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Soluble urokinase plasminogen activator receptor is associated with kidney disease and its progression in sickle cell anemia

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Running Title: suPAR in SCD

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Sickle Genome Project (SGP) individuals are available via St. Jude Cloud (https://platform.stjude.cloud/data/cohorts; accession no.: SJC-DS-1006) upon request and subsequent approval by the SGP steering committee. The WGS data are also available through dbGaP (phs002470.v1.p1). Individual phenotype data may be made available on request to the corresponding authors

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A rapid decline in estimated glomerular filtration rate (eGFR) predicts an increased risk of mortality in patients with sickle cell anemia (SCA).(1) Identifying early biomarkers and mechanisms for SCA-related chronic kidney disease (CKD) are critical to guide screening and intervention strategies. Soluble urokinase-type plasminogen activator receptor (suPAR) is released into circulation by enzymatic cleavage of urokinase-type plasminogen activator receptor (uPAR) from vascular endothelial cells or from activated white blood cells (WBC), such as neutrophils, T-lymphocytes, and monocytes.(2, 3) In podocytes, suPAR binds integrin αvβ3 resulting in foot process effacement and the development of focal segmental glomerulosclerosis (FSGS).(2) In tubular epithelial cells, suPAR binds integrin β6 increasing Smad3 signaling and interstitial fibrosis.(4) In addition to being a potential CKD biomarker, neutralizing antibodies inhibiting suPAR function are reno-protective in diabetic rats.(5)

SCA is a chronic inflammatory disease with WBC exhibiting an activated phenotype.(6) FSGS and interstitial fibrosis, pathologies associated with suPAR-related CKD in the general population, are common histopathologic features observed in kidneys of SCA mice and patients.(1) The associations of higher WBC counts with suPAR concentrations and of suPAR with CKD and its progression are unclear but may highlight high-risk patients and serve as a therapeutic target for SCA-related CKD.

We demonstrate that suPAR is independently associated with kidney dysfunction in transgenic mice, children and adults with SCA and predicts a rapid decline in kidney function on longitudinal follow-up.

Animal procedures were approved by the Institutional Animal Care and Use Committee at University of Illinois Chicago (UIC). Blood was collected from transgenic sickle mice

(Townes model, Jackson Laboratory; Bar Harbor, Maine) and WBC count (Advia 120 Hematology System; Siemens, Germany), cystatin C (CysC) (Thermo Fisher; Waltham, MA) and suPAR (R&D Systems; Minneapolis, MN) concentrations were measured.

Institutional review board approval and written informed consent were obtained before recruitment and biosample collection from both SCA (hemoglobin (Hb)SS or HbSβ^0-thalassemia genotype) cohorts during an outpatient visit when the patients were not experiencing a vaso-occlusive pain episode. All biosamples were frozen at -80^{0}C. Children were enrolled in the Sickle Cell Clinical Research and Intervention Program (SCCRIP); data through 12/2020 were considered. Plasma suPAR was measured using ViroGates (Brikerod, Denmark) ELISA kit and eGFR calculated utilizing the Chronic Kidney Disease in Children Study (CKID) Under 25 formula.(7) Baseline clinical and laboratory data from the closest values within three months of suPAR collection were used except for urine albumin-to-creatinine ratio (uACR), which was determined using the closest value within a year.

Between 10/2009-6/2018, 212 SCA adults were recruited from UIC and had blood samples available for measuring serum suPAR (R&D Systems; Minneapolis, MN) by ELISA. 147 patients had peripheral blood mononuclear cells (PBMCs) isolated for gene expression studies. There was a trend for lower eGFR in the subgroup of SCA patients with PBMCs vs. those without PBMCs (129 vs. 144mL/min/1.73m^2, respectively; P=0.07). Baseline clinical and laboratory data were collected at enrollment. The Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation (2021) was used to calculate eGFR.(8) CKD stage was defined according to the National Kidney Foundation guidelines.(9) All follow-up outpatient eGFR (n=2,831) and uACR (n=820) values were included in the slope analyses.

Genotyping and defining high-risk apolipoprotein L1 (*APOL1)* status was performed as previously described.(10) Messenger RNA from PBMCs was profiled using Affymetrix Human gene 2.0 ST array and data processing as previously described.(11) Gene expression levels of *PLAUR*, encoding uPAR, and *GPLD1*, encoding glycosylphosphatidylinositol specific phospholipase D1, were regressed on CKD stage, adjusting for age, sex, hydroxyurea (HU), lymphocytes and monocytes in logarithmic scale, and erythroid gene signature as a proxy of the amount of erythroid progenitors in circulation in PBMCs.(12)

Associations between suPAR and mouse Hb genotype, WBC, and CysC were conducted by the test for linear trend for univariable analyses. The independent associations of WBC and CysC with suPAR were performed using linear regression. The univariable associations with suPAR was performed using linear mixed effects models accounting for relatedness via kinship matrix (SCRRIP; R version 4.2.1; library GMMAT)(13) and the test for linear trend (UIC). In both cohorts, $P<0.0033$ was statistically significant after Bonferroni correction (0.05/15). Variables with $P<0.1$ in univariable analyses were considered for a joint model; backwards selection was implemented to select the final regression model for variables independently associated with suPAR. We evaluated the effect of suPAR on baseline eGFR with a linear mixed effects model (SCRRIP; R library GMMAT) adjusting for age, sex, HU and chronic transfusion use, and *APOL1*. The association of suPAR with eGFR decline was assessed using linear mixed effects model by restricted maximum likelihood, conditioning on random mean patient effects and adjusting for time (years from baseline) and suPAR-by-time interaction, with and without the following covariates: age, sex, diabetes, systolic blood pressure, body mass index, HU and angiotensin-converting enzyme/angiotensin receptor blocker use, baseline eGFR, and *APOL1* (UIC; R version 4.0.3). The association of suPAR with uACR progression was assessed similarly

with albuminuria as the dependent variable and adjusting for baseline uACR instead of baseline eGFR.

Circulating suPAR concentrations were higher in HbSS (n=20) versus HbAA (n=14) mice and associated with WBC counts and CysC levels (**Figure 1**). We did not observe differences in suPAR between male (n=10; 2016±604pg/mL) and female (n=10; 1994±666pg/mL) HbSS mice (P=0.9). The WBC count (natural log β=0.37±0.08; P<0.001) and CysC (natural log β=0.41±0.18; P=0.03) were independently associated with suPAR concentrations in multivariable analysis.

Baseline characteristics of the two cohorts are provided in **Supplementary Table 1**. In children, higher suPAR concentration was associated with female sex, chronic transfusion therapy, increased body mass index, increased WBC, decreased HbF, and not being on HU in univariable analysis (**Supplementary Table 2**). A lower eGFR, increased WBC, and female sex were independently associated with elevated suPAR concentrations (**Table 1**). Baseline eGFR decreased -14.84mL/min/1.73m^2 per one-unit increase in log-transformed suPAR (SE=5.95; P=0.013). This relationship persisted adjusting for age, sex, and HU and chronic transfusion use (β=-14.13; SE=6.13; P=0.021), and further adjustment for high-risk *APOL1* status (β=-12.76; SE=6.18; P=0.039).

In adults, higher suPAR concentration was associated with female sex, diabetes, increased WBC, lower Hb concentration, and lower eGFR in univariable analyses (**Supplementary Table 2**). A lower eGFR, increased WBC, female sex, diabetes, and lower HbF were independently associated with suPAR concentration (**Table 1**). With a median follow-up of 7.0 (interquartile range, 4.9-9.9) years, the estimated average annual change in eGFR was -2.0 (95% CI, -2.1 to -1.8) mg/mL/1.73m^2. We observed a more rapid decline in eGFR with higher

suPAR levels in the adjusted model (**Table 2**). The estimated average annual change in uACR was +28 (95% CI, +15 to +40) mg/g creatinine. A higher baseline suPAR level was independently associated with rapid uACR increase in unadjusted and adjusted models (**Table 2**).

Gene expression in PBMCs was assessed in 147 HbSS patients (median age 35 years, 54% female, 58% on HU, 22% with eGFR<90mg/mL/1.73m^2, 47% with uACR≥100mg/g) and 27 African Americans without SCA. Expression of *PLAUR* (β=0.3±0.06; P=7.3x10^{-6}) and *GPLD1* (β=0.18±0.04; P=8.7x10^{-6}) were increased in SCA patients versus controls. In SCA patients, increased *GPLD1* expression was independently associated with higher CKD stage (β=0.03±0.01; P=0.0035).

The source of circulating suPAR in SCA is unclear but may be from cleavage of uPAR from the endothelium or secretion from activated WBCs. WBCs from patients with sickle cell disease have increased *PLAUR* expression, the gene encoding uPAR, compared to WBCs from healthy controls after exposure to phytohemagglutinin or interleukin-2.(14) Our data demonstrates that expression of both *PLAUR* and *GPLD1*, the gene encoding the enzyme that cleaves uPAR into circulation, are increased in PBMCs from SCA patients versus controls. Furthermore, increased *GPLD1* expression is associated with worsening CKD stage. Female sex was associated with higher suPAR concentrations in both our pediatric and adult SCA cohorts. This has also been observed in the general population and partly attributed to body fat and visceral fat distribution but not to estradiol or total testosterone levels.(15)

In a cross-sectional study of 77 sickle cell disease adults, higher suPAR was associated with lower eGFR on univariable analysis.(14) Our study demonstrates that elevated suPAR concentrations are associated with kidney dysfunction in SCA at a young age, which remained

significant adjusting for high-risk *APOL1* status. Additionally, HbSS mice as young as three months old had higher suPAR concentrations compared to HbAA mice, highlighting suPAR as an early biomarker of kidney damage. To our knowledge, this is also the first study demonstrating higher suPAR concentrations predict a more rapid decline in eGFR and increase in uACR on longitudinal follow-up.

Our study is limited by suPAR being measured at a single time point. The relatively small sample size limits our ability to evaluate whether *APOL1* risk status or diabetes are effect modifiers for suPAR levels on CKD progression. The effects of sexual maturity on suPAR may help us better understand differences by sex. We observed lower suPAR concentrations with HU use in children but not adults on univariable analysis. This may be due to differences in HU dosing strategies or compliance. Chronic transfusions are used for SCA patients with severe complications and may explain our observation with higher suPAR in univariable analysis. suPAR may be a systemic endothelial damage biomarker, as observed in cardiac disease and severe COVID infection.(3) Gene expression studies were performed in a subset of SCA adults with a more severe phenotype due to costs which may explain the trend for a lower eGFR. Future, larger studies with multiple suPAR assessments, sexual maturation data, and evaluation of multiorgan injury can help address these limitations and identify suPAR thresholds for SCA patients at high-risk for CKD.

In conclusion, our study demonstrates the association of suPAR with decreased kidney function in children and adults with SCA as well as in a murine model of SCA. Kidney-specific therapies are urgently needed, and suPAR-mediated kidney inflammation and damage may serve as both a biomarker and a targetable pathway to prevent SCA-related CKD.

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Table 1: Clinical and laboratory variables independently associated with soluble urokinase plasminogen activator receptor concentrations in children and adults with Sickle Cell Anemia				
	St Jude Children's Research Hospital		**University of Illinois at Chicago**	
		P value		**P value**
eGFR (mL/min/1.73m^2)	-0.0017± 0.00062	0.0048	-0.0054 ± 0.0013	6.8 x 10^{-5}
WBC (natural log)	0.14 ± 0.026	1.56 x10^{-7}	0.3 ± 0.08	2.9 x 10^{-4}
Female	0.10 ± 0.03	0.0013	0.17 ± 0.06	0.0029
Diabetes	–	–	0.58 ± 0.23	0.013
Hemoglobin F (natural log)	–	–	-0.061 ± 0.031	0.049

β-coefficient ± standard error values provided
eGFR, estimated glomerular filtration rate; WBC, white blood cell count

Table 2: Association of baseline Soluble urokinase plasminogen activator receptor with estimated glomerular filtration decline and urine albumin to creatinine ratio progression in adults with sickle cell anemia

	Unadjusted Model	P value	Adjusted Model*	P value
eGFR decline (mL/min/1.73m^2 per year)	-7.7×10^{-5} (-1.7×10^{-4} to 1.1×10^{-5})	0.085	-1.1×10^{-4} (-1.9×10^{-4} to -1.7×10^{-5})	0.019
uACR increase (mg/g creatinine per year)	0.012 (0.005 – 0.019)	0.00070	0.013 (0.007 – 0.019)	5.2×10^{-5}

β-coefficient (95% confidence interval) provided for eGFR decline and CKD progression, respectively

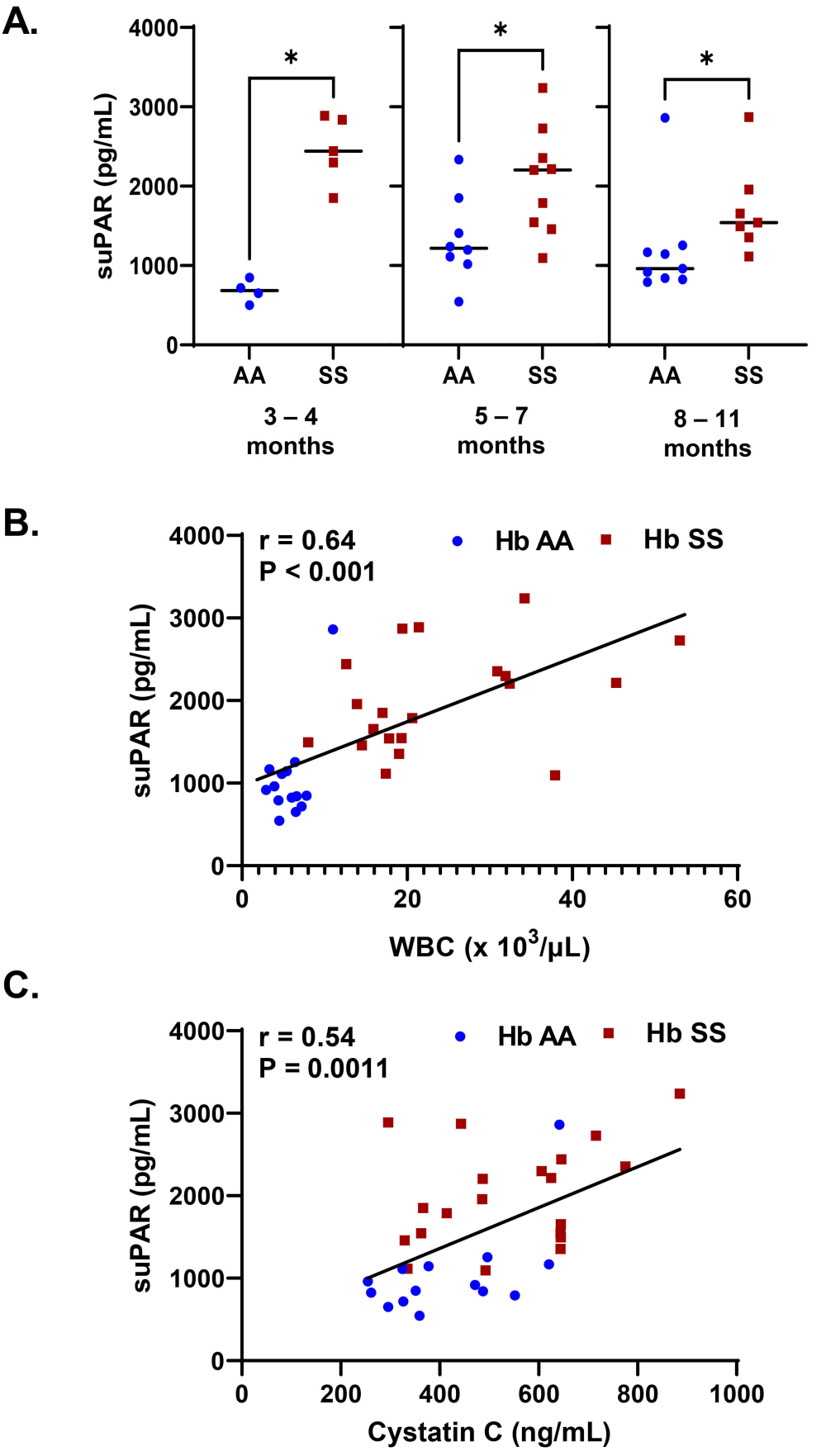
eGFR, estimated glomerular filtration rate; uACR, urine albumin-to-creatinine ratio; CKD, chronic kidney disease

β-coefficient represents the change in eGFR decline (mL/min/1.73m^2 per year) or in urine ACR increase (mg/g creatinine per year) that is associated with one pg/mL increase in suPAR.

*Adjusted for age, sex, diabetes, systolic blood pressure, body mass index, hydroxyurea and angiotensin converting enzyme inhibitor or angiotensin receptor blocker use, baseline eGFR or uACR, and *APOL1* high risk status.

Figure Legend

Figure 1: Soluble urokinase plasminogen activator receptor concentrations in transgenic sickle mice. A comparison of soluble urokinase plasminogen activator receptor concentrations in transgenic hemoglobin SS versus hemoglobin AA mice by **(A)** age, **(B)** white blood cell (WBC) count, and **(C)** cystatin C levels. * indicates $P < 0.05$.


A.
suPAR (pg/mL)
4000
3000
2000
1000
0
*
*
*
AA
SS
AA
SS
AA
SS
3 – 4 months
5 – 7 months
8 – 11 months
B.
r = 0.64
P < 0.001
Hb AA
Hb SS
suPAR (pg/mL)
WBC (x 10³/µL)
0
20
40
60
C.
r = 0.54
P = 0.0011
Hb AA
Hb SS
suPAR (pg/mL)
Cystatin C (ng/mL)
0
200
400
600
800
1000

Supplementary Table 1: Patient characteristics				
	N	**St Jude Children's Research Hospital**	**N**	**University of Illinois at Chicago**
Age (years)	245	11 (7 – 17)	212	29 (24 – 39)
Age (years) females	123	11 (11 – 17)	119	31 (25 – 39)
Age (years) males	122	11 (6 – 17)	93	28 (23 – 37)
Females, N (%)	245	123 (50%)	212	119 (56%)
Diabetes, N (%)	–	–	212	4 (2%)
Hydroxyurea, N (%)	245	206 (84%)	212	118 (56%)
Hydroxyurea dose (mg/kg/day)	–	–	118	14 (11 - 20)
RAAS blocker, N (%)	245	4 (2%)	212	35 (17%)
Chronic transfusion, N (%)	245	67 (27%)	212	23 (11%)
Systolic blood pressure (mmHg)	190	111 (103 – 118)	211	119 (110 – 128)
Body mass index (kg/m^2)	243	18 (16 - 22)	208	23 (21 – 26)
WBC (x 10^3/µL)	245	4.8 (3.0 – 6.7)	212	5.3 (3.6 – 7.1)
Hemoglobin (g/dL)	245	9.1 (8.1 – 9.9)	212	8.9 (8.0 – 9.7)
Absolute reticulocyte count (x10^3/µL)	–	–	209	328 (233 – 430)
Hemoglobin F (%)	226	14.8 (7.1 – 23.5)	209	6.1 (2.8 – 9.8)
LDH (u/L)	216	467 (373 – 589)	197	352 (264 – 460)
eGFR (mL/min/1.73m^2)	245	117 (100 – 133)	212	124 (109 – 133)
eGFR by strata, N (%) **≥ 120** **90 – 119** **60 – 89** **< 60**	245	 111 (45%) 101 (41%) 32 (13%) 1 (0.4%)	212	 130 (61%) 48 (23%) 21 (10%) 13 (6%)
uACR (mg/g creatinine)	60	22 (11 – 60)	179	43 (13 – 231)
uACR by strata, N (%) **< 30** **30 – 299** **≥ 300**	60	 36 (60%) 22 (37%) 2 (3%)	179	 77 (43%) 64 (36%) 38 (21%)
***APOL1* high risk, N (%)**	245	21 (9%)	208	28 (13%)

Median (interquartile ranges) provided, unless N (%) specified; RAAS, renin-angiotensin-aldosterone system, WBC, white blood cell count; LDH, lactate dehydrogenase; eGFR, estimated glomerular filtration rate; uACR, urine albumin-to-creatinine ratio

Supplementary Table 2: Association of clinical and laboratory variables with soluble urokinase plasminogen activator receptor concentrations in children and adults with sickle cell anemia.

	St Jude Children's Research Hospital	P value	University of Illinois at Chicago	P value
Age (years)	0.0036 ± 0.0033	0.27	0.005 ± 0.003	0.064
Females	0.11 ± 0.034	0.0019	0.18 ± 0.06	0.0027
Diabetes	–	–	0.77 ± 0.22	0.00054
Hydroxyurea	-0.16 ± 0.047	0.00047	-0.15 ± 0.06	0.81
RAAS blocker	–	–	-0.009 ± 0.083	0.9
Chronic Transfusion	0.17 ± 0.038	9.0×10^{-6}	0.21 ± 0.098	0.028
Systolic blood pressure (mmHg)	0. 41 ± 0.21	0.046	0.23 ± 0.26	0.38
Body mass index (kg/m^2)	0.27 ± 0.074	0.00024	0.16 ± 0.18	0.37
WBC (x 10^3/μL)	0.0055 ± 0.00090	5.9×10^{-10}	0.27 ± 0.06	1.8×10^{-5}
Hemoglobin (g/dL)	-0.12 ± 0.082	0.15	-0.09 ± 0.02	6.5×10^{-5}
Absolute reticulocyte count (x10^3/μL)	–	–	-0.05 ± 0.06	0.45
Hemoglobin F (%)	-0.056 ± 0.012	4.1×10^{-6}	-0.08 ± 0.03	0.013
LDH (u/L)	0.028 ± 0.053	0.60	0.04 ± 0.09	0.63
eGFR (mL/min/1.73m^2)	-0.0017 ± 0.00067	0.012	-0.005 ± 0.001	8.8×10^{-7}
uACR (mg/g creatinine)	0.033 ± 0.070	0.63	0.045 ± 0.018	0.013
***APOL1* high risk (%)**	0.14 ± 0.062	0.026	-0.13 ± 0.09	0.15

β-coefficient ± standard error values provided

RAAS, renin-angiotensin-aldosterone system, WBC, white blood cell count; LDH, lactate dehydrogenase; eGFR, estimated glomerular filtration rate; uACR, urine albumin-to-creatinine ratio

To approximate normality, suPAR, body mass index, uACR, LDH, and systolic blood pressure were log-transformed; WBC, hemoglobin, and hemoglobin F were square-root-transformed in children and suPAR, systolic blood pressure, body mass index, WBC, hemoglobin F, LDH, and uACR were log-transformed in adults with SCA.