How to Cite this article: Aihua Wu, Martin Wolley, Alexandra Matthews, Diane Cowley, Paul Welling, Robert Fenton, and Michael Stowasser, In Primary Aldosteronism Acute Potassium Chloride Supplementation Suppresses Abundance and Phosphorylation of the Sodium-Chloride Cotransporter, Kidney360, Publish Ahead of Print, 2022, 10.34067/KID.0003632022

Article Type: Original Investigation

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Aihua Wu, Martin Wolley, Alexandra Matthews, Diane Cowley, Paul Welling, Robert Fenton, and Michael Stowasser

Key Points:
*KCl intake induced reduction in NCC and pNCC in uEVs from PA patients during significantly raised level of endogenous aldosterone.

*Low plasma K+ (secondary to aldosterone excess) may dominate in terms of NCC regulation in the setting of PA.

Abstract:
Background: Elevated abundance of sodium-chloride cotransporter (NCC) and phosphorylated NCC (pNCC) are potential markers of primary aldosteronism (PA), but these effects may be driven by hypokalaemia.

Methods: We measured plasma potassium in PA patients. If potassium was <4.0 mmol/L, patients were given sufficient oral potassium chloride (KCl) over 24 hours to achieve as close to 4.0 mmol/L as possible. Clinical chemistries were assessed and urinary extracellular vesicles (uEVs) were examined to investigate effects on NCC.

Results: Among 21 PA patients who received a median total dose of 6.0 [2.4, 16.8] g KCl, increases were observed in plasma potassium (from 3.4 to 4.0 mmol/L, P<0.001), aldosterone (305 to 558 pmol/L, P=0.01) and renin (1.2 to 2.5 mU/L, P<0.001), while decreases were detected in uEV levels of NCC (median fold change (post/basal) [FC]= 0.71 [0.09, 1.99], P=0.02), pT60-NCC (FC=0.84 [0.06, 1.66], P=0.05) and pT55/60-NCC (FC=0.67 [0.08, 2.42], P=0.02). By contrast, in 10 PA patients who did not receive KCl, there were no apparent changes in plasma potassium, NCC abundance and phosphorylation status, but increases were observed in plasma aldosterone (from 178 to 418 pmol/L, P=0.006) and renin (2.0 to 3.0 mU/L, P=0.009). Plasma potassium correlated inversely with uEV levels of NCC (R²=0.11, P=0.01), pT60-NCC (R²=0.11, P=0.01) and pT55/60-NCC (R²=0.11, P=0.01).

Conclusion: Acute oral KCl loading replenished plasma potassium in PA patients and suppressed NCC abundance and phosphorylation despite a significant rise in plasma aldosterone. This supports the view that potassium supplementation in humans with PA overrides the aldosterone stimulatory effect on NCC. The increased plasma aldosterone in PA patients without KCl supplementation may be due to aldosterone response to posture change.

Disclosures: P. Welling reports the following: Advisory or Leadership Role: American Journal of Physiology, Renal Editorial Board; American Society of Physiology, Chair, Finance Committee, and Council Member. R. Fenton reports the following: Advisory or Leadership Role: Associate editor for American Journal of Physiology Renal; Editorial board member of J Am Soc Neph. The remaining authors have nothing to disclose.

Funding: Commonwealth Government of Australia: Aihua Wu; Leducq Foundation: Aihua Wu, Martin J. Wolley, Alexandra Matthews, Diane Cowley, Paul A. Welling, Robert A. Fenton, Michael Stowasser, Potassium in Hypertension

Author Contributions: Aihua Wu: Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Project administration; Resources; Software; Validation; Visualization; Writing - original draft; Writing - review and editing Martin Wolley: Conceptualization; Funding acquisition; Investigation; Supervision; Writing - review and editing Alexandra Matthews: Methodology Diane Cowley: Methodology Paul Welling: Funding acquisition; Writing - review and editing Robert Fenton: Funding acquisition; Writing - review and editing Michael Stowasser: Conceptualization; Supervision; Writing - review and editing

Data Sharing Statement: All data is included in the manuscript and/or supporting information.

Clinical Trials Registration:

Registration Number:

Registration Date:

The information on this cover page is based on the most recent submission data from the authors. It may vary from the final published article. Any fields remaining blank are not applicable for this manuscript.

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In Primary Aldosteronism Acute Potassium Chloride Supplementation Suppresses Abundance and Phosphorylation of the Sodium-Chloride Cotransporter

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Key point

- KCl intake induced reduction in NCC and pNCC in uEVs from PA patients during significantly raised level of endogenous aldosterone.
- Low plasma K⁺ (secondary to aldosterone excess) may dominate in terms of NCC regulation in the setting of PA.

Abstract

Background: Elevated abundance of sodium-chloride cotransporter (NCC) and phosphorylated NCC (pNCC) are potential markers of primary aldosteronism (PA), but these effects may be driven by hypokalaemia.

Methods: We measured plasma potassium in PA patients. If potassium was <4.0 mmol/L, patients were given sufficient oral potassium chloride (KCl) over 24 hours to achieve as close to 4.0 mmol/L as possible. Clinical chemistries were assessed and urinary extracellular vesicles (uEVs) were examined to investigate effects on NCC.

Results: Among 21 PA patients who received a median total dose of 6.0 [2.4, 16.8] g KCl, increases were observed in plasma potassium (from 3.4 to 4.0 mmol/L, \( P<0.001 \)), aldosterone (305 to 558 pmol/L, \( P=0.01 \)) and renin (1.2 to 2.5 mU/L, \( P<0.001 \)), while decreases were detected in uEV levels of NCC (median fold change\(_{\text{post/basal}}\) [FC]=0.71 [0.09, 1.99], \( P=0.02 \)), pT60-NCC (FC=0.84 [0.06, 1.66], \( P=0.05 \)) and pT55/60-NCC (FC=0.67 [0.08, 2.42], \( P=0.02 \)). By contrast, in 10 PA patients who did not receive KCl,
there were no apparent changes in plasma potassium, NCC abundance and phosphorylation status, but increases were observed in plasma aldosterone (from 178 to 418 pmol/L, \( P=0.006 \)) and renin (2.0 to 3.0 mU/L, \( P=0.009 \)). Plasma potassium correlated inversely with uEV levels of NCC (\( R^2=0.11, P=0.01 \)), pT60-NCC (\( R^2=0.11, P=0.01 \)) and pT55/60-NCC (\( R^2=0.11, P=0.01 \)).

**Conclusion:** Acute oral KCl loading replenished plasma potassium in PA patients and suppressed NCC abundance and phosphorylation despite a significant rise in plasma aldosterone. This supports the view that potassium supplementation in humans with PA overrides the aldosterone stimulatory effect on NCC. The increased plasma aldosterone in PA patients without KCl supplementation may be due to aldosterone response to posture challenge.
Introduction

Primary aldosteronism (PA) is a common and potentially curable form of hypertension. Hypokalemia was considered as a mandatory feature of PA until the more recent recognition of the high prevalence of PA among normokalemic hypertensives.\textsuperscript{1-4} The thiazide-sensitive sodium-chloride (Cl\textsuperscript{-}) cotransporter (NCC) is the major transporter on the apical surface of epithelial cells for electroneutral Na\textsuperscript{+}/Cl\textsuperscript{-} reabsorption in the early distal convoluted tubule (DCT). Aldosterone and its analogues were thought to be major regulators of NCC through the mineralocorticoid receptor,\textsuperscript{5-9} but whether these effects in humans are primarily driven by mineralocorticoid induced hypokalaemia is unclear.

Urinary extracellular vesicles (uEVs) are frequently used as a non-invasive source of renal biomarker discovery,\textsuperscript{10, 11} and are a reliable tool to monitor specific physiological responses and disease mechanisms.\textsuperscript{12} In humans, PA is associated with increased abundance of pNCC in uEVs.\textsuperscript{13} Examination of uEVs from PA patients undergoing 4 days co-administration of fludrocortisone acetate and oral NaCl loading, with oral KCl supplements to correct or prevent hypokalaemia during testing, provided evidence that NCC is mineralocorticoid-sensitive.\textsuperscript{14} However, recent studies demonstrated K\textsuperscript{+} supplementation reduced the degree of upregulation of NCC abundance induced by mineralocorticoids in mice kidney and uEVs from PA patients,\textsuperscript{14-16} suggesting that higher plasma K\textsuperscript{+} induced by higher KCl intake may counterbalance mineralocorticoid-induced NCC upregulation.

In the Hypertension Unit of Princess Alexandra Hospital, Brisbane, Australia, before undergoing seated saline suppression testing (SSST) as a means of confirming or
excluding PA, patients whose plasma K⁺ are lower than 4.0 mmol/L are given oral potassium supplements (in the form of potassium chloride [KCl]) in variable amounts to achieve or maintain normokalaemia during SSST the next day. By taking advantage of the clinical SSST protocol, the current study aimed to utilize uEVs to address the hypothesis that oral KCl supplementation in PA patients with elevated endogenous aldosterone will suppress NCC abundance and phosphorylation. Concomitantly, we examined the renal outer medullary potassium channel (ROMK) to assess whether altering plasma K⁺ in a setting of aldosterone excess affected K⁺ excretion through ROMK.

**Methods**

**Ethical issues**

The clinical procedures of posture responsiveness testing and seated saline testing were performed in the Hypertension Unit of the Princes Alexandra Hospital, Brisbane, Australia. The laboratory investigations were performed in the Endocrine Hypertension Research Centre, The University of Queensland Diamantina Institute, Brisbane, Australia. Ethical approval was granted by the Metro South Human Research Ethics Committee (HREC/18/QPAH/103).

**Recruitment**

Hypertensive patients with raised plasma aldosterone-to-renin ratios (ARRs) who were admitted for seated saline suppression testing (SSST, as a means of confirming or excluding PA) were invited to participate and provided informed written consent. A total
of 38 (F24/M14) patients were invited and all agreed to participate. All subjects completed the KCl replacement experiment prior to SSST.

**KCl replacement**

At least four weeks prior to admission, medications affecting plasma aldosterone and renin levels were withdrawn and replaced by other anti-hypertensive medications (e.g. verapamil, prazosin or doxazosin, moxonidine and/or hydralazine). Patients were admitted to hospital to ensure the dietary (normal hospital diet) and posture requirements were met and to facilitate monitoring of plasma $K^+$ levels and other parameters. Detailed clinical routine procedures and sampling time-points for the current study are listed in Table 1. In brief, prior to the SSST, patients commenced a 24-hour urine collection at home in the morning of the day of admission. The following day (Day 0), aldosterone responsiveness to upright posture was determined by measuring plasma aldosterone at 7AM following overnight recumbency and again at 10AM following 3 hours of upright posture. On the day of SSST (Day 1), an infusion of 2L 0.9% saline over 4 hours was commenced at 8AM, at least 2 hours after rising from bed and at least 30 min after assuming a seated position.

The baseline (7AM) plasma $K^+$ results were available at 10AM on Day 0. If plasma $K^+$ concentration at 7AM was less than 4.0 mmol/L, participants were given sufficient slow-release KCl (Span-K) up to four times a day (Q6H) in an attempt to achieve as close to 4.0 mmol/L as possible by the next morning (Day 1) before SSST.
For the current study, blood collected at 7AM during recumbent posture on Day 0 were adopted as the baseline blood measurement (basal) and blood collected on Day 1 just before commencement of the SSST at 8AM during seated posture was adopted as the post-test blood measurement.

**Urine collection and uEV isolation**

Two sterilized 200 ml containers were given to the 38 participants for urine collection. Urine collection time-points are listed in Table 1. Briefly, 25 to 200 ml of mid-stream morning urine was collected after baseline blood collection and before KCl supplementation on Day 0 (baseline urine), and at 6-7AM on Day 1 before commencement of SSST (post-test urine). On Day 0, twenty-six participants who were unable to produce enough urine volume (≥25 ml) at 7AM collected urine between 7AM and 10AM but before KCl replacement commenced; two participants (patients 26 and 34) were on low dose (1.2g) of KCl supplementation before baseline urine collection. Collected urine samples were immediately treated with protease inhibitor cocktail (Roche cOmplete, EDTA-free, 1 tablet per 50 ml urine) before aliquoting and freezing at -80°C. uEVs were isolated using progressive ultracentrifugation techniques with dithiothreitol (DTT) treatment as previously described\(^\text{14}\). Obtained uEV pellets were resuspended in 70-110 μl 1x PBS containing 0.1% v/v SDS, followed by on-ice sonicating with approximately 5-10 cycles at 50% power (10s on/off on Bioruptor Pico) and 10 min centrifugation at 17,000g to pellet insoluble residues. Total protein concentration of the obtained supernatant was measured by spectrophotometer (Thermo Scientific Nanodrop Lite).
Sample measurements

Measurements of blood and 24-hour urine were performed by Pathology Queensland Laboratory at Princess Alexandra Hospital immediately after collection was completed. Plasma aldosterone was determined by LC-MS/MS,\textsuperscript{17} direct renin concentration was determined by chemiluminescent immunoassay,\textsuperscript{18} plasma cortisol was determined by immunoassay. Spot urine creatinine concentration was measured using a creatinine urinary detection assay kit (EIACUN, Invitrogen) at Endocrine Hypertension Research Centre. Spot urinary Na\textsuperscript{+} and K\textsuperscript{+} were measured by Pathology Queensland Chemistry Department.

Immunoblotting

uEVs were treated with 5x Laemmli buffer (1/4, v/v) and incubated at 60°C for 10 min before sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Ten μg of each sample were loaded and separated on 4-15% Criterion\textsuperscript{TM} TGX\textsuperscript{TM} Precast Midi Protein gels (5671083 or 56710985 depending on loading volume, Bio-Rad) and were transferred to Turbo polyvinyl difluoride Midi membrane (1704157, Bio-Rad) under 2.5A and 25V for 7 mins on Bio-Rad Turbo transferring system. Each blot was duplicated for proteins with similar size. Blots were then blocked followed by overnight 4°C incubation with the following antibodies: rabbit anti-NCC (1/1000; AB3553, Merck Millipore), rabbit anti-T55/60pNCC (1/1000),\textsuperscript{19} rabbit anti-T60pNCC (1/2500),\textsuperscript{19} rabbit anti-ROMK (1/500),\textsuperscript{20} rabbit anti-ALG-2-interacting protein X (ALIX) (1/2000; ABC40, Merck Millipore), rabbit anti-tumor susceptibility gene 101 (TSG101) (1/2000; MASBC649, Merck Millipore) and rabbit anti-tetraspanin CD9 (CD9) (1/1000, ab92726,
Abcam). NCC and pNCCs were measured by the dominant band between 100-150kDa. 
ROMK was measured by a complex glycosylated band detected between 50-55kDa, core-
glycosylated band at 40kDa and unglycosylated band detected at 37kDa.\textsuperscript{20} uEV marker 
proteins ALIX, TSG101 and CD9 were measured by the dominant bands at 96kDa, 
45kDa and 25kDa, respectively. HRP conjugated goat anti-rabbit IgG antibody (12-348, 
Merck Millipore) was used as the secondary antibody at 1/20,000 and 1/500 dilution 
respectively for luminol-based enhanced chemiluminescence (1705061, Bio-rad) before 
exposure in configuring signal accumulation mode by Bio-Rad ChemiDoc XRS+ Imager 
with Image Lab software. Images that did not exceed saturation were exported for 
analyses.

**Analyses inclusion criteria**

Due to the different physiological states, only PA confirmed patients were included in all 
analyses. Immunoblotting detection of at least two EV-enriched proteins (ALIX, TSG101 
or CD9) in each sample was considered successful isolation of uEVs, and patients in 
whom uEVs were successfully isolated from both basal and post-test samples were 
included in the comparison analyses. Samples included in uEV protein analyses are listed 
in Table S1.

**Statistical analyses**

Calculations were processed with R. Due to blood at baseline being hemolyzed, patient 
10 was excluded from the comparisons of blood biochemical parameters. Therefore, 
comparisons of blood biochemical parameters were performed in a total of 37 subjects.
Because the volume of spot urine collected from patient 15, 18, 29, 31 and 35 remained inadequate after uEV isolation, measurements of creatinine, Na\(^+\) and K\(^+\), the comparisons of spot urinary electrolytes-to-creatinine ratios were performed in a total of 32 (PA27/LRH5) subjects. Paired Wilcoxon tests were performed to compare the differences of biochemical parameters in blood samples collected at 7AM on Day 0 and 8AM on Day 1, and in spot urine electrolytes-to-creatinine (Na\(^+\)/creatinine and K\(^+\)/creatinine) at basal and post-test. In immunoblotting analyses, absolute abundances of analysed proteins were analysed with ImageJ software. To minimise errors due to alterations in actual uEV biogenesis or excretion rates, relative protein abundances (derived by dividing the absolute abundances of proteins of interest by the sum abundance of ALIX, TSG101 and CD9 in each patient’s sample) were used for comparisons. Wilcoxon tests were performed to assess baseline differences in analysed proteins. Paired T-tests were performed to assess the changes in the relative abundances of analysed proteins (log10 transformed). Pearson’s correlations were sought between log10 transformed relative abundances of analysed proteins and biochemical parameters. If baseline urines for uEV were collected post 10AM, 10AM biochemical results were used. A \(P\) value <0.05 was considered statistically significant. Data are presented as median [range], unless stated otherwise.

**Results**

**-Participants’ characteristics**

A total of 38 (24 females [F] and 14 males [M]) hypertensive patients with repeatedly raised ARRs were recruited and all agreed to participate (Table 2). SSST was positive in
32 thereby confirming the diagnosis of PA, and negative in 6 in which PA was excluded. Due to the different physiological states, only PA confirmed patients were included in analyses. The report numbers of participants at each stage of study are depicted in Figure 1. Among the 32 (F19/M12) PA patients, the median age was 45 [25, 70] (median [range]) years old, with the median body mass index of 30.3 [20.8, 47.5] kg/m², median blood pressure of 146 [115, 174] / 89 [68, 110] mmHg, and were on a median of 2 [0,4] anti-hypertensive medications without affecting plasma aldosterone and renin levels.

Baseline blood sample from patient 10 was hemolyzed, but her plasma K+ at 10AM on D0 was 3.8 mmol/L, hence oral KCl supplementation was given and commenced at 12 noon on the same day. Of the remaining 31 (F18/M12) PA patients, twenty-one (F13/M8) whose baseline plasma K+ were lower than 4.0mmol/L therefore were given KCl supplementation (hereafter referred to as KCl group), and 10 (F6/M4) did not received KCl (hereafter referred to as non-KCl group). Among the 10 in the non-KCl group, nine had baseline K+ levels that were either ≥ 4.0mmol/L (N=6) or only just below 4.0mmol/L (N=3), while remaining 1 patient (patient 32) had a baseline K+ of 3.1 but was not given KCl despite meeting protocol criteria.

-Baseline biochemical differences

Baseline plasma K+ was significantly higher (P<0.001) in the non-KCl group (4.0[3.1, 4.4] mmol/L) than that in the KCl group (3.4 [2.8, 3.9] mmol/L) (Figure 2), whilst plasma levels of aldosterone (178 [59, 356] vs. 305 [57, 1630] pmol/L, P=0.08), ARR (81 [31, 513] vs. 171 [34, 2480] pmol/mU, P=0.03) and cortisol (229 [136, 373] vs. 320 [178, 470] nmol/L, P=0.02) were relatively lower in the non-KCl group than in the KCl group.
There were no statistical differences in 24-hour urinary levels of Na\(^+\), K\(^+\) and the Na\(^+\)/K\(^+\), and in spot urinary Na\(^+\)/creatinine, K\(^+\)/creatinine, ratio of Na\(^+\)/K\(^+\) and creatinine between the two groups.

**Physiological effects of KCl supplementation in PA**

In the KCl group (Figure 3a), plasma K\(^+\) increased \((P<0.001)\) from a median of 3.4 [2.8, 3.9] to 4.0 [3.1, 4.7] mmol/L in response to a median of 6.0 [2.4, 16.8] g total dose of KCl, though plasma K\(^+\) fell in two subjects (from 3.9 to 3.8 mmol/L in patient 11 despite 3.6g KCl, and from 3.4 to 3.1 mmol/L in patient 24 despite 4.8g KCl). Spot urine K\(^+\)/creatinine increased from 5.0 [2.2, 15.2] to 9.8 [4.5, 25.9] mmol/mmol \((p<0.001)\). Increases were also detected in plasma aldosterone (from 305 [57, 1630] to 558 [313, 1140] pmol/L, \(P=0.01\)) and direct renin concentration (from 1.2 [0.5, 3.9] to 2.5 [0.6, 6.6] mU/L, \(P<0.001\)). There were no apparent changes in plasma levels of ARR \((P=0.31)\) and cortisol \((P=0.92)\), spot urine Na\(^+\)/creatinine \((P=0.25)\), spot urine Na\(^+\)/K\(^+\) \((P=0.39)\), and spot urine creatinine concentration \((P=0.83)\).

Among the 10 (F6/M4) in the non-KCl group (Figure 3b), there were no apparent changes in plasma K\(^+\) (basal 4.0 [3.1, 4.4] vs post 3.8 [3.5, 4.4] mmol/L, \(P=0.54\)), but increase in the spot urine K\(^+\)/creatinine were observed, from 3.6 [2.8, 9.2] to 6.3 [4.3, 10.8] mmol/mmol \((P=0.04)\). Like those in the KCl group, there were increases in plasma levels of aldosterone (from 178 [59, 356] to 418 [316, 700] pmol/L, \(P=0.006\)) and renin (from 2.0 [0.5, 4.4] to 3.0 [0.5, 7.2] mU/L, \(P=0.009\)), and an additional increase in plasma ARR from 81 [31, 513] to 131 [48, 843] pmol/mU \((P=0.01)\). There were no
apparent changes in plasma cortisol \( (P=0.06) \), spot urine \( \text{Na}^+ / \text{creatinine} \) \( (P=0.20) \), spot urine \( \text{Na}^+ / \text{K}^+ \) \( (P=0.73) \), and spot urine creatinine \( (P=0.65) \).

- **uEV characterisation**

There was insufficient sample quantity to allow for characterisation of the uEVs by transmission electron microscopy and nanoparticle tracking analysis, but we have previously used these approaches to examine uEV pools isolated from humans by the same protocol, and seen very clear uEV structures and the uEVs’ size distribution was within the EVs appropriate range \( (30-1000\text{nm} \text{ in diameter}) \).\(^{21, 22}\) Immunoblotting detection of at least two EV-enriched proteins (ALIX, TSG101 or CD9) in each sample was considered successful isolation of uEVs, and patients in whom uEVs were successfully isolated from both basal and post-test samples were included in the comparison analyses (Figure S1 and Table S1). The observation of a shift in the ALIX bands in multiple samples may be due to ALIX truncation by the endosomal sorting complex required for transport (ESCRT) machinery,\(^{23}\) or related to the presence of Tamm Horsfall protein.\(^{24}\) During the test, no apparent changes were observed in spot urine creatinine concentration (Figure 3) and in absolute abundance of the EV-enriched proteins (Figure S2), rendering an effect of potassium on uEV concentration less likely.\(^{12, 24}\)

- **Baseline uEV protein differences**

Baseline proteins were compared by allocation of oral KCl supplementation and sex (Figure 4). PA in the KCl group \( (N=21, F13/M8) \) had higher abundances of NCC \( (1.35 \text{ fold}) \).
P=0.04) and total ROMK (1.65 [0.34, 4.32], P=0.04) than PA in the non-KCl group (N=9, F5/M4) (NCC: 0.50 [0.25, 1.98], pT60-NCC: 0.45 [0.05, 1.82], total ROMK: 0.87 [0.14, 1.90]) (Figure 4a). No sex-related differences were detected in analysed proteins when total females were compared with total males (Figure 4b). By dividing females into two groups based on age of 45 into “likely pre-menopausal” and “likely post-menopausal”, 25 females who were younger than 45-years-old exhibited a significantly higher level of NCC (1.71 [0.53, 4.57], P=0.04) compared to that in females who were 45 of age and older (0.50 [0.12, 3.26]), and higher level of ROMK (1.65 [1.06, 4.32], P=0.04) compared to that in males (1.34 [0.14, 2.07]) (Figure 4c).

-Acute KCl reduces NCC abundance and phosphorylation

In the KCl group, there were significant decreases in the abundances of NCC (median fold change (FC)=0.71 [0.09, 1.99] (post/basal), P=0.02), pT60-NCC (FC=0.84 [0.06, 1.66], P=0.05) and pT55/60-NCC (FC=0.67 [0.08, 2.42], P=0.02). By contrast, in the non-KCl group, no significant changes in NCC, pT60-NCC and pT55/60-NCC were seen. There were no significant changes in uEV levels of ROMK in either the KCl or non-KCl group (Figure 5 and Figure S2).

-K⁺ inversely correlates with NCC abundance and phosphorylation

The uEV levels of NCC (R²=0.11, P=0.01), pT60-NCC (R²=0.11, P=0.01) and pT55/60 (R²=0.11, P=0.01) were negatively correlated with plasma [K⁺] among all PA participants during the test (Figure 6a, e, h). In addition, NCC positively correlated with
plasma aldosterone \((R^2=0.14, P=0.004)\), ARR \((R^2=0.26, p<0.001)\), and negatively correlated with plasma renin \((R^2=0.23, P<0.001)\) (Figure 6b, d, c). pT60-NCC positively correlated with plasma aldosterone \((R^2=0.1, P=0.01)\) and ARR \((R^2=0.14, P=0.004)\) (Figure 6f, g). Furthermore, ROMK weakly positively correlated with spot urine \(\text{Na}^+\)/creatinine \((R^2=0.09, P=0.04)\) and \(\text{K}^+\)/creatinine \((R^2=0.09, P=0.04)\) (Figure 6i, j). \(\Delta\text{KCl}\) dose strongly negatively correlated with fold change of NCC \((R^2=0.3, P=0.004)\) (Figure 7a). There were no significant associations between \(\Delta\text{plasma K}^+\) and fold change of NCC \((R^2=0.11, P=0.09)\) and between \(\Delta\text{spot urine K}^+\)/creatinine and fold change of pT55/60-NCC \((R^2=0.17, P=0.05)\) (Figure 7b, c). There were no additional associations of the analysed proteins with other biochemical parameters (Figure S3).

**Discussion**

Aggressively correcting hypokalaemia by 24-hour oral KCl supplementation prior to SSST increased median plasma \([\text{K}^+]\) in the KCl group, whilst in the non-KCl group plasma \([\text{K}^+]\) remained unchanged. However during the 24-hour period, increases in plasma aldosterone were observed in both KCl (1.8-fold) and non-KCl (2.2-fold) groups, which is likely due to difference in posture between baseline (recumbent) and post-test (upright) sampling as evidenced by the observed rises in direct renin concentration in both groups.\(^{26}\) Upright posture has a significant stimulatory effect on renin beginning in 15 minutes and peaking between 60-120 minutes, and aldosterone secretion directly correlates with the elevation in the renin activity during the 120 minutes upright posture.\(^{27-30}\) Although there are posture (or angiotensin II)-unresponsive forms of PA, in which adrenocorticotropin hormone assumes a dominant role over angiotensin II in
regulating aldosterone production,\textsuperscript{31} in the current study, plasma aldosterone levels in most patients in the non-KCl group were responsive to the upright posture (Table 2). Furthermore, blood samples were collected at 7AM at baseline and just before 8AM at post-test, and there were no statistically changes in plasma cortisol in both groups, making it unlikely that the changes in plasma aldosterone reflected its circadian rhythm. In the KCl group, the rise in plasma $K^+$ may also have contributed to the rise in plasma aldosterone, but no apparent correlation was detected (Figure S4).

By using uEV we observed a significant suppressive effect of oral KCl supplementation on NCC abundance and phosphorylation (pT55/60-NCC and pT60-NCC) in PA patients despite an increase in endogenous aldosterone, whilst no obvious changes were detected in PA patients who did not receive KCl. uEV analysis is thought to offer only an indirect way to assess NCC abundance in the DCT and might reflect changes in NCC trafficking rather than changes in NCC expression. A recent large-scale unbiased analysis demonstrated that uEV proteins track the abundance of the parent protein in the kidney, thus supporting the use of uEV protein changes to monitor specific physiological responses and disease mechanisms.\textsuperscript{12} Moreover, human uEVs reproduced the inverse correlations of plasma $K^+$, and the positive association of plasma aldosterone with kidney NCC and pT60-NCC that have been observed in in vivo studies and clinical studies of NCC in uEVs.\textsuperscript{14, 21, 32-34} The negative correlation between $\Delta$KCl dose and NCC fold change suggests that at least part of the variance in NCC may be a result of the KCl dose. And this variance has also been observed in two groups of PA patients underwent 4-day administration exogenous mineralocorticoid.\textsuperscript{14, 15} The negative correlation between $\Delta$KCl dose and NCC fold change, the greater change of spot urine $K^+$/creatinine in the KCl
group and negative correlations of plasma K$^+$ with NCC abundance and phosphorylation infers that reduced NCC phosphorylation may be related to promoting kaliuresis to avoid acute hyperkalemia induced by KCl supplementation.\textsuperscript{35-38}

Interestingly, PA patients in the non-KCl group exhibited relatively low baseline plasma aldosterone (~half of that in PA in KCl group) and doubled their aldosterone levels post-test. However, the significant rise in plasma aldosterone in PA non-KCl subjects did not lead to overall increased NCC abundance and phosphorylation. In contrast, baseline NCC and pT60-NCC were significantly higher in PA patients in the KCl-group than non-KCl group in association with relatively low plasma K$^+$. These observations support the hypothesis that elevated endogenous aldosterone per se does not result in increased NCC abundance and phosphorylation.

In the current study, alteration of plasma K$^+$ level was achieved by oral KCl supplementation, which leaves a question as to whether plasma K$^+$ independently suppresses NCC abundance and phosphorylation, or it acts concurrently with Cl$^-$. Our recent study has demonstrated that significantly increased plasma Cl$^-$ in PA patients during acute saline infusion appeared to only cause changes in NCC and pNCC due to urine dilution.\textsuperscript{21} Therefore it is less likely that plasma Cl$^-$ contributes to reduction in uEV levels of NCC abundance and phosphorylation. The putative renal “K$^+$ switch” mechanism is a relatively cohesive model that may provide a mechanism linking K$^+$ intake and NCC regulation.\textsuperscript{14, 32, 39-41} The “switch” activates NCC in response to low K$^+$ intake, and “turns off” NCC in response to high K$^+$ intake. Hence, while aldosterone stimulates ENaC to promote distal Na$^+$ reabsorption and K$^+$ excretion in the aldosterone-
sensitive distal nephron, the $K^+$ concentration itself regulates NCC activity to alter the $Na^+$ amount delivered downstream to ENaC, at least in the conventional model.\textsuperscript{42, 43}

The current study provides further evidence that $K^+$ regulate NCC activity during a state of increased endogenous aldosterone in PA patients. Although other interventions including adrenalectomy and spironolactone reduced NCC in adrenal intact animals,\textsuperscript{44, 45} and conditional tubule knockdown of the mineralocorticoid receptor also reduces NCC,\textsuperscript{46} these effects might also be attributable to plasma $K^+$. Because this was an observational study undertaken opportunistically while patients were admitted for a clinical procedure (SSST), hospital 24-hour urine measurements at both baseline and completion of the test period (which would have meant delaying the SSST and therefore extending the hospital admission for a further 24 hours) were not clinically feasible. We instead used the creatinine normalized spot urine values of $Na^+$ and $K^+$ as indicators of changes in distal nephron function. The significant increase in urinary $K^+/creatinine$ accompanied by the trend to an increase of urinary $Na^+/creatinine$ in PA who received oral KCl is in accordance with the phenomenon of $K^+$-induced natriuresis. However, we were unable to assess if the kaliuresis induced by oral KCl supplementation was ENaC-dependent as reported by others,\textsuperscript{47} because ENaC (which has low abundance and is difficult to measure in uEVs) was not measured in this study.

ROMK weakly positively correlated with spot urinary $Na^+/creatinine$ and spot urinary $K^+/creatinine$, but we detected no overall significant changes in ROMK among patients who received KCl. The visible reductions in ROMK upon KCl replacement seen in some subjects were not accompanied by reduced spot urinary $K^+/creatinine$. Because ROMK is
also expressed in the thick ascending limb of Henle’s loop, it may be hard to detect K\(^+\)-dependent and aldosterone effects on ROMK, which are restricted to the aldosterone-sensitive segments. Although there is a ROMK isoform that is predominantly expressed in the distal nephron,\(^{48}\) the commercially unavailable antibody specific to the ROMK isoform makes it infeasible to minimize the interference with the thick ascending limb of Henle’s loop expressed ROMK. Furthermore, animal studies have revealed a separate, flow-dependent BK channel that can partially compensate for inactive ROMK,\(^{49}\) but this was not assessed in the current study due to the limited availability of uEV material.

Estrogen in women may protect from cardiovascular and renal diseases before menopause.\(^{50,51}\) Although the sex dimorphic regulation of NCC is disputed, it has been observed that estrogen restores NCC abundance in ovariectomized female rats and stimulates NCC abundance and phosphorylation.\(^{52-54}\) In the current study, the lack of sex-related differences in NCC and its phosphorylated forms at baseline may be related to the inclusion of females who were 45 of age and older. By dividing females into two groups based on age into “likely pre-menopausal” and “likely post-menopausal”, we were able to demonstrate differences, possibly sex hormone-related, in NCC.

In conclusion, the current study reports for the first time that acute oral KCl supplementation in PA patients is associated with suppressed NCC abundance and phosphorylation in uEVs, despite a significant rise in plasma aldosterone, and this effect may be KCl dose-dependent. These observations support (1) the speculation that elevation of endogenous aldosterone per se does not result in increased NCC abundance
and phosphorylation; (2) that in PA, the effects of low plasma K\(^+\) (secondary to aldosterone excess) may dominate in terms of NCC regulation.

**Disclosures**

P. Welling reports the following: Advisory or Leadership Role: American Journal of Physiology, Renal Editorial Board; American Society of Physiology, Chair, Finance Committee, and Council Member. R. Fenton reports the following: Advisory or Leadership Role: Associate editor for American Journal of Physiology Renal; Editorial board member of J Am Soc Neph. The remaining authors have nothing to disclose.

**Funding**

AW was supported by a scholarship from the Commonwealth Government of Australia. This work is supported by a grant from the Leducq Foundation (Potassium in Hypertension Network).

**Author contributions**

Aihua Wu: Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Project administration; Resources; Software; Validation; Visualization; Writing - original draft; Writing - review and editing. Martin Wolley: Conceptualization; Funding acquisition; Investigation; Supervision; Writing - review and editing. Alexandra Matthews: Methodology. Diane Cowley: Methodology. Paul Welling: Funding acquisition; Writing - review and editing. Robert Fenton: Funding acquisition; Writing -
review and editing. Michael Stowasser: Conceptualization; Supervision; Writing - review and editing.

**Data sharing statement**

All data is included in the manuscript and/or supporting information.

**Supplemental table of contents**

**Supplementary Figure S1 (A-J).** Immunoblots of analysed proteins in all participants.

**Supplementary Figure S2.** Changes of absolute abundances of uEV proteins among the 26 PA patients within the 24-hour experiment period.

**Supplementary Figure S3.** Correlations of the analysed proteins with biochemical parameters.

**Supplementary Figure S4.** Correlation between changes in plasma K⁺ and plasma aldosterone in PA patients receiving KCl replacement.

**Supplementary Table S1.** Detection of EV-enriched proteins in each uEV isolate, and sample inclusion in uEV analyses.
References


### Tables

**Table 1** Detailed clinical procedures and sampling time-point for hypertensive patients with raised plasma ARRs who were admitted for seated saline suppression testing. ARR, aldosterone-to-renin ratio; SSST, seated saline suppression testing; KCl, potassium chloride, given as slow-released KCl (Span-K).

<table>
<thead>
<tr>
<th>6AM</th>
<th>Admission day</th>
<th>The day of posture responsiveness testing (Day 0)</th>
<th>The day of SSST (Day 1)</th>
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<td></td>
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<td>1. Home 24-hour urine test started</td>
<td>1. Urine for uEVs was collected after getting up (post-test spot urine and uEV for the current study).</td>
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<td>7AM</td>
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<td>1. Home 24-hour urine test ended. 2. Blood was collected following overnight recumbency (Results were adopted as baseline blood measurements for the current study). 3. Urine for uEVs was collected after bleeding (baseline spot urine and uEV for the current study).</td>
<td>2. Before SSST, blood was collected 30 min after assuming a seated posture (Results were adopted as post-test blood measurements for the current study). 3. SSST commenced at 8AM.</td>
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<td>10AM</td>
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<td>4. Blood results at 7AM were available. For the current study, if plasma [K⁺] &lt;4.0mmol/L, participants were given sufficient oral KCl up to Q6h to achieve as close to 4.0mmol/L as possible by the next morning at 7AM. 5. Blood was collected following 3-hour of upright posture.</td>
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<td>12PM</td>
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<td>SSST completed.</td>
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<td>Participants discharged.</td>
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<td>3PM</td>
<td>Patients were invited and consented to participate the study.</td>
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- At least 4 weeks before admission, medication affected plasma aldosterone or renin levels were replaced by other anti-hypertension drugs, e.g., verapamil, prazosin, moxonidine, and/or hydralazine.
- Patients were admitted to hospital to ensure the dietary (hospital normal diet) and posture requirements were met and to facilitate measurement of plasma K⁺ levels and other parameters.
- Oral KCl supplementation: if participants’ plasma K⁺ level at 7AM on Day 0 was less than 4.0mmol/L, participants were given sufficient slow-release KCl (Span-K) up to 4 times per day (Q6H) to achieve as close to 4.0mmol/L as possible by the next morning at 7AM.
Table 2 Detailed participants' clinical features. No., number; BMI body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; eGFR, estimated glomerular filtration rate; plasma [K⁺], plasma potassium concentration; anti-HTN, anti-hypertensive; SSST, seated saline suppression testing; Moxo, moxonidine; Prazo, prazosin; Vera, verapamil; Hydralazine; Span-K, slow-release potassium chloride; Dx, diagnosis of primary aldosteronism; F, female; M, male; U, posture-unresponsive; R, posture-responsive; PA, primary aldosteronism; LRH, low renin essential hypertension. KCl, the group of patients who received KCl supplementation; Non-KCl, the group of patients who did not receive KCl. *Bloods for eGFR and plasma creatinine were collected on the day of admission, bloods for plasma K⁺ were collected on a recumbent posture at 7AM on Day 0; ** 7AM blood sample was haemolysed, plasma K⁺ level at 10AM was 3.8 mmol/L hence KCl supplementation was given and commenced at 12 noon.

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**Note:** The table includes sex (F = female, M = male), age, height, weight, BMI, waist circumference (WC), waist-to-hip ratio (WHR), breast circumference (BC), bone density, and bone density measures. KCl and Non-KCl indicate whether KCl is included or excluded.
**Figure captions**

**Figure 1.** Flow diagram of report numbers of participants at each stage of study. Comparisons of blood biochemical parameters were performed between 21 (F13/M8) in the KCl group (excluding patient 10) and 10 (F6/M4) in the non-KCl group. Home 24-hour urinary parameters were compared between 20 (F12/M8) in the KCl group (excluding patient 7 and 9) and 9 (F5/M4) in the non-KCl group (excluding patient 28). Spot urinary parameters were compared between 18 (F12/M6) in the KCl group (excluding patient 15, 18 and 35) and 9 (F5/M4) in the non-KCl group (excluding patient 31). A total of 30 (F18/M12) PA patients were included in the baseline analyses of NCC abundance and phosphorylation, and 26 (F16/M10) PA patients were included in paired comparisons. HTN, hypertension; ARRs, aldosterone-to-renin ratios; SSST, seated saline suppression testing; N, sample size; KCl, potassium chloride; PA, primary aldosteronism; uEV, urinary extracellular vesicles.

**Figure 2.** Baseline (7AM) biochemical differences between PA in the KCl group and those in the non-KCl group. KCl, PA patients who received oral KCl (Span-K) supplementation; Non-KCl, PA patients who did not receive oral KCl supplementation; K⁺, potassium; Aldo, aldosterone; ARR, aldosterone-to-renin ratio; Timed U.Na⁺/Cr, 24-hour urinary sodium-to-creatinine ratio; Timed U.K⁺/Cr, 24-hour urinary potassium-to-creatinine ratio; Timed U.Na⁺/K⁺, 24-hour urinary sodium-to-potassium ratio. Spot U.Na⁺/Cr, spot urinary sodium-to-creatinine ratio; Spot U.K⁺/Cr, spot urinary potassium-to-creatinine ratio; Spot U.Na⁺/K⁺, spot urinary sodium-to-potassium ratio; P values
based on unpaired Wilcoxon test; Median, the median value of each parameter in each
group.

**Figure 3.** Physiological changes within the 25-hour experimental period in the KCl group
(a.) and the non-KCl group (b.). Basal, baseline values measured at 7AM on D0 on a
recumbent posture; Post, post-test values measured at 8AM on D1 during seated posture;
K⁺, potassium; Aldo, aldosterone; ARR, aldosterone-to-renin ratio; Spot U.Na⁺/Cr, spot
urinary sodium-to-creatinine ratio; Spot U.K⁺/Cr, urinary potassium-to-creatinine ratio;
Spot U.Na⁺/K⁺, urinary sodium-to-potassium ratio. *P* values based on paired Wilcoxon
test; Median, the median value of each parameter at baseline or post-test.

**Figure 4.** Baseline differences in uEV levels of analysed proteins in primary
aldosteronism participants according to allocation of oral KCl supplementation (a.), sex
(b. and c.). KCl, participants in the KCl group; Non-KCl, participants in the non-KCl
group; F, female participants; M, male participants; Female<45yo, females who were
younger than 45-year-old; Female≥45yo, females who were 45-year-old and older;
Rel.abundance, relative protein abundance (the ratio of protein absolute abundance to the
sum abundance of ALIX, TSG101 and CD9); *P* values based on unpaired Wilcoxon test.

**Figure 5.** Changes of relative abundances of analysed proteins in PA participants who
received (a.) and those who did not receive oral KCl supplementation (b.). Basal, baseline;
Post, post-test; Rel.abundance, relative protein abundance relative protein abundance (the
ratio of protein absolute abundance to the sum abundance of ALIX, TSG101 and CD9); *P*
values based on paired T-test (log10 transformed relative protein abundance was applied).
**Figure 6.** Notable correlations among all PA participants during the test. Basal, baseline values (colored in dark grey), biochemical parameter measured at 7AM on D0 in recumbent posture; Post, post-test values (colored in silver), biochemical parameter measured at 8AM on D1 during seated posture; potassium; ARR, aldosterone-to-renin ratio; Spot U.Na⁺/Cr, spot urinary sodium-to-creatinine ratio; Spot U.K⁺/Cr, spot urinary potassium-to-creatinine ratio; rel.ab, relative protein abundance (the ratio of protein absolute abundance to the sum abundance of ALIX, TSG101 and CD9).

**Figure 7.** Notable correlations of ΔKCl dose (a.), Δplasma [K⁺] (b.) and Δspot urine K⁺/creatinine (c.) with fold changes of NCC abundance and phosphorylation in PA participants.
HTN patients with raised ARRs admitted for SSST were invited and all consented
N=38

Allocated to sufficient oral KCl supplementation
N=25
Allocated to no oral KCl supplementation
N=13

SSST diagnosis
- SSST positive (PA confirmed): N=22
- SSST negative (PA excluded): N=3

SSST diagnosis
- SSST positive (PA confirmed): N=10
- SSST negative (PA excluded): N=3

Analysed N=22
- Excluded from analysis (N=3, due to different pathophysiological states, all PA excluded patients were excluded)

Data available for analyses:
- Blood biochemistry N=21
- Home 24-Hour urine N=20
- Spot urine N=18
- Baseline uEV proteins N=21
- Paired uEV proteins N=20

Analysed N=10
- Excluded from analysis (N=3, due to different pathophysiological states, all PA excluded patients were excluded)

Data available for analyses:
- Blood biochemistry N=10
- Home 24-Hour urine N=9
- Spot urine N=9
- Baseline uEV proteins N=9
- Paired uEV proteins N=6
Figure 3

a. Plasma K⁺ (mmol/L)  
P = 0.0011

Plasma Aldo (pmol/L)  
P = 0.014

Plasma Renin (mU/L)  
P = 9.5e-05

Plasma ARR (pmol/mU)  
P = 0.31

Plasma Cortisol (nmol/L)  
P = 0.92

Spot U.Na⁺/Cr (mmol/mmol)  
P = 0.18

Spot U.K⁺/Cr (mmol/mmol)  
P = 0.00013

Spot U.Na⁺/K⁺ (mmol/mmol)  
P = 0.73

Spot U.Cr (mmol/L)  
P = 0.65

b. Plasma K⁺ (mmol/L)  
P = 0.54

Plasma Aldo (pmol/L)  
P = 0.0059

Plasma Renin (mU/L)  
P = 0.31

Plasma ARR (pmol/mU)  
P = 0.65

Plasma Cortisol (nmol/L)  
P = 0.064

Spot U.Na⁺/Cr (mmol/mmol)  
P = 0.2

Spot U.K⁺/Cr (mmol/mmol)  
P = 0.039

Spot U.Na⁺/K⁺ (mmol/mmol)  
P = 0.73

Spot U.Cr (mmol/L)  
P = 0.65
Figure 4

(a) NCC

(b) NCC

(c) NCC
Figure 5

(a) and (b) show scatter plots comparing basal and post-test relative abundances for NCC, pT60-NCC, pT55/60-NCC, and Total ROMK. The plots are arranged in columns, with each column representing a different condition. The y-axis represents relative abundance, while the x-axis categorizes data as Basal and Post. Each plot includes statistical significance values (P-values) indicating the difference between basal and post-test measurements. For example, the NCC condition in (a) has a P-value of 0.017, suggesting a significant change from basal to post-test. Similarly, in (b) the P-values range from 0.37 to 0.89, indicating no significant change for the conditions tested.
**Figure 6**

(a) $R^2 = 0.11, P = 0.013$

(b) $R^2 = 0.14, P = 0.0036$

(c) $R^2 = 0.23, P = 0.00014$

(d) $R^2 = 0.26, P = 5 \times 10^{-5}$

(e) $R^2 = 0.11, P = 0.014$

(f) $R^2 = 0.14, P = 0.0038$

(g) $R^2 = 0.11, P = 0.012$

(h) $R^2 = 0.085, P = 0.042$

(i) $R^2 = 0.088, P = 0.038$
**Figure 7**

(a) $R^2 = 0.3, P = 0.0041$

(b) $R^2 = 0.11, P = 0.094$

(c) $R^2 = 0.17, P = 0.053$
Supplementary material

Table of contents

- Supplementary Figure S1 (A-J)
- Supplementary Figure S2
- Supplementary Figure S3
- Supplementary Figure S4
- Supplementary Table S1
Supplementary Figure S1. Immunoblots of analysed proteins in all participants. Each image was merged by a chemiluminescent blot image with a colorimetric image of the same blot using Image Lab software. pT53/58-NCC of the fifth image was a chemiluminescent blot image. The control on each blot is a uEV pool containing resuspension of mixed uEVs isolated from a large amount of urine collected from two healthy volunteers between 9AM and 4PM on multiple days.
Supplementary Figure S1

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Supplementary Figure S2. Changes of absolute abundances of uEV proteins among the 26 PA patients within the 24-hour experiment period.
Supplemental Figure S3. Correlations of the analysed proteins with biochemical parameters.
Supplemental Figure S4. Correlation between changes in plasma $K^+$ and plasma aldosterone in PA patients receiving KCl replacement.
### Supplemental Table S1. Detection of EV-enriched proteins in each uEV isolate, and sample inclusion in analyses.

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+, detected in uEV isolate; -, not detected in uEV isolate; NA, not applicable (inadequate urine for uEV isolation or patients who were diagnosed of LRH that excluded in analyses). Yes, included in analyses; No, not included in analyses.