**Soluble urokinase-type plasminogen activator receptor in sickle cell disease-associated chronic kidney disease**

**Supplemental Material**

## Materials and Methods

### *Samples collection*

The study was approved by the review board (IRB) of Howard University and all subjects provided written informed consent prior to the sample collection. Whole blood and urine samples were collected from 77 SCD patients and 10 healthy control subjects. The patient samples were collected during routine clinic visits when patients were at a steady state. The demographic characteristics of the cohort are shown in the Table 1.

### *ELISAs*

suPAR concentrations were measured in the plasma and urine samples by ELISA (ViroGates). Urinary creatinine concentrations were measured by Creatinine Parameter Assay kit (R&D Systems). Urinary suPAR concentrations were normalized by urinary creatinine concentrations. Plasma plasmin activity was evaluated using a fluorometric assay kit (Abcam). Plasma NE was detected by a quantitative sandwich ELISA assay (Abcam). uPA activity was evaluated by quantitative enzyme activity assay (Abcam).

### *Peripheral Blood Mononuclear Cells isolation and activation*

Peripheral Blood Mononuclear Cells (PBMCs) were isolated from whole blood using centrifugation on Ficoll Paque (GE Healthcare). PBMCs were washed three times with phosphate buffered saline (PBS) and resuspended in PBS to the concentration of $5x10^6$ cells/ml. PBMCs were

activated with phytohemagglutinin P (PHA) (0.5 μg/ml, Sigma-Aldrich) for 48 hours followed by human interleukin-2 (IL-2,10 U/ml, R&D Systems) for 24 hours.

### *THP-1 Differentiation*

Human THP-1 promonocytic cells were obtained from ATCC and differentiated into macrophages with 25 nM PMA (phorbol 12-myristate 13-acetate, Sigma-Aldrich) for 72 hours, and treated with either purified mutated human hemoglobin (HbS) or normal human hemoglobin (HbA) (5 μM, Sigma-Aldrich).

### *RNA Sequencing*

Total RNA was isolated from activated and non-activated PBMC, and THP1-derived macrophages using TRIzol™ Reagent (Invitrogen), depleted of cytoplasmic and mitochondrial ribosomal RNA, and strand-specific libraries were constructed using the Illumina® TruSeq Stranded Total RNA Gold kit. RNA strand-specific libraries were constructed using TruSeq Stranded Total RNA Gold kit (Illumina) and sequenced on an Illumina NextSeq 500 using 75 bp paired-end sequencing on two v2.5 150 cycle High-Output kits, generating 40-50 million paired-end reads per sample. The sequencing data were mapped to human reference genome using Dragen RNA v.3.8.4 software (Illumina). Comparisons were conducted using Dragen differential expression software v. 3.6.3 software for DESeq2 analysis (Illumina). Gene counts produced by Dragen were further normalized using a differential expression workflow for RNA Seq data based on the DESeq2 package from Bioconductor in R (https://github.com/genepattern/DESeq2).

### *Real-time RT-PCR*

Total RNA was isolated from PBMCs and THP-1 derived macrophages using using TRIzol™ Reagent (Invitrogen). First-strand cDNA was prepared from total RNA using the MultiScribe™

Reverse Transcriptase (Thermo-Fisher Scientific). RT-PCR was performed in triplicates using Roche LightCycler 480 SYBR Green I Master Mix (Roche Diagnostics) containing FastStart Taq Polymerase and double-strand DNA specific SYBR Green I dye (Fisher Scientific) was used for real-time RT-PCR by LightCycler® 480 System (Roche). The PLAUR gene, encoding uPAR, was amplify with primers: forward - 5'- AACGAGGGCCCAATCCTGGAGCTTGAA-3', reverse – 5'-GGTTTTTCGGTTCGTGAGTGC-3'. Human ribosomal protein RPS18 was amplified with primers: forward - 5'-GCAGAATCCACGCCAGTACAAG-3', reverse - 5'-GCTTGTTGTCCAGACCATTGGC-3' and was used for result normalization.

***Flow cytometry***

PBMCs ($5\times 10^5$ cells per sample) were blocked with 10% goat serum (Sigma -Aldrich) following incubation with human Fc block reagent (BD Biosciences) at $4^0$C. Cells were incubated with either mouse anti-human CD87-PE (uPAR) or IgG1-PE isotype control antibodies (both from BD Biosciences). Flow cytometry analysis was performed on FACS-Verse Cytometer (Becton Dickinson).

***Immunofluorescent staining***

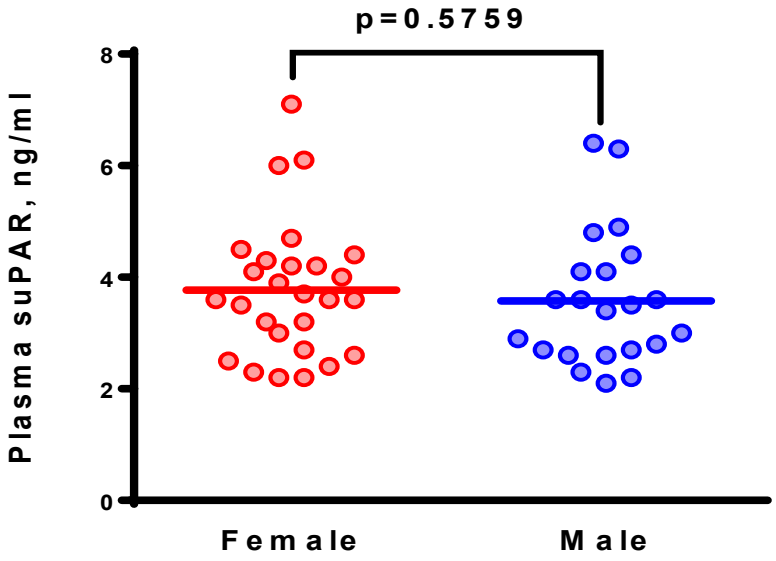
THP1-derived macrophages were treated with either HbS or HbA for 72 hours, fixed in 0.5% glutaraldehyde, and incubated with either mouse anti-human CD87-PE (uPAR) or IgG1-PE isotype control antibodies (both from BD Biosciences). Nuclei were counterstained with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI, Sigma-Aldrich). Images were acquired by Olympus IX51 microscope with Olympus DP72 camera. Quantification of uPAR expression was done in four different fields with 100 x original magnification.

***CKD characteristics***

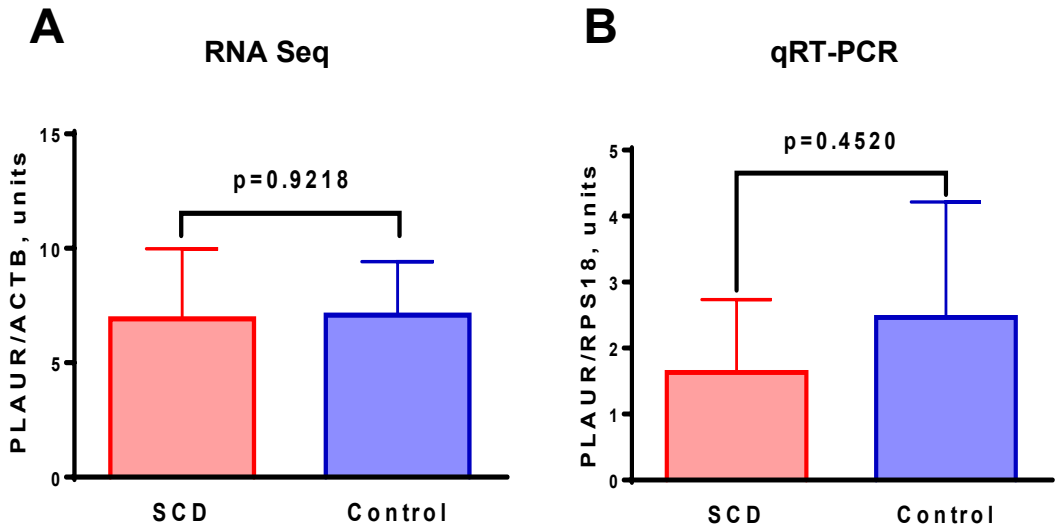
Levels of creatinine (CRE) in plasma, and albumin (ALB) and creatinine in urine were measured by ELISA. Estimated Glomerular Filtration Rate (eGFR) was calculated using the formular $eGFR = 141 \times min\,(CRE/\kappa, 1)^{\alpha} \times max(Cr/\kappa, 1)^{-1.209} \times 0.993^{Age} \times 1.018\ [if\ female] \times 1.159\ [if\ black]$. Where: CRE is serum creatinine in mg/dL, κ is 0.7 for females and 0.9 for males, α is -0.329 for females and -0.411 for males, min indicates the minimum of CRE /κ or 1, and max indicates the maximum of CRE /κ or 1. CKD stage was defined according to the National Kidney Foundation, Kidney Disease Outcomes Quality Initiatives (K/DOQI) guidelines: stage 0 - eGFR>60 ml/min/1.73m$^2$ and AL/CRE<30 mg/g; stage 1- eGFR>90 ml/min/1.73m$^2$ and AL/CRE≥30 mg/g; stage 2 – eGFR 60–89 ml/min/1.73m$^2$ and AL/CRE≥30 mg/g, stage 3 – eGFR 30 – 59 /1.73m$^2$; stage 4 – eGFR 15 – 29 ml/min/1.73m$^2$ and stage 5 – eGFR < 15 ml/min/1.73m$^2$

***Statistical analysis***

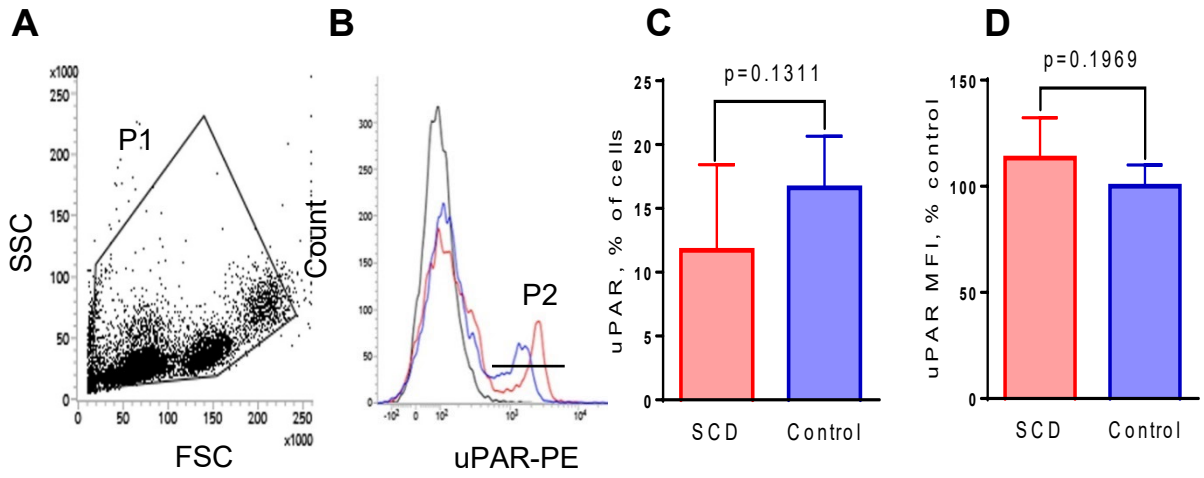
Data were analyzed and graphed using GraphPad Prism 6 software. All bar graphs showed mean ±SD. Unpaired Student's t-test was used to evaluate the statistical difference between two groups, and p values less than 0.05 were considered statistically significant. Pearson correlation analyses and Receiver Operating Characteristic (ROC) analysis were performed using GraphPad Prism 6 software.



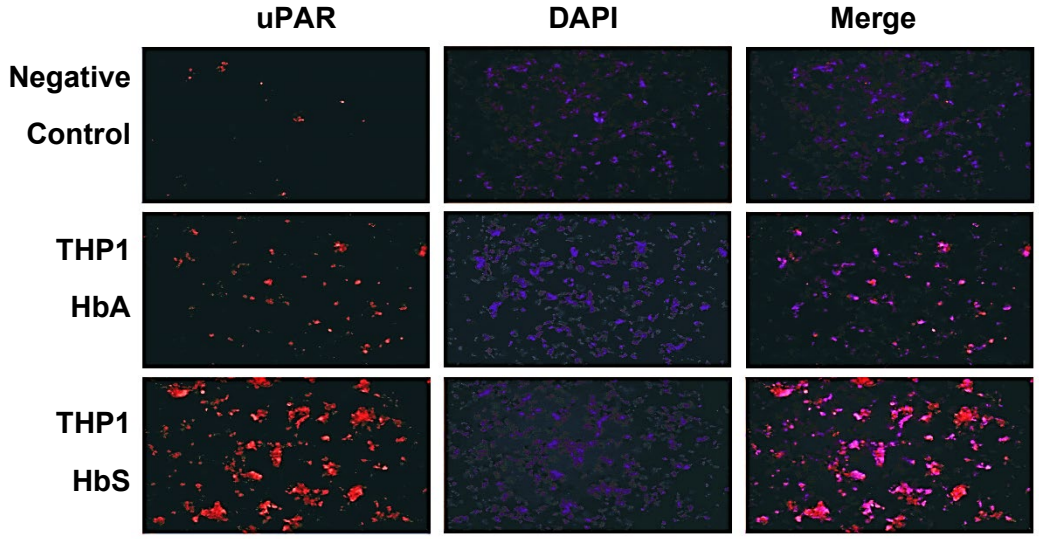

**Supplemental Figure 1. Similar plasma suPAR levels in SCD female and male patients**
Plasma suPAR levels were measured in SCD patients without CKD using suPARnostic ELISA Kit (ViroGates). Levels of PsuPAR are 3.77±0.2345 ng/ml for females (n=27), and 3.573±0.2567 ng/ml for males (n=22). Results for each patient and means for groups are shown.



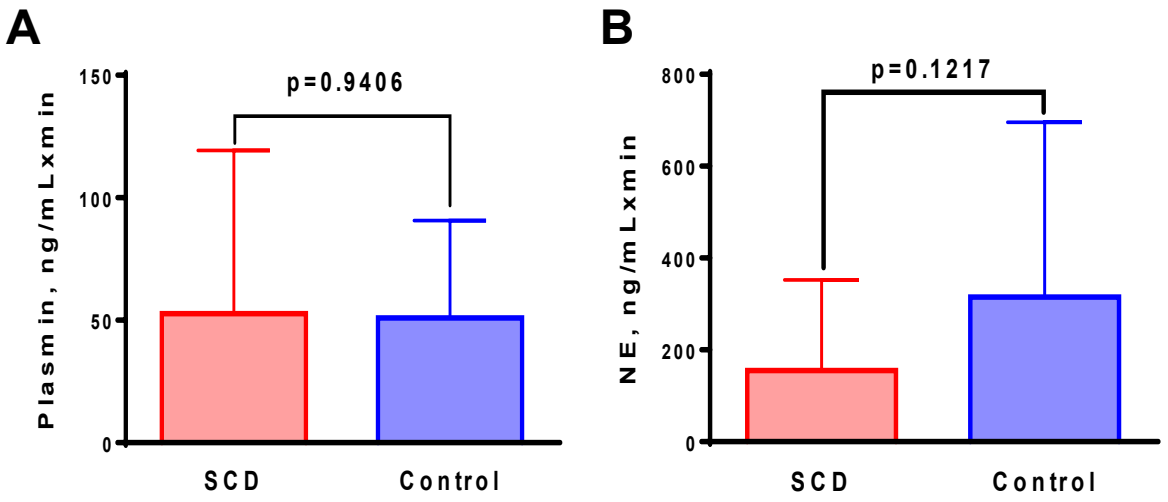

**Supplemental Figure 2. No significant difference in PLAUR gene expression in PBMCs collected from SCD patients and control subjects.**
(A) Analysis of PLAUR gene expression SCD and control PBMCs using RNA sequencing (Illumina). Actin beta was used for normalization (n=6). (B) Real-time RT-PCR for PLAUR gene expression. RPS18 was used for normalizations (n=3). Results are shown as mean ± SD. $p<0.05$ is considered statistically significant.



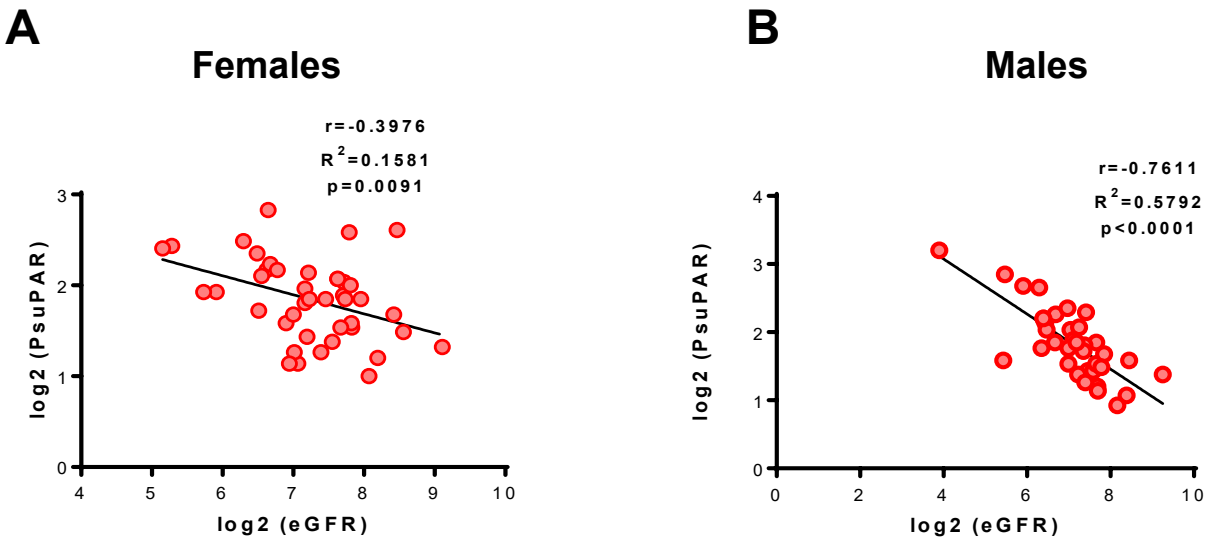

**Supplemental Figure 3. Flow cytometry of PBMC collected from SCD patients and control subjects.**
(A) Front scatter (FSC) vs. side scatter (SSC) plot represents PBMCs cell populations and P1 gate strategy. (B) Representative flow cytometry data for uPAR expression for cells located within P1 gate. Iso-control (black line), SCD PBMCs (red line) and control PBMCs (blue line). uPAR positive cells are shown in gate P2. (C) Percent of uPAR positive cells on SCD and control PBMCs within gate P2. (D) Mean of fluorescence intensity of uPAR on SCD and control PBMC. n=5. The results are shown as mean ± SD, $p<0.05$ is considered statistically significant.



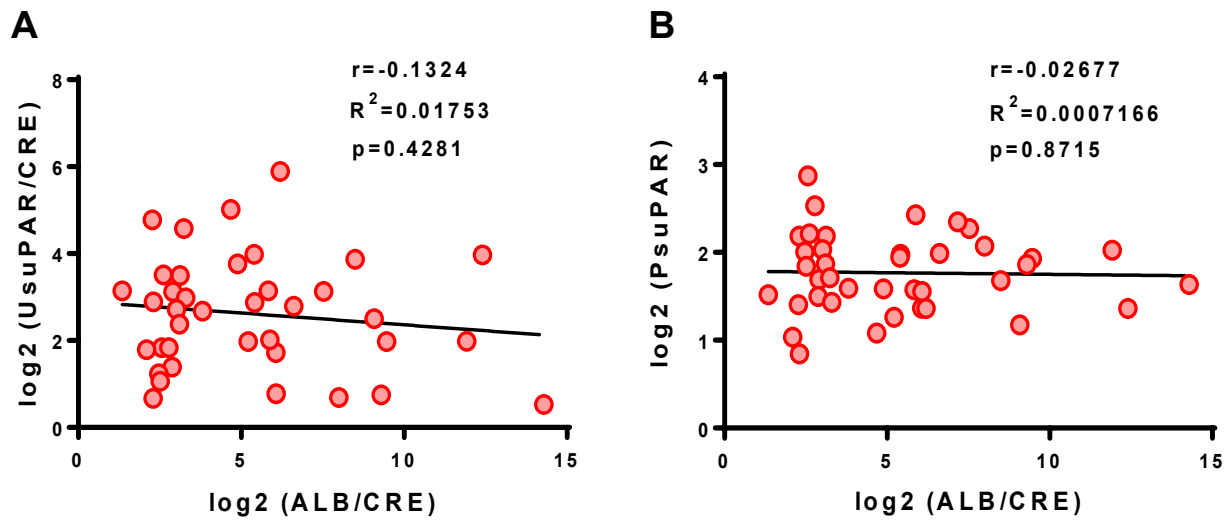

**Supplemental Figure 4. Higher levels of uPAR expression in human macrophages treated with mutated hemoglobin S compared to normal hemoglobin treatment.**
THP1 cells were differentiated into macrophages with 25 nM PMA (phorbol 12-myristate 13-acetate) for 72 hours and treated with either purified mutated hemoglobin (HbS) or normal hemoglobin (HbA) (5 µM). Immunofluorescent staining of THP1-derived macrophages with anti CD87-PE antibodies (uPAR, red color). DAPI (blue color) is used for nuclear staining. Mouse isotype IgG-PE antibodies are used as a negative control.




**Supplemental Figure 5. Similar activity of plasmin and neutrophil elastase (NE) in plasma of SCD patients and control subjects**
Plasma plasmin (A) and neutrophil elastase (NE) (B) activities were measured by ELISA. SCD n=24, control n=10. The results are shown as mean ± SD. $p<0.05$ is considered statistically significant.




**Supplemental Figure 6. eGFR strongly correlates with plasma suPAR in both female and male SCD patients.**
Pearson correlation of log2 (eGFR) with plasma log2 (suPAR) in female (A, n=41) and male (B, n=36) patients. Pearson correlation was performed using GraphPad Prism 6.




**Supplemental Figure 7. Correlation of urinary and plasma suPAR with albuminuria.** Pearson correlation of urinary (A) and plasma (B) log2 (suPAR) levels with albuminuria (log2 (ALB/CRE)). ALB- urinary albumin, CRE- urinary creatinine n=40. Pearson correlation was performed using GraphPad Prism 6.