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Genetic variants and cell-free hemoglobin processing in sickle cell nephropathy

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ABSTRACT

Intravascular hemolysis and hemoglobinuria are associated with sickle cell nephropathy. ApoL1 is involved in cell-free hemoglobin scavenging through association with haptoglobin-related protein. *APOL1* G1/G2 variants are the strongest genetic predictors of kidney disease in the general African-American population. A single report associated *APOL1* G1/G2 with sickle cell nephropathy. In 221 patients with sickle cell disease at the University of Illinois at Chicago, we replicated the finding of an association of *APOL1* G1/G2 with proteinuria, specifically with urine albumin concentration (β=1.1, *P*=0.003), observed an even stronger association with hemoglobinuria (OR=2.5, *P*=4.3x10^{-6}), and also replicated the finding of an association with hemoglobinuria in 487 patients from the Walk-Treatment of Pulmonary Hypertension and Sickle cell Disease with Sildenafil Therapy study (OR=2.6, *P*=0.003). In 25 University of Illinois sickle cell disease patients, concentrations of urine kidney injury molecule-1 correlated with urine cell-free hemoglobin concentrations (r=0.59, *P*=0.002). Exposing human proximal tubular cells to increasing cell-free hemoglobin led to increasing concentrations of supernatant kidney injury molecule-1 (*P*=0.01), reduced viability (*P*=0.01) and induction of *HMOX1* and *SOD2*. *HMOX1* rs743811 associated with chronic kidney disease stage (OR=3.0, *P*=0.0001) in the University of Illinois cohort and end-stage renal disease (OR=10.0, *P*=0.0003) in the Walk-Treatment of Pulmonary Hypertension and Sickle cell Disease with Sildenafil Therapy cohort. Longer *HMOX1* GT-tandem repeats (>25) were associated with lower estimated glomerular filtration rate in the University of Illinois cohort (*P*=0.01). Our findings point to an association of *APOL1* G1/G2 with kidney disease in sickle cell disease, possibly through increased risk of hemoglobinuria, and associations of *HMOX1* variants with kidney disease, possibly through reduced protection of the kidney from hemoglobin-mediated toxicity.

Introduction

Sickle cell nephropathy is a prevalent complication of sickle cell disease (SCD) and is associated with early mortality.[1-5] The strongest genetic association with chronic kidney disease (CKD) in the general African-American population is homozygosity or compound heterozygosity for two common variants of *APOL1*, G1 (S342G and I384M substitutions) and G2 (N388 and Y389 deletions),[6-11] which is observed in 10-15% of African-Americans.[6,12] Similar to the genetic persistence of the Hb S mutation, which protects against mortality from malaria, the *APOL1* G1 and G2 variants are believed to have been selected by affording protection from *Trypanosoma brucei rhodesiense* infection.[6,13] ApoL1 complexes with haptoglobin-related protein to form the trypanosome lytic factor, and this complex can scavenge cell-free hemoglobin.[14] Many other genetic variants have been associated with CKD in African-Americans including *MYH9*, which encodes the non-muscle myosin IIA heavy chain and is a component of the cytoskeleton of podocytes.[15]

Two non-replicated genetic association studies have been reported in sickle cell nephropathy. The first identified four variants and three haplotypes in *BMPR1B*, a bone morphogenetic protein receptor gene, which were associated with estimated glomerular filtration rate (eGFR).[16] The second found that individuals with the *APOL1* G1/G2 risk variants (defined as being homozygous or compound heterozygous for the G1 and/or G2 risk variants using a recessive model) were 3.4-times more likely to have dipstick-defined proteinuria and that variants in *MYH9* were independently associated with proteinuria after adjusting for *APOL1* variant status.[17] Furthermore, a significant interaction between the *APOL1* G1/G2 risk variants and an *MYH9* risk haplotype was observed in predicting eGFR.

Intravascular hemolysis is a potential cause of oxidative injury and endothelial damage in SCD. Under normal conditions, plasma cell-free hemoglobin represents approximately 10% of the hemoglobin from red blood cell turnover[18] (average concentration 0.2 μM, range <0.06–0.7).[19] Intravascular hemolysis that exceeds the ability of haptoglobin and haptoglobin-related protein-ApoL1 complexes to bind cell-free hemoglobin results in hemoglobinuria[20] and, based on animal models, cell-free hemoglobin-mediated damage to the proximal tubule may be a mechanism of kidney damage.[21] Cell-free hemoglobin rapidly converts to the less stable methemoglobin followed by release of heme[22] and free heme may also elicit damage to the kidney.[23,24] Circulating cell-free hemoglobin is increased more than 10-fold in SCD,[25] with average

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concentrations of 3.5 μM (range 0.4–10.9 μM) at steady state and 5.3 μM (range 1.0–25.3 μM) during vaso-occlusive crises.[19] Markers of hemolysis have been associated with kidney disease in some[26-30] but not all[31-33] SCD cohorts. Hemoglobinuria, determined by urine dipstick analysis, has been observed in 15-42% of adults with SCD,[34-36] and is associated with elevated markers of hemolysis and risk of CKD progression.[36] Not all patients with SCD develop hemoglobinuria and not all patients with SCD and hemoglobinuria develop progressive CKD, suggesting that inherent susceptibilities to hemoglobinuria and CKD may differ.

We investigated the association of previously identified variants in *BMPR1B* with eGFR, variants in *APOL1* and *MYH9* with urine albumin concentrations, and the association of these variants with hemoglobinuria in a cohort of adult SCD patients treated at the Comprehensive Sickle Cell Center at the University of Illinois at Chicago (UIC). We then examined the effect of cell-free hemoglobin on cultured renal tubular cells and the expression of candidate genes to protect from potentially toxic effects of hemoglobin.

Methods

The study was approved by the institutional review boards of the participating institutions and the subjects provided written informed consent.

Urine hemoglobin and kidney injury molecule-1 measurements

Random urine samples were collected from UIC SCD patients with an eGFR >60 mL/min/1.73 m^2 during a routine clinic visit between March and May 2013, as previously described.[36,37] Urine concentrations of hemoglobin (Bethyl laboratories, Montgomery, TX, USA) and kidney injury molecule-1 (KIM-1) (R&D Systems, Minneapolis, MN, USA) were measured using enzyme-linked immunosorbent assays (ELISA). Urine albumin and creatinine values were determined by the UIC Clinical Pathology Laboratories using methods approved by Clinical Laboratory Improvement Amendments. Albuminuria was defined as a urine albumin to creatinine ratio ≥30 mg/g creatinine.

Human tubular cell culture studies

Human kidney-2 (HK-2) proximal tubular cells (ATCC, Manassas, VA, USA) were cultured in Keratinocyte Serum-Free Medium (Life Technologies, Grand Island, NY, USA) at 37°C in a 100%-humidified atmosphere containing 5% CO_2-95% air. After an initial 24 h of incubation, lyophilized hemoglobin (Sigma-Aldrich, St. Louis, MO, USA) was added to the culture medium and after another 24 h of incubation, supernatant and HK-2 cells were harvested. The absorption spectrum of lyophilized hemoglobin was determined using an Ocean Optics Spectrophotometer (Dunedin, FL, USA). Cell count and viability were determined using a Countess Automated Cell Counter (Invitrogen, Life Technologies, Grand Island, NY, USA) with the trypan blue method and KIM-1 concentrations were measured in the supernatant using ELISA (R&D Systems, Minneapolis, MN, USA).

To determine whether cell-free hemoglobin interacts and binds with HK-2 cells, cell-free hemoglobin was labeled with fluoroscein isothiocyanate (FITC) as follows. We added 200 μL of 5 mg/mL FITC dissolved in 95% ethanol gradually to 9.3 mg of cell-free hemoglobin dissolved in 0.7 mL of 200 μm of borate buffer pH 10.0 with vigorous vortexing. The mixture was incubated for 1 h at 37°C followed by an additional 24 h of incubation at 4°C. The mixture was then extensively dialyzed against 10 mM of Tris-HCl buffer pH 7.4 (Tris: hydroxymethylaminomethane) to remove and sequester excess FITC. For binding studies, FITC-labeled hemoglobin (5–20 μM) was added to formalin-fixed HK-2 cells in LabTek chamber slides (Thermo Fisher Scientific, Waltham, MA, USA) and incubated for 1 h on an electric ice tray maintained at 4°C (ICE 120, TECA Corporation, Chicago, IL, USA). The cell layers were washed, fixed in 95% ethanol, and mounted with 1:1 phosphate-buffered saline:glycerol for fluorescence microscopy and digital photography (Nikon Corporation, New York, NY, USA). Competitive binding was also performed on additional chamber slides with FITC-labeled cell-free hemoglobin and unlabeled cell-free hemoglobin.

Total RNA was extracted using Trizol (Invitrogen, Carlsbad, CA, USA) from HK-2 cells after 48 h of culture. Approximately 1 μg of total RNA was reverse-transcribed using the High Capacity cDNA Reverse Transcription Kit (Life Technologies, Grand Island, NY, USA) and employing random primers according to the manufacturer's instructions. After generating cDNA, quantitative polymerase chain reaction (PCR) was performed using the following primers for candidate genes involved in reactive oxygen species or heme metabolism: (i) *HMOX1*: Hs01110250_m1, (ii) *SOD1*: Hs00533490_m1, (iii) *SOD2*: Hs00167309_m1, (iv) *GSS*: Hs00609286_m1, (v) *GSR*: Hs00167317_m1, (vi) *CAT*: Hs00156308_m1, (vii) *NFE2L2*: Hs00975961_g1, (viii) *CP*: Hs00236810_m1, and (ix) *GAPDH*: Hs02758991_g1, (Catalogue #4331182) (Life Technologies, Grand Island, NY, USA) with TaqMan Fast Advanced Master Mix (Life Technologies) according to the manufacturer's instructions. The PCR products were detected using Fast SYBR green technology (Life Technologies). GAPDH mRNA quantification was used as the internal calibrator and the standard curve method was used to determine relative mRNA quantitation. All measurements were performed in triplicate. Cell lysates were obtained from the HK-2 cells after 24 h of exposure to incremental doses of lyophilized hemoglobin by the freeze-thaw method and protein concentrations of differentially expressed genes (Hmox1 EIA kit, Enzo® Life Sciences, Farmingdale, NY, USA; SOD2 ELISA kit, abcam®, Cambridge, MA, USA) were measured.

Study cohorts

The UIC cohort comprised 247 adults with SCD receiving routine medical care at UIC from whom DNA samples were obtained between August 2010 and June 2013. Clinical and laboratory data were extracted from the electronic medical record charting system, Cerner PowerChart, from clinic visits closest to the date of sample collection. The Walk-Treatment of Pulmonary Hypertension and Sickle Cell Disease with Sildenafil Therapy (Walk-PHaSST) cohort consisted of 540 SCD patients from nine centers in the USA and one in the UK; these patients were recruited between February 2008 and June 2009. The UIC was a participating site for Walk-PHaSST and patients from UIC were excluded from this cohort.

Measures of hemoglobinuria and chronic kidney disease

Hemoglobinuria was defined as urine dipstick-positive for blood with <2 red blood cells per high power field on microscopy on two consecutive urinalyses in the UIC cohort and <5 red blood cells per high power field in the Walk-PHaSST cohort. A different definition for hemoglobinuria was applied in the Walk-PHaSST cohort because only a single urinalysis result was available and the lowest category provided in this cohort for red blood cells in the urine was 0–5 red blood cells per high power field. The eGFR was

calculated using the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) formula[38] and CKD stage was defined according to the National Kidney Foundation, Kidney Disease Outcomes Quality Initiatives (K/DOQI) guidelines.[39] Urine albumin concentrations were determined from a baseline visit along with the average of three consecutive urine albumin concentration measurements and end-stage renal disease (ESRD) was defined as an eGFR <15 mL/min/1.73 m^2 or the need for hemodialysis.

Genotyping in the UIC and Walk-PHaSST cohorts

Genotyping was carried out using an Affymetrix Axiom genome-wide Pan-African GeneChip array at the Core Genomics Facility at UIC in DNA isolated from peripheral blood mononuclear cells from the 247 patients in the UIC SCD cohort, as previously described.[40] All samples had a genotype call rate >95%. Single nucleotide polymorphisms (SNP) deviating from Hardy-Weinberg equilibrium (P<0.0001) or with a minor allele frequency <0.01 were removed. Two population outliers, based on principal components analysis of genotype data, and one random sample in each of 24 sample pairs having proportion of identity-by-descent >5% were excluded from the study, resulting in 221 samples. Validation studies were conducted in the Walk-PHaSST cohort. Genotyping was performed for 540 samples from the Walk-PHaSST cohort of sickle cell patients using the Illumina Human 610-Quad SNP array as previously described.[41] All samples had a genotype call rate >95%. Six population outliers and 39 samples exhibiting proportion of identity-by-descent >5% were excluded from the analysis. An additional eight samples that overlapped UIC cohort samples were also removed, resulting in 487 samples.

The G1 and G2 variants in *APOL1* were investigated for relationships with hemoglobinuria and CKD. The S342G and I384M substitutions are in almost complete linkage disequilibrium and are termed G1; the deletion of two amino acids, N388 and Y389, is termed G2. The G2 variant was further imputed to 1000 genome project phase 1 data for the UIC cohort and both G1 and G2 variants imputed for the Walk-PHaSST cohort. Estimated genotypes with the highest probabilities were used for analysis. Homozygosity or compound heterozygosity for the G1 and G2 variants of *APOL1* (defined as *APOL1* G1/G2) were examined for their association with hemoglobinuria and markers of kidney disease. We also tested additive and dominant genetic models for the G1 and G2 variants for these phenotypes. Variants in *BMPR1B* (rs2240036, rs4145993, rs17022863, rs1434549, rs1470409, rs4331783) and *MYH9* (rs5756129, rs11912763, rs16996648, rs5750248, rs1557529, rs5756152, rs8141189, rs1005570. rs16996672, rs933224) that had been previously identified in the literature as being associated with sickle cell nephropathy[16,17] were also examined for associations with eGFR and urine albumin concentrations, respectively. The imputation qualities for *BMPRIB* and *MYH9* are provided in *Online Supplementary Table S1*.

HMOX1 and *SOD2* were candidate genes identified as having differential expression from the PCR experiments. SNP located within ±10 Kb of *HMOX1* and *SOD2* were imputed to 1000 genome data with African and European subjects as the reference panel using Beagle[42] version 4. SNP with minor allele frequency >0.1 and imputation dosage r^2>0.3 were selected for analysis. Tag-SNP were identified based on the phased genotypes of the UIC cohort using a greedy algorithm[43] with a linkage disequilibrium threshold set at r^2=0.5. Allele dosages were associated with hemoglobinuria and markers of kidney disease in the UIC cohort and validated in the Walk-PHaSST cohort. The multi-allele polymorphism of GT-tandem repeats in the promoter region of *HMOX1*, which may affect heme oxygenase activity[44] and clinical outcome in SCD patients,[45] was also determined in the SCD patients from the UIC cohort. The 5'-flanking region containing (GT)n repeats of *HMOX1* was amplified by PCR with a 5-carboxyfluorescein–labeled forward primer (AGAGCCTGCAGCTTCTCAGA) and standard reverse primer (ACAAAGTCTGGCCATAGGAC), as described previously.[46] Samples were amplified using AccuPrime™ SuperMix II (Life Technologies, Grand Island, NY, USA) with an initial denaturation step at 94°C for 2 min, followed by 35 cycles of 30 s at 94°C, 30 s at 60°C, and 1 min at 68°C. The PCR products were mixed together with GeneScan 600 LIZ size standard and analyzed using a 3730XL ABI sequencer. Data were subsequently analyzed using the software package Genemapper 4.1. Amplification and fragment analysis were performed in the DNA Services Facility at the UIC. The *HMOX1* GT-tandem repeat numbers were categorized into two allele classes (S if there were ≤25 repeats or L if there were >25 repeats) and we examined the association of SS/SL/LL variants with hemoglobinuria and kidney disease markers using additive, dominant, and recessive models.

Statistical methods

Clinical variables are presented as median values and interquartile range (IQR) and experimental data as mean values and standard error. The comparison of urine KIM-1 by albuminuria status was performed using the Kruskall-Wallis test and correlations between urine KIM-1 and urine albumin or urine hemoglobin concentrations were determined using linear regression analysis. Comparisons of HK-2 cell viability, supernatant KIM-1 concentrations, and PCR analysis with cell-free hemoglobin concentrations were performed using ANOVA. Systat 11 (Systat Software Corporation, Chicago, IL, USA) was used for the statistical analyses. We evaluated 35 tag-SNP in the UIC cohort (14 in *HMOX1*, 21 in *SOD2*) and a P value <0.0014 was statistically significant after the Bonferroni correction. For eGFR and urine albumin concentration, linear regression analysis was used adjusting for age, gender, hemoglobin genotype, and hydroxyurea treatment. For hemoglobinuria and ESRD phenotypes, logistic regression was used adjusting for age, gender, hemoglobin genotype, and hydroxyurea treatment. The first principal component on genotype data was also included in the model to account for variation in European ancestry. P values were estimated using the χ^2 test with one degree of freedom. Bias reduction was used for parameter estimation for the ESRD phenotype due to the small numbers of patients with ESRD. For CKD stage, ordinal logistic regression was used adjusting for age, gender, hemoglobin genotype, and hydroxyurea treatment. P values were estimated using the Wald test and combined P values were estimated using a Z-score approach.[47] In subgroup analysis, we also compared associations of the genetic variants with the average urine albumin concentration over three consecutive determinations and CKD stage redefined by the average urine albumin concentration.

Results

Association of previously described genetic variants with sickle cell nephropathy

The study sample from the UIC cohort comprised 221 patients and the Walk-PHaSST cohort comprised 487 patients. The patients' clinical characteristics are summarized in Table 1.

Homozygosity or compound heterozygosity of the *APOL1* G1/G2 variants, observed in 14% of the UIC cohort, was associated with urine albumin concentration (n=203, β 1.1, P=0.003) (Table 2) in concurrence with the reported association with dipstick-defined proteinuria.[17] We were unable to replicate the previously reported asso-

ciations of *MYH9* variants with proteinuria[17] or *BMPR1B* variants with eGFR.[16] *APOL1* G1/G2 was also associated with eGFR (β -11.6, *P*=0.039), CKD stage (OR 2.6, *P*=0.022) and ESRD (OR 6.5, *P*=0.036). Given our recent report of the association of hemoglobinuria with albuminuria and CKD in SCD patients,[36] we also tested the association of *APOL1* G1/G2 variants with hemoglobinuria and found this to be the strongest association (OR 2.5, $P=4.3\times10^{-6}$) (Figure 1). Associations of the *APOL1* G1/G2 variants with urine albumin concentration (β 1.4, *P*=0.0001) and CKD stage (OR 3.8, *P*=0.005) were strengthened when using the average urine albumin concentration over three consecutive visits in 170 patients. Homozygosity or compound heterozygosity of the *APOL1* G1/G2 variants was observed in 16% of the Walk-PHaSST cohort and validation studies showed a significant association with hemoglobinuria (OR 2.6, *P*=0.003) (Figure 1). The associations of the G1/G2 risk variants with hemoglobinuria using an additive model (UIC: *P*=0.0004, Walk-PHaSST: *P*=0.01) and a dominant model

Table 1. Characteristics of the patients in the UIC and Walk-PHaSST cohorts.

Variable	N.	UIC	N.	Walk-PHaSST
Age	221	35 (27-46)	487	37 (25-47)
Sex (male : female)	221	90 : 131	487	222 : 265
Genotype				
Hb SS		157 (71%)		360 (74%)
Hb SC	221	40 (18%)	487	93 (19%)
Hb S^0-thalassemia		8 (4%)		9 (2%)
Hb S^+-thalassemia		15 (7%)		18 (4%)
Other		1 (0.5%)		7 (1%)
ACE-inhibitor/ARB use	221	26 (12%)	-	-
Hydroxyurea use	221	108 (49%)	487	167 (34%)
eGFR (mL/min/1.73 m^2)	215	138 (103-152)	482	131 (103-149)
Urine albumin concentration (mg/g creatinine)	203	26 (8-142)	283	24 (6-184)
Chronic kidney disease:				
Absent		100 (49%)		139 (47%)
Stage I		68 (33%)		99 (34%)
Stage II	205	15 (7%)	294	15 (5%)
Stage III		12 (6%)		31 (11%)
Stage IV		1 (0.5%)		3 (1%)
Stage V (ESRD)		9 (4%)		7 (2%)
Hemoglobinuria	200	38 (19%)	426	90 (21%)

Continuous variables are presented as median values (interquartile range). UIC: University of Illinois at Chicago; Walk-PHaSST, Walk-Treatment of Pulmonary Hypertension and Sickle Cell Disease with Sildenafil Therapy; ACE: angiotensin-converting enzyme; ARB: angiotensin receptor blocker; eGFR: estimated glomerular filtration rate, ESRD, end-stage renal disease.

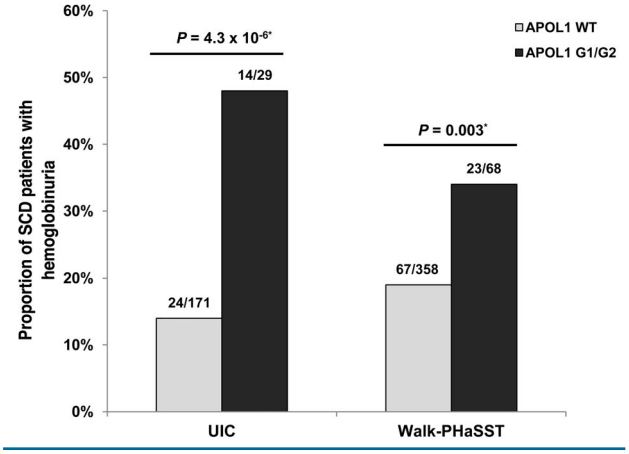

Figure 1. Significant association between the *APOL1* G1/G2 risk variants and hemoglobinuria in the UIC and Walk-PHaSST cohorts. Higher proportions of sickle cell disease patients who were homozygous or compound heterozygous for the *APOL1* G1 and/or G2 variants had hemoglobinuria (UIC: 48% *vs.* 14%, respectively; Walk-PHaSST: 34% *vs.* 19%, respectively). SCD, sickle cell disease; UIC, University of Illinois at Chicago; Walk-PHaSST, Walk-Treatment of Pulmonary Hypertension and Sickle Cell Disease with Sildenafil Therapy; WT, wild-type; *APOL1* G1/G2, homozygosity or compound heterozygosity for the G1 and/or G2 variants. *P value after adjustment for age, gender, hydroxyurea therapy, and hemoglobin genotype.

Table 2. Association of previously reported risk variants with markers of kidney disease in adult patients from the UIC cohort.

Gene	Kidney phenotype	SNP	Risk allele	N	β-coefficient	*P* value
APOL1	Urine albumin concentration (mg/g creatinine)		G1/G2	203	1.1	0.0032
		rs5756129	T	203	0.01	1.0
		rs11912763	A	203	-0.17	0.5
		rs16996648	C	203	0.10	0.6
		rs5750248	T	203	-0.07	0.7
MHY9	Urine albumin concentration (mg/g creatinine)	rs1557529	A	203	0.04	0.8
		rs5756152	A	203	0.02	0.9
		rs8141189	A	203	0.05	0.8
		rs1005570	A	203	-0.05	0.8
		rs16996672	T	203	-0.01	1.0
		rs2240036	C	215	-0.75	0.8
		rs4145993	T	215	-1.28	0.6
BMPR1B	eGFR (mL/min/1.73 m^2)	rs17022863	G	215	1.26	0.7
		rs1434549	T	215	-0.61	0.8
		rs1470409	A	215	-1.48	0.6
		rs4331783	A	215	-0.72	0.8

G1: reference allele: A, alternative allele: G; Imputation r^2 in Walk-PHaSST: 0.82; G2: reference allele: AATAATT, alternative allele A; Imputation r^2 in UIC: 0.86, in Walk-PHaSST: 0.77. UIC: University of Illinois at Chicago; SNP: single nucleotide polymorphism; eGFR: estimated glomerular filtration rate.

(UIC: *P*=0.2, Walk-PHaSST: *P*=0.3) were less significant than with the recessive model.

Urine hemoglobin and kidney injury molecule-1 concentrations

Twenty-five SCD patients (median age of 32 years, 14 females) provided urine samples during a clinic visit. Thirteen of these patients were part of the UIC cohort of genotyped patients and one was a compound heterozygote for *APOL1* G1/G2. Patients with albuminuria had higher urine KIM-1 concentrations (12 ng/g creatinine, IQR 10–22 ng/g creatinine) than patients without albuminuria (4 ng/g creatinine, IQR 3–5 ng/g creatinine) (*P*=0.007) and a direct correlation was observed between the concentrations of urine albumin (r=0.49, *P*=0.01) (Figure 2A) and urine hemoglobin (r=0.59, *P*=0.002) (Figure 2B) with urine KIM-1, a marker of tubular injury.

Cultured human proximal tubular (HK-2) cells

Cultured HK-2 cells were exposed to incremental doses of cell-free hemoglobin for 24 h. The absorption spectrum of the lyophilized hemoglobin had a major peak at 640 nm, consistent with methemoglobin. The number of HK-2 cells was stable (Figure 3A) but cell viability was decreased (n=3, *P*=0.01) (Figure 3B) with increasing cell-free hemoglobin concentration. Using fluorescent microscopy, we observed binding of FITC-labeled hemoglobin to the HK-2 cells which was competitively displaced by increasing concentrations of unlabeled hemoglobin, providing evidence for a direct interaction between cell-free hemoglobin and HK-2 cells (Figure 4). Consistent with cell-free hemoglobin causing injury to proximal tubular cells, increasing KIM-1 concentrations were observed in the supernatant of the HK-2 cells exposed to increasing concentrations of cell-free hemoglobin (n=4, *P*=0.01) (Figure 5).

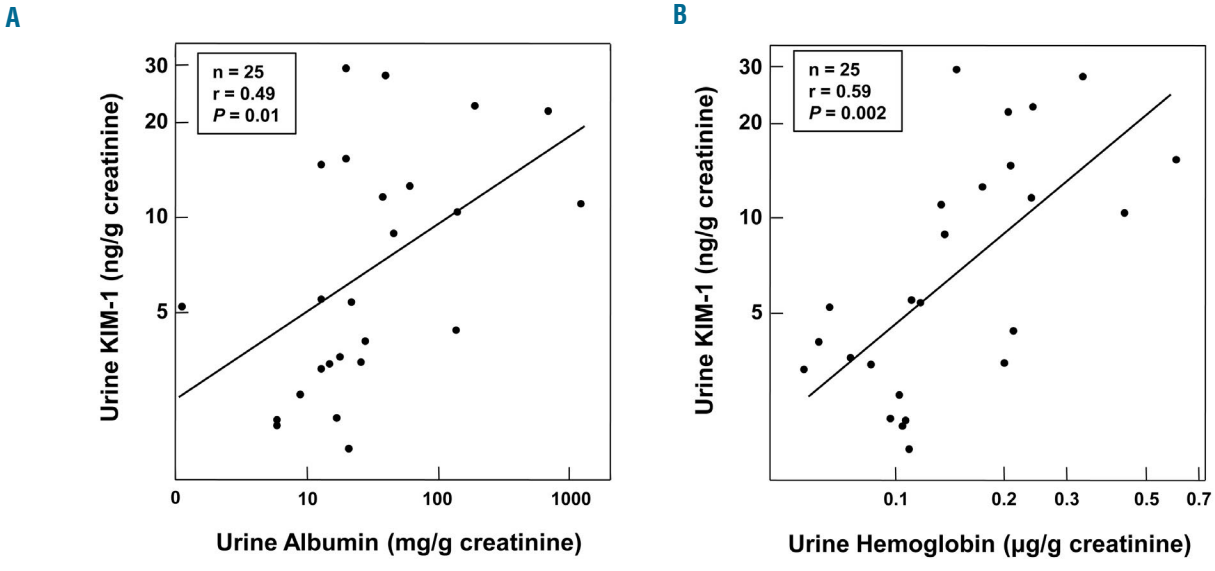

Figure 2. (A) Direct correlation between urine KIM-1 and urine albumin concentrations. In 25 patients with sickle cell disease, increased kidney injury molecule-1 (KIM-1) concentrations were observed with increasing urine albumin concentrations. (B) Direct correlation between urine KIM-1 and urine hemoglobin concentrations. In 25 patients with sickle cell disease, increased urine kidney injury molecule-1 (KIM-1) concentrations were observed with increasing urine hemoglobin concentrations.

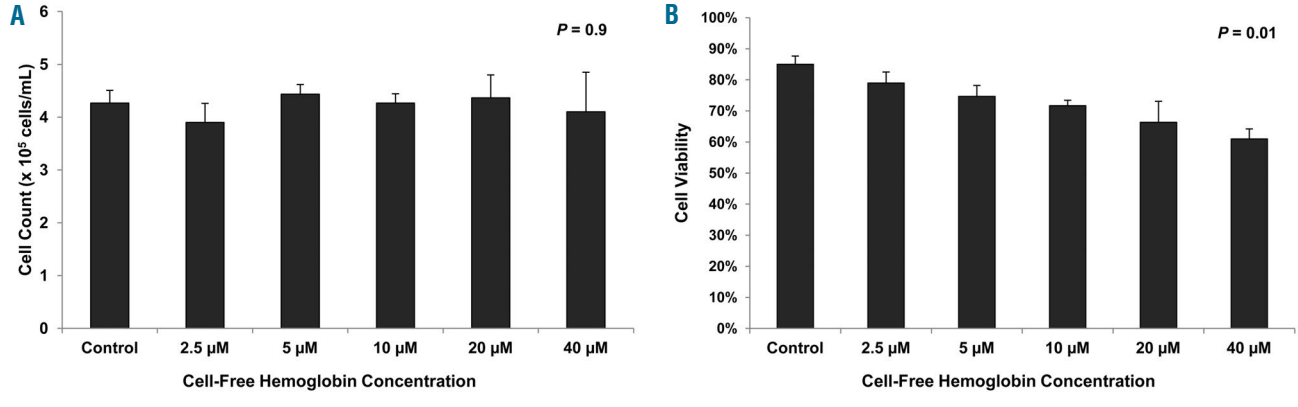

Figure 3. (A) Effect of cell-free hemoglobin exposure on HK-2 cell counts. After 24 h of exposure to incremental doses of cell-free hemoglobin, HK-2 cell counts were not significantly different (n = 3). (B) Effect of cell-free hemoglobin exposure on HK-2 cell viability. HK-2 cell viability tended to decrease progressively after 24 h of exposure to increasing dose of cell-free hemoglobin (n = 3).

To determine the response of human proximal tubular cells to cell-free hemoglobin, we measured relative gene expression of candidate genes involved in heme metabolism or in the metabolism of reactive oxygen species. We detected increased relative expression of *HMOX1* (n=4, *P*=0.0001) (Figure 6A) and *SOD2* (n=4, *P*=0.004) while no significant changes in gene expression were detected for *SOD1*, *GSS*, *GSR*, *CAT*, *NFE2L2*, and *CP* (*data not shown*). The increase in *HMOX1* expression correlated with increased Hmox1 protein concentrations in the cell lysates (Figure 6B) while no significant increase in SOD2 protein content was observed (*data not shown*).

Based on the HK-2 cell gene expression data, we examined 14 tag-SNP in the *HMOX1* gene and 21 tag-SNP in the *SOD2* gene. In the UIC cohort, we identified a SNP (rs743811; 2,767 bp downstream of *HMOX1*; minor allele frequency=0.14) which was significantly associated with CKD stage (OR 2.8, *P*=0.0003) (Table 3A). This association persisted when using the CKD stage classified using the average urine albumin concentration from three consecutive measurements (OR 2.9, *P*=0.0003). In the Walk-PHaSST cohort, the *HMOX1* rs743811 SNP was significantly associated with ESRD (OR 9.8, *P*=0.0004) (Table 3A). These relationships persisted after adjusting for the *APOL1* G1/G2 risk variants in the UIC and Walk-PHaSST cohorts (Table 3B). On combined meta-analysis of the two cohorts, *HMOX1* rs743811 was significantly associated with CKD stage and ESRD (Table 3B). Using gene expres-

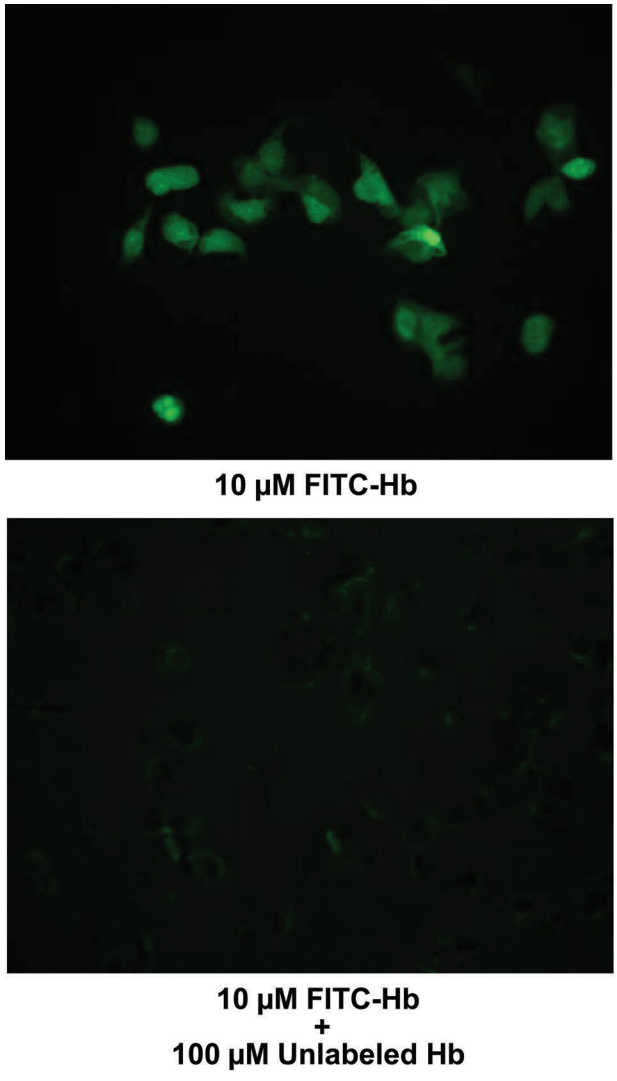

Figure 4. Exposure of HK-2 cells to labeled hemoglobin. Binding of fluoroscein isothiocyanate (FITC)-labeled cell-free hemoglobin (Hb) to HK-2 cells was observed after 1 h of incubation while reduced fluorescence was observed in a competitive assay of FITC-labeled Hb with unlabeled Hb (n = 4).

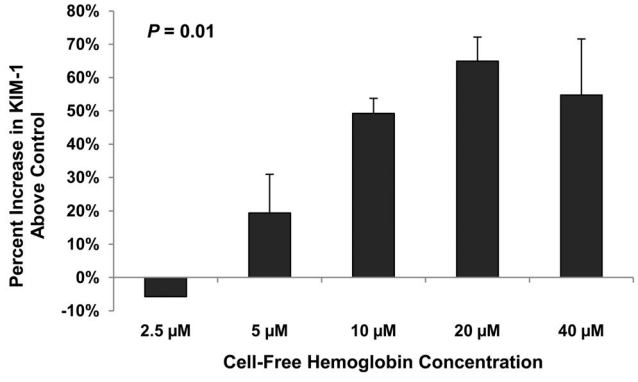

Figure 5. KIM-1 concentrations from HK-2 cells exposed to cell-free hemoglobin. Increasing kidney injury molecule-1 (KIM-1) concentrations were detected in the supernatant of HK-2 cells exposed for 24 h to increasing doses of cell-free hemoglobin (n = 4).

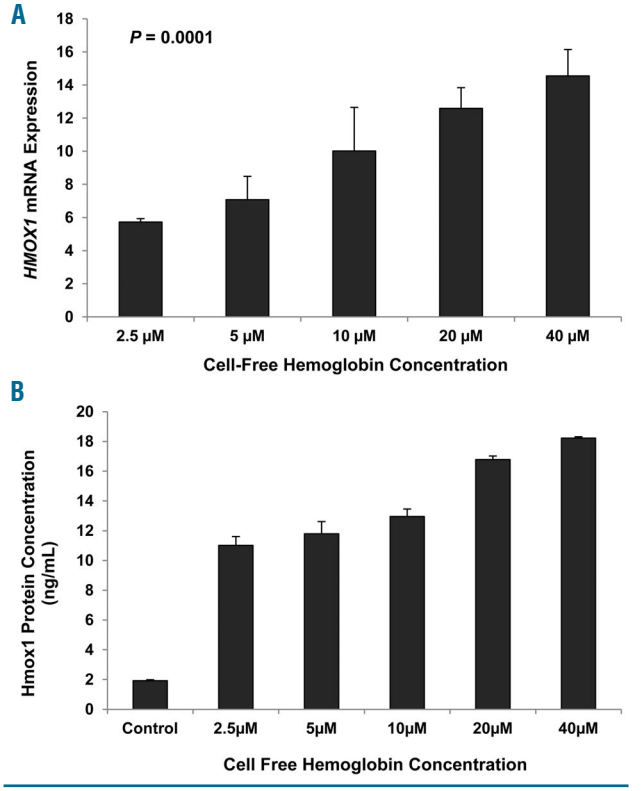

Figure 6. (A) *HMOX1* expression. Increased expression of *HMOX1* mRNA was detected in HK-2 cells after 24 h of exposure to increased dose of cell-free hemoglobin. (B) Hmox1 protein concentrations. Increased concentrations of Hmox1 protein were detected in the HK-2 cell lysates after 24 h of exposure to increasing doses of cell-free hemoglobin.

sion data from peripheral blood mononuclear cells of SCD patients from a previous study,[40] we did not observe an association of the *HMOX1* rs743811 SNP with transcript levels of *HMOX1*.

We also determined the GT-tandem repeat polymorphism in 237 SCD patients from the UIC cohort (Figure 7A). The longer allele (>25 GT-repeats) was associated with decreased eGFR using an additive model (β -9.2, *P*=0.012) (Figure 7B) and a dominant model (β -8.8, *P*=0.025), while a trend was observed using a recessive model (β -27.3, *P*=0.07). We did not observe an association between *HMOX1* GT-tandem repeat length and *HMOX1* expression in the peripheral blood mononuclear cells of SCD patients. No significant association was found for SOD2 tag-SNP.

Discussion

We confirmed a significant association of *APOL1* G1/G2 with urine albumin concentration and also observed significant associations with eGFR, CKD stage, and ESRD in the UIC SCD cohort. We also observed a strong association of *APOL1* G1/G2 with hemoglobinuria and replicated this finding in the Walk-PHaSST cohort. We observed that a marker of tubular cell injury, KIM-1, directly correlated

Table 3A. Association of a polymorphism in *HMOX1* with sickle nephropathy in adult patients from the UIC and Walk-PHaSST cohorts.

		UIC cohort				Walk-PHaSST cohort			Combined
Gene Variant	Phenotype	N.	OR/β	*P* value	Gene variant	Phenotype	N.	OR/β	*P* value	*P* value
	Hemoglobinuria	200	1.2	0.6		Hemoglobinuria	426	1.4	0.2	0.1
HMOX1	eGFR	215	-11.3	0.0030	*HMOX1*	eGFR	482	-3.8	0.1	0.0038
rs743811	Albuminuria	203	2.2	0.019	rs743811	Albuminuria	283	1.2	0.5	0.043
MAF = 0.14	CKD stage	205	2.8	0.00033	MAF = 0.15	CKD Stage	294	1.4	0.2	0.00067
	ESRD	215	2.3	0.2		ESRD	482	9.8	0.00037	0.00023

Table 3B. Association of a polymorphism in *HMOX1* with sickle nephropathy in adult patients from the UIC and Walk-PHaSST cohorts after adjustment for the *APOL1* G1/G2 variants.

		UIC cohort				Walk-PHaSST cohort			Combined
Gene Variant	Phenotype	N	OR/β	*P* value	Gene variant	Phenotype	N.	OR/β	*P* value	*P* value
	Hemoglobinuria	200	1.3	0.5		Hemoglobinuria	426	1.4	0.2	0.1
HMOX1	eGFR	215	-11.7	0.0020	*HMOX1*	eGFR	482	-3.8	0.1	0.0031
rs743811	Albuminuria	203	2.3	0.014	rs743811	Albuminuria	283	1.2	0.5	0.038
MAF = 0.14	CKD stage	205	3.0	0.00013	MAF = 0.15	CKD Stage	294	1.4	0.2	0.00041
	ESRD	215	2.5	0.2		ESRD	482	10.0	0.00032	0.00022

Reference allele: T, alternative allele: C. Imputation dosage r^2=1 in both the UIC and Walk-PHaSST cohorts. UIC, University of Illinois at Chicago; Walk-PHaSST, Walk-Treatment of Pulmonary Hypertension and Sickle Cell Disease with Sildenafil Therapy; eGFR, estimated glomerular filtration rate; CKD, chronic kidney disease; MAF, minor allele frequency; ESRD, end-stage renal disease

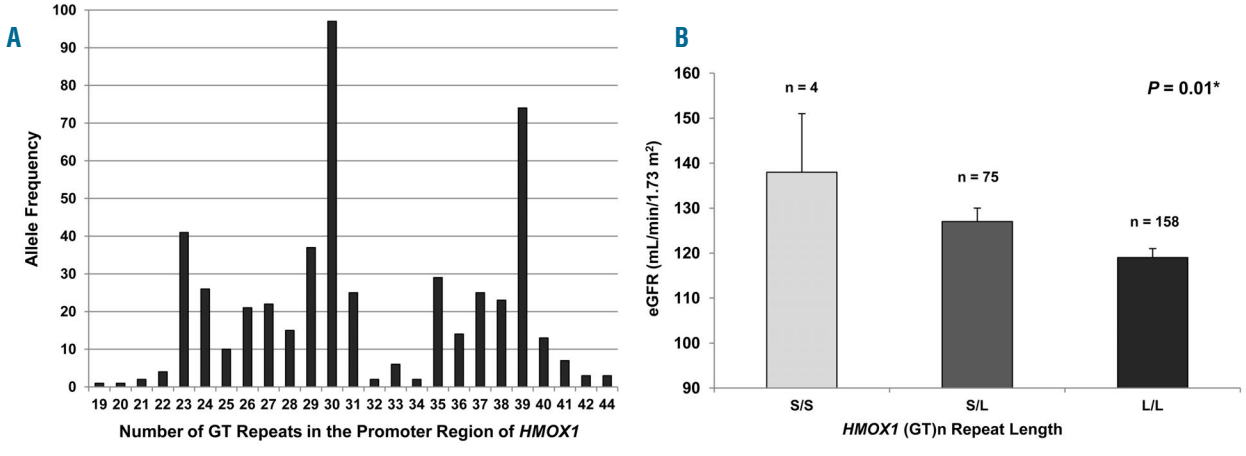

Figure 7. (A) Distribution of GT-tandem repeats in the promoter region of *HMOX1*. Consistent with the literature, a trimodal distribution of GT-repeats in the promoter region was observed in 237 sickle cell disease patients from the University of Illinois at Chicago cohort. (B) GT-tandem repeats in the promoter region of *HMOX1* are associated with estimated glomerular filtration rate. Using an additive model, the L allele (>25 GT repeats) was associated with a lower estimated glomerular filtration rate (eGFR) compared to the S allele (≤25 GT repeats) (L/L: 119±2 mL/min/1.73 m²; S/L: 127±3 mL/min/1.73 m²; S/S: 138±13 mL/min/1.73 m²). **P* value after adjustment for age, gender, hydroxyurea therapy, and hemoglobin genotype

with increasing cell-free hemoglobin concentration in the urine of SCD patients. Consistent with these clinical data, we found that supernatant KIM-1 concentrations increased while cell viability decreased in human proximal tubular cells (HK-2 cells) cultured with increasing concentrations of cell-free hemoglobin. Expression of *HMOX1*, which is instrumental in heme catabolism, was increased in the HK-2 cells after exposure to cell-free hemoglobin and a variant in *HMOX1* (rs743811) significantly associated with CKD stage in the UIC cohort and ESRD in the Walk-PHaSST cohort. Furthermore, the longer GT-tandem repeat promoter length polymorphism was significantly associated with decreased eGFR in the UIC cohort.

ApoL1 circulates in the blood bound to haptoglobin-related protein and this complex binds cell-free hemoglobin with high affinity as part of the trypanosome lytic factor.[14] ApoL1 is also a component of high-density lipoprotein (HDL), which is an avid scavenger of plasma heme[48,49] and hemoglobin.[50,51] The fate of hemoglobin bound to HRP/ApoL1/HDL is not clear, but our findings raise the possibility that ApoL1 has a role in clearing cell-free hemoglobin from the plasma and that the G1/G2 variants have decreased ability to bind or clear plasma cell-free hemoglobin. Intravascular hemolysis is constantly present, even in the non-SCD population, and there is a basal requirement for hemoglobin scavenging. Although the association of hemoglobinuria and kidney disease may only be a minor component of the kidney impairment associated with this African variant of *APOL1*, consideration of other hemolytic diseases and our further studies here are consistent with a role for hemoglobinuria in the pathogenesis of CKD.[20,52-57]

KIM-1 is a transmembrane protein whose expression and release by proximal tubular cells is increased after exposure to inflammatory stimuli.[58] Consistent with other studies,[59,60] we observed that concentrations of urine KIM-1 were increased in SCD patients with albuminuria. A unique finding of our study is that KIM-1 concentration significantly correlated with cell-free hemoglobin concentration in the urine of patients with SCD and in the supernatant of HK-2 cells exposed to cell-free hemoglobin. We were also able to demonstrate through our fluorescent studies that cell-free hemoglobin directly interacts with the surface of HK-2 cells. Increased expression of *HMOX1* and *SOD2* mRNA in the HK-2 cells is likely a result of increased heme exposure and oxidative stress from the cell-free hemoglobin. Findings by other investigators support a role for cell-free hemoglobin in mediating damage to proximal tubules. CKD was observed in 64% of patients with paroxysmal nocturnal hemoglobinuria at baseline, with 43% having proteinuria with preserved eGFR and 21% having more advanced stages of CKD characterized by eGFR <60 mL/min/1.73 m^2.[61] Increased hemosiderin deposits in the tubules are almost universally observed and tubular dysfunction occurs in 67% of patients with paroxysmal nocturnal hemoglobinuria.[62] Similarly, in animal studies with induced hemolysis, hemosiderin deposits are found primarily in the proximal tubular cells and the deposition and oxidative damage to the tubular cells are exacerbated in mice deficient in haptoglobin[20,56] or hemopexin.[55]

The *APOL1* G1/G2 variants have been primarily linked to glomerular diseases in African-Americans and in the context of hemolytic disease, such as SCD, increased cell-free hemoglobin may result in vasculopathy and direct podocyte injury resulting in damage to the glomerular filtration barrier and albuminuria. Albuminuria itself can be toxic to the tubular cells and lead to elevated KIM-1 concentrations in the urine. Alternatively, impaired reabsorption of albumin by proximal tubule injury may lead to higher urine concentrations of albumin. Under normal physiological conditions, the nephron filters about 3 g of albumin per day and the proximal tubular cells are responsible for reabsorbing approximately 90% of this load.[63] Transgenic sickle mice models and longitudinal studies may help improve our understanding of the timing between hemoglobinuria, glomerular and tubular damage, and increases in urine concentrations of albumin and KIM-1.

A novel finding of our study is that a SNP in *HMOX1*, rs743811, was significantly associated with CKD stage in the UIC SCD cohort, ESRD in the Walk-PHaSST SCD cohort, and with CKD stage and ESRD in a combined meta-analysis of both cohorts. Heme oxygenase-1 is an inducible enzyme, highly expressed in erythrocyte-phagocytosing macrophages; it catabolizes heme to biliverdin and carbon monoxide. Using immunoperoxidase staining, increased Hmox1 was detected in the renal tubules of biopsy specimens from a patient with paroxysmal nocturnal hemoglobinuria[52] and a patient with SCD.[64] Furthermore, increased expression of *HMOX1* has been observed in a porcine proximal tubular cell line exposed to urine from a patient with paroxysmal nocturnal hemoglobinuria[52] and in the kidneys of sickle cell mice.[64,65] A protective role for Hmox1 is suggested by several[52,66] but not all[67] studies. The *HMOX1* GT-tandem repeat polymorphism has been previously shown to correlate with Hmox1 activity.[44] Prior studies investigating the *HMOX1* GT-tandem repeat polymorphism in kidney disease produced conflicting results with one study of patients with coronary artery disease showing an association between longer GT repeats and an increased risk of CKD progression, defined as sustained serum creatinine doubling or developing ESRD,[68] while a study of patients with autosomal dominant polycystic kidney disease or IgA nephropathy did not show any effect on the development of ESRD.[69] In children with SCD, shorter *HMOX1* GT repeats were associated with lower rates of hospitalization for acute chest syndrome.[45] Consistent with the shorter GT-tandem repeat length polymorphism having a protective effect in SCD,[45] we observed increased eGFR with the shorter allele. We did not observe an association between the *HMOX1* rs743811 or the GT-tandem repeat variant with *HMOX1* expression in peripheral blood mononuclear cells. This may be explained by differences between expression of *HMOX1* in peripheral blood mononuclear cells and the kidneys or the variants may alter Hmox1 activity, which should be investigated in future studies.

There are several limitations to our study. The concentrations of hemoglobin, KIM-1, and urine albumin to creatinine ratios were determined from random urine samples and future studies collecting urine in a controlled and longitudinal manner are warranted. The samples sizes in our cohorts were small and may be underpowered to identify SNP associated with markers of kidney disease. Genotyping was performed using different platforms in the UIC and Walk-PHaSST cohorts requiring imputation. The differences in genotyping platforms and imputation quality, along with clinical heterogeneity and variation in phenotyping between the UIC and Walk-PHaSST cohorts,

may explain the observed differences in the odds ratios between the two cohorts. We did not observe an association of the *HMOX1* SNP, rs743811, with gene expression in peripheral blood mononuclear cells. This may be due to differences in expression of *HMOX1* between peripheral blood mononuclear cells and the kidney or because the SNP may be influencing the function or structure of Hmox1. Our candidate gene approach was limited to well-characterized proteins involved in the metabolism of reactive oxygen species and heme.

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Authorship and Disclosures

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