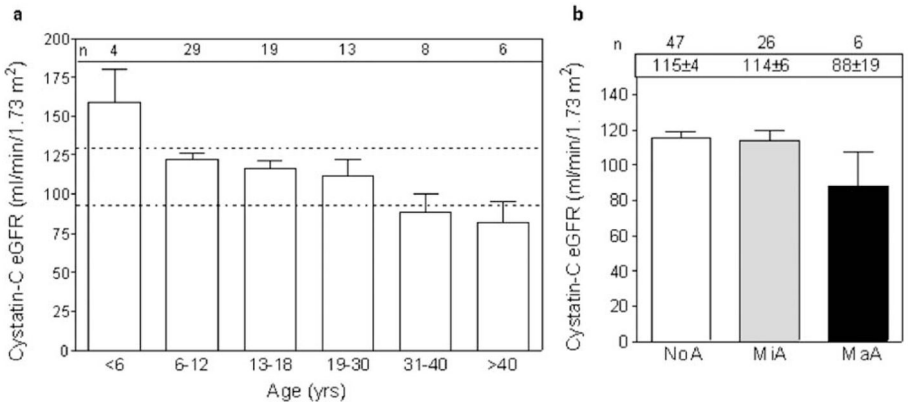

Figure 2.

Plasma Cystatin C Estimated GFR (eGFR) in patients with SCA (a) across different age groups and (b) different albuminuria groups. Values between dashed lines in panel (a) represent normal GFR values. Numbers of patients in each age group (n) is listed above both panels and the mean ± SEM of eGFR in the NoA, MiA, and MaA groups are listed above the columns in panel (b).

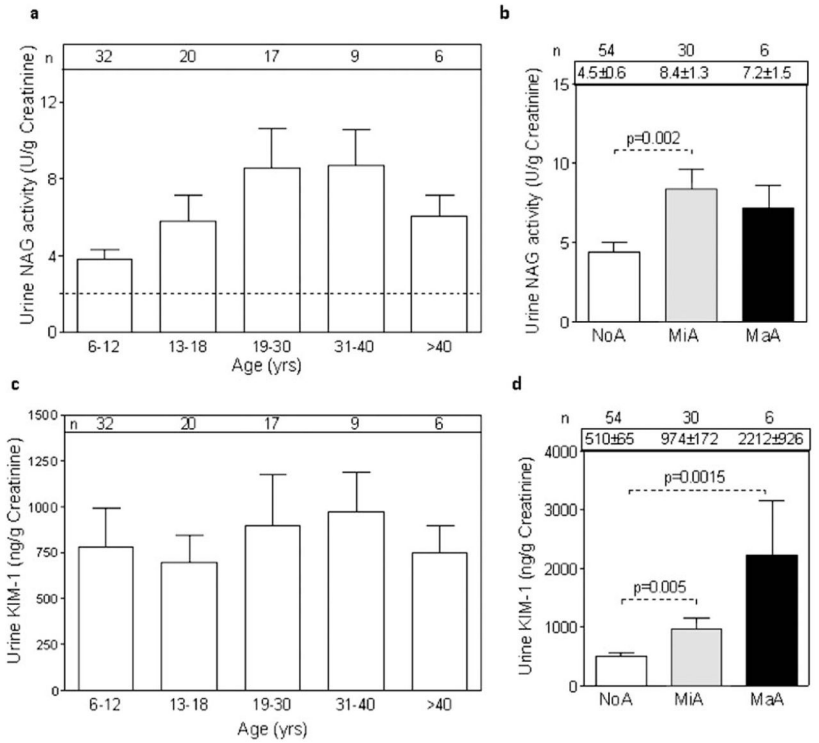

Figure 3.

Urine *N*-acetyl-b-D-glucosaminidase (NAG) Activity and Kidney Injury Molecule-1 (KIM-1) Levels in Patients with SCA. (a) NAG activity across different age groups and (b) different albuminuria groups. Values below the dashed line represent normal NAG levels reported. (c) KIM-1 levels across different age groups, and (d) different albuminuria groups. Numbers of patients in each age group (*n*) is listed above the graphs and the mean ± SEM of NAG activity and KIM-1 levels in the NoA, MiA, and MaA groups are listed above the columns in panel b and d, respectively. Statistically significant differences between the albuminuria groups, adjusted for age are shown with the corresponding *P* value.

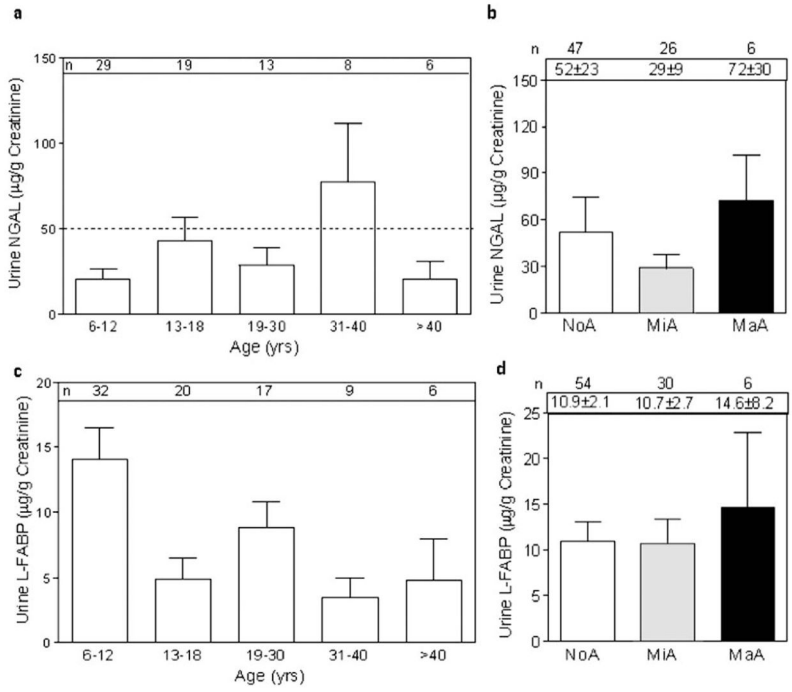

Figure 4.

Urine neutrophil gelatinase-associated lipocalin (NGAL) and liver-type fatty acid binding protein (L-FABP) levels in patients with SCA. (a) NGAL levels across different age groups and (b) different albuminuria groups. (c) L-FABP levels across different age groups and (d) different albuminuria groups. Numbers of patients in each age group (*n*) is listed above the graphs. The mean ± SEM of NGAL and L-FABP levels in the NoA, MiA, and MaA groups are listed above the columns in panel (b) and (d), respectively.

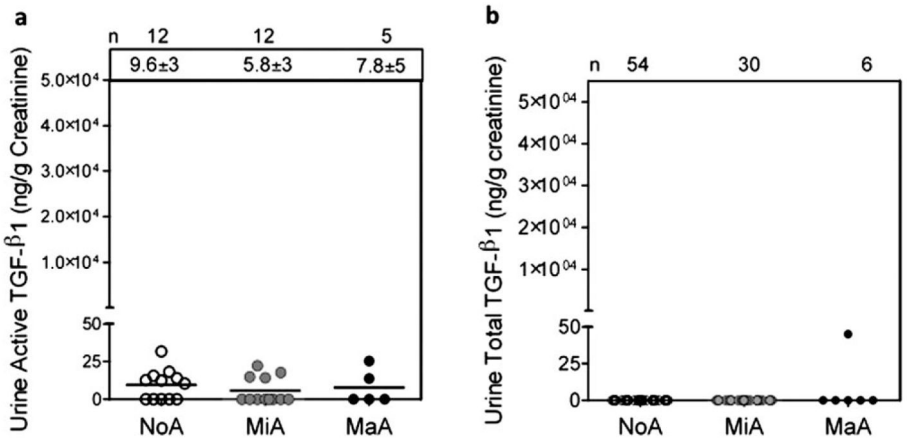

Figure 5.

Urine TGF-β Levels in patients with SCA with different levels of albuminuria. (a)Total TGF-β was measured in a dozen urine samples in the NoA, MiA groups and all samples in the MaA groups. Mean ± SEM are depicted above the groups. (b) Active TGF-β levels in the urine in all SCA patients. Number of urine samples analyzed from each group is shown above the graph. No active TGF-β was detectable in 89 samples and one sample showed detectable levels of TGF-β. The standards used for both types of ELISAs showed the expected levels of TGF-β (The correlation coefficient (r^2) for the standard curve for the ELISA assays was 0.988 for panel (a) and 0.99 for panel (b).

TABLE I

Characteristics of Patients with Sickle Cell Anemia (Hb SS Disease) Versus Those with Other Forms of Sickle Cell Disease (Hb SC Disease and Hb Sβ+ Thalassemia)

	Hb SS			Other (Hb SC, Hb Sβ+ Thalassemia)[a]		
Parameter	**Mean ± SEM**	**Range/%**	***N***	**Mean ± SEM**	**Range/%**	**N**
Median age (years)	18.0 ± 1.3	3–56	90	18.4 ± 2.5	4–48	26
Gender (Male:Female)	40:50	–	90	17:9	–	26
On Hydroxyurea (HU)	49	54%	90	4	15%	26
On chronic transfusion	18	20%	90	0	0%	26
On HU and chronic transfusions	5	5.5%	90	0	0%	26
Not On HU or chronic transfusions	18	20%	90	22	85%	26
Hemoglobin (g/dL)	9.2 ± 0.12	7.0–13	89	12.1 ± 0.21	10.2–14.3	26
Reticulocyte %	9.2 ± 0.57	2.4–24.7	76	2.9 ± 0.21	1.1–5.6	22
C-reactive protein (mg/L)	2.4 ± 0.73	0.3–28.4	42	0.66 ± 0.16	0.4–2.4	12
Blood pressure (Systolic)	113 ± 1.4	96–157	90	119 ± 2.3	96–157	26
Blood pressure (Diastolic)	63.6 ± 0.92	46–96	90	72.2 ± 2.4	53–111	26
Urine albumin (mg/g creatinine)	132.4 ± 60.3	3.1–5145	90	20.8 ± 3.4	2.8–62.6	26
Urine Sp. gravity	1.011 ± 0.0003	1.0–1.03	90	1.011± 0.001	1.01–1.02	23
Blood urea nitrogen (mg/dL)	9.4 ± 0.57	4–36	87	9.6 ± 0.58	4–16	26
Serum creatinine (mg/dL)	0.57 ± 0.03	0.3–1.4	87	0.67 ± 0.04	0.3–1.21	26
Urine creatinine (mg/dL)	0.57 ± 0.03	0.19–2.33	90	0.53 ± 0.06	0.12–1.2	26
Indirect bilirubin (μmol/L)	2.7 ± 0.26	0.7–10.4	64	1.5 ± 0.47	0.4–9.3	18

[a] Other group includes 16 subjects with Hb SC disease and 10 subjects with Hb $S\beta^{+}$ thalassemia.

TABLE II

Characteristics of Hb SS Patients with Normo-Albuminuria, Micro-Albuminuria, and Macro-Albuminuria

	Normo-albuminuria			Micro-albuminuria			Macro-albuminuria		
Parameter	**Mean ± SEM**	**Range/%**	***n***	**Mean ± SEM**	**Range/%**	***n***	**Mean ± SD**	**Range/%**	***n***
Median age (in yrs)	12 ± 1.5	3–47	54	20 ± 2.4	4–56	30	24.5 ± 6.2	9–52	6
Gender (Male:Female)	26:29		54	12:18		30	2:4		6
On Hydroxyurea (HU)	29	54%	54	16	53%	30	4	67%	6
On Chronic Transfusion	11	20%	54	6	20%	30	1	17%	6
On HU and Chr. Transfusion	3	6%	54	2	7%	30	0	0%	6
On neither HU/Transfusions	11	20%	54	6	20%	30	1	17%	6
Hemoglobin (g/dL)	9.2 ± 0.16	7.1–13	53	9.3 ± 0.21	7.1–11.6	30	8.8 ± 0.48	7–10	6
Reticulocyte (%)	8.7 ± 0.69	2.4–24.7	45	9.8 ± 1.13	2.9–22.9	26	9.5 ± 1.6	6.3–15.5	5
C-Reactive Protein (mg/L)	3.1 ± 1.1	0.3–28.4	27	1.2 ± 0.3	0.3–3.7	14	0.8	–	1
Blood Pressure (Systolic)	110.7 ± 1.35	82–126	54	116 ± 2.8	94–167	30	120.8 ± 8.6	101–160	6
Blood pressure (Diastolic)	62.4 ± 1.11	50–84	54	64.8 ± 1.7	46–96	30	68.8 ± 3.7	59–81	6
Urine Albumin (mg/g creat.)	15.96 ± 0.97	3.1–28.9	54	64.19 ± 8.6	30.3–256	30	1521 ± 745	346–5145	6
Urine Sp. Gravity	1.011 ± 0.001	1.0–1.03	51	1.011± 0.001	1.010–1.020	26	1.011 ± 0.002	1.00–1.020	5
Blood Urea Nitrogen (mg/dL)	9.0 ± 0.6	4–30	53	9.4 ± 0.86	5–26	28	13.8 ± 4.75	4–36	6
Serum creatinine (mg/dL)	0.53 ± 0.03	0.3–1.1	53	0.61 ± 0.05	0.3–1.3	28	0.82 ± 0.18	0.4–1.4	6
Urine Creatinine (mg/mL)	0.57 ± 0.03	0.22–1.2	54	0.59 ± 0.08	0.2–2.3	30	0.5 ± 0.11	0.19–0.94	6
Indirect Bilirubin (μmol/L)	2.6 ± 0.31	0.8–10.4	41	2.97 ± 0.5	0.7–8.8	20	2.67 ± 1.5	0.9–5.7	3