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Hinrichs, Gitte Rye; Michelsen, Jannie Solmunde; Zachar, Rikke; Friis, Ulla Glenert; Svenningsen, Per; Birn, Henrik; Bistrup, Claus; Jensen, Boye L

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ALBUMINURIA IN KIDNEY TRANSPLANT RECIPIENTS IS ASSOCIATED WITH INCREASED URINARY SERINE PROTEASES AND ACTIVATION OF THE EPITHELIAL SODIUM CHANNEL

AUTHORS

Gitte R. Hinrichs¹, Jannie S. Michelsen², Rikke Zachar¹, Ulla G. Friis¹, Per Svenningsen¹, Henrik Birn³, Claus Bistrup², Boye L. Jensen¹

AFFILIATIONS

¹University of Southern Denmark, Department of Cardiovascular and Renal Research, Odense, Denmark
²Odense University Hospital, Department of Nephrology, Odense, Denmark
³Aarhus University, Department of Biomedicine, and Aarhus University Hospital, Department of Renal Medicine, Aarhus, Denmark

AUTHORS CONTRIBUTIONS

Gitte R. Hinrichs, Claus Bistrup and Boye L Jensen: Participated to the conception and design of the work, performance of the research, data analysis, the interpretation of the data, drafting of the work and writing of the manuscript. Jannie S. Michelsen: Participated in performance of the research and data collection.

Rikke Zachar and Per Svenningsen: Contributed with data analysis, in particular the exosome analysis and writing of the manuscript. Ulla G. Friis: Contributed with patch clamp experiment and drafting of the manuscript. Henrik Birn: Contributed to interpretation of the data and writing of the manuscript.

RUNNING HEAD

ENaC activity in albuminuric kidney transplant recipients
CORRESPONDING AUTHOR

Gitte Rye Hinrichs MD

Department of Cardiovascular and Renal Research

Institute of Molecular Medicine

University of Southern Denmark

J. B. Winsløws Vej 21,3., DK-5000 Odense C, Denmark.

Telephone: +45 6550 3796. Fax: +45 6613 3479

E-mail: ghinrichs@health.sdu.dk
ABSTRACT

Albuminuria predicts adverse renal outcome in kidney transplant recipients. The present study addressed the hypothesis that albuminuria is associated with increased urine serine proteases with the ability to activate the epithelial sodium channel (ENaC) and with greater extracellular volume and higher blood pressure. In a cross-sectional design, kidney transplant recipients with (n=18) and without (n=19) albuminuria were included for office blood pressure measurements, estimation of volume status by bioimpedance, and collection of spot urine and plasma samples. Urine was analyzed for serine proteases and for ability to activate ENaC current in vitro. Urine exosome protein was immunoblotted for prostasin and γ-ENaC protein.

In present study it was found, that compared to non-albuminuria (8.8 mg/g creatinine), albuminuric (1722mg/g creatinine) kidney transplant recipients had a higher systolic and diastolic blood pressure, despite receiving significantly more antihypertensives, and a greater urinary total plasminogen, active plasmin, active urokinase-type plasminogen activator and prostasin protein abundance which correlated significantly with u-albumin. Fluid overload correlated with systolic blood pressure, urinary albumin/creatinine and plasminogen/creatinine. Urine from albuminuric kidney transplant recipients evoked a greater amiloride- and aprotinin-sensitive inward current in single collecting duct cells (murine cell line M1). γENaC subunits at 50 and 75 kDa showed increased abundance in urine exosomes from albuminuric kidney transplant recipients when compared to controls. These findings show, that albuminuria in kidney transplant recipients is associated with hypertension, ability of urine to proteolytically activate ENaC current and increased abundance of γENaC. ENaC activity could contribute to hypertension and adverse outcome in posttransplant proteinuria.

KEYWORDS: Aldosterone, allograft, exosome, hypertension, proteinuria
Kidney transplantation is a superior treatment in patients with end stage renal disease due to survival and quality of life benefits (18, 24, 44, 56). Chronic allograft dysfunction is a multifactorial process associated with interstitial fibrosis, renal tubular atrophy and graft loss. The most important non-immunological factors that may adversely impact long-term renal graft function are hypertension (21, 26, 33, 34) and proteinuria (1, 14, 16, 25, 30) both being strong predictors of graft dysfunction. Both proteinuria and hypertension are modifiable risk factors, and reduction in hypertension has been associated with improved graft survival (27, 33). High salt intake is positively associated with blood pressure (BP) in kidney transplant recipients (KTRs) (42, 53) and thus an independent risk factor, suggesting hypertension is salt-sensitive in KTRs. Studies have implied a significant association between dietary salt intake and proteinuria (57); however, the mechanisms underlying this association are not fully understood. Interestingly, the urinary albumin excretion is greater in salt-sensitive hypertensive patients when compared to salt resistant hypertensive patients (31). Evidence for a pathophysiological connection between proteinuria and salt-sensitive BP comes from studies in several proteinuric conditions demonstrating a concurrent aberrant filtration of plasma serine proteases, in particular urokinase-type plasminogen activator (uPA), the zymogen plasminogen, and prostasin into the tubular fluid. These serine proteases can activate the amiloride-sensitive epithelial sodium channel (ENaC) in the collecting ducts by extracellular proteolytic release of an inhibitory peptide tract from the γ-subunit (2, 4, 7, 8, 47, 49). Soluble serine proteases, plasmin, prostasin and uPA in the tubular fluid, may turn ENaC from “moderate” to a highly active state in albuminuria and enhance sodium and water reuptake (6, 20, 23, 38, 39, 49, 52). While this pathophysiological mechanism cannot explain the occurrence of albuminuria, it could potentially maintain a vicious cycle once albuminuria manifests in post-transplant nephropathy with increased sodium reabsorption leading to an expansion of the extracellular volume (ECV) and hypertension with further aggravation of albuminuria. The present study was designed to address the hypothesis that post-transplant nephropathy is associated with aberrant presence of proteases in urine with ability to activate ENaC current and that the amounts of proteases would relate directly to BP and fluid overload. The hypothesis was addressed in a cross-sectional observational study design where urine samples from KTRs...
with or without albuminuria were compared to identify urine serine proteases in albuminuric KTRs and to
demonstrate their in vitro effect on ENaC. Secondary outcomes were to analyze the association between
urinary serine proteases, fluid overload and BP in KTRs.

MATERIAL AND METHODS

Study design

The study was an investigator-initiated, cross-sectional, observational study conducted at the outpatient
clinic at the Department of Nephrology, Odense University Hospital, Denmark. The study was approved by
The Ethics Committee of The Region of Southern Denmark (Project-ID: S-20150015) and the Danish Data
Protection Agency (ID: 2008-58-0035). All patients received oral and written information about the study
and gave written informed consent to participate. The study was performed in accordance with the Helsinki
Declaration.

Study population

The study included stable ambulatory KTRs presenting consecutively for a routine follow-up at the
outpatient clinic at the Department of Nephrology, Odense University Hospital between February 2016 to
November 2016. The KTRs were recruited according to the following inclusion criteria: (I) age between 18-
75 years, (II) kidney transplantation > 1 year ago, (III) follow at the outpatient clinic, (IV) severe
albuminuria (urine albumin/creatinine-ratio >300mg/g) or normoalbuminuria (urine albumin/creatinine-ratio
<30mg/g) as per KDIGO guidelines. The exclusion criteria were: (I) lack of compliance or understanding of
the study, (II) clinical relevant organic or systemic disease including malignancy or (III) treatment with anti-
mineralocorticoids, including amiloride or aldosterone antagonists.

Data collection

Blood pressure recordings Office BP was recorded three times by a validated automatic oscillometric
device (OMRON, model HBP – 1300) (9) after 30 min of rest in the sitting position. The average of the
measurements was recorded.
Evaluation of fluid status  Body composition was evaluated using a Body Composition Monitor (BCM) (Fresenius Medical Care Deutschland GmbH), applied to the patient after 30 min of rest. Electrodes were placed on one hand and one foot with the patient in a supine position. The height and weight of the patients were registered, and the measurements were initiated. The BCM device uses bioimpedance spectroscopy with a spectrum of 50 frequencies between 5 and 1000kHz, to measure EVC, intracellular (ICV), total body water and calculate fluid overload (overhydration (OH)). The values obtained were normalized to body surface area of 1.73m² (10, 29).

Blood and urine sampling  Blood samples were obtained after 30 min of rest in a sitting position, centrifuged at 1500g at 4°C for 15 min and the plasma was frozen and stored at -80°C. Immediately upon voiding, two tablets of complete (protease inhibitor cocktail, Cat; 11836145001, Sigma Aldrich, St. Louis, Missouri, USA) were added to 100 ml of urine which was frozen at -80°C. Other spot urine samples were centrifuged at 13.000 rpm for 1 min and the supernatant were frozen in -80°C as aliquots.

Analyses

Urine and plasma analysis  Urine electrolytes, creatinine, albumin and plasma-sodium, potassium, albumin and eGFR were analyzed at the Department of Biochemistry and Pharmacology, Odense University Hospital using automated, standardized assays. Urinary osmolality was analyzed by osmometer (The Advanced™ OSMOMETER Model 3D3, Advanced Instruments, INC). Urine total plasmin(ogen) (referred to plasminogen and plasmin concentration) and plasma aldosterone were analyzed using commercial ELISA Kits (Human Plasminogen Total Antigen, IHPLGKT-TOT, Innovative Research, Inc., Novi, Michigan, U.S.A and MS E-5200, Labor Diagnostika Nord GmbH & Co. KG, Germany, respectively) in accordance with the manufactures provisions. EDTA-plasma (100 µl) incubated with plasma from a nephrectomized sheep for 3 h and plasma renin concentration (PRC) was measured by radioimmunoassay of ANGI through the antibody-trapping method of Poulsen and Jørgensen as previously described (41). Concentrations were measured by the rate of ANGI formation and standardized in terms of international units per liter (IU·l⁻¹) by the activity of the WHO International Standard (ref. no. 68-356; National Institute for Biological Standards
and Control, Hertfordshire, UK) of which samples of 0.05 IU/l were included in every run of the renin assay.

In the period of measurement, 1 IU of the WHO standard corresponded to 32 ± 5 ng AngI per hour.

Between-assay coefficient of variation was 15%.

**Urinary exosomes** Frozen urine samples were thawed overnight at 4°C and vortexed thoroughly. Seventy ml of the samples were centrifuged at 3,000 g and 4°C for 30 min (Sorvall RC 26 Plus). Exosomes were isolated from the supernatant by ultracentrifugation at 45,000 rpm (220,000 g, 4°C) for 100 min (Beckman Ultracentrifuge L-70). The pellet was resuspended in 2 x 100 µl resuspension buffer (sucrose: 0.3 M; imidazole: 25 mM; EDTA-disodium salt: 1 mM; pH 7.2; cOmplete mini tablet (Roche)) and stored at -80°C until western blot-analysis were performed.

**Western blotting** Crude urine samples or exosomes were subjected to western blotting. The loaded amount was normalized to urinary creatinine. The urine was mixed with 4x SDS sample buffer (NuPAGE, invitrogen) and 10 X reducing buffer (NuPAGE, invitrogen) and heated for 5 min. Samples were loaded onto NuPAGE 4-12% Bis-Tris Gel, separated by electrophoresis, and subsequently transferred to Immobilon-P transfer membrane (Merck Milipore). The membrane was blocked for one hour with 5% milk or 3% BSA in TRIS-buffered saline with Tween 20 (TBST). The membrane was incubated with primary antibody: anti-prostasin (cat. No. 15527-1-AP; Proteintech Group, IL, USA, 1:1000); anti-plasminogen (ab 6189-100; Abcam, MA, USA, 1:5000); anti-urokinase (ab8473; Lot. No. GR106505-1, Abcam, MA, USA, 1:2000); anti-apoptosis-linked gene 2 interacting protein (ALIX; ab 53538, Santa Cruz Biotechnology, Dallas, Texas, USA, 1: 1:500), anti-aquaporin 2((AQP2), C-17)(ab 9882, Santa Cruz, 1:2000), or anti γ-ENaC (in house, mAb 4C11; 1:1000) (38) for one hour at room temperature or overnight at 4°C. Following wash 2 x 5 min and 1 x 15 min with TBST the membrane was incubated with HRP-conjugated secondary antibodies (Dako, Denmark 1:2000). After repeated wash in TBST, the signals were developed by enhanced chemiluminescence (PerkinElmer, Inc, Waltham, MA, USA) and recorded using Molecular Imager ChemiDoc™XRS⁺ (Bio-Rad). For densitometry, band density was analyzed using Image Lab software (Bio-rad). The obtained values were compared to the positive control or to the mean of the control group.
Whole cell patch-clamp experiments

Murine cortical collecting ducts cells (M1, ATCC, Boras, Sweden) were used for whole-cell patch clamp experiments as previously described (27). Briefly, M1 cells were maintained in DMEM: F12 (Life Technologies, Taastrup, Denmark) with 5 µmol/L dexamethasone (Sigma, ST. Louis, Mo) and 5% FCS (Life Technologies), and incubated at 37°C / 5% CO2. Experiments were conducted at room temperature in the tight-seal whole-cell configuration 24-30 h after seeding the cells. The patch pipettes were heat-polished with resistances of 5-7 MΩ. Seal resistance range was 1-15 GΩ. High resolution membrane currents were recorded with an EPC-9 patch-clamp amplifier (HEKA) controlled by pulse v8.11 software on a Power Macintosh G3 computer. The current was monitored by the response to a voltage step of -160 mV for 200 ms from a holding potential of -60 mV. This pulse was repeated every 3 s throughout the entire experiment. After 30-60 s, the cell was gently flushed with urine and the current monitored.

Urinary protease activity

Urinary protease activity was measured using a quantitative protease assay (EnzChek Peptidase/protease Assay Kit (33758)) as previously described (7). Urine was subjected to aprotinin-coated sepharose beads to enrich for serine proteases (49). Pure human plasmin was used to produce a standard curve. Fifty µL of eluate was used for the fluorescence assay.

Statistical evaluation

Normally distributed data (D'Agostino & Pearson omnibus or KS normality test) were presented as mean ± SEM. Data that were not normally distributed were log-transformed and presented in semi-logarithmic diagrams with geometric means. The albuminuric group and controls were compared by unpaired Student’s T-test. If log-transformed data were not normally distributed, the statistical analyses were performed using non-parametric correlation (Spearman) and nonparametric t-test (Mann-Whitney test). Correlations were evaluated using Pearson correlation. One-way analysis of variance (ANOVA) followed by Bonferroni’s Multiple Comparison post-hoc test was used when comparing means of three groups. P < 0.05 was considered statistically significant. GraphPad Prism 6.07 for Windows was used to produce graphic presentations.

RESULTS
**Patient characteristics**

KTRs were classified as albuminuric (albumin-creatinine ratio (ACR) > 300 mg/g) or non-albuminuric controls (ACR < 30 mg/g) (Table 1). There was no significant difference between the groups with respect to age, gender distribution, time from transplantation or numbers of patients receiving calcineurin inhibitors. In the albuminuria group, the majority of grafts originated from deceased donors whereas in the control group, the majority received kidneys from living donors. KTRs with albuminuria had significantly higher systolic- and diastolic BP despite significantly more antihypertensive medications (Table 1) and displayed reduced p-albumin concentration (Table 1). There were no significant differences in eGFR, plasma electrolytes, plasma aldosterone and renin concentration between groups (Table 1). The mean weight, body mass index, extracellular water (ECW), intracellular water (ICW), ECV/ICV ratio (E/I) and degree of volume expansion did not differ significantly between groups although there was a trend towards greater volume expansion in albuminuric KTRs (P = 0.08).

**Albuminuria correlated with augmented urine excretion of serine proteases**

The total urinary plasminogen/creatinine ratio was significantly higher in the albuminuric KTRs compared to controls (Figure 1A). Urinary plasmin(ogen) excretion related directly and significantly with urinary albumin excretion (P=0.001, R=0.69, Figure 1B). The relationship between the degree of albuminuria and urinary plasminogen levels after excluding the control patients was also significant (data not shown, P=0.001, R=0.67). Plasma plasminogen was significantly higher in the albuminuric group compared to controls (Figure 1C). Urinary protease activity was analyzed in random subsets of aprotinin-affinity-purified urine samples from both groups (n=12) and compared to a pure plasmin standard curve. There was no significant difference in protease activity between the albuminuric group compared to control KTRs (Figure 1D, P = 0.29, Students t test). Western blotting identified plasminogen in urine samples from albuminuric KTRs displaying bands consistent with intact plasminogen (~100 kDa), plasmin heavy A chain (~ 60 kDa) and the active plasmin B light chain (~ 26 kDa). No plasminogen, heavy A or B light chains were detected in controls (Figure 2A). Purified human active plasmin served as a positive control and displayed bands at ~ 60...
and ≈ 26 kDa while human plasma displayed predominantly the intact zymogen plasminogen that migrated just below ≈ 100 kDa (Figure 2A). Semi-quantitative analysis by densitometry showed significantly higher densities of the ≈ 100 kDa bands in the albuminuric group when compared to controls (Figure 2B). Western blotting identified uPA in urine from albuminuric KTRs with bands at ≈ 60, ≈ 50- and ≈ 25 kDa compatible with pro-uPA zymogen (≈ 60 kDa), two-chain active uPA (≈ 50 kDa) and low molecular weight uPA (≈ 30 kDa), while no uPA was identified in urine from control patients (Figure 2C). When quantitated by densitometry the excretion of the active two-chain uPA was significantly greater in albuminuric KTRs (Figure 2D). Western blotting for prostasin displayed a ≈ 40 kDa band in urine from albuminuric KTRs (n=11) and in 5 out of 13 KTRs from the control group (Figure 2E). The excretion of prostasin was higher in urine from albuminuric KTRs when compared to controls (Figure 2F) and correlated significantly with urinary albumin excretion (data not shown, P = 0.0004, R = 0.44).

Urine from albuminuria transplant recipients activates ENaC in vitro

In vitro activation of inward current likely carried by ENaC, was demonstrated by the detection of an increase in whole-cell inward current in single M1-cells when exposed to urine from KTRs (Figure 3). Urine from albuminuric KTRs (n=5) resulted in ≈10 times increase in inward current (P< 0.0001) while no significant increase was observed with urine from control KTRs. The increase in inward current with urine from albuminuric KTRs was abolished with amiloride (2 µmol/L), Figure 3B) and aprotinin (700 µg/ml), Figure 3B).

Urine exosome γ-ENaC protein abundance and proteolysis

The purified exosomal urine fractions were all positive for the exosome marker ALIX with no difference between groups (Figure 4A and 4E). A homogenate of a human kidney cortex pool (HCP) also displayed ALIX (Figure 4A). Principal-specific AQP2 was identified in the exosomal fraction with bands migrating at 25 and 37 kDa corresponding to unglycosylated and mature AQP2, respectively, and with no significant difference in abundance between the albuminuric KTRs and controls (Figure 4B and 4E). Western blotting
for prostasin revealed a ≈40 kDa band with similar intensity in all KTRs, and an additional 50 kDa band in albuminuric KTRs and in HCP with higher abundance in the albuminuric KTRs when compared to controls (Figure 4C, and 4E, densitometry of 50 kDa band not shown). Immunoblotting of human kidney cortex tissue homogenate pool (HCP) for γENaC with an antibody directed against the inhibitory peptide tract (58) resulted in four distinct bands at ~90, 75, 50 and 37kDa likely corresponding to full length intact and glycosylated γENaC, furin-cleaved γENaC, and γENaC cleaved by a distally acting protease only (Figure 4D). No full-length γENaC was detected in the exosome fractions from the albuminuric or the control KTRs. Exosomal fractions revealed a 75 kDa band corresponding to furin-cleaved ENaC, the abundance of this band was significantly higher in the albuminuric KTRs (Figure 4E). A 50 kDa band was observed consistently and with a significantly higher abundance in the albuminuric KTRs (Figure 4E). The 37 kDa band seen in kidney cortex tissue was inconsistently observed in exosomes with faint signal in some of the albuminuric KTRs.

**Albuminuria/protease activity and relation with extracellular fluid volume and blood pressure**

*Pre-hoc* hypotheses were analyzed regarding a direct relation between urinary serine protease protein and fluid overload and hypertension. Significant positive correlations were observed between fluid overload and the urine albumin/creatinine-ratio (P = 0.01; r=0.21) (Figure 5A) as well as between fluid overload and urine plasminogen/creatinine ratio (P=0.02; r=0.16) (Figure 5B). Fluid overload also correlated significantly with systolic BP (P=0.0006; r=0.31) (Figure 5C).
DISCUSSION

The present study was designed to test whether albuminuria in KTRs was associated with urinary serine proteases, gain of protease activity and ability of urine to activate ENaC current in vitro. Subsequent aim was to examine a potential association between urinary serine proteases and in vivo water accumulation and hypertension. The data showed (I) significantly higher systolic and diastolic BP and greater use of antihypertensive medication in KTRs with albuminuria; (II) a direct significant relation between systolic BP and fluid overload, u-albumin/creatinine and urine plasminogen/creatinine in KTRs; (III) an increased urinary excretion of the serine proteases plasmin, uPA, and prostatin in albuminuric KTRs that correlated with u-albumin; (IV) activation of amiloride- and aprotinin-sensitive inward current in collecting duct cells by urine from albuminuric KTRs only; and (V) increased abundance of active, cleaved γENaC protein in urinary exosomes from albuminuric KTRs. These results are consistent with proteolytic activation of ENaC leading to impaired Na⁺ excretion, fluid retention, and hypertension in albuminuric KTRs.

The prevalence of albuminuria/proteinuria in KTRs is high and a well-established, independent, prognostic marker for progressive chronic allograft dysfunction, graft loss, and cardiovascular disease (CVD), being the main causes of transplant failure (46). Post-transplant proteinuria has been attributed to glomerulonephritis, nephrosclerosis, renal vein thrombosis, chronic rejection, allograft glomerulopathy and reflux nephropathy (15, 40, 55). Hypertension is another important prognostic marker of chronic allograft dysfunction and CVD (22, 33) with a prevalence of 50-90% (21, 32, 34, 35). These observations were corroborated in the present study that included KTRs by the degree of albuminuria and showed that albuminuric KTRs received significantly more antihypertensive medication and had higher BP levels compared to normo-albuminuric KTRs. The pathogenesis of posttransplant hypertension is multifactorial but data support, that impaired renal sodium excretion contributes. Calcineurin inhibitors (CNIs) increased NCC activity (19) and NCC abundance and this correlated to blood pressure response of thiazide use (50). To our knowledge, involvement of ENaC and effect of ENaC blockers has not previously been investigated. In the present study, the majority of albuminuric KTRs had received grafts from deceased donor which is in accord with the well-known adverse impact on graft kidney function. However, eGFR was not significantly different...
between groups. Is posttransplant hypertension sodium dependent? In a cross-sectional study, there was a positive association between sodium intake and blood pressure (53) and dietary sodium restriction reduced blood pressure and albuminuria in KTRs (13). Moreover, extracellular volume expansion in KTRs was associated with increased blood pressure and increased sodium intake in agreement with a contribution from enhanced renal sodium and water reabsorption (11). In the non-transplant setting, patients with salt-sensitive hypertension display greater urinary albumin excretion which is in agreement with a general coupling.

Together the evidence supports that hypertension is sodium dependent (5, 13, 53). In the present study, there was no significant difference in fluid overload between albuminuric KTRs and controls, however, it should be emphasized, that usual diuretic and antihypertensive treatment were maintained. Nevertheless, there was a direct relation between fluid overload and systolic BP, and between fluid load and urinary albumin/creatinine and plasminogen/creatinine ratios. Albuminuria was associated with an increased excretion of serine proteases uPA, plasmin and prostasin which have the ability to induce “second hit” cleavage of γENaC causing the putative release of an inhibitory peptide tract. In non-transplant CKD patients, there was a correlation between body water and urinary plasminogen and proteinuria (45) consistent with impaired sodium excretion and a possible involvement of ENaC. We have previously demonstrated a correlation between proteinuria and urinary serine proteases in preeclampsia (7), type 2 diabetes (8), nephrotic syndrome in adult, (49) and pediatric patients (4, 47). Moreover, amiloride was an efficient add on to achieve blood pressure control in subjects with type 2 diabetes (37), and increased natriuresis in response to 2 days high doses of amiloride (3). However, the potential risk of hyperkalemia is evident with reduced kidney function (51) The findings in KTRs are in line with a similar effect from aberrant filtration of enzymes as a consequence of a damaged glomerular filtration barrier. The proteases may mutually activate, leading to an amplified enzymatic activity as the ultrafiltrate is concentrated along the nephron and collecting ducts (48). Soluble prostasin was present also in the urine of some normo-albuminuric KTRs. This is likely due to the physiological expression of prostasin in principal cells leading to its release with exosomes (2, 12, 43, 54).

Such interpretation was supported by a significant increase in total urinary prostasin in albuminuric KTRs, while the abundance of exosome-associated prostasin was not significantly different between groups, suggesting constant membrane abundance and shedding rate of GPI-anchored prostasin (12). Aldosterone
has little impact on kidney prostasin abundance (2, 36), and plasma aldosterone was not different between
groups. Therefore, aldosterone does not account for differences in exosomal γ-ENaC abundance (28).
Assuming that the increased exosomal excretion of cleaved γENaC moieties reflects principal cell surface
abundance, this indicates an increased collecting duct ENaC abundance. Of note, the detected γENaC
products contain the inhibitory peptide tract and are not equivalent to full channel activation which would
remove the epitope and yield a lower signal abundance. As the excretions of the exosomal marker ALIX as
well as AQP2 were similar between groups, the increase in exosomal γENaC in albuminuric KTRs was not
related to a difference in overall and in principal cell exosome release rate. The present findings imply that
distally acting K⁺-sparing diuretics such as amiloride and spironolactone should be tested for superior effect
in KTRs. No published studies have specifically addressed differential natriuretic potencies of diuretic
classes in KTRs.

Summary and perspective

Findings in albuminuric KTRs are consistent with pathophysiological activation of ENaC, impaired Na⁺
excretion, fluid retention and hypertension. The study is limited by the cross-sectional design and the small
number of patients, but it provides a tentative explanation for sodium sensitive hypertension in albuminuric
KTRs which may be tested in future, interventional trials.

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DISCLOSURE
The authors declare no conflicts of interest

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FIGURE 1 (A) Urinary total plasminogen/creatinine-ratio was significantly higher in KTRs with albuminuria (n= 18) compared to normoalbuminuric transplant recipients (controls, n= 13), six values in the control group were under the detection range of the assay and were not included (geometric means 1721 vs 7,66 µg/g, P < 0,0001 by Student t test). Since data were log-normally distributed, the Y-axis is logarithmic. (B) Urinary plasmin(o)gen correlated significantly with urine albumin from spot urine samples (P=0.001, R=0.69), six samples from the control group exhibited u-plasmin(o)gen values below detection range and these were included in the analysis with a value of zero. (C) Plasma plasmin(o)gen concentration was significantly higher in the albuminuric group (geometric means 186637 vs 61376 ng/ml, P = 0,004 by Student t test). (D) Serine proteases were purified from 0,7 ml crude urine samples from 12 albuminuric KTRs and 12 controls by aprotinin-coated sepharose beads. Eluate was used for fluorescence serine protease activity assay (50 µL). Diluted series of pure human plasmin served as standard curve. Broken line A indicates the mean of serine protease activity from the albuminuric group and B from controls (89285 vs 48471 Fluorescein counts).

FIGURE 2 Western immunoblot of spot urine samples normalized for creatinine and separated by SDS-PAGE from kidney transplant recipients (KTRs) with - and without albuminuria. (A) Intact plasminogen (83-88kDa), active plasmin (60kDa), plasmin heavy A chain (~ 60kDa) and plasmin light B chain (~26kDa) was present in urine from albuminuria patients. The matched control group showed no detectable plasmin(o)gen. Purified human active plasmin (P1) served as a positive control and displayed bands at 60 and 26 kDa while human plasma (P2) displayed predominantly the intact zymogen plasminogen that migrated just below 100 kDa. Plasminogen band densities were significantly higher in the albuminuric group compared to controls (B) (P = 0,0007, Mann-Whitney test). (C) Urine urokinase-type plasminogen activator (uPA) was detected as a band migrating at ~50kDa in samples predominantly from the albuminuric group. Omitting the primary antibody and only probing with the secondary antibody did not produce any visible bands. (D) Density of the 50kDa band was significantly higher in the albuminuric patients (P = 0,03, Mann-Whitney test). (E) Western immunoblot of urine samples normalized to creatinine for prostasin displayed band migrating at
-40kDa in all the tested albuminuric recipients (n=11) and in 5 out of 13 tested samples from the control group (Figure 2E). Densitometry of prostasin protein bands was significantly higher in urine samples from albuminuric KTRs (F) (P = 0.0005, Student t test). Human placenta homogenate served as positive control for prostasin (P, Figure 2E).

**FIGURE 3** Patch Clamp. A-B, Original current trace obtained in murine cortical collecting duct cells (M1) by patch clamp recordings from single cells. A, The grey trace, displays a recording from a M1 cell before superfusion with urine and the black trace displays a recording from the same cell after superfusion with urine from an albuminuric kidney transplant recipient. B, Bar graph showing the average changes in current obtained in patch clamp recordings in response to superfusion with urine from controls (black) and albuminuric KTRs (grey). The same urine samples from the albuminuric KTRs were tested with separate cells after addition of amiloride (dark grey) and after aprotinin (light grey). pA, picoampere; (pF values, mean ± SEM: Controls 27.42 ± 4.83; albuminuric KTRs 12.63 ± 0.83; albuminuric KTRs + amiloride 13,77 ± 1.9; albuminuric KTRs + aprotinin 16.12 ± 4.46)

**FIGURE 4** (A-D) Western immunoblotting of exosomes contained in urine-pellet after ultracentrifugation of urine samples from kidney transplant patients with albuminuria (KTRs) and without albuminuria (Controls). The loaded amount of protein was normalized for urine creatinine before SDS-PAGE. Aliquots from the same human kidney cortex homogenate pool was run as a positive control on most gels (HCP) (A,E). Human kidney cortex pool (HCP). The exosome marker ALIX showed a single significant band migrating at approximately 100kDa, detected in all samples and with no difference between groups (P = 0.86, Student t test). (B, E) Immunoblot for the principal-specific aquaporin-2 (AQP-2); there was no significant difference in AQP2 abundance between the groups (P = 0.86, Student t test). (C, E) Immunoblotting for prostasin demonstrated a band of the expected molecular size that migrated ~40kDa in all patients with no significant difference between groups (P = 0.074, Student t test). (D) Immunoblotting for γENaC with an antibody directed against the inhibitory peptide tract showed a band migrating at approximately 75kDa corresponding to furin-cleaved ENaC in exosomes, whereas the intact 100 kDa protein was observed only in human kidney cortex pool (HCP). The abundance of the 75kDa band was significantly higher in the albuminuric group by
densitometry (4E, lower) \((P = 0.003, \text{Mann-Whitney test})\). Three patients from the albuminuric group displayed a band at 37kDa consistent with proteolytically cleaved \(\gamma\)ENaC. The band at 50kDa was observed most consistently, and with a significantly higher abundance in the albuminuric transplant recipients \((P = 0.001, \text{Student } t \text{ test})\).

FIGURE 5 (A-C) Fluid overload (OH) measured using body composition monitor (BCM-Fresenius) correlated significantly with urinary albumin-creatinine ratio \((P = 0.014; r=0.21)\) (7 controls had values under the detection level of \(u\)-albumin, and these values are not included), plasminogen-creatinine ratio \((P=0.02; r=0.16)\), and systolic BP \((P=0.0006; r=0.31)\).
Table 1. Baseline characteristics of albuminuric kidney transplant recipients (KTRs, ACR > 300mg/g) and normoalbuminuric KTRs (controls, ACR < 30mg/g)

<table>
<thead>
<tr>
<th></th>
<th>Albuminuric KTRs</th>
<th>Controls</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Recipient characteristics</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of participants</td>
<td>18</td>
<td>19</td>
<td></td>
</tr>
<tr>
<td>Sex distribution (female/male)</td>
<td>11 female/7 male</td>
<td>10 female/9 male</td>
<td></td>
</tr>
<tr>
<td>Median age (years)</td>
<td>52.1 ± 4</td>
<td>54.8 ± 3</td>
<td>0.58 ns</td>
</tr>
<tr>
<td>Time from transplantation (months)</td>
<td>97.9</td>
<td>75.5</td>
<td>0.39 ns</td>
</tr>
<tr>
<td>Donor (deceased/living related)</td>
<td>12 deceased/6 living</td>
<td>8 deceased/11 living</td>
<td></td>
</tr>
<tr>
<td><strong>Clinical and biochemical parameters</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>147 ± 4</td>
<td>128 ± 2.7</td>
<td>0.0005 ***</td>
</tr>
<tr>
<td>Diastolic BP (mmHg)</td>
<td>83 ± 3</td>
<td>75 ± 1.9</td>
<td>0.05 *</td>
</tr>
<tr>
<td>Heart rate (bpm)</td>
<td>69 ± 3</td>
<td>67 ± 3</td>
<td>0.64 ns</td>
</tr>
<tr>
<td>Urine albumin/creatinine (mg/g)</td>
<td>1722 ± 243.4</td>
<td>8.8 ± 2.1</td>
<td>&lt;0.0001 ****</td>
</tr>
<tr>
<td>Urinary Na⁺/K⁺ ratio</td>
<td>2.7 ± 0.5</td>
<td>3.0 ± 0.3</td>
<td>0.57 ns</td>
</tr>
<tr>
<td>Urinary osmolality (mosm/kg)</td>
<td>380.7 ± 33</td>
<td>437 ± 42</td>
<td>0.3 ns</td>
</tr>
<tr>
<td>Plasma albumin (g/L)</td>
<td>39.9 ± 0.9</td>
<td>42.5 ± 0.6</td>
<td>0.02 *</td>
</tr>
<tr>
<td>Plasma natrium (mmol/L)</td>
<td>139.5</td>
<td>141</td>
<td>0.38 ns</td>
</tr>
<tr>
<td>Plasma kalium (mmol/L)</td>
<td>4.2 ± 0.1</td>
<td>4.1 ± 0.1</td>
<td>0.91 ns</td>
</tr>
<tr>
<td>eGFR (ml/min/1.73m²)</td>
<td>38.1 ± 5</td>
<td>48.5 ± 3.3</td>
<td>0.09 ns</td>
</tr>
<tr>
<td>Plasma renin (mIU/L)</td>
<td>42.6 ± 1.2</td>
<td>30.4 ± 1.2</td>
<td>0.26 ns</td>
</tr>
<tr>
<td>Plasma aldosterone (ng/L)</td>
<td>124.5 ± 6.1</td>
<td>118.7 ± 6.8</td>
<td>0.53 ns</td>
</tr>
</tbody>
</table>

**Medication**
<table>
<thead>
<tr>
<th>Calcineurin inhibitors (numbers of patients)</th>
<th>13/18</th>
<th>17/19</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antihypertensive medications (numbers)</td>
<td>2.4 ± 0.3</td>
<td>1.3 ± 0.3</td>
</tr>
<tr>
<td>Angiotensin-converting enzyme</td>
<td>7/18</td>
<td>5/19</td>
</tr>
<tr>
<td>Angiotensin receptor blockers</td>
<td>7/18</td>
<td>2/19</td>
</tr>
<tr>
<td>Alpha- receptor blockers</td>
<td>2/18</td>
<td>2/19</td>
</tr>
<tr>
<td>Beta receptor blockers</td>
<td>5/18</td>
<td>7/19</td>
</tr>
<tr>
<td>Calcium channel blockers</td>
<td>10/18</td>
<td>5/19</td>
</tr>
<tr>
<td>Furosemide</td>
<td>10/18</td>
<td>5/19</td>
</tr>
<tr>
<td>Thiazide</td>
<td>3/18</td>
<td>1/19</td>
</tr>
</tbody>
</table>

**Body Composition Monitor**

<table>
<thead>
<tr>
<th></th>
<th>75.8 ± 4.8</th>
<th>76.3 ± 3.8</th>
<th>0.93</th>
<th>ns</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight (kg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>26 ± 1.1</td>
<td>25.9 ± 0.9</td>
<td>0.99</td>
<td>ns</td>
</tr>
<tr>
<td>Total body weight (L/1,73m²)</td>
<td>35.3 ± 1.5</td>
<td>34.3 ± 1.7</td>
<td>0.66</td>
<td>ns</td>
</tr>
<tr>
<td>Extracellular volume (L/1,73m³)</td>
<td>16.8 ± 0.7</td>
<td>16.3 ± 0.8</td>
<td>0.61</td>
<td>ns</td>
</tr>
<tr>
<td>Intracellular volume (L/1,73m²)</td>
<td>18.5 ± 0.9</td>
<td>18 ± 0.9</td>
<td>0.72</td>
<td>ns</td>
</tr>
<tr>
<td>Fluid overload (L/1,73m²)</td>
<td>1.2 ± 0.3</td>
<td>0.6 ± 0.1</td>
<td>0.08</td>
<td>ns</td>
</tr>
</tbody>
</table>

Normally distributed data are presented as mean ± SEM; ns, non-significant. See text for details. *Numbers of antihypertensive drugs including diuretics. See text for details.*
Figure 2

A

B

C

D

E

F
Figure 3

A

B

Increase in inward current (%)

Control n=5  Albuminuric KTRs n=5  Albuminuric KTRs + Amlod n=5  Albuminuric KTRs + Aprotinin n=4
Figure 4

A

ALG-2-interacting protein (ALIX)

ALG-2-interacting protein (ALIX)

B

Mature Aquaporin-2

Unglycosylated Aquaporin-2

C

Prostasin

Prostasin

D

Furin-cleaved γENaC

Protease-cleaved γENaC

E

ALX density (100-kDa)

AQG2 density (~290kDa)

Prostasin density (39kDa)

γENaC density (~50kDa)
Figure 5

A

B

C

Fluid overload (L/Liter)

u-albumin/creatinin (mg/g)

Fluid overload (L/Liter)

u-plasminogen/creatinin (ug/g)

Fluid overload (L/Liter)

Sys BP (mmHg)