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Increased urine semaphorin-3A is associated with renal damage in hypertensive patients with chronic kidney disease: a nested case–control study

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Abstract

Background—Semaphorins are guidance proteins implicated in several processes such as angiogenesis, organogenesis, cell migration, and cytokine release. Experimental studies showed that semaphorin-3a (SEMA3A) administration induces transient massive proteinuria, podocyte foot process effacement and endothelial cell damage in healthy animals. While SEMA3A signaling has been demonstrated to be mechanistically involved in experimental diabetic

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Conflict of interest Dr. Ganesan Ramesh filed a provisional patent for the discovery of semaphorin 3A as a biomarker of injury and disease. All other authors declared no competing interest.

glomerulopathy and in acute kidney injury, to date its role in human chronic kidney disease (CKD) has not been investigated.

Methods—To test the hypothesis that SEMA3A may play a role in human CKD, we performed a cross-sectional, nested, case–control study on 151 matched hypertensive patients with and without CKD. SEMA3A was quantified in the urine (USEMA) by ELISA. Glomerular filtration rate was estimated (eGFR) by the CKD-EPI formula and albuminuria was measured as albumin-to-creatinine ratio (ACR).

Results—USEMA levels were positively correlated with urine ACR ($p = 0.001$) and serum creatinine ($p < 0.001$). USEMA was higher in patients with both components of renal damage as compared to those with only one and those with normal renal function ($p < 0.007$ and <0.001, respectively). The presence of increased USEMA levels (i.e. top quartile) entailed a fourfold higher risk of combined renal damage ($p < 0.001$) and an almost twofold higher risk of macroalbuminuria ($p = 0.005$) or of reduced eGFR, even adjusting for confounding factors ($p = 0.002$).

Conclusions—USEMA is independently associated with CKD in both diabetic and non diabetic hypertensive patients. Further studies may help clarify the mechanisms underlying this association and possibly the pathogenic changes leading to the development of CKD.

Keywords

Albuminuria; Chronic kidney disease; Diabetes; Hypertension; Semaphorin-3A

Introduction

Greater knowledge of the cellular and molecular mechanisms underlying the development and progression of hypertensive and diabetic renal damage may lead to the identification of new biomarkers of disease progression and eventually to more effective treatment. Semaphorins are a large family of guidance proteins that have been implicated in several cellular developmental processes [1, 2]. Semaphorin-3a (SEMA3A) has been shown to be involved in a number of rather different biological mechanisms. Indeed, SEMA3A is known to regulate immune response by suppressing both T and B cell autoimmunity [1]. SEMA3A levels have been inversely related to disease activity and to the degree of renal damage in systemic lupus erythematosus and rheumatoid arthritis (RA) patients [3, 4]. Interestingly, both SEMA3A and its receptor neuropilin-1 are expressed in the developing kidney [5–7], and SEMA3A remains expressed in adult podocytes and collecting tubules [8]. While SEMA3A inhibits ureteric bud branching by downregulating the glial cell-line-derived neurotrophic factor [6], the regulation of SEMA3A expression in the kidney and its pathophysiological role are unknown.

In animal studies, administering SEMA3A induces acute and transient massive proteinuria [9]. Furthermore, SEMA3A is secreted into the urine in response to hypoxia, and preliminary studies suggest that urine SEMA (USEMA) could be a promising acute kidney injury (AKI) biomarker in critically ill patients [10]. While it has been hypothesized that SEMA3A signaling may be implicated in microvascular lesions and mesangiolysis in experimental diabetic nephropathy [11], to date its role in chronic kidney disease (CKD) has

never been investigated. We therefore decided to investigate the relationship between USEMA and the presence and degree of renal damage in a cross-sectional, case–control study on hypertensive patients with or without diabetes.

Subjects and methods

Study design and selection of patients

The cohort object of the present study was derived from the Italy-Developing Education and awareness on MicroAlbuminuria in patients with hypertensive Disease (I-DEMAND) study. Details on the design of the study, inclusion criteria and study procedures have been previously published [12, 13]. In brief, participants included patients between 18 and 80 years of age recruited in 87 centers of specialized care (Internal Medicine, Cardiology, Nephrology, Diabetology) with treated or untreated hypertension documented for at least 1 year. Regarding renal involvement, exclusion criteria were acute renal failure or rapid deterioration of renal function in patients with chronic renal failure, serum creatinine more than 3 mg/dl, secondary hypertension (with the exception of nephroparenchymal hypertension) and clinical signs of urinary tract infection. Glomerular filtration rate was estimated (eGFR) using the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation [14]. Reduced GFR (eGFR+) was defined as GFR <60 ml/min/1.73 m^2. Albuminuria was evaluated by measuring the urinary albumin-to-creatinine ratio (ACR) from a single urine specimen. Albuminuria was measured by immunonephelometry on an Immage Immunochemistry System (Beckman Coulter Inc., Fullerton, CA, USA) using the manufacturer's reagents. Macroalbuminuria (AlbU+) was defined as ACR ≥35 mg/mmol [15]. CKD was defined as either macroalbuminuria or GFR<60 ml/min/1.73 m^2.

In the present case–control study, a group of 151 age- (±5 years), gender-, diabetes-, and body mass index (BMI) (±1 point)-matched hypertensive patients were extracted from the original I-DEMAND database (n = 4,151) and pair-matched with a nested, case–control methodology to form four groups on the basis of the presence/absence of eGFR reduction, macroalbuminuria, both or neither.

Urinary biomarker measurement

Urine samples were spun at 10,000 rpm for 5 min and 50 μl supernatant was used for USEMA quantification by enzyme-linked immunosorbent assay (ELISA) (Cat # MBS732622, My Biosource, San Diego, CA, USA). Briefly, USEMA standard and samples were added to antibody-coated 96-well plates, after which 100 μl of conjugate was placed in each well. The plates were incubated at 37 °C for 1 h after mixing thoroughly, then washed and incubated with tetramethylbenzidine (TMB) substrate for 20 min and reaction was arrested by adding sulfuric acid. The color change was measured using a plate reader (BioTek Synergy HT, BioTek Instruments Inc., Winooski, VT, USA) at a wavelength of 450 nm. The concentration of SEMA3A in the samples was then determined by comparing the optical density of the samples to the standard curve. The minimum detectable level of SEMA3A is typically <0.1 ng/ml. All measurements were made in duplicate and in a blinded fashion.

Statistical analysis

Continuous variables are expressed as mean ± standard deviation (SD) or median with interquartile range as appropriate, and categorical variables are expressed as a percentage. Data that were not normally distributed, such as urinary SEMA3A/creatinine (USemaCR), ACR and triglycerides, were logarithmically transformed before analysis. Comparisons among groups were made by analysis of variation (ANOVA) for continuous variables and χ^2 test for categorical variables. Logistic regression analysis was used to describe the relationship between SEMA3A and the presence of CKD and its components. Odds ratios (OR) and 95 % confidence intervals (CI) were calculated by exponentiation of logistic regression coefficients. Statistical analyses were performed using Statview for Windows (SAS Institute Inc., version 5.0.1, Cary, NC, USA). A p value <0.05 was considered statistically significant.

Results

The study sample was composed of 151 patients aged 60 ± 6 years, 48 % males, and 49 % with type II diabetes. The main clinical characteristics of the study population as a whole and when analyzed on the basis of GFR and ACR values are shown in Table 1. Due to the study design, anthropometric, clinical and hemato-chemical characteristics were similar among the four groups of patients, except for waist circumference, serum uric acid and USEMA which increased along with the severity of renal damage.

Of note, USEMA increased significantly in patients with renal dysfunction even after adjustment for urinary creatinine excretion (USemaCR) (Fig. 1). USemaCR levels were positively correlated with urinary ACR (r 0.26, p = 0.001), and serum creatinine (r 0.27, p < 0.001), and inversely related to eGFR (r −0.29, p < 0.001). Patients with USemaCR above the median (i.e. >59.77 pg/mg) revealed a tendency to higher ACR levels (83 ± 131 vs. 50 ± 91, p = 0.065), significantly higher serum creatinine levels (1.41 ±0.54 vs. 1.11 ± 0.40, p < 0.001) and lower eGFR (54 ± 21 vs. 70 ± 22, p < 0.001) as compared to those with USemaCR below the median. The concomitant occurrence of both components of CKD was found more frequently in patients with USEMA above the median (39 vs. 12 %, p < 0.001).

Patients with either feature of renal damage showed significantly higher USEMA levels as compared with those without CKD. In particular, logarithmically transformed USemaCR was higher in patients with increased albuminuria (4.29 ± 0.86 vs. 3.78 ± 1.18 pg/mg, p = 0.003), or eGFR below 60 ml/min (4.27 ± 1.05 vs. 3.74 ± 1.02 pg/mg, p = 0.002). Conversely, there were no significant differences in USEMA levels on the basis of gender or presence/absence of diabetes. Moreover, the risk of being male, or of having diabetes was similar in all four USemaCR quartiles (data not shown). The presence of increased USEMA levels was significantly related to the occurrence of each component of renal damage we investigated. In particular, being in the USemaCR top quartile (i.e. ≥123.61 pg/mg; logarithmically transformed ≥4.817 pg/mg) entailed a fourfold (OR 4.15, 95 % CI 2.02–8.54, p < 0.001) higher risk of combined renal damage and an almost twofold higher risk of having macroalbuminuria or reduced eGFR even after taking into consideration potentially confounding factors such as serum uric acid and waist circumference (Table 2).

Discussion

The main finding of the present report is the relationship between the amount of USEMA and the severity of renal dysfunction in a group of hypertensive patients selected on the basis of presence/absence of diabetes, increased albuminuria and decreased eGFR. While it was recently reported that USEMA levels significantly increased shortly after cardiopulmonary bypass in pediatric patients who developed AKI [10], to the best of our knowledge this is the first time that its presence and degree has been studied in the urine of CKD patients. Our results extend, to the setting of CKD, literature findings which have thus far mostly indicated an association between increased USEMA and AKI both in animals and in humans. In particular, a single injection of recombinant SEMA3A has been shown to cause transient massive proteinuria, and glomerular damage in mice [9]. Recent studies have shown that USEMA levels increase within the first 2 h after the start of cardiopulmonary surgery in pediatric patients [10].

An increase in USEMA excretion during CKD might be due to greater systemic production, or overexpression at the renal level, two mechanisms which may lead to an abnormal leak of SEMA3A into the urine. As a matter of fact, SEMA3A serum levels have been found to be inversely related to disease activity and to the degree of renal damage in systemic lupus erythematosus patients [3, 4]. Moreover, in experimental models of ischemia–reperfusion in mice, circulating SEMA3A levels have been shown to rapidly downregulate after acute insult [10]. On the other hand, podocyte SEMA3A is known to play a crucial role during glomerular development, and in regulating endothelial cell migration and survival [5]. Since SEMA3A remains expressed in adult podocytes and collecting tubules [8], its urinary accumulation during renal pathological changes might be a consequence of increased local production. In fact, enhanced SEMA3A signaling has been shown in the podocyte of mice with advanced diabetic glomerulopathy [11] and it has been hypothesized that it may play a role in the pathogenesis of microvascular lesions and mesangiolysis in diabetic nephropathy.

It remains to be established whether the increased amount of USEMA we found in our CKD patients merely reflects local injury to glomerular structures or, rather, if it plays a mechanistic role in the development of renal damage. Previous experimental data suggest a pathogenetic role of exogenously administered SEMA3A in the induction of functional and ultrastructural changes at the glomerular filtration barrier level in vivo [9]. In fact, acute systemic SEMA3A injection has been reported to downregulate podocin and nephrin expression and to produce extensive fusion and effacement of podocyte processes, as well as glomerular endothelial cell swelling, thus resulting in massive proteinuria in healthy mice [9]. Furthermore, SEMA3A is thought to regulate integrin function in endothelial cells and has recently been demonstrated to act as a potent vascular permeability factor [16].

In the present study we found a significant relationship between USEMA and clinical signs of CKD such as albuminuria and eGFR. While eGFR is usually taken as a measure of glomerular function, albuminuria is thought to be a marker of both glomerular and tubular damage [17]. Therefore, increased urinary leak of SEMA may be secondary to multifactorial and complex mechanisms of damage. This hypothesis is strengthened by the results of multiple logistic regression, which suggest that the relationship between USEMA,

albuminuria and eGFR is independent of each other. Surprisingly, we did not find any differences in USEMA between diabetic and non diabetic patients. While enhanced SEMA3A signaling has been shown in the podocyte of mice with diabetic glomerulopathy [11] there are no data comparing USEMA levels in different renal diseases in the literature.

There are both limitations and strengths to our work that must be acknowledged. Among the former is the cross-sectional nature of the study design which obviously limits our ability to understand the pathogenetic mechanisms underlying the reported relationship. Unfortunately, SEMA3A serum measurements were not performed in our study and therefore we cannot rule out that increased USemaCR is a consequence of a gain in SEMA3A systemic production. Nor were we able to fully characterize the occurrence of increased USEMA with respect to different renal phenotypes since this abnormality seems to cluster with both isolated AlbU+ and eGFR reduction. Undoubtedly, further studies with a different clinical approach might help clarify the pathogenetic mechanisms underlying the reported associations. In this respect our study should be considered as hypothesis-generating. On the other hand, the nested case–control approach we used in our investigation could be considered a powerful means for looking at the association between USEMA and CKD components since it allowed us to compare several subgroups with similar clinical characteristics but different degrees and types of renal involvement from among a large cohort of patients.

In conclusion, for the first time we report an association between CKD components and USEMA, a protein believed to be implicated in cellular developmental processes, cytokine release, immune modulation and AKI. Our data should encourage further experimental and clinical research to clarify the role (pathogenetic and predictive) of USEMA in the context of CKD in diabetic and hypertensive patients.

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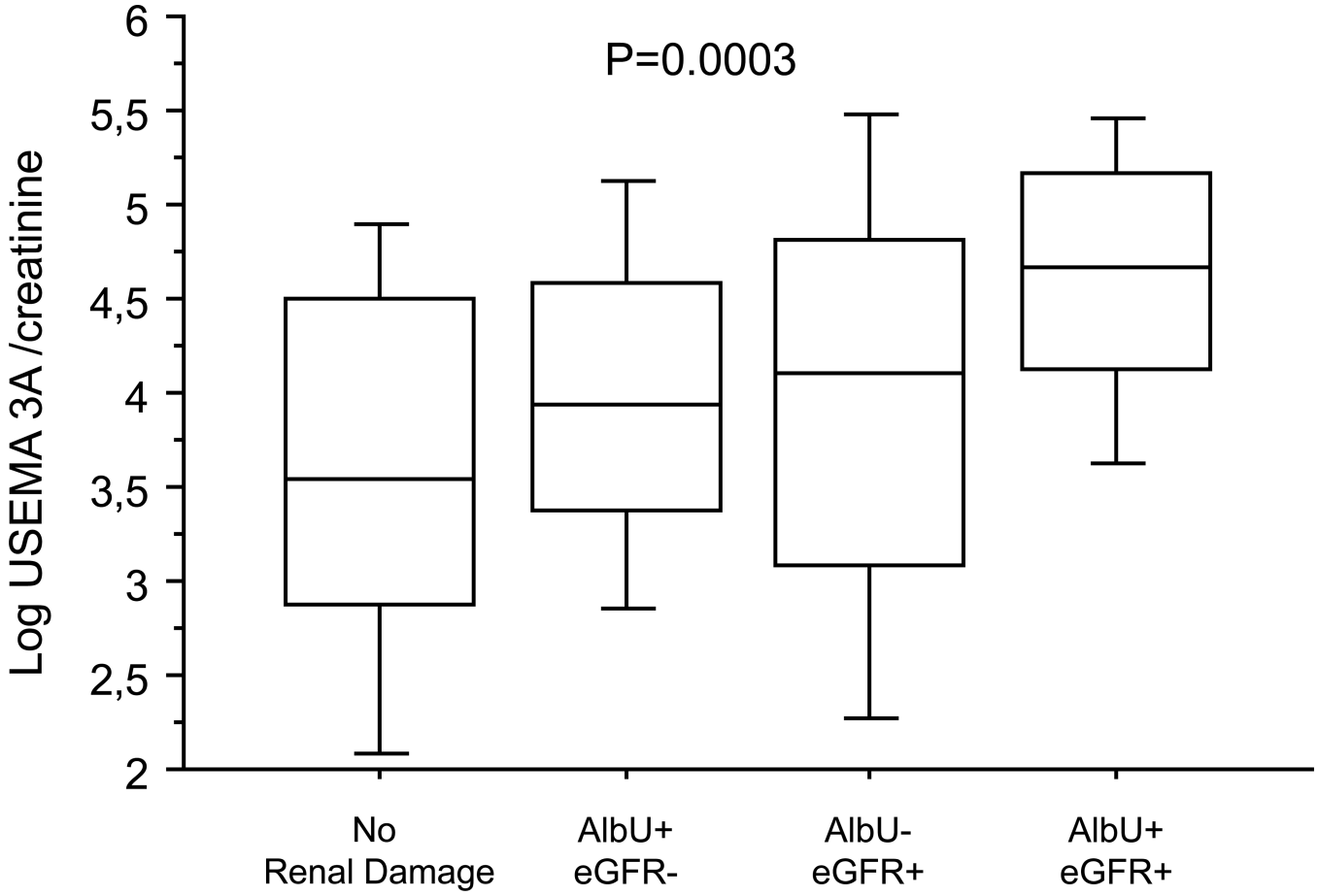

Fig. 1.

USEMA3A in hypertensive patients according to the presence/absence of macroalbuminuria and reduced GFR. To provide more detailed information about data distribution, the results are presented as *box*-and-*whisker plots*. The *central box* encloses the middle 50 % of the data; the *horizontal line* inside the *box* represents the median. *Vertical lines* (whiskers) extend from each end of the *box* and cover the distance between the 10th and 90th percentiles. *eGFR* estimated glomerular filtration rate, *eGFR*− eGFR ≥60 ml/min, *AlbU*− normoalbuminuria, *eGFR*+ eGFR <60 ml/min, *AlbU*+ macroalbuminuria. Data were logarithmically transformed before statistical analysis. *$p < 0.007$ as compared to those with only one marker of renal damage. **$p < 0.0001$ as compared to those with normal renal function

Table 1

Clinical characteristics of the study patients according to albuminuria and eGFR

Variable	All	eGFR− AlbU−	eGFR− AlbU+	eGFR+ AlbU−	eGFR+ AlbU+	p for trend
N	151	39	32	41	39	
eGFR (ml/min)	62 ± 23	82 ± 13	83 ± 16	50 ± 8	38 ± 12	<0.001
ACR	66.8 ± 114.1	1.0 ± 0.6	101.7 ± 83.0	0.8 ± 0.6	173.3 ± 154.7	<0.001
Diabetes (%)	49	49	44	49	51	0.938
Sex (% males)	48	49	44	49	49	0.969
Age (years)	60 ± 6	60 ± 1	60 ± 10	61 ± 2	61 ± 7	0.831
BMI (kg/m^2)	28.6 ± 4.8	27.7 ± 3.9	28.9 ± 6.0	28.7 ± 4.0	29.2 ± 5.4	0.672
WC (cm)	100 ± 12	96 ± 9	98 ± 14	100 ± 11	104 ± 13	0.042
Systolic BP (mmHg)	141 ± 18	138 ± 15	140 ± 21	140 ± 17	147 ± 20	0.143
Diastolic BP (mmHg)	83 ± 10	83 ± 9	81 ± 10	83 ± 8	86 ± 12	0.185
Mean BP (mmHg)	103 ± 12	101 ± 10	100 ± 13	102 ± 10	106 ± 13	0.133
Pulse pressure (mmHg)	58 ± 14	55 ± 10	59 ± 17	57 ± 15	61 ± 13	0.269
Fasting serum glucose (mg/dl)	130 ± 50	118 ± 37	148 ± 68	124 ± 47	134 ± 45	0.081
Serum creatinine (mg/dl)	1.3 ± 0.5	0.9 ± 0.2	0.9 ± 0.2	1.4 ± 0.3	1.8 ± 0.5	<0.001
Serum uric acid (mg/dl)	5.8 ± 1.7	5.1 ± 1.6	5.3 ± 1.4	5.9 ± 1.6	6.8 ± 1.6	0.006
Triglycerides (mg/dl)	165 ± 99	167 ± 128	158 ± 80	152 ± 60	182 ± 114	0.599
Cholesterol (mg/dl)	208 ± 43	204 ± 35	214 ± 39	206 ± 40	208 ± 57	0.803
HDL-cholesterol (mg/dl)	50 ± 13	51 ± 12	52 ± 11	46 ± 12	50 ± 16	0.222
LDL-cholesterol (mg/dl)	126 ± 39	122 ± 33	136 ± 41	131 ± 37	115 ± 43	0.147
USEMA (pg/ml)	61 ± 42	46 ± 32	53 ± 34	58 ± 30	85 ± 54	<0.001
USemaCR (pg/mg)	90 ± 91	69 ± 95	71 ± 61	93 ± 96	125 ± 97	0.0250
LogSEMA	1.66 ± 0.38	1.51 ± 0.41	1.62 ± 0.32	1.65 ± 0.40	1.87 ± 0.27	<0.001
LogUSemaCR	4.02 ± 1.07	3.60 ± 1.13	3.93 ± 0.84	3.97 ± 1.20	4.60 ± 0.75	<0.001
Antihypertensive treatment (%)	97	95	97	95	100	0.561
RAAS inhibitors (%)	86	75	87	90	87	0.551
Diuretics (%)	42	31	37	51	46	0.262
Antiplatelet therapy (%)	39	36	44	37	41	0.889
Statins (%)	42	49	34	36	49	0.442

Variable	All	eGFR− AlbU−	eGFR− AlbU+	eGFR+ AlbU−	eGFR+ AlbU+	p for trend
Glucose lowering drugs (%)	46	41	56	41	46	0.556

Data are mean ± standard deviation (SD) or percentage

eGFR estimated glomerular filtration rate, *eGFR−* eGFR ≥60 ml/min, *eGFR+* eGFR <60 ml/min, *AlbU−* normoalbuminuria, *AlbU+* macroalbuminuria, *ACR* urinary albumin creatinine ratio, *BMI* body mass index, *WC* waist circumference, *BP* blood pressure, *HDL* high density lipoprotein, *LDL* low density lipoprotein, *USEMA* urinary Semaphorin-3A, *UsemaCR* urinary semaphorin 3A creatinine ratio, *RAAS* renin–angiotensin–aldosterone system

Table 2

Odds ratios of renal damage for USEMA increase

Variable	eGFR+		AlbU+		Combined renal damage	
	OR (95 % CI)	p	OR (95 % CI)	p	OR (95 % CI)	p
Model 1	1.63 (1.18–2.26)	0.0033	1.61 (1.16–2.25)	0.0043	2.84 (1.69–4.75)	<0.0001
Model 2	3.06 (1.18–7.91)	0.0213	4.56 (1.68–12.37)	0.0029	16.00 (3.24–78.89)	0.0007
Model 3a	2.10 (1.30–3.40)	0.0025				
Model 3b	–	–	1.83 (1.20–2.80)	0.0052	–	–
Model 3c	–	–	–	–	4.15 (2.02–8.54)	0.0001

Model 1 for each 1 pg/mg increase of USemaCR
Model 2 USemaCR highest quartile (i.e. ≥123.61 pg/mg, ≥4.817 pg/mg when logarithmically transformed)
Model 3a for each 1 pg/mg increase of USemaCR also in model Log ACR, SUA and WC
Model 3b Model 1 + eGFR, SUA and WC
Model 3c Model 1 + eGFR, Log ACR, SUA and WC
eGFR estimated glomerular filtration rate, *eGFR+* eGFR <60 ml/min, *AlbU+* macroalbuminuria, *USemaCR* urinary semaphorin 3A to creatinine ratio, *OR* odds ratio, *CI* confidence intervals, *WC* waist circumference, *Log ACR* logarithmically transformed albumin-to-creatinine ratio, *SUA* serum uric acid