HHS Public Access

Author manuscript

Kidney Int Rep. Author manuscript; available in PMC 2018 September 01.

Published in final edited form as:

Kidney Int Rep. 2017 September ; 2(5): 893–904. doi:10.1016/j.ekir.2017.05.008.

Trial of Amiloride in Type 2 Diabetes with Proteinuria

Mark L. Unruh[1,2], **V. Shane Pankratz**[1], **John E. Demko**[3], **Evan C. Ray**[4], **Rebecca P. Hughey**[3,4], and **Thomas R. Kleyman**[3,4,5]

[1]Nephrology Division, Department of Internal Medicine, University of New Mexico, Albuquerque NM

[2]New Mexico VA Health Care System, Albuquerque, NM

[3]Renal-Electrolyte Division, Department of Medicine, University of Pittsburgh, Pittsburgh, Pennsylvania, USA

[4]Department of Cell Biology, University of Pittsburgh, Pittsburgh, Pennsylvania, USA

[5]Department of Pharmacology and Chemical Biology, University of Pittsburgh, Pittsburgh, Pennsylvania, USA

Abstract

Introduction—Renal Na^+ retention and extracellular fluid volume expansion are hallmarks of nephrotic syndrome, which occurs even in the absence of activation of hormones that stimulate renal Na^+ transporters. Plasmin-dependent activation of the epithelial Na^+ channel (ENaC) has been proposed to have a role in renal Na^+ retention in the setting of nephrotic syndrome. We hypothesized that the ENaC inhibitor amiloride would be an effective therapeutic agent in inducing a natriuresis and lowering blood pressure in individuals with macroscopic proteinuria.

Methods—We conducted a pilot double-blind randomized cross-over study comparing the effects of daily administration of either oral amiloride or hydrochlorothiazide (HCTZ) to patients with type 2 diabetes and macroscopic proteinuria. Safety and efficacy were assessed by monitoring systolic blood pressure (SBP), kidney function, adherence, weight, urinary Na^+ excretion and serum electrolytes. Nine subjects were enrolled in the trial.

Results—No significant difference in SBP or weight was seen between HCTZ and amiloride ($p \geq 0.15$). Amiloride induced differences in serum K^+ ($p < 0.001$), with a 0.88±0.30 mmol/L greater acute increase observed. Two subjects developed acute kidney injury and hyperkalemia when treated with amiloride. Four subjects had readily detectable levels of urinary plasminogen plus

Corresponding Author: Mark Unruh MD MS, Solomon, Gardner & Sterling Chair, Chair, Department of Internal Medicine, University of New Mexico School of Medicine MSC 10-5550, 1 University of New Mexico, Albuquerque, NM 87131-0001, Phone: (505) 272-0407, Fax: (505) 272-2349.

Disclosures

none

plasmin (uPl), and five did not. Changes in SBP in response to amiloride did not differ between individuals with vs. those without detectable uPl.

Conclusion—In summary, among patients with type 2 diabetes, normal renal function and proteinuria, there were reductions in SBP in groups treated with HCTZ or amiloride. Acute kidney injury and severe hyperkalemia were safety concerns with amiloride.

Keywords

Proteinuria; nephrotic syndrome; amiloride; plasmin; plasminogen; hyperkalemia

Introduction

Proteinuria is a reflection of glomerular damage, but it also is a risk factor for cardiovascular disease, stroke, and end-stage kidney disease [1–3]. It has also been associated with extracellular volume expansion and high blood pressure in various human populations [4–6]. Multiple studies have examined the role of proteinuria as a risk factor for the development of elevated blood pressure. A study involving normotensive adult men and women from Okinawa found the annual frequency for development of hypertension to be 2.4-fold higher in individuals with non-nephrotic range proteinuria at baseline [7]. Examination of nine potential biomarkers for hypertension risk in the normotensive, healthy male and female offspring of the Framingham Heart Study participants found that urinary albumin/Cr, a marker of proteinuria, determined from a single void morning urine sample predicted the development of hypertension with an odds ratio of 1.21 [8]. Another study found that higher levels of urinary albumin, despite being considered within the normal range, predicted incident hypertension in a population of healthy non-diabetic female nurses [9].

The relationship between proteinuria and blood pressure is complicated as hypertension can cause renal damage resulting in increased proteinuria, and the development of essential hypertension does not require pre-existing proteinuria [10–12]. Studies involving type 2 diabetics reflect this complicated relationship between proteinuria and hypertension. In the natural course of type 2 diabetes, microalbuminuria and elevations in blood pressure are thought to occur at around the same time. Blood pressure in microalbuminuric diabetics is more sensitive to dietary salt intake than in normoalbuminuric patients despite both groups having similar aldosterone and plasma renin activity levels [13]. However, proteinuria is not consistently identified as a risk factor for incipient elevation in blood pressure and in some studies elevated blood pressure predicts the advent of microalbuminuria [14–16]. Analyses of normotensive normoalbuminuric subjects in the Diabetes Control and Complications Trial study found that higher urinary albumin levels, though still in the normal range, predicted incident hypertension [17]. A similar finding was seen in a non-diabetic cohort [18]. These disparate results regarding hypertension predicting microalbuminuria versus microalbuminuria predicting hypertension may be related to the cut-off that defined microalbuminuria.

Studies have suggested that activation of Na^+ transporters in the distal nephron is responsible for the enhanced renal Na^+ retention that is seen proteinuric states, and that Na^+ retention in this setting does not require activation of the renin-angiotensin-aldosterone

system [19–22] In rats with experimentally induced nephrotic syndrome, proteinuria-associated Na^+ retention is attenuated by the epithelial Na^+ channel (ENaC) blocker, amiloride, suggesting a role for ENaC in this process [22, 23]. Recent work has suggested that enhanced ENaC activation by filtered proteases may contribute to renal Na^+ retention in nephrotic syndrome [24–27]. Proteases activate ENaC by cleaving two of the channel subunits (α and γ) at multiple sites flanking imbedded inhibitory tracts, releasing these tracts and transitioning the channel to higher activity states (for review, see [28]). The protease furin, constitutively expressed in the trans-Golgi network, has an important role in this process. Furin cleaves the α subunit twice, releasing an inhibitory tract and transitioning channels from a low to a moderate activity state [29, 30]. Furin cleaves the γ subunit at a site preceding its inhibitory tract. Numerous other proteases, including plasmin, have been shown cleave the γ subunit distal to its inhibitory tract, thus releasing this tract and transitioning ENaC to a high activity state [25, 31].

Plasminogen is filtered by damaged glomeruli, and can be converted to its active form, plasmin, by urokinase in kidney tubules [24, 32–34]. It has been suggested that tubular plasmin may be an important factor contributing to renal Na^+ retention in proteinuric states by either directly or indirectly (via activation of the tubular protease prostasin) cleaving and activating ENaC [26, 27, 35]. Urine from patients with diabetes, preeclampsia, and nephrotic syndrome, often containing above 100 μg plasminogen plus plasmin (uPl) per gram of creatinine (uPl/Cr), activates ENaC in vitro [36–38]. If proteinuria leads to ENaC activation by plasmin and renal Na^+ retention, ENaC inhibitors such as amiloride should provide an effective tool to induce a natriuresis and improve blood pressure in this setting. In addition to blocking ENaC [39], amiloride is also a urokinase inhibitor that will reduce the conversion of plasminogen to plasmin [40].

While previous studies have examined the role of amiloride in low-renin hypertension [41, 42] and as an additional agent to the conventional treatment of hypertension [43], there is limited clinical information regarding the impact of ENaC inhibitors on blood pressure and volume status in the setting of proteinuria [44]. In this setting, amiloride should block ENaC-dependent Na^+ retention and subsequent volume expansion and hypertension. We performed a pilot study to determine the effect size and safety of amiloride as a therapeutic agent compared to hydrochlorothiazide (HCTZ) in individuals with type 2 diabetes, normal renal function and proteinuria. The primary outcome for this pilot was change in systolic blood pressure (SBP). Our goal was to also confirm previous studies that urinary plasminogen and plasmin correlates urinary albumin in this clinical setting [38].

Materials and Methods

Participants

Inclusion criteria for this study included a history of type 2 diabetes, age 18 to 80 years, presence of systolic hypertension or pre-hypertension at time of screening (average SBP ≥120 mmHg and <180), urinary protein/creatinine ratio ≥ 100 mg/g or albumin/Cr ≥ 100 mg/g at screening, and HbA1C≤9% (as glucosuria would confound the endpoints related to natriuresis and diuresis). Patients were excluded from study participation with serum K^+ level <3.5 mEq/L or >5.0 mEq/L at screening, history of hyperkalemia (serum K>5.5

mEq/L) in the last two years, estimated GFR <60 mL/min/1.73m^2 as determined by MDRD 4-variable equation, contraindication to use of HCTZ or amiloride, symptomatic heart failure, acute cardiac issues, cirrhosis, organ transplantation, dementia, evidence of poor adherence by missed clinic visits, and large arm circumference.

Recruitment of patients

The study sample consisted of type 2 diabetic male and female subjects recruited through the University of New Mexico Hospital (UNMH) clinics, Nephrology Clinic, Endocrinology Clinic, General Medicine Clinic, Pharmacy Clinic and the University of New Mexico Clinical and Translational Science Center (CTSC) Patient Recruitment Services. Type 2 diabetic participants with macroscopic proteinuria who were seen in the UNMH clinics of the research investigators or identified from Patient Recruitment Services were invited to participate in this pilot trial. The study protocol 13-017 was approved by the University of New Mexico Institutional Review Board. The study was recorded in clincaltrials.gov NCT01804777.

Objectives and Study Design

The study employed a randomized, controlled, double-blind, single-center, crossover design comparing orally administered amiloride in escalating doses of 10 to 20 mg daily with HCTZ in doses of 12.5 mg to 25 mg daily for 2 week treatment periods (Fig. 1). Responses to therapy were measured at the end of each treatment period with the assumption that any carry-over effects from the previous treatment would be eliminated during a four-week wash out period. The UNMH Research Pharmacist performed randomization and blinding, and provided the study medications. End-point measurements were performed at the end of each two-week active treatment period, with the exception of the 24-hour urine Na^+, which occurred approximately four days after initiation of the study drug in order to measure natriuresis prior to escape.

Outcomes

This pilot study examined feasibility and estimated preliminary effect sizes for the primary and secondary outcomes. To determine safety and feasibility of amiloride use in type 2 diabetics with proteinuria, participants were closely monitored for development of hyper/hypokalemia, GI intolerance, and acute kidney injury (increase of serum creatinine >0.5 mg/dL [45]). Adherence was measured using pill counts and self-report, and the target for adherence was >80% of pills used and high-levels of adherence by self-report. The primary outcome was a change in clinic SBP, as measured by the OMRON Digital Blood Pressure Monitor, and measured as the average of three serial BP measurements taken one minute apart after 5 minutes of sitting quietly. To demonstrate effect sizes on clinically relevant hypertension outcomes such as volume status and urinary Na^+ excretion, changes in volume status were assessed by measurements of weight and % total body water using a body composition analyzer [46]. Na^+ excretion assessed with the 24-hour urine excretion of Na^+.

Sample Size

The pilot study was designed to enroll 20 participants. Assuming a standard deviation of change in SBP = 5 mmHg, alpha = 0.05, we estimated having approximately 22% power to detect a difference in SBP change between the two treatments if they differed by 2 mmHg. Regardless of the significance of a difference, the targeted sample size would have enabled the estimation of the effects of the intervention on SBP with a precision of about ± 2.4 mmHg, thus enabling the design of a future study with acceptable statistical power if the magnitude of the estimates appeared to be clinically meaningful in this pilot study.

Randomization

The randomization was performed centrally by the UNMH Research Pharmacy (RP). Patients were allocated to the different treatment sequences using random numbers. The RP was responsible for coordinating and managing the investigational drug inventory, storage, distribution, and record keeping for this clinical research study and conducted this clinical trial by following established standard operating procedures for drug preparation and delivery (blinding of study patients and investigators).

Visit Schedule and Study measures

After the screening visit, a one-month run-in period assessed changes in blood pressure after withdrawal of diuretics (loop, thiazide, and K-sparing) and adherence to the study diet. All other antihypertensive medications and dosages were maintained during the study. During the run-in period, subjects met with the CTSC study nutritionist to discuss eating habits and were educated on how to record their food intake, given examples of food choices, and established the study diet. Each participant was advised to adhere to an individualized diet containing 1.1 g/kg protein per day, 70 mmol of Na^+ (4 gram), and 50 mmol of K^+ (2 gram) as prescribed by the dietician. Nutrition analysis of food diary records was performed using Nutrition Data System for Research (NDSR) 2012. The CTSC nutritionist contacted subjects at day 14 and at day 30 to confirm compliance. Quality control reviews of dietary records were used to minimize missing nutrient values and errors. Participants were advised not to make any dietary changes during the course of the study except under guidance from the study dietician.

BP, weight, body composition, adherence, and adverse effects of therapy were recorded during clinic visits. The time of day for these visits was not standardized. Body composition was assessed by Bioelectrical Impedance Analysis (BIA). BIA determines the electrical impedance, or opposition to the flow of an electric current through body tissues which can then be used to calculate an estimate of total body water (TBW). Adherence to study medication was assessed using pill-counts and self-report. At the end of the study period, the participants had a close-out visit with the study dietician and physician to return diet and blood pressure regimen to their usual-care in collaboration with the participant’s primary physician.

Immunoblotting for urinary plasminogen and plasmin

Urine specimens collected at the initiation of the study were used to determine relative amounts of plasminogen and plasmin, normalized to creatinine (uPl/Cr). Urinary proteins

were precipitated and de-salted with chloroform/methanol [47]. The volume of urine precipitated was adjusted to optimize detection of plasminogen and plasmin from each patient by immunoblotting. Precipitated proteins were suspended in BioRad 2x Laemmli Sample Buffer, heated for 2 min at 90°C, and subjected to SDS-PAGE on BioRad Criterion TGX precast 10% polyacrylamide gels. In order to separate plasminogen (Mr 88 kDa) and plasmin (Mr 75 kDa) bands on the gel, BioRad Precision Plus Protein™ All Blue Standards were included in a gel lane, and proteins were electrophoresed until the 37 kDa standard reached the bottom of the gel. Proteins were electrophorectically transferred to nitrocellulose (Merck-Millipore) and incubated with a mouse anti-plasminogen antibody (MAB2596, R&D Systems, Inc.) overnight and HRP-tagged secondary antibody (Jackson Labs) for 90 min before incubation with Perkin Elmer Western Lightning Plus ECL and collection of the signal with a BioRad Versadoc, as previously described [48]. Bands for plasminogen or plasmin were quantified using BioRad Quantity One software. Purified plasminogen (Sigma) was run on each SDS-gel in order to determine levels of plasminogen and plasmin in patient samples based on a standard curve. Immunoblot analyses were performed 3 to 6 times for each patient sample and used to calculate mean and SD.

Statistical Analysis

We summarized patient characteristics as mean ± standard deviation (S.D.) for quantitative measures, and as number (percent) for qualitative features. We assessed the primary study outcomes of feasibility by estimating the proportions of patients with hyperkalemia and the rates of study adherence for participants who were receiving each of the study treatments. Change in SBP and other study outcomes, within and between the low- and high-dose two-week treatment periods were estimated and compared using mixed model analysis of variance to account for the repeated per-subject assessments. In order to determine whether uPl/Cr has a role in amiloride treatment effects, we performed an additional series of mixed model analyses of variance, comparing the degree to which uPl/Cr status associated with treatment outcomes. This was achieved by testing for an interaction between uPl/Cr and treatment group. We estimated the degree of change observed for each treatment within groups stratified on the basis of uPl/Cr above or below 100 μg/g. Estimated effects from the mixed models are presented as mean±standard error (S.E.), and all reported p-values reflect two-sided tests of significance. Analyses were performed in SAS (Cary, NC) version 9.4.

Results

1485 patients with diabetes were screened for study participation using endocrine and nephrology clinics, and a CTSC registry. The majority of these were excluded due to low levels of proteinuria or an elevated hemoglobin A1c. Of those remaining after initial screening, 32 were eligible for study participation and agreed to an in-person screening visit. Of those, 23 were excluded due to elevated hemoglobin A1c and low levels of proteinuria. Table 1 shows the participant characteristics at baseline. Their age (mean ± S.D.) was 58.4 ± 10.0, and five (56%) were women. Their BMI was 32.2 ± 6.2 kg/m^2 and albumin/Cr was 1120 ± 780 mg/g. The participants had an eGFR of 87.4 ± 20.4 ml/min/1.73m^2 and SBP of 132 ± 13 mmHg. Seven (78%) of the patients were on angiotensin converting enzyme inhibitor or angiotensin receptor blocker, and the number of anti-hypertensives was 1.6

± 1.3. Adherence to study protocols was medium to high for 7 of the 9 patients completing treatment with HCTZ. Adherence was similar for those completing the amiloride treatment protocol, with 5 of 7 patients reporting medium to high adherence.

Table 2 and Figure 2 summarize the mean SBP estimates observed at treatment initiation and over the subsequent four weeks of treatment, where patients received the lower amiloride or HCTZ dose for two weeks and subsequently received the higher amiloride or HCTZ dose. The within-person decline in SBP, estimated from the linear mixed effects model over the first two weeks post treatment was 8.0±4.4 mmHg in the HCTZ group, and 5.1±4.5 mmHg in the amiloride group, with the resulting treatment difference between the amiloride and HCTZ treatment groups of 2.9±6.3 mmHg. Over the full four weeks of treatment, those in the HCTZ group experienced an estimated decline of 10.2±4.4 mmHg and the amiloride group experienced an estimated decline of 0.5±4.6 mmHg, with those treated with HCTZ experiencing a 10.2±7.5 mmHg greater decline in SBP over the four weeks of therapy than those treated with amiloride. However, these changes in SBP over time did not differ significantly by treatment ($p=0.15$).

Serum K^+ increased with amiloride treatment (Table 2 and Figure 2). The estimated mean within-person increase in serum K^+ was 0.9± 0.2 mmol/L in the amiloride group over the first two weeks of treatment, while it was unchanged (0.0±0.2 mmol/L) in the HCTZ group, with a difference between the amiloride and HCTZ treatment groups of 0.9±0.3 mmol/L. For those treated over the full four weeks of therapy, levels of serum K^+ were 0.6±0.2 mmol/L higher in the amiloride group than at baseline, while levels decreased by 0.1±0.2 mmol/L from baseline in the HCTZ group, with serum K^+ levels increasing over the four-week treatment period in the amiloride group by 0.7±0.3 mmol/L more than in the HCTZ group. Changes in serum K^+ levels during treatment were significantly different between the amiloride and HCTZ ($p<0.001$).

Table 2 and Figure 2 also summarize weights observed in the two study groups at baseline and at two-week intervals during treatment. The estimated mean within-person decline in weight was 0.9±0.6 kg over the first 14 days with low dose amiloride, while for those treated with HCTZ the estimated decline was 0.6±0.6 kg. Average weight losses of 0.1±0.7 kg and 0.3±0.6 kg were observed under high dose treatment with amiloride and HCTZ, respectively. Differences in weight changes between treatment groups were not statistically significant ($p=0.46$). Our analyses excluded the two participants who dropped out of the study due to hyperkalemia and acute kidney injury (see below).

Immunoblotting of urine samples was performed to assess levels of plasminogen and plasmin, which were combined and normalized to urinary creatinine (uPl/Cr, Figure 3). We defined high uPl/Cr as ≥ 100 μg/g and low uPl/Cr as < 100μg/g. Of the nine study participants, four had high uPl/Cr at the start of the study, as assessed by immunoblotting (Figure 3). Surprisingly, five subjects had low or negligible levels. The individual measurements of either plasminogen or plasmin would have arrived at identical groupings of high vs. low uPl/Cr.

Over the low dose phase of the study, the SBP of those with high urinary Pl/Cr fell an estimated 11.8±6.7 mmHg with amiloride and 10.1±6.7 mmHg with HCTZ, while the SBP of those with low Pl/Cr rose by 1.4±6.4 mmHg with amiloride and fell by 6.7±6.0 mmHg with HCTZ. With high dose therapy, the SBP of those with high Pl/Cr fell by 0.9±7.1 mmHg with amiloride and by 9.0±6.7 mmHg with HCTZ, while the SBP of those with low Pl/Cr levels rose by 1.1±6.3 mmHg with amiloride and fell by 11.8±6.0 mmHg with HCTZ. Although these differences are intriguing, we are not able to conclude that uPl/Cr correlates with treatment effects on SBP (p=0.23).

Differences in treatment effect on serum K^+ in those with different Pl/Cr levels were smaller than those observed for SBP. For instance, among those treated with amiloride, serum K^+ rose by 0.8±0.3 mmol/L in the low dose study phase for those with low uPl/Cr and rose by 1.0±0.3 mmol/L for those with high uPl/Cr. As with SBP, we are not able to conclude that uPl/Cr correlates with the effect of amiloride on serum K^+ (p=0.70).

In the low dose study phase, the weight of those with high uPl/Cr levels fell by 0.7±0.8 kg with amiloride and by 1.1±0.9 kg with HCTZ, while the weight of those with low uPl/Cr fell by 1.0±0.8 kg with amiloride and by 0.2±0.8 kg with HCTZ. In the high dose study phase, the weight of those with high levels of uPl/Cr levels rose by 0.3±0.9 kg with amiloride and fell by 0.9±0.9 kg with HCTZ, while the weight of those with low levels of plasminogen fell by 0.5±0.8 kg with amiloride and rose by 0.1±0.8 kg with HCTZ. These differences were not statistically significant (p=0.60).

In the amiloride treatment arm, urinary albumin/Cr decreased by 10% from baseline during the low dose phase of the trial, and by 15% during the high dose phase of the trial. The estimated effects of HCTZ were 7% and 14% reductions in albumin/Cr over the low and high dose phases, respectively. These treatment effects on albumin/Cr were not statistically different from pretreatment albumin/Cr values. Amounts of Na^+ excreted in urine over 24 h measured prior starting a diuretic, 4 days after initiation of the diuretic (HCTZ or amiloride), and at the end of the two-week period on the diuretic did not differ significantly.

Our assessment of treatment feasibility suggests that there are risks associated with amiloride treatment in this patient population. Of the nine subjects who received amiloride, two experienced a serious adverse event (hyperkalemia and acute kidney injury). While on the higher dose of amiloride, one participant was found to have both elevated serum K^+ (6.8 mEq/L) and an acute rise in serum Cr (1.57 mg/dL, up from a baseline of 1.26) in a protocol blood collection, with no physical complaints. The subject experienced decreases in blood pressure of up to 14 mmHg and decreases in weight of up to 3.4 kg over the course of amiloride therapy. He was promptly directed to the UNMH Emergency Room where he was admitted overnight for treatment and monitoring. In addition to discontinuing the amiloride, he received IV fluids and standard hyperkalemia therapy. This participant experienced rapid correction of serum K^+ and Cr with IV volume resuscitation, consistent with pre-renal azotemia. The second participant experienced hyperkalemia (serum K^+ of 7.8 mEq/L) and acute kidney injury (serum Cr was 1.51 mg/dL, up from a baseline of 0.76) on the lower dose of amiloride. This individual also was hospitalized and amiloride was discontinued.

She received IV fluids and medical therapy for hyperkalemia and experienced rapid recovery.

Discussion

A growing literature suggests that ENaC activation occurs in proteinuric patients due to proteases within the urinary space that cleave and activate ENaC, and that this process contributes to extracellular volume expansion and increased blood pressure [24–27, 35, 49]. In this setting, filtered plasminogen (inactive precursor) is converted to plasmin (active protease) by urokinase that is expressed in tubular epithelial lumen [24, 32–34, 50].

We expected that amiloride would substantially reduce the SBP and volume status by blocking both ENaC and urokinase [39, 40], when compared to HCTZ. We anticipated enrolling twenty subjects, but the trial was stopped early due to safety concerns related to two episodes of acute kidney injury and hyperkalemia. It was our expectation that the selection of patients with normal kidney function, with a serum K^+ in a range from 3.5–5.0 and the use of a standardized diet would mitigate hyperkalemia. We observed a significant increase in serum K^+ levels among those being treated with amiloride. This is in contrast to our primary outcomes, where there were no significant reductions in weight or in SBP with amiloride when compared to HCTZ (Table 2). Our inability to detect significant effects on blood pressure and weight may, in part, be due to the small sample size that resulted from stopping the trial early. It is interesting to note that a recent study comparing type 1 diabetics with and without nephropathy observed significant reductions in SBP in both groups with short term (2 day) amiloride (20 or 40 mg/d) administration, whereas mean arterial pressure was significantly reduced only in the group with nephropathy [44]. Another study of 80 individuals with type 2 diabetes and resistant hypertension reported a beneficial effect with amiloride (5 or 10 mg/d), with a 6 mmHg reduction in SBP [51]. The study included individuals with and without proteinuria, and reported a 9% incidence of hyperkalemia (K^+ > 5.5 meq/L).

The severity of hyperkalemia in our pilot was striking given the multiple steps taken to mitigate hyperkalemia. The study protocol excluded patients with a history of hyperkalemia and an eGFR < 60. The study also had a moderate Na^+ and low K^+ diet that was instituted prior to randomization. There was evidence for adherence to the low K^+ diet as demonstrated by self-reported dietary records, where among the 49 instances of self-reported diet, the average ± standard deviation K^+ intake was 1.6±0.6 g, and only 5 instances of intake above 2.5 g were reported. It is not surprising that the addition of a K^+-sparing diuretic to an ACE-I or ARB in individuals with some impairment in renal function increases the risk of significant hyperkalemia [52, 53]. It remains unclear whether the risk of hyperkalemia would be adequately attenuated by novel agents to enhance intestinal K^+ excretion when using amiloride in diabetics also receiving ACE-I or ARBs [54]. The combination of a loop or thiazide diuretic and/or a lower dose of amiloride could also reduce the risk of hyperkalemia in this setting [55].

While we expected to readily detect plasminogen and plasmin in the urine of diabetics with proteinuria, we were surprised to find that five of nine subjects had low or negligible levels,

based on immunoblot analyses. However, there was a correlation between the albumin/Cr and uPl/Cr, in agreement with findings from other groups [36–38]. The differences in urinary Pl/Cr levels allowed us to examine whether high uPl/Cr associated with a response to amiloride. We found that individuals with high uPl/Cr responded to low-dose amiloride with a fall in SBP by 11.8±6.7 mmHg. This is in contrast to an estimated increase in SBP by 1.4±6.4 mmHg under low dose amiloride among those with low or negligible levels of urinary Pl/Cr. Lack of statistical significance (p=0.16) may be attributable to low sample size. These findings suggest that, with a larger study population, we might observe significant reductions in SBP and weight with amiloride in individuals excreting readily detectable uPl/Cr in their urine.

The findings from this report should be interpreted in light of several limitations. First, the use of other anti-hypertensive medications such as an ACE-I/ARB may have attenuated the effects of amiloride by reducing levels of hormones that are known to activate ENaC [56, 57]. These anti-hypertensives may also have contributed to the episodes of hyperkalemia observed in this study [52, 53]. Second, the blood pressure of our study sample may have been too low at randomization to demonstrate a significant effect of amiloride. The blood pressure of the study sample was lower at randomization than at baseline, perhaps owing to regression to the mean or to our moderate Na^+ diet and dietary monitoring. Third, the initial dosing of amiloride was based on that used in Liddle's syndrome. It may be that a lower dose of amiloride would have a better safety profile. Lastly, this pilot explored whether amiloride had a differential effect among those with measurable uPl/Cr. A larger study prospectively measuring uPl/Cr would be needed to assess whether amiloride should be used in the subpopulation of proteinuric diabetic patients with higher levels.

In summary, among individuals with normal renal function and proteinuria, we did not find evidence that amiloride was superior to HCTZ with regard to reductions in blood pressure or weight. Furthermore, acute kidney injury and severe hyperkalemia was a safety concern with amiloride at a dose of 10 or 20 mg in patients with diabetes and proteinuria using ACE/ARB blockade. Our study was stopped early for harm, and the evaluations of the primary study outcomes, blood pressure and weight, were inconclusive. Data collected from this study provide important preliminary data for future studies of amiloride in the context of proteinuria and plasminuria. uPl/Cr varies between individuals with proteinuria. Further studies are needed to determine whether uPl/Cr provides a more robust biomarker than albuminuria for those individuals with proteinuria who develop Na^+ retention and increases in BP [38].

Acknowledgments

This work was supported by a grant from Dialysis Clinics, Inc. and by grants from the National Institutes of Health (T32 DK061296, T35 DK065521, P30 DK079307, K08 DK110332) and University of New Mexico CTSC (UL1TR00449). The study was recorded in clincaltrials.gov NCT01804777.

References

1. Hsu CY, Iribarren C, McCulloch CE, et al. Risk factors for end-stage renal disease: 25-year follow-up. Arch Intern Med. 2009; 169:342–350. [PubMed: 19237717]

2. Lambers Heerspink HJ, Brantsma AH, de Zeeuw D, et al. Albuminuria assessed from first-morning-void urine samples versus 24-hour urine collections as a predictor of cardiovascular morbidity and mortality. Am J Epidemiol. 2008; 168:897–905. [PubMed: 18775924]

3. Ninomiya T, Perkovic V, Verdon C, et al. Proteinuria and stroke: a meta-analysis of cohort studies. Am J Kidney Dis. 2009; 53:417–425. [PubMed: 19070947]

4. Agarwal R, Andersen MJ. Correlates of systolic hypertension in patients with chronic kidney disease. Hypertension. 2005; 46:514–520. [PubMed: 16103271]

5. Kim BJ, Lee HJ, Sung KC, et al. Comparison of microalbuminuria in 2 blood pressure categories of prehypertensive subjects. Circ J. 2007; 71:1283–1287. [PubMed: 17652896]

6. Schork A, Woern M, Kalbacher H, et al. Association of Plasminuria with Overhydration in Patients with CKD. Clin J Am Soc Nephrol. 2016; 11:761–769. [PubMed: 26933188]

7. Inoue T, Iseki K, Higashiuesato Y, et al. Proteinuria as a significant determinant of hypertension in a normotensive screened cohort in Okinawa, Japan. Hypertens Res. 2006; 29:687–693. [PubMed: 17249524]

8. Wang TJ, Gona P, Larson MG, et al. Multiple biomarkers and the risk of incident hypertension. Hypertension. 2007; 49:432–438. [PubMed: 17242302]

9. Forman JP, Fisher ND, Schopick EL, et al. Higher levels of albuminuria within the normal range predict incident hypertension. J Am Soc Nephrol. 2008; 19:1983–1988. [PubMed: 18579639]

10. Feld LG, Brentjens JR, Van Liew JB. Renal injury and proteinuria in female spontaneously hypertensive rats. Ren Physiol. 1981; 4:46–56. [PubMed: 7302359]

11. Rossi GP, Bernini G, Desideri G, et al. Renal damage in primary aldosteronism: results of the PAPY Study. Hypertension. 2006; 48:232–238. [PubMed: 16801482]

12. Wang G, Lai FM, Kwan BC, et al. Podocyte loss in human hypertensive nephrosclerosis. Am J Hypertens. 2009; 22:300–306. [PubMed: 19131934]

13. Trevisan R, Bruttomesso D, Vedovato M, et al. Enhanced responsiveness of blood pressure to sodium intake and to angiotensin II is associated with insulin resistance in IDDM patients with microalbuminuria. Diabetes. 1998; 47:1347–1353. [PubMed: 9703338]

14. Coonrod BA, Ellis D, Becker DJ, et al. Predictors of microalbuminuria in individuals with IDDM. Pittsburgh Epidemiology of Diabetes Complications Study. Diabetes Care. 1993; 16:1376–1383. [PubMed: 8269796]

15. Mathiesen ER, Ronn B, Storm B, et al. The natural course of microalbuminuria in insulin-dependent diabetes: a 10-year prospective study. Diabet Med. 1995; 12:482–487. [PubMed: 7648820]

16. Pambianco G, Costacou T, Ellis D, et al. The 30-year natural history of type 1 diabetes complications: the Pittsburgh Epidemiology of Diabetes Complications Study experience. Diabetes. 2006; 55:1463–1469. [PubMed: 16644706]

17. de Boer IH, Kestenbaum B, Rue TC, et al. Insulin therapy, hyperglycemia, and hypertension in type 1 diabetes mellitus. Arch Intern Med. 2008; 168:1867–1873. [PubMed: 18809813]

18. Deschenes G, Feraille E, Doucet A. Mechanisms of oedema in nephrotic syndrome: old theories and new ideas. Nephrol Dial Transplant. 2003; 18:454–456. [PubMed: 12584259]

19. Meltzer JI, Keim HJ, Laragh JH, et al. Nephrotic syndrome: vasoconstriction and hypervolemic types indicated by renin-sodium profiling. Ann Intern Med. 1979; 91:688–696. [PubMed: 496101]

20. Ichikawa I, Rennke HG, Hoyer JR, et al. Role for intrarenal mechanisms in the impaired salt excretion of experimental nephrotic syndrome. J Clin Invest. 1983; 71:91–103. [PubMed: 6848563]

21. de Seigneux S, Kim SW, Hemmingsen SC, et al. Increased expression but not targeting of ENaC in adrenalectomized rats with PAN-induced nephrotic syndrome. American journal of physiology Renal physiology. 2006; 291:F208–217. [PubMed: 16403831]

22. Lourdel S, Loffing J, Favre G, et al. Hyperaldosteronemia and activation of the epithelial sodium channel are not required for sodium retention in puromycin-induced nephrosis. J Am Soc Nephrol. 2005; 16:3642–3650. [PubMed: 16267158]

23. Deschenes G, Wittner M, Stefano A, et al. Collecting duct is a site of sodium retention in PAN nephrosis: a rationale for amiloride therapy. Journal of the American Society of Nephrology: JASN. 2001; 12:598–601. [PubMed: 11181809]

24. Svenningsen P, Bistrup C, Friis UG, et al. Plasmin in nephrotic urine activates the epithelial sodium channel. J Am Soc Nephrol. 2009; 20:299–310. [PubMed: 19073825]
25. Passero CJ, Mueller GM, Rondon-Berrios H, et al. Plasmin activates epithelial Na+ channels by cleaving the gamma subunit. J Biol Chem. 2008; 283:36586–36591. [PubMed: 18981180]
26. Ray EC, Rondon-Berrios H, Boyd CR, et al. Sodium retention and volume expansion in nephrotic syndrome: implications for hypertension. Adv Chronic Kidney Dis. 2015; 22:179–184. [PubMed: 25908466]
27. Svenningsen P, Andersen H, Nielsen LH, et al. Urinary serine proteases and activation of ENaC in kidney--implications for physiological renal salt handling and hypertensive disorders with albuminuria. Pflugers Arch. 2015; 467:531–542. [PubMed: 25482671]
28. Kleyman TR, Carattino MD, Hughey RP. ENaC at the Cutting Edge: Regulation of Epithelial Sodium Channels by Proteases. J Biol Chem. 2009; 284:20447–20451. [PubMed: 19401469]
29. Hughey RP, Bruns JB, Kinlough CL, et al. Epithelial sodium channels are activated by furin-dependent proteolysis. J Biol Chem. 2004; 279:18111–18114. [PubMed: 15007080]
30. Carattino MD, Sheng S, Bruns JB, et al. The epithelial Na+ channel is inhibited by a peptide derived from proteolytic processing of its alpha subunit. J Biol Chem. 2006; 281:18901–18907. [PubMed: 16690613]
31. Bruns JB, Carattino MD, Sheng S, et al. Epithelial Na+ channels are fully activated by furin- and prostasin-dependent release of an inhibitory peptide from the gamma-subunit. J Biol Chem. 2007; 282:6153–6160. [PubMed: 17199078]
32. Lau SO, Tkachuck JY, Hasegawa DK, et al. Plasminogen and antithrombin III deficiencies in the childhood nephrotic syndrome associated with plasminogenuria and antithrombinuria. J Pediatr. 1980; 96:390–392. [PubMed: 7359230]
33. Kristensen P, Eriksen J, Dano K. Localization of urokinase-type plasminogen activator messenger RNA in the normal mouse by in situ hybridization. J Histochem Cytochem. 1991; 39:341–349. [PubMed: 1899685]
34. Wagner SN, Atkinson MJ, Wagner C, et al. Sites of urokinase-type plasminogen activator expression and distribution of its receptor in the normal human kidney. Histochem Cell Biol. 1996; 105:53–60. [PubMed: 8824906]
35. Passero CJ, Hughey RP, Kleyman TR. New role for plasmin in sodium homeostasis. Curr Opin Nephrol Hypertens. 2010; 19:13–19. [PubMed: 19864949]
36. Andersen H, Friis UG, Hansen PB, et al. 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. 2015; 30:781–789. [PubMed: 25609736]
37. Buhl KB, Friis UG, Svenningsen P, et al. Urinary plasmin activates collecting duct ENaC current in preeclampsia. Hypertension. 2012; 60:1346–1351. [PubMed: 22987920]
38. Buhl KB, Oxlund CS, Friis UG, et al. Plasmin in urine from patients with type 2 diabetes and treatment-resistant hypertension activates ENaC in vitro. J Hypertens. 2014; 32:1672–1677. discussion 1677. [PubMed: 24805959]
39. Kleyman TR, Cragoe EJ Jr. Amiloride and its analogs as tools in the study of ion transport. J Membr Biol. 1988; 105:1–21. [PubMed: 2852254]
40. Vassalli JD, Belin D. Amiloride selectively inhibits the urokinase-type plasminogen activator. FEBS Lett. 1987; 214:187–191. [PubMed: 3106085]
41. Hood SJ, Taylor KP, Ashby MJ, et al. The spironolactone, amiloride, losartan, and thiazide (SALT) double-blind crossover trial in patients with low-renin hypertension and elevated aldosterone-renin ratio. Circulation. 2007; 116:268–275. [PubMed: 17606839]
42. Eide IK, Torjesen PA, Drolsum A, et al. Low-renin status in therapy-resistant hypertension: a clue to efficient treatment. J Hypertens. 2004; 22:2217–2226. [PubMed: 15480108]
43. Saha C, Eckert GJ, Ambrosius WT, et al. Improvement in blood pressure with inhibition of the epithelial sodium channel in blacks with hypertension. Hypertension. 2005; 46:481–487. [PubMed: 16116042]
44. Andersen H, Hansen PB, Bistrup C, et al. Significant natriuretic and antihypertensive action of the epithelial sodium channel blocker amiloride in diabetic patients with and without nephropathy. J Hypertens. 2016; 34:1621–1629. [PubMed: 27214087]

45. Weisbord SD, Mor MK, Resnick AL, et al. Prevention, incidence, and outcomes of contrast-induced acute kidney injury. Arch Intern Med. 2008; 168:1325–1332. [PubMed: 18574090]

46. Jebb SA, Cole TJ, Doman D, et al. Evaluation of the novel Tanita body-fat analyser to measure body composition by comparison with a four-compartment model. Br J Nutr. 2000; 83:115–122. [PubMed: 10743490]

47. Wessel D, Flugge UI. A method for the quantitative recovery of protein in dilute solution in the presence of detergents and lipids. Anal Biochem. 1984; 138:141–143. [PubMed: 6731838]

48. Hughey RP, Mueller GM, Bruns JB, et al. Maturation of the epithelial Na+ channel involves proteolytic processing of the alpha- and gamma-subunits. J Biol Chem. 2003; 278:37073–37082. [PubMed: 12871941]

49. Kastner C, Pohl M, Sendeski M, et al. Effects of receptor-mediated endocytosis and tubular protein composition on volume retention in experimental glomerulonephritis. Am J Physiol Renal Physiol. 2009; 296:F902–911. [PubMed: 19193726]

50. Piedagnel R, Tiger Y, Lelongt B, et al. Urokinase (u-PA) is produced by collecting duct principal cells and is post-transcriptionally regulated by SV40 large-T, arginine vasopressin, and epidermal growth factor. J Cell Physiol. 2006; 206:394–401. [PubMed: 16155905]

51. Oxlund CS, Buhl KB, Jacobsen IA, et al. Amiloride lowers blood pressure and attenuates urine plasminogen activation in patients with treatment-resistant hypertension. J Am Soc Hypertens. 2014; 8:872–881. [PubMed: 25492830]

52. Turgutalp K, Bardak S, Helvaci I, et al. Community-acquired hyperkalemia in elderly patients: risk factors and clinical outcomes. Ren Fail. 2016; 38:1405–1412. [PubMed: 27494301]

53. Mavrakanas TA, Gariani K, Martin PY. Mineralocorticoid receptor blockade in addition to angiotensin converting enzyme inhibitor or angiotensin II receptor blocker treatment: an emerging paradigm in diabetic nephropathy: a systematic review. Eur J Intern Med. 2014; 25:173–176. [PubMed: 24315413]

54. Bakris GL, Pitt B, Weir MR, et al. Effect of Patiromer on Serum Potassium Level in Patients With Hyperkalemia and Diabetic Kidney Disease: The AMETHYST-DN Randomized Clinical Trial. JAMA. 2015; 314:151–161. [PubMed: 26172895]

55. Brown MJ, Williams B, Morant SV, et al. Effect of amiloride, or amiloride plus hydrochlorothiazide, versus hydrochlorothiazide on glucose tolerance and blood pressure (PATHWAY-3): a parallel-group, double-blind randomised phase 4 trial. Lancet Diabetes Endocrinol. 2016; 4:136–147. [PubMed: 26489809]

56. Pearce D, Soundararajan R, Trimpert C, et al. Collecting duct principal cell transport processes and their regulation. Clin J Am Soc Nephrol. 2015; 10:135–146. [PubMed: 24875192]

57. Rossier BC, Baker ME, Studer RA. Epithelial sodium transport and its control by aldosterone: the story of our internal environment revisited. Physiol Rev. 2015; 95:297–340. [PubMed: 25540145]

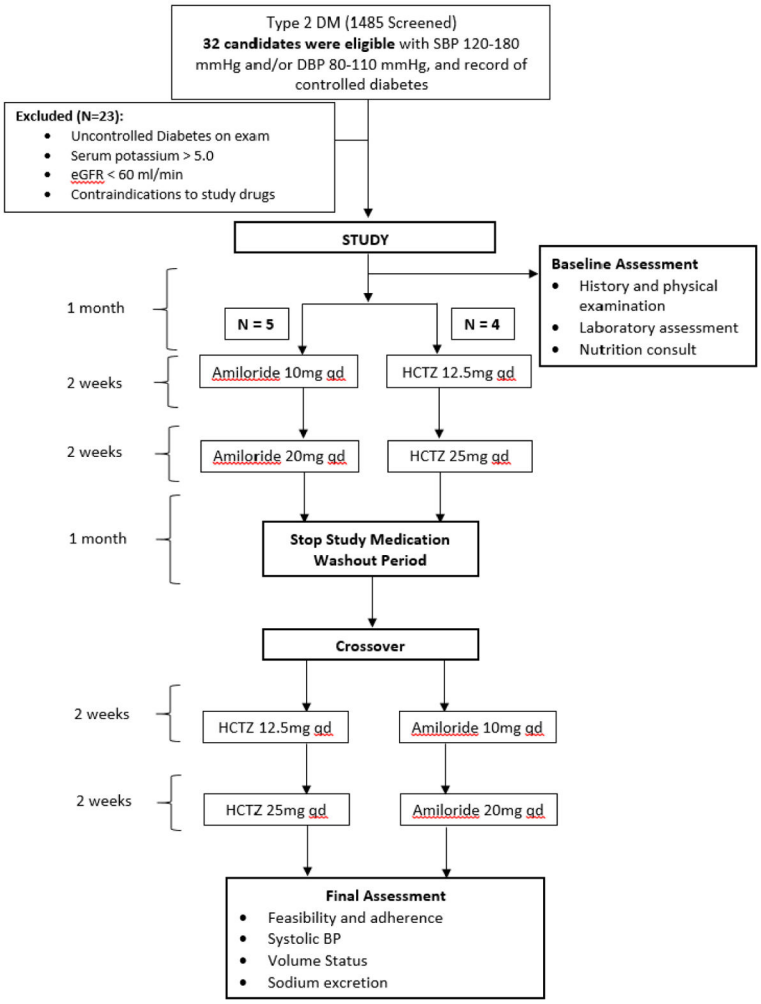


Figure 1.
Flowchart illustrating the design of the randomized trial.

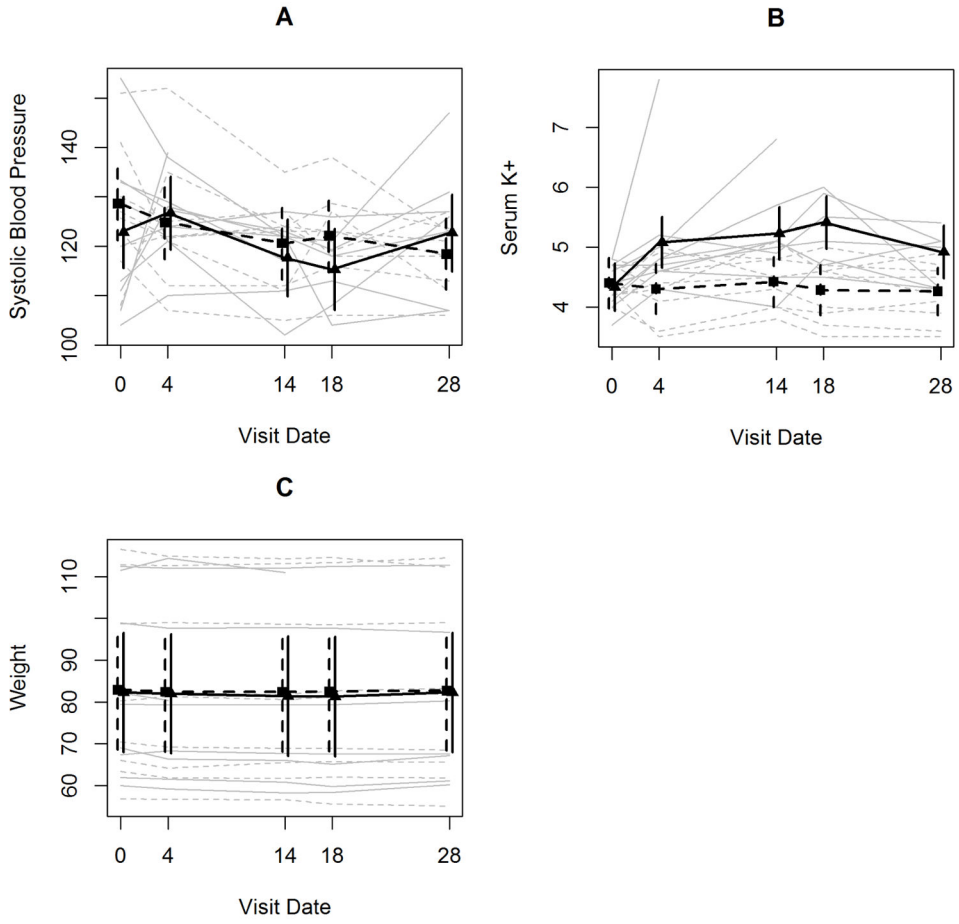
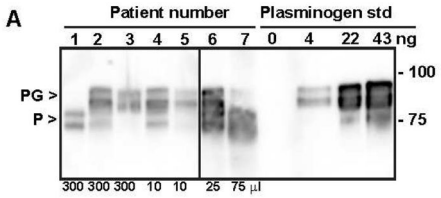
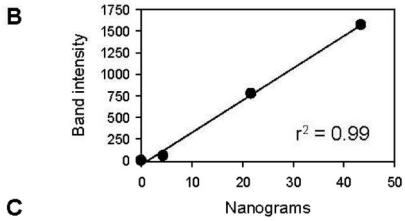


Figure 2. Trends in study outcomes over the course of the randomized trial

Plots of observed trends in outcomes for each study participant (grey lines) and of estimated trends in means (heavy black lines). 95% confidence intervals are shown for the time-specific estimates (vertical lines). Solid lines are for results observed under treatment with amiloride and dashed lines are for results observed under treatment for HCTZ. (A) presents SBP results, (B) serum K^+ results and (C) weight results





C

Patient	[Total] (µg/gCr)	SD	n	[PG] (µg/gCr)	SD	n	[P] (µg/gCr)	SD	n
1	21.78	6.34	3	Negligible			21.78	6.34	3
2	95.22	24.30	3	57.85	13.17	4	32.64	13.39	3
3	36.56	8.99	3	36.56	8.99	3	Negligible		
4	2001.14	232.40	3	957.85	296.48	6	757.55	53.16	3
5	573.05	93.47	3	346.38	122.43	4	192.92	31.03	3
6	1125.57	381.71	4	492.76	199.92	4	632.80	298.20	4
7	1386.46	331.82	3	265.49	60.85	3	936.77	246.72	6
8	Negligible			Negligible			Negligible		
9	Negligible			Negligible			Negligible		

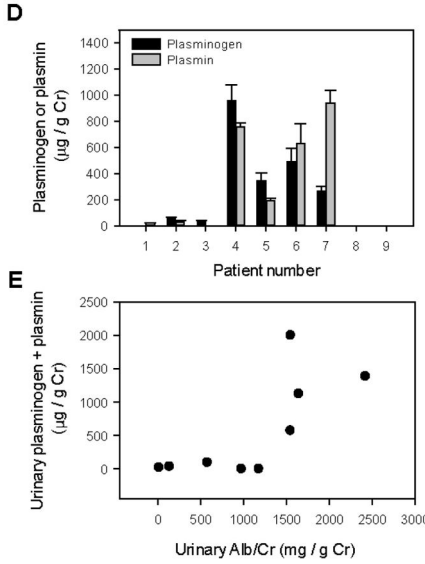


Figure 3. Quantitation of uPl

Aliquots of urine from nine patients were subjected to SDS-PAGE and immunoblotting with anti-plasminogen/plasmin antibodies (n=3–6). Urine volumes analyzed were optimized for detection of plasminogen and plasmin by immunoblotting (10–300 µl), and are listed at the bottom of the figure. Varying amounts of pure plasminogen (4 to 43 ng) were included on the same blot to create a standard curve and establish levels of plasminogen and plasmin for each patient. A representative immunoblot (A) and corresponding standard curve (B) are shown for seven patients (two patients consistently lacked a signal). Line between samples 5 and 6 indicate where the blot was cut. Arrowheads indicate plasminogen (PG) and plasmin (P). Data from 3–6 analyses were normalized to urine creatinine and values presented in panel (C) and as a bar graph in panel (D). (E) Correlation between uPl/Cr and urinary albumin/Cr.

Table 1

Characteristics of participants upon enrollment into the study. Of the 9 participants in the trial, 5 were randomized to Amiloride followed by HCTZ (Amiloride – HCTZ) and 4 were randomized to HCTZ followed by Amiloride (HCTZ – Amiloride).

	All Subjects		Amiloride - HCTZ		HCTZ - Amiloride	
	n	**%**	**n**	**%**	**n**	**%**
Females	5	55.6	4	80.0	1	25.0
Caucasian	4	44.4	2	40.0	2	50.0
Asian	1	11.1	0	0.0	1	25.0
Hispanic	5	55.6	4	80.0	1	25.0
	Mean	**Standard Deviation**	**Mean**	**Standard Deviation**	**Mean**	**Standard Deviation**
Age (years)	58.4	10.0	56.4	13.2	61.0	4.3
BMI (kg/m^2)	32.2	6.2	28.3	2.5	37.1	6.1
Albumin/Cr (mg/g)	1120	780	1070	650	1170	1030
Weight (kg)	84.0	21.3	68.7	7.6	103.1	16.1
Creatinine (mg/dL)	0.8	0.3	0.7	0.2	1.0	0.2
Potassium (mmol/L)	4.4	0.4	4.2	0.4	4.6	0.2
Mean SBP (mm Hg)	132	13	123	20	126	19
Mean DBP (mm Hg)	80.3	8.1	75.1	3.9	75.0	11.3
EGFR (mL/min)	87.4	20.4	92.6	23.0	77.4	14.8

Table 2

Summaries of outcomes over the course of the study. Baseline estimates are simple averages and standard deviations obtained across study participants at the appropriate time period. ΔLow and ΔHigh estimates are estimates of change from baseline for the study participants at the end of the low and high dose periods, respectively. Summaries are presented overall and stratified by uPl/Cr.

Treatment	Dose	N	Systolic Blood Pressure (mmHg)		Serum K^+ (mmol/L)		Weight (kg)	
All patients			**mean**	**standard deviation (standard error for Δ)**	**mean**	**standard deviation (standard error for Δ)**	**mean**	**standard deviation (standard error for Δ)**
Amiloride	Baseline	9	123	16	4.4	0.4	82.5	20.5
	ΔLow	8	−5.1	4.5	0.9	0.2	−0.9	0.6
	ΔHigh	7	−0.1	4.6	0.6	0.2	−0.1	0.7
HCTZ	Baseline	9	127	13	4.4	0.3	83.0	21.8
	ΔLow	9	−8.0	4.4	0.0	0.2	−0.6	0.6
	ΔHigh	9	−10.2	4.4	−0.1	0.2	−0.3	0.6
High Plasminogen			mean	standard deviation/error (for Δ)	mean	standard deviation/error (for Δ)	mean	standard deviation/error (for Δ)
Amiloride	Baseline	4	135	14	4.5	0.3	91.3	24.9
	ΔLow	4	−11.8	6.7	1.0	0.3	−0.7	0.8
	ΔHigh	3	−0.9	7.1	0.9	0.3	0.3	0.9
HCTZ	Base	4	131	19	4.5	0.3	93.8	25.4
	ΔLow	4	−10.1	6.7	0.1	0.3	−1.1	0.9
	ΔHigh	4	−9.0	6.7	−0.1	0.3	−0.9	0.9
Low Plasminogen			mean	standard deviation/error (for Δ)	mean	standard deviation/error (for Δ)	mean	standard deviation/error (for Δ)
Amiloride	Baseline	5	113	10	4.2	0.4	75.5	15.3
	ΔLow	4	1.4	6.4	0.8	0.3	−1.0	0.8
	ΔHigh	4	1.1	6.3	0.4	0.3	−0.5	0.8
HCTZ	Base	5	123	5	4.3	0.2	74.4	16.0
	ΔLow	5	−6.7	6.0	0.0	0.3	−0.2	0.8
	ΔHigh	5	−11.8	6.0	−0.2	0.3	0.1	0.8