Association of Plasminuria with Overhydration in Patients with CKD

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Abstract

Background and objectives Hypervolemia is a common feature of patients with CKD and associated with hypertension. Recent work has shown stimulation of sodium retention by urinary plasmin during nephrotic syndrome. However, it is unclear whether plasminuria plays a role in patients with stable CKD and non-nephrotic proteinuria.

Design, setting, participants, & measurements In this cross-sectional study, we analyzed the fluid status of 171 patients with CKD consecutively presenting to our outpatient clinic from 2012 to 2013 using bioimpedance spectroscopy (Body Composition Monitor [BCM]; Fresenius Medical Care, Germany) and its associations to the urinary excretion of plasminogen and plasmin from a spot urine sample. Two–electrode voltage clamp measurements were performed in *Xenopus laevis* oocytes expressing human epithelial sodium channel to investigate whether plasmin in concentrations found in urine can activate the channel.

Results Overhydration >5% and overhydration >10% of the extracellular volume were found in 29% and 17% of the patients, respectively, and overhydration was associated with edema, hypertension, higher stages of CKD, and proteinuria. Proteinuria was the strongest independent predictor for overhydration (+0.58 L/1.73 m^2 per 10-fold increase; $P<0.001$). Urinary excretion of plasmin(ogen) quantified by ELISA correlated strongly with proteinuria ($r=0.87$) and overhydration ($r=0.47$). Using a chromogenic substrate, active plasmin was found in 44% of patients and correlated with proteinuria and overhydration. Estimated urinary plasmin concentrations were in a range sufficient to activate epithelial sodium channel currents *in vitro*. In multivariable analysis, urinary excretion of plasmin(ogen) was associated with overhydration similar to proteinuria.

Conclusions Hypervolemia in patients with CKD is strongly associated with proteinuria, even in the non-nephrotic range. Protein-rich urine contains high amounts of plasminogen and active plasmin, rendering plasminuria as a possible link between proteinuria and hypervolemia.

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Introduction

Sodium retention and edema are hallmarks of renal disease, particularly in patients with nephrotic syndrome. The pathogenesis of edema formation is still a matter of debate, and both underfill and overfill mechanisms have been put forward (1). Recent evidence points to the crucial role of proteinuria in promoting sodium retention through activation of the epithelial sodium channel (ENaC) (2), which is known to be an important player in sodium homeostasis and BP control (3). Studies with protein-rich urine from both nephrotic rats and patients have shown a stimulation of ENaC currents *in vitro* that was attributed to occurrence of the serine protease plasmin in the urine (4,5). It is an emerging concept that serine proteases contribute to ENaC regulation by cleaving specific sites in the extracellular domains of the α- and γ-subunits (6–10), resulting in the release of inhibitory peptides and activation of the channel (11–13). Proteolytic activation of ENaC by serine proteases as shown in native renal tissue (14) is enhanced by low-salt diet or aldosterone infusion (15) and might involve membrane-anchored prostasin (16) and/or tissue (urinary) kallikreins (17). Under pathophysiologic conditions of proteinuria, ENaC is thought to be aberrantly activated by plasmin that arises from filtered plasminogen and conversion by the tubular urokinase–type plasminogen activator (4,6).

Plasmin–induced ENaC activation and sodium retention may occur in patients with preeclampsia (5) and diabetic patients who are proteinuric (18,19). Recently, immunohistochemical studies in human nephrectomy specimens indicated that the γ-subunit of ENaC was processed proteolytically exclusively in patients with proteinuria (20). However, these data were generated from a small number of patients ($n=6$), and therefore, it remains unclear whether activation of ENaC by plasmin plays a role in the majority of patients with CKD who are proteinuric.

In this study, we analyzed the extracellular volume status with a focus on surrogates of sodium retention in a cohort of patients with nondialysis-dependent CKD to identify determinants of hypervolemia and test the role of urinary excretion of plasminogen and plasmin.

Materials and Methods

Patients

This cross-sectional study included stable ambulatory patients with CKD presenting consecutively for a routine follow-up at the outpatient department of the University Hospital of Tuebingen between September of 2012 and April of 2013. Patients with CKD stable for at least the last 6 months were included after they provided written informed consent (n=171). Patients were excluded when they had acute deterioration of kidney function (rise in plasma creatinine concentration by ≥1.5-fold), declined to participate (n=2), or failed to give a spot urine sample (n=17). Patients were classified according to the Kidney Disease Improving Global Outcomes classification of CKD. In addition, participants evaluated as potential kidney donors served as controls, and inpatients suffering from acute nephrotic syndrome were included for comparison. The study was in compliance with the Declaration of Helsinki and approved by the local ethics committee of the University of Tuebingen (259/2012MPG23).

Evaluation of Fluid Status

The fluid status of each patient was assessed by clinical examination and analysis of body composition using the Fresenius Body Composition Monitor (BCM). This device uses bioimpedance spectroscopy with a spectrum of 50 frequencies between 5 and 1000 kHz to estimate extracellular water (ECW) and intracellular water (ICW) and calculate the amount of overhydration (OH) (21,22). Reference values for OH are age independent and between −1 and +1 L. Values obtained for OH, ECW, and ICW were normalized to a body surface area of 1.73 m^2. In addition, ultrasonography was performed to analyze filling of the inferior cava vein and exclude pleural effusion or ascites, which are not determined by the BCM.

Laboratory Assays

Blood and spot urine samples were drawn from each patient during the outpatient visit in the morning. Some patients (n=69) also provided a 24-hour collected urine sample. Laboratory parameters were determined using automated Siemens autoanalyzers (Advia 1800 and Immulite 2000). N-terminal pro brain natriuretic peptide (NT-pro-BNP) was corrected for Modification of Diet in Renal Disease GFR according to the formula by Luchner *et al.* (23). Serum aldosterone and plasma renin activity were measured manually using RIA methods (Immunotech, Prague, Czech Republic and Zentech, Angleur, Belgium). Urinary albumin, IgG, and α1-microglobulin were measured using a nephelometric method with a Siemens BN ProSpec Analyzer.

Urinary plasmin(ogen) and urokinase concentrations were determined using ELISA kits as specified by the manufacturer (Loxo, Heidelberg, Germany). While the ELISA detects both plasminogen and plasmin (as indicated by plasmin[ogen]), urinary activity of plasmin was measured using the chromogenic S-2302 (Haemochrom, Essen, Germany); 50 μl urine was incubated with 2 mM S-2302 for 8 hours at 37°C with or without antiplasmin (final concentration of 20 μg/ml; Merck GmbH, Darmstadt, Germany). The difference of the ODs at 405 nm between these two conditions reflected the specific activity of plasmin.

For Western blot analysis, SDS-PAGE was performed with 12 μg urinary proteins per lane after depletion of albumin and IgG (Qiagen, Germantown, MD). Goat plasmin(ogen) antibody recognizing both intact plasminogen and plasmin was used as the primary antibody (AB6189; Abcam, Inc., Cambridge, MA). Bands were developed by chemiluminescence on a ChemiDoc Touch System (Bio-Rad, Hercules, CA).

Two–Electrode Voltage Clamp Measurements Using Human ENaC–Expressing Oocytes

Oocytes were collected from *Xenopus laevis* with the approval of the animal welfare officer for the University of Erlangen-Nürnberg as described (7,9,10). Defolliculated stages 5 and 6 oocytes were injected with complementary RNA encoding human α-, β-, and γ-ENaC (0.2 ng complementary RNA per subunit of ENaC). ENaC–mediated whole–cell currents were measured using the two–electrode voltage clamp technique as previously described (7,9,10).

Statistical Analyses

Data are provided as geometric means with the interquartile range. Plasma and urinary concentrations were $\log_{10}$ transformed to approximate normal distribution. Differences between the GFR and albuminuria strata and differences between whole–cell current measurements were analyzed with ANOVA. Adjusted differences between the GFR and albuminuria strata were analyzed using analysis of covariance. The association of continuous and categorical parameters with fluid status was analyzed by univariate parametric or nonparametric correlation. Categorical data were tested for significance using the chi-squared test. Multivariable linear regression analyses were performed to identify independent determinants. Selection of the variables entering the final least squares model was derived from forward stepwise linear regression of parameters that were univariately correlated with the dependent variable and had a P value <0.05. Identified parameters were confirmed by backward stepwise analysis. The residuals of each model were tested for normality. Ridge regression was applied to analyze the relationship between the highly collinear parameters proteinuria and plasmin(ogen)uria. Data analysis was performed using the statistical software packages JMP 11 and JMP 12 Pro (SAS Institute Inc., Cary, NC) and GraphPad Prism 4.03 (GraphPad Software, La Jolla, CA).

Results

Study Cohort

The study consecutively included 171 patients with CKD of various etiologies and stable disease and ten inpatients with acute nephrotic syndrome. Healthy candidates (n=15)

who were potential kidney donors were included as a control group. The characteristics of the study cohort are provided in Table 1.

Fluid Status of the Cohort and Its Determinants

We applied bioimpedance spectroscopy using the Fresenius BCM to assess the fluid status of each patient. In patients with CKD, median ECW and ICW values were 15.8 L/1.73 m^2 (interquartile range, 14.7–16.9) and 17.7 L/1.73 m^2 (interquartile range, 16.2–19.5), respectively, corresponding to an increased ECW-to-ICW ratio of 0.89 (interquartile range, 0.82–0.98) (Table 1). Median OH was +0.2/1.73 m^2 (interquartile range, −0.5–+1.2), which corresponded to 1.0% (interquartile range, −3.6–+7.0) of the ECW. OH>+1, >+2, or >+3 L/1.73 m^2 was found in 28%, 13%, and 4% of the patients, respectively. In patients with CKD and edema (n=63 or 37%), hypertension (n=139 or 81%), or loop diuretic use (n=66 or 39%), OH was higher by +1.45, +0.34, and +0.82 L/1.73 m^2, respectively, compared with in individuals without this finding. OH was strongly associated with the albuminuria stages of CKD (Figure 1A) and to a lesser extent, the GFR stages, which lost statistical significance on adjustment for proteinuria (Figure 1B). Furthermore, OH correlated positively with age, systolic BP, plasma creatinine concentration, and NT-pro-BNP concentration, whereas an inverse relationship to body mass index (BMI), serum aldosterone concentration, and plasma renin activity was found (Table 2). Among urinary parameters, only proteinuria was correlated with OH, whereas urinary Na^+ excretion or the Na^+-to-K^+ ratio determined from either spot urine or collected urine was not significantly correlated.

Table 1. Patient characteristics of the study cohort

Characteristic	Healthy, n=15	Patients with CKD, n=171	Patients with Acute Nephrotic Syndrome, n=10
Median age, yr	50 (42–62)	60 (48–72)	53 (41–69)
Sex distribution, women/men	73%/ 27%	42%/58%	40%/60%
BMI, kg/m^2	29.2 (25.5–30.4)	28.7 (25.6–32.1)	23.3 (20.5–26.3)
MDRD GFR, ml/min per 1.73 m^2	96 (76–106)	45 (30–68)	35 (20–69)
Proteinuria, mg/g crea	95 (77–185)	520 (146–1485)	6660 (5379–8023)
Albuminuria, mg/g crea	14 (7–16)	110 (29–850)	6050 (4841–7568)
Presence of edema, %	0	37	100
Systolic BP, mmHg	130 (117–135)	135 (125–150)	153 (120–165)
Medication			
Antihypertensive drug classes	No medication	3 (1–4)	4 (2–4)
ACE inhibitors	No medication	42%	10%
AT blockers	No medication	36%	60%
Loop diuretics	No medication	39%	60%
Thiazide diuretics	No medication	30%	30%
ENaC blockers	No medication	2%	0
MR blockers	No medication	3%	10%
ECW, L/1.73 m^2	14.8 (14.1–15.5)	15.8 (14.7–16.9)	18.0 (16.6–19.4)
ICW, L/1.73 m^2	17.4 (16.6–18.8)	17.7 (16.2–19.5)	18.3 (16.3–19.8)
OH, L/1.73 m^2	0 (−0.9–+0.2)	+0.2 (−0.5–+1.2)	+3.8 (+1.5–+5.7)
ECW-to-ICW ratio	0.85 (0.80–0.88)	0.89 (0.82–0.98)	1.03 (0.91–1.12)
Proportion with OH>+1 L/1.73 m^2	1 (7%)	48 (28%)	9 (90%)
Proportion with OH>+2 L/1.73 m^2	0	22 (13%)	7 (70%)
Proportion with OH>+3 L/1.73 m^2	0	7 (4%)	6 (60%)
Diagnoses	NA	Diabetic/hypertensive nephropathy and glomerulosclerosis (36%), GN (35%), interstitial disease (2%), polycystic kidney disease (4%), unknown (23%)	Membranous GN (30%), IgA GN (30%), membranoproliferative GN (10%), thrombotic microangiopathy (10%), microscopic polyangiitis (10%), unknown (10%)

Values reported are n (percentages) for categorical variables and medians (interquartile ranges) for continuous variables. MDRD GFR is the GFR determined using the MDRD formula. BMI, body mass index; MDRD, Modification of Diet in Renal Disease; crea, creatinine; ACE, angiotensin-converting enzyme; AT, angiotensin; ENaC, epithelial sodium channel; MR, mineralocorticoid receptor; ECW, extracellular water; ICW, intracellular water; OH, overhydration; NA, not applicable.

Multivariable analysis revealed that, in addition to BMI, presence of edema, corrected NT-pro-BNP, and plasma renin activity, proteinuria was the strongest independent predictor of OH (Table 3). There were no two-way or multiway interactions among these variables. Sex, BMI, presence of edema, and proteinuria were independent predictors of ECW (Table 3).

Characterization of Urinary Plasmin(ogen) and Plasmin Excretion

Because of the proposed role of plasmin in activating ENaC during proteinuria, we further characterized the urinary excretion of plasminogen and plasmin. Median plasmin(ogen) excretion in patients with CKD was 19 μg/g creatinine (interquartile range, 2–227) and correlated

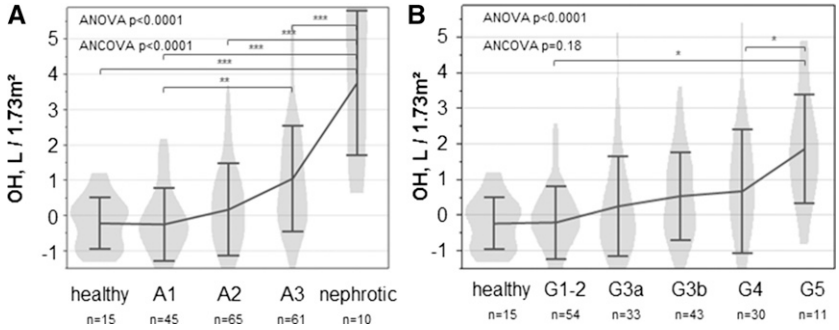

Figure 1. | Overhydration (OH) in patients with CKD in relation to GFR and albuminuria stages. OH in relation to different stages of (A) albuminuria and (B) GFR. For comparison, values of *n*=15 healthy donors and *n*=10 patients with acute nephrotic syndrome are depicted. Values depicted are contours of data density, mean, and SD. Analysis of covariance (ANCOVA) was adjusted for significant covariates derived from multiple linear regression (corrected plasma N-terminal pro brain natriuretic peptide ([NT-pro-BNP,] edema, body mass index, plasma renin activity, and proteinuria). A1–A3 denote albuminuria stages corresponding to <30, 30–300, and >300 mg/g creatinine. G1–G5 denote GFR stages corresponding to >60 (G1–G2), 45–59 (G3a), 30–44 (G3b), 15–29 (G4), and <15 ml/min per 1.73 m^2 (G5). *P<0.05; **P< 0.01; ***P<0.001.

Table 2. Univariate correlations of the different parameters with fluid status in patients with CKD (*n*=171)

Parameter	OH, L/1.73 m^2		ECW, L/1.73 m^2		ICW, L/1.73 m^2	
	r	*P* Value	*r*	*P* Value	*r*	*P* Value
Age, yr	0.20	0.01	0.28	<0.001	−0.18	0.02
Sex: 1, man; 2, woman	−0.19	0.13	−0.21	<0.001	−0.52	<0.001
BMI, log kg/m^2	−0.23	0.003	0.38	<0.001	0.07	0.36
Edema: 0, not present; 1, present	0.47	<0.001	0.49	<0.001	−0.07	0.21
Use of loop diuretics: 0, no; 1, yes	0.24	<0.001	0.31	<0.001	−0.12	0.12
No. of antihypertensive drug classes	0.16	0.05	0.44	<0.001	−0.12	0.16
Systolic BP, log mmHg	0.23	0.002	0.32	<0.001	0.06	0.41
Plasma creatinine concentration, log mg/dl	0.34	<0.001	0.27	<0.001	−0.04	0.62
MDRD GFR, log ml/min per 1.73 m^2	−0.21	<0.001	−0.26	0.02	−0.12	0.03
Proteinuria, log mg/g crea	0.45	<0.001	0.29	<0.001	−0.03	0.71
Urinary Na^+ excretion, log mmol/g crea	0.06	0.17	0.27	0.02	0.25	0.44
Log urinary Na^+-to-K^+ ratio	0.09	0.24	0.13	0.09	0.03	0.68
Plasma NT-pro-BNP concentration, log pg/ml	0.55	<0.001	0.19	0.01	−0.41	<0.001
Corrected plasma NT-pro-BNP concentration, log pg/ml	0.50	<0.001	0.14	0.04	−0.39	<0.001
Serum aldosterone concentration, log pg/ml	−0.22	0.004	−0.11	0.16	0.03	0.71
Plasma renin activity, log ng Ang L/ml per hour	−0.23	0.003	−0.12	0.12	0.06	0.43
Plasma albumin, log g/dl	−0.12	0.13	0.04	0.59	0.07	0.39
Diameter of inferior cava vein during expiration, log mm/1.73 m^2	0.16	0.05	0.02	0.79	0.11	0.20

MDRD GFR is the GFR determined using the MDRD formula. Corrected plasma NT-pro-BNP concentration is the concentration of NT-pro-BNP corrected for MDRD GFR. Values reported are Pearson *r* or Spearman rho values and levels of significance. OH, overhydration; ECW, extracellular water; ICW, intracellular water; BMI, body mass index; MDRD, Modification of Diet in Renal Disease; crea, creatinine; Na^+, sodium; K^+ potassium; NT-pro-BNP, N-terminal pro brain natriuretic peptide; Ang, angiotensin II.

strongly with proteinuria (Figure 2A). In 44% of patients with CKD, active plasmin was detectable in the urine (0.47 relative units/g creatinine; interquartile range, 0.05–2.58) (Figure 2B) and correlated highly with proteinuria (r=0.63; P<0.001); this was not observed in the healthy controls (Figure 2B). Patients with acute nephrotic syndrome showed the highest plasmin(ogen) excretion (2459 μg/g creatinine; interquartile range, 1079–5850) and plasmin activity (17 relative units/g creatinine; interquartile range, 4–34). Western blot analysis revealed plasmin and smaller fragments in the urine, which had higher values among patients with higher stages of albuminuria and reached highest values in patients who were nephrotic (Figure 3A). Plasminogen was barely detectable in the urine (in contrast to plasma), suggesting a high degree of conversion to plasmin and subsequently, smaller fragments. Urinary plasmin(ogen) concentrations were three orders of magnitude higher in patients with albuminuria stage A3 or nephrotic syndrome (Figure 3B).

The relationship between urinary plasmin(ogen) excretion and markers of glomerular or tubular proteinuria was investigated by measuring albumin, IgG, and α1-microglobulin. Urinary plasmin(ogen) was strongly correlated to albumin and IgG (r=0.69–0.84) and to lesser extent, α1-microglobulin (r=0.61). There was a weak correlation to the urinary urokinase concentration (r=0.40) that modestly increased with higher albuminuria stages (Figure 3C).

Activation of ENaC Currents by Plasmin

To study whether urinary plasmin concentrations found in patients are sufficient to stimulate sodium retention *via* activation of ENaC, two–electrode voltage clamp measurements using human ENaC–expressing oocytes were performed. As shown in Figure 3D, ENaC–mediated whole–cell

Table 3. Correlates of fluid status as determined by multivariable regression

Covariate	Estimate (95% Confidence Interval)	*P* Value	Adjusted Incremental r^2
OH adjusted r^2=0.49; P<0.001			
y Intercept	3.76 (0.55 to 6.97)	0.02	
Proteinuria, log mg/g crea	0.58 (0.30 to 0.85)	<0.001	0.19
Corrected plasma NT-pro-BNP concentration, log pg/ml	0.65 (0.36 to 0.94)	<0.001	0.36
Edema: 1, present	0.48 (0.29 to 0.67)	<0.001	0.43
BMI, log kg/m^2	−4.14 (−6.15 to 2.13)	<0.001	0.47
Plasma renin activity, log ng Ang L/ml per hour	−0.20 (−0.47 to 0.07)	0.15	0.49
ECW adjusted r^2=0.55; P<0.001			
y Intercept	5.51 (2.06 to 8.97)	0.002	
Sex: 1, man	0.80 (0.62 to 0.99)	<0.001	0.32
Edema: 1, present	0.59 (0.39 to 0.79	<0.001	0.48
BMI, log kg/m^2	6.45 (4.23 to 8.67)	<0.001	0.55
Proteinuria, log mg/g crea	0.37 (0.07 to 0.66)	0.02	0.55
ICW adjusted r^2=0.45; P<0.001			
y Intercept	23.05 (21.70 to 24.40)	<0.001	
Sex: 1, man	1.55 (1.23 to 1.87)	<0.001	0.25
Corrected plasma NT-pro-BNP concentration, log pg/ml	−1.27 (−0.79 to 1.76)	<0.001	0.38
Age, yr	−0.05 (−0.03 to 0.07)	<0.001	0.45

Parameters were selected by a stepwise approach with P<0.05 for retention. OH, overhydration; crea, creatinine; NT-pro-BNP, N-terminal pro brain natriuretic peptide; BMI, body mass index; Ang, angiotensin I; ECW, extracellular water; ICW, intracellular water.

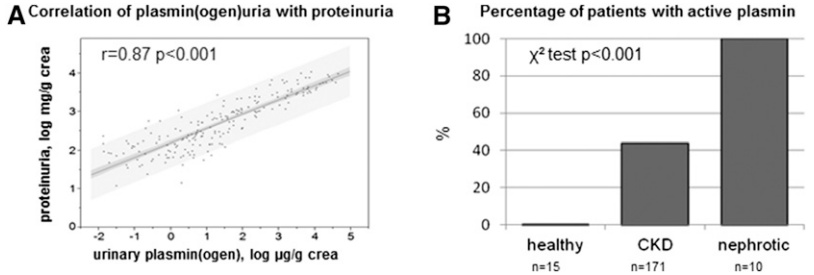

Figure 2. | Urinary plasmin(ogen)uria and plasmin activity. (A) Correlation of plasmin(ogen)uria with proteinuria in n=171 patients with CKD and (B) percentage of patients with CKD with active plasmin compared with healthy persons and patients with nephrotic syndrome. The shaded area represents the confidence interval of fit and prediction. crea, creatinine.

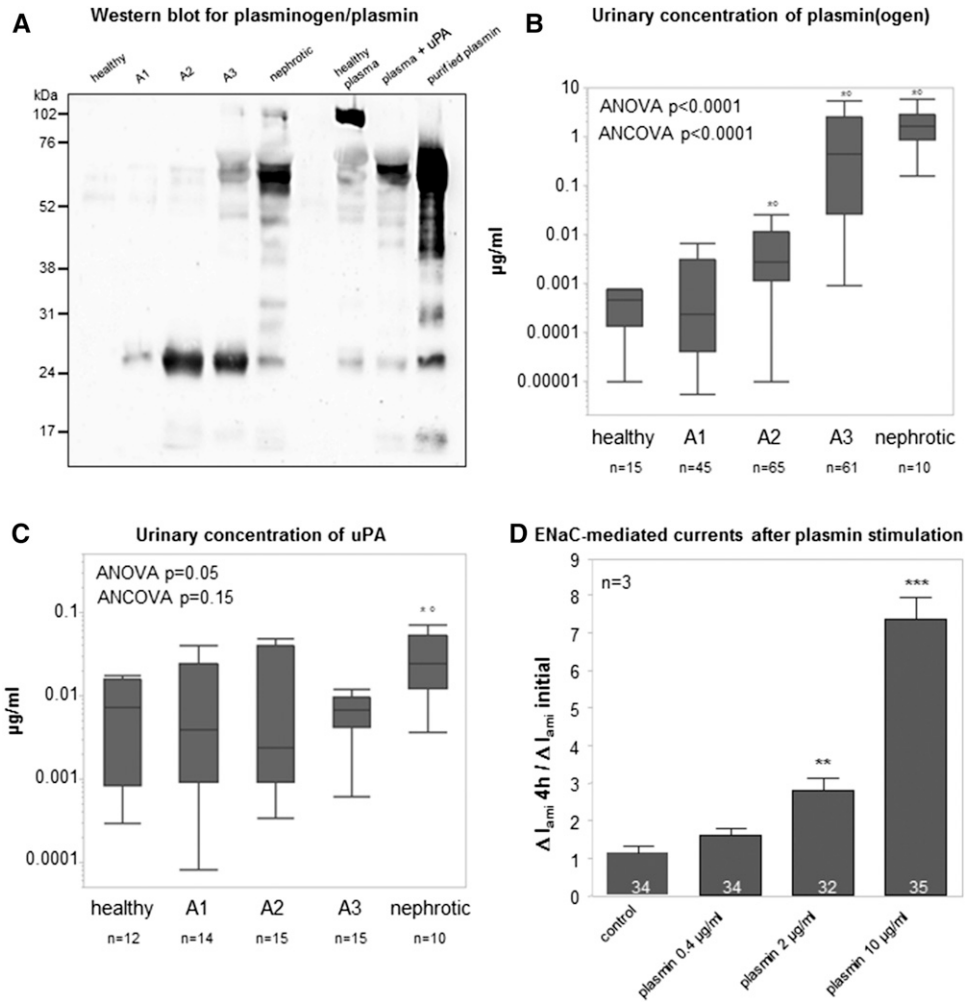

Figure 3. | Characterization of urinary plasmin(ogen) in patients with CKD and its relation to urinary urokinase concentration and stimulation of epithelial sodium channel (ENaC) currents *in vitro*. (A) Western blot of representative urine samples and plasma showing both plasminogen (91 kD) and plasmin (72 kD) with additional low molecular mass fragments. Note the increasing pattern of plasmin as albuminuria stages are increased and the absence of plasmin in the plasma. Addition of urokinase–type plasminogen activator (uPA) to plasma resulted in the occurrence of a plasmin band. A lane with purified plasmin served as the control. (B and C) Urinary plasmin(ogen) and urokinase concentrations by ELISA stratified by the albuminuria stages. For comparison, values of healthy donors and patients with acute nephrotic syndrome are included. Analysis of covariance (ANCOVA) was adjusted for proteinuria, corrected plasma NT-pro-BNP, edema, body mass index, and plasma renin activity (parameters selected from multivariable analysis). A1–A3 denote albuminuria stages corresponding to <30, 30–300, and >300 mg/g creatinine. *Significant difference to the healthy group; °Significant difference to the previous group. (D) ENaC–mediated whole–cell currents can be activated *in vitro* by plasmin used in concentrations similar to those observed in urine samples from patients. Amiloride–sensitive whole–cell currents (ΔI_{ami}) were measured in *Xenopus laevis* oocytes heterologously expressing human ENaC before (ΔI_{ami} initial) and after exposure of the oocytes for 4 hours (ΔI_{ami} 4h) to a control solution or solutions containing different concentrations of plasmin. Columns represent the relative stimulatory effect on ΔI_{ami} calculated as the ratio of ΔI_{ami} 4h to ΔI_{ami} initial. Numbers inside the columns indicate the numbers of individual oocytes measured; *n* indicates the number of different batches of oocytes. *P<0.05; **P< 0.01; ***P<0.001.

currents were stimulated by exogenous human plasmin In a concentration-dependent manner, reaching the highest values in concentration ranges that corresponded to the urinary plasmin(ogen) concentration of patients with stage 3 albuminuria and nephrotic syndrome.

Association of Urinary Plasmin(ogen) and Plasmin Excretion with Fluid Status

There was a strong univariate relationship between urinary plasmin(ogen) excretion and extracellular volume or OH (Figure 4). Urinary plasmin(ogen) excretion also correlated positively with NT-pro-BNP (r=0.40; P<0.001). In multivariable analysis, urinary plasmin(ogen) and plasmin were an independent predictor of OH similar to proteinuria (Table 4). Because of collinearity, both protein and plasminuria could replace each other in the models, and they lost their significance when included in the same model (Supplemental Table 1). Ridge regression to discriminate the contribution of each parameter revealed parallelism of both parameters throughout the whole model (Supplemental Figure 1).

Discussion

This study shows that the urinary excretion of protein is the strongest predictor of hypervolemia in an unselected CKD cohort with stable disease. This was most evident in patients with acute nephrotic syndrome who exhibited

Table 4. Multivariable regression analysis with proteinuria, urinary excretion of plasmin(ogen), and plasmin activity as independent variables

Predictor	OH (95% Confidence Interval); *P* Value		ECW (95% Confidence Interval); *P* Value	
	Univariate	Multivariable[a]	Univariate	Multivariable[b]
Proteinuria, log mg/g crea	0.99 (0.69 to 1.29); *P*<0.001	0.58 (0.30 to 0.85); *P*<0.001	0.78 (0.39 to 1.17); *P*<0.001	0.37 (0.07 to 0.66); *P*=0.02
Urinary plasmin(ogen) excretion, log μg/g crea	0.42 (0.30 to 0.54); *P*<0.001	0.23 (0.11 to 0.34); *P*<0.001	0.36 (0.20 to 0.52); *P*<0.001	0.18 (0.06 to 0.30); *P*=0.004
Urinary plasmin activity, log RU/mg crea	0.37 (0.16 to 0.57); *P*<0.001	0.21 (0.04 to 0.37); *P*=0.01	0.30 (0.04 to 0.55); *P*=0.02	0.16 (−0.02 to 0.34); *P*=0.07

Values reported are estimates (95% confidence intervals) and *P* values from univariate and multivariable regression analyses. OH, overhydration; ECW, extracellular water; crea, creatinine; RU, relative unit.
[a]Adjusted for body mass index, edema, plasma renin activity, and corrected plasma N-terminal pro brain natriuretic peptide (NT-pro-BNP).
[b]Adjusted for sex, body mass index, and edema.

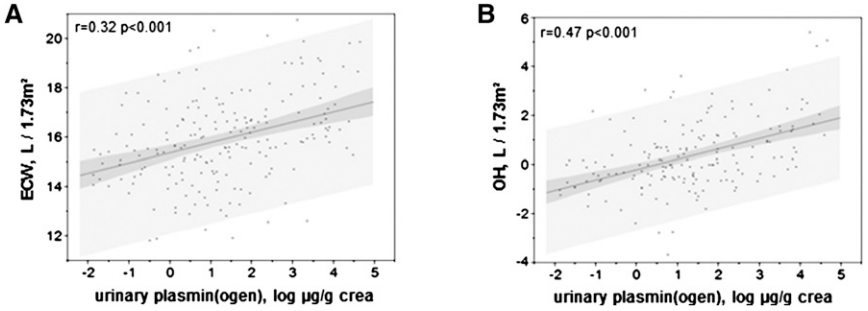

Figure 4. | Correlation of urinary excretion of urinary plasmin(ogen) with extracellular volume and overhydration (OH). The shaded area represents the confidence interval of fit and prediction. crea, creatinine; ECW, extracellular water.

extreme values for both proteinuria and hypervolemia. A novel finding of this study is the continuous relationship between proteinuria and hypervolemia, which also applies to patients with CKD and low-range proteinuria who are commonly referred to as being non-nephrotic. It is remarkable that the protein excretion determined from a single spot urine sample was highly predictive of hypervolemia, whereas measures of urinary sodium excretion were not. As a clinical consequence of hypervolemia, patients who were proteinuric more often had edema and hypertension, which prompted treatment with loop diuretics and a higher number of antihypertensive drugs (Table 2).

The data are consistent with the notion that plasminogen along with its active form, plasmin, may be involved in sodium retention during proteinuria. Importantly, we showed that plasmin concentrations in the range of those observed in urine samples of patients with CKD were sufficient to activate ENaC currents *in vitro*. In previously published studies, higher plasmin concentrations have been used to show its activating effect on ENaC (9,10,24,25). However, the exposure time to plasmin in those studies was relatively short (5–30 minutes), whereas in this study, plasmin at comparatively low concentrations showed a stimulating effect on ENaC after 4 hours of exposure. *In vivo*, even lower plasmin concentrations may be sufficient to activate ENaC, because the channels are constantly exposed to the plasmin–containing tubular fluid.

Plasminogen is a large protein, with 91 kD (26) secreted as inactive zymogen from the liver and activated on limited proteolysis by distinct enzymes (urokinase–type and tissue plasminogen activators). Because of its size, it is largely withheld by the intact glomerulus. However, after glomerular injury, larger amounts of plasminogen can be filtered and activated in the tubulus lumen. Urokinase–type plasminogen activator lining the tubular epithelium is thought to be the principal enzyme responsible for the generation of active urinary plasmin (2). However, serine protease inhibitors, such as antiplasmin (27) or uristatin (28), have been found to enter the tubular lumen during proteinuria, adding to the complexity of the situation. It is noteworthy that, in a proteomic study analyzing active serine proteases in the urine of healthy participants, minute amounts of both active plasmin and plasminogen were found (29). In agreement with this study, we also detected plasminogen in the urine of healthy participants using ELISA; however, no activity was noted (Figure 2). We hypothesize that glomerular disease increases the amount of urinary plasminogen to become pathophysiologically relevant.

Aldosterone is the major hormone regulating the final urinary Na^+ concentration by stimulation of ENaC. In

addition to its effects on gene expression and membrane abundance, aldosterone has also been found to stimulate channel activity by proteolysis, possibly by upregulation of the serine protease prostasin (30), which was formerly referred to as channel activating protease 1 (31). The contribution of ENaC to sodium retention is thought to be small when aldosterone secretion is suppressed (*e.g.*, by a high-salt diet or volume expansion as observed in our study). In this situation, proteolytic ENaC activation by aberrantly filtered serine proteases, such as plasmin, may become important, and it may lead to nonphysiologic sodium retention and exacerbate hypervolemia. The findings of our study are clearly in favor of a primary overfill in proteinuric renal disease, which is corroborated by increased NT-pro-BNP secretion.

The limitations of this study are caused by its associative character. It cannot be ruled out that proteins or mechanisms other than plasminogen account for the strong association with hypervolemia. Because of the high collinearity between proteinuria and urinary plasminogen as well as plasmin excretion, these parameters could replace each other in multivariable models and could not be separated by using more sophisticated statistical approaches. Other studies in the field, such as the ones by Buhl *et al.* (5,19), which showed an activating effect of protein-rich urine on ENaC activity *in vitro*, were also associative. Nevertheless, the data provide strong evidence that proteinuria exerts a strong influence on hypervolemia in a continuous relationship. Thus far, studies on plasminuria have concentrated on a limited number of patients with single-disease etiologies (preeclampsia [5] or diabetes mellitus [18,19]). The results of this large study were derived from an unselected CKD cohort with different etiologies, making the results highly generalizable.

Identification of patients with high plasminogen excretion might be helpful to select those who could benefit from a treatment with amiloride, a blocker of ENaC and urokinase. Thus, in addition to directly inhibiting the channel, amiloride may reduce tubular plasmin generation from filtered plasminogen. In a recent study by Oxlund *et al.* (32), the addition of amiloride to a triple-antihypertensive regimen lowered the BP in patients with treatment-resistant hypertension who were proteinuric and was paralleled by a tendency for reduced urinary plasminogen activation.

In conclusion, hypervolemia in patients with CKD is strongly associated with proteinuria, even in the nonnephrotic range. Protein-rich urine contains high amounts of plasminogen and active plasmin, rendering plasminuria a possible link between proteinuria and hypervolemia.

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Disclosures

None.

References

1. Bockenhauer D: Over- or underfill: Not all nephrotic states are created equal. *Pediatr Nephrol* 28: 1153–1156, 2013
2. Svenningsen P, Andersen H, Nielsen LH, Jensen BL: Urinary serine proteases and activation of ENaC in kidney–implications for physiological renal salt handling and hypertensive disorders with albuminuria. *Pflugers Arch* 467: 531–542, 2015
3. Rossier BC: Epithelial sodium channel (ENaC) and the control of blood pressure. *Curr Opin Pharmacol* 15: 33–46, 2014
4. Svenningsen P, Bistrup C, Friis UG, Bertog M, Haerteis S, Krueger B, Stubbe J, Jensen ON, Thiesson HC, Uhrenholt TR, Jespersen B, Jensen BL, Korbmacher C, Skøtt O: Plasmin in nephrotic urine activates the epithelial sodium channel. *J Am Soc Nephrol* 20: 299–310, 2009
5. Buhl KB, Friis UG, Svenningsen P, Gulaveerasingam A, Ovesen P, Frederiksen-Møller B, Jespersen B, Bistrup C, Jensen BL: Urinary plasmin activates collecting duct ENaC current in preeclampsia. *Hypertension* 60: 1346–1351, 2012
6. Svenningsen P, Friis UG, Bistrup C, Buhl KB, Jensen BL, Skøtt O: Physiological regulation of epithelial sodium channel by proteolysis. *Curr Opin Nephrol Hypertens* 20: 529–533, 2011
7. Diakov A, Bera K, Mokrushina M, Krueger B, Korbmacher C: Cleavage in the gamma-subunit of the epithelial sodium channel (ENaC) plays an important role in the proteolytic activation of near-silent channels. *J Physiol* 586: 4587–4608, 2008
8. Rossier BC, Stutts MJ: Activation of the epithelial sodium channel (ENaC) by serine proteases. *Annu Rev Physiol* 71: 361–379, 2009
9. Haerteis S, Krappitz M, Diakov A, Krappitz A, Rauh R, Korbmacher C: Plasmin and chymotrypsin have distinct preferences for channel activating cleavage sites in the γ subunit of the human epithelial sodium channel. *J Gen Physiol* 140: 375–389, 2012
10. Haerteis S, Krappitz A, Krappitz M, Murphy JE, Bertog M, Krueger B, Nacken R, Chung H, Hollenberg MD, Knecht W, Bunnett NW, Korbmacher C: Proteolytic activation of the human epithelial sodium channel by trypsin IV and trypsin I involves distinct cleavage sites. *J Biol Chem* 289: 19067–19078, 2014
11. Kleyman TR, Carattino MD, Hughey RP: ENaC at the cutting edge: Regulation of epithelial sodium channels by proteases. *J Biol Chem* 284: 20447–20451, 2009
12. Haerteis S, Schaal D, Brauer F, Brüschke S, Schweimer K, Rauh R, Sticht H, Rösch P, Schwarzinger S, Korbmacher C: An inhibitory peptide derived from the α-subunit of the epithelial sodium channel (ENaC) shows a helical conformation. *Cell Physiol Biochem* 29: 761–774, 2012
13. Kashlan OB, Blobner BM, Zuzek Z, Carattino MD, Kleyman TR: Inhibitory tract traps the epithelial Na+ channel in a low activity conformation. *J Biol Chem* 287: 20720–20726, 2012
14. Jacquillet G, Chichger H, Unwin RJ, Shirley DG: Protease stimulation of renal sodium reabsorption in vivo by activation of the collecting duct epithelial sodium channel (ENaC). *Nephrol Dial Transplant* 28: 839–845, 2013
15. Frindt G, Palmer LG: Acute effects of aldosterone on the epithelial Na channel in rat kidney. *Am J Physiol Renal Physiol* 308: F572–F578, 2015
16. Carattino MD, Mueller GM, Palmer LG, Frindt G, Rued AC, Hughey RP, Kleyman TR: Prostasin interacts with the epithelial Na+ channel and facilitates cleavage of the γ-subunit by a second protease. *Am J Physiol Renal Physiol* 307: F1080–F1087, 2014
17. Patel AB, Chao J, Palmer LG: Tissue kallikrein activation of the epithelial Na channel. *Am J Physiol Renal Physiol* 303: F540–F550, 2012
18. Andersen H, Friis UG, Hansen PB, Svenningsen P, Henriksen JE, Jensen BL: Diabetic nephropathy is associated with increased urine excretion of proteases plasmin, prostasin and urokinase and activation of amiloride-sensitive current in collecting duct cells. *Nephrol Dial Transplant* 30: 781–789, 2015
19. Buhl KB, Oxlund CS, Friis UG, Svenningsen P, Bistrup C, Jacobsen IA, Jensen BL: Plasmin in urine from patients with type 2 diabetes and treatment-resistant hypertension activates ENaC in vitro. *J Hypertens* 32: 1672–1677, 2014
20. Zachar RM, Skjødt K, Marcussen N, Walter S, Toft A, Nielsen MR, Jensen BL, Svenningsen P: The epithelial sodium channel γ-subunit is processed proteolytically in human kidney. *J Am Soc Nephrol* 26: 95–106, 2015
21. Moissl UM, Wabel P, Chamney PW, Bosaeus I, Levin NW, Bosy-Westphal A, Korth O, Müller MJ, Ellegård L, Malmros V, Kaitwatcharachai C, Kuhlmann MK, Zhu F, Fuller NJ: Body fluid

volume determination via body composition spectroscopy in health and disease. *Physiol Meas* 27: 921–933, 2006
22. Chamney PW, Wabel P, Moissl UM, Müller MJ, Bosy-Westphal A, Korth O, Fuller NJ: A whole-body model to distinguish excess fluid from the hydration of major body tissues. *Am J Clin Nutr* 85: 80–89, 2007
23. Luchner A, Weidemann A, Willenbrock R, Philipp S, Heinicke N, Rambausek M, Mehdorn U, Frankenberger B, Heid IM, Eckardt KU, Holmer SR: Improvement of the cardiac marker N-terminal-pro brain natriuretic peptide through adjustment for renal function: A stratified multicenter trial. *Clin Chem Lab Med* 48: 121–128, 2010
24. Svenningsen P, Uhrenholt TR, Palarasah Y, Skjødt K, Jensen BL, Skøtt O: Prostasin-dependent activation of epithelial Na+ channels by low plasmin concentrations. *Am J Physiol Regul Integr Comp Physiol* 297: R1733–R1741, 2009
25. Passero CJ, Mueller GM, Rondon-Berrios H, Tofovic SP, Hughey RP, Kleyman TR: Plasmin activates epithelial Na+ channels by cleaving the gamma subunit. *J Biol Chem* 283: 36586–36591, 2008
26. UniProt Consortium: PLG–Plasminogen Precursor–Homo sapiens (Human). Available at: http://www.uniprot.org/uniprot/P00747. Accessed May 5, 2015
27. Vaziri ND, Gonzales EC, Shayestehfar B, Barton CH: Plasma levels and urinary excretion of fibrinolytic and protease inhibitory proteins in nephrotic syndrome. *J Lab Clin Med* 124: 118–124, 1994
28. Nacken R, Artunc F, Bertog M, Haerteis S, Korbmacher C: Protease inhibitors in nephrotic urine may prevent proteolytic activation of the epithelial sodium channel (ENaC) by urinary proteases. *Acta Physiol Suppl* 694: P085, 2013
29. Navarrete M, Ho J, Krokhin O, Ezzati P, Rigatto C, Reslerova M, Rush DN, Nickerson P, Wilkins JA: Proteomic characterization of serine hydrolase activity and composition in normal urine. *Clin Proteomics* 10: 17, 2013
30. Narikiyo T, Kitamura K, Adachi M, Miyoshi T, Iwashita K, Shiraishi N, Nonoguchi H, Chen LM, Chai KX, Chao J, Tomita K: Regulation of prostasin by aldosterone in the kidney. *J Clin Invest* 109: 401–408, 2002
31. Vallet V, Chraibi A, Gaeggeler HP, Horisberger JD, Rossier BC: An epithelial serine protease activates the amiloride-sensitive sodium channel. *Nature* 389: 607–610, 1997
32. Oxlund CS, Buhl KB, Jacobsen IA, Hansen MR, Gram J, Henriksen JE, Schousboe K, Tarnow L, Jensen BL: Amiloride lowers blood pressure and attenuates urine plasminogen activation in patients with treatment-resistant hypertension. *J Am Soc Hypertens* 8: 872–881, 2014

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