# **Supplementary Materials**

Wu and Wolley *et al.*, Acute intravenous NaCl and volume expansion reduces sodiumchloride-cotransporter abundance and phosphorylation in urinary extracellular vesicles

# **Table of Contents**

Supplementary Spreadsheet. The LC-MS/MS proteomics data.

Supplementary Methods. Detailed methods in the current study.

Figure S1. Flow diagram of report numbers of individuals at each stage of study.

**Figure S2**. A heatmap clustering of 294 differentially expressed proteins during SSST between PA and LRH subjects.

Figure S3. Functional enrichment analyses of the 878 DEPs.

**Figure S4**. Correlations between biochemical parameters and the 12 differentially expressed renal transmembrane proteins.

Figure S5. Immunoblots of analysed proteins.

Figure S6. Correlations between biochemical parameters and NCC, pNCC and AQP2.

Figure S7. Boxplots of changes in the total contents in the urine during SSST.

**Figure S8**. Correlations between EV markers (quantified by immunoblotting and MS) and spot urine creatinine.

**Table S1**. Participants' clinical features and anti-hypertensive drugs at baseline.

**Table S2**. NTA measures of particle size and concentration of nine uEV samples.

Table S3. Abbreviation list.

#### **Supplementary Methods**

• Recruitment

Hypertensive patients who were admitted for SSST in the Hypertension Unit of Princess Alexandra Hospital were invited to participate and provide informed written consent. A total of 44 (F29/M15) patients were invited and all agreed to participate. All subjects were hypertensive with repeatedly raised ARRs and underwent SSST to confirm or exclude the diagnosis of PA.

• Sample size

We previously reported several uEV proteins increased over 2.5-fold in responding to alterations of mineralocorticoid and salt loading, and plasma aldosterone to be suppressed on at least of 101 pmol/L in 24 patients with PA subjects. For proteins that are detectable by MS and WB, we accepted a p<0.05 with 80% power using two-tailed test for bidirectional result. Due to the co-isolation and fragmentation of the isobaric precursor ions, a TMT multiplex labelling strategy reduces missing data points, providing high-quality data for statistical analysis from a limited number of clinical samples (generally one peptide with  $\leq$ 5 replicates per condition) [20, 21]. Assuming SSST would induce at least a 50%-fold change, we required at least 10 subjects analysed on MS and 16 subjects analysed on WB.

• The SSST

At least four weeks prior to SSST, 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 undergoing

SSST were admitted to hospital to ensure the dietary (normal hospital diet) and posture requirements were met and to facilitate monitoring of plasma K<sup>+</sup> levels and other parameters. An infusion of 2 L of 0.9% saline over 4 hours was commenced at 8AM, 30 min after assuming a seated position. Two aliquots of bloods were collected just before 8AM (baseline) and at completion of the infusion at 12 noon (post-SSST). Clinical routine measurements of plasma aldosterone (by LC-MS/MS), direct renin concentration (by chemiluminescent immunoassay), cortisol (by immunoassay) and K<sup>+</sup> levels were performed by Pathology Queensland Laboratory right after the SSST. Due to limited amounts of bloods, plasma concentrations of Cl- and HCO3- were analysed by Pathology Queensland Laboratory in 41 participants, including 31 (F18/M13) PA and 10 (F10/M0) LRH subjects, plasma copeptin was measured in 33 participants (PA25, F14/M11; LRH8, F8/M0). Blood pressure was recorded during SSST. Demonstration of aldosterone production that is relatively autonomous of its chronic regulator angiotensin II requires failure of plasma aldosterone to suppress to below 162 pmol/L, provided that direct renin concentration at completion was less than 8.4 mU/L and plasma cortisol concentration was lower at completion than that basally.

# • Urine collection and isolation of uEVs

Participants were provided with two sterilised 200ml containers for urine collection. 50-200 ml of mid-stream second morning urine were collected before SSST at 7AM (basal) and 1 hour post completion (post) of the saline infusion. Collected urine samples were immediately treated with protease inhibitor cocktail (Roche cOmplete, EDTA-free) and phosphatase inhibitors (Sigma-Aldrich, Pierce<sup>TM</sup>) before aliquoting and freezing at -80°C. Nine participants provided an additional urine collection at 8AM on the day before SSST

to be used as a baseline sample, as participants felt they would be unable to produce enough urine volume on the day of SSST. Due to limited spot urine samples, spot urine creatinine concentration was measured in 39 participants (PA32, F18/M14; LRH7, F7/M0) using a creatinine urinary detection assay kit (EIACUN, Invitrogen). uEVs were isolated using progressive ultracentrifugation techniques with 200 mg/mL dithiothreitol treatment. For LC-MS/MS the obtained uEVs were resuspended in 200 µl uEV isolation buffer containing 1x phosphate-buffered saline (PBS), protease and phosphatase inhibitors and 0.5% SDS. For immunoblotting analysis, uEVs were resuspended in approximately 110 µl of 1x PBS containing 0.5% SDS. Resuspended uEVs were ultrasonic-homogenised for 5 cycles of 10 seconds on/off on ice before being frozen at -80°C pending subsequent analyses.

• Characterisation of uEVs

uEVs were characterized by size distribution measured by nanoparticle-tracking analysis (NTA) and the presence of marker proteins of EV (e.g., ALIX, TSG101). Due to the limited amount of uEVs obtained from participants, nine uEVs isolated from two healthy volunteers at different times on multiple days were characterised by both NTA and the presence of marker proteins using immunoblotting, and patients' uEVs were characterised by the presence of marker proteins by LC-MS/MS and immunoblotting. For NTA, a NanoSight NS500 instrument (Nanosight Ltd, Amesbury, UK) with NanoSight NTA v3 software was used. Before each session, the acquisition parameter settings were determined using the NTA latex standard (Malvern Polystyrene Latex Microsphere 100nm) in a 1/250 dilution in ultrapure water (Pureau, AU), and fixed for all measurements during the session [camera level 10, slider shutter 696, slider gain 73, detection threshold 5]. All uEV samples were analysed on 5 captures of 60 seconds. Sample dilution was initiated at 1/500, while

alternative dilutions were applied to obtain the recommended number of particles (50-100) per image.

## • TMT labelled LC-MS/MS

- Sample preparation and digestion

Peptides for LC-MS/MS were generated using filter-aided sample preparation. In short, uEV samples were centrifuged at 16000×g for 1 h at 4°C and supernatants assayed for protein concentration using the Pierce BCA Protein Assay Kit (Thermo Scientific, IL, USA). 10 µg of individual exosome proteins were loaded onto a Vivacon-30 kDa spin column (Sartorius, Goettingen, Germany) by centrifugation at 16000×g. Spin columns were washed three times with UA buffer containing 8 M urea (Thermofisher, IL, USA) and 100 mM triethylammonium bicarbonate (Fisher Scientific, Leicestershire, UK). The proteins were then reduced using 50 mM of dithiothreitol (Thermofisher, IL, USA) for 1 h at 56 °C and alkylated with 50 mM 2-chloroacetamide for 20 minutes in the dark at room temperature, with centrifugation after each addition to remove excessive reagents. The spin columns were then washed one more time with UA buffer before adding 40 µl of Lys-C solution (FUJIFILM Wako, Osaka, Japan) (enzyme: protein = 1:50, dissolved in UA buffer). After 3h incubation at 37°C, 400 µl of trypsin solution (Promega, WI, USA) (enzyme: protein = 1:25, dissolved in 100 mM triethylammonium bicarbonate) was added, and the spin column was incubated at 37°C overnight. Peptides were collected by centrifuging the spin column in a new collection tube. Peptide concentration was measured using the Pierce Fluorometric Peptide Assay Kit (Thermofisher, IL, USA) according to the manufacturer's protocol.

- TMT labelling and fractionation

Peptides were labelled with TMT 10plex isobaric labeling reagents (Thermofisher, IL, USA). In brief, 0.4mg of each TMT tag (127N, 127C, 128N, 128C, 129N, 129C, 130N and 130C) was dissolved in 164µl acetonitrile, before adding to 400µl of peptide solution containing 6.4 µg peptides (Supplemental Table 2). A universal control channel containing an equal peptide amount from each sample was used and labelled with TMT tag 126 for comparisons between samples. After one hour at room temperature, individual labelling reactions were quenched by addition of 32 µl of 5% hydroxylamine. Individually labelled samples were combined and vacuum-dried in a SpeedVac, before fractionation using a Pierce high pH RP-fractionation kit (Thermofisher, IL, USA) according to the manufacturer's protocol. In total three sets of TMT labelling were performed. Nine labelling channels were used in each set, with four patients (basal and post) occupying eight channels and one channel for the universal control.

## - LC-MS/MS analysis

The TMT labelled samples were analyzed by nano liquid chromatography (nLC, EASY LC-1200, Thermo Fisher) coupled to a MS/MS system (Q Exactive Plus, Thermo Scientific) through an EASY-Spray nano-electrospray ion source (Thermo Scientific). A pre-column (Acclaim®PepMap 100, 75 µm x 2 cm, C18, 3 µm, 100 Å, Thermo Scientific) was used to trap peptides and an analytical column (EASY-Spray Column, PepMap, 75 µm x 25 cm, C18, 3 µm, 100 Å, Thermo Scientific) was used to separate peptides. For nLC separation, buffer A was 100% H2O/0.1% formic acid and buffer B was 80% ACN/0.1% formic acid. A linear gradient from 5% to 22% buffer B for 40 min, and then from 22% to 35% for 20 min was used for peptide separation. Precursor scans were performed at scan range of 300-1,600, a resolution of 70,000, maximum injection time of 100 ms and automatic gain

control of  $3 \times 10^6$ . MS/MS scans were up to 10 data-dependent acquisition scans performed at a resolution of 35,000, maximum injection time of 100 ms and automatic gain control of  $1 \times 10^5$ . Isolation window was set at 2 Da. Higher-energy-collisional-dissociation normalized collision energy was set at 33%. Fixed first mass was set at 110. Dynamic exclusion was set at 30 s. Rejection of precursor ions with charge state +1 and above +8 was employed.

#### - LC-MS/MS data analysis

LC-MS/MS raw files belonging to one TMT set were searched together against a UniProt human protein database (downloaded on 25<sup>th</sup> January 2019) using both the Sequest and Mascot algorithms through Proteome Discoverer software (v2.3). Quantification of peptides and proteins was done through the reporter ion quantification module of Proteome Discoverer, with corrections for TMT isotopic interferences enabled. Basal cursor mass tolerance was set as 10 ppm and fragment mass tolerance was set as 0.02 Da, and a maximum of 2 missed cleavage sites. Carbamidomethylation of cysteine was set as a static modification. Protein N-terminal acetylation, methionine oxidation, TMT labelling of peptide N-terminus and lysine were set as variable modifications. Peptide false discovery rate was calculated by Percolator and confined to  $\leq 1\%$ . Protein ratios obtained with the aid of the universal control channel TMT126 were log-transformed for paired *t*-test to determine the proteins that showed significant changes (p<0.05 with 95% confidence interval) post SSST. Differentially expressed protein (DEP) was identified as that with p<0.05, FDR<0.1 and fold change of  $\geq 1.20$  or  $\leq 0.83$ .

• Immunoblotting validation

Defrosted uEVs were mixed thoroughly before 10 min centrifugation at 15000×g at 4°C to pellet insoluble residues. The supernatant was collected and total protein concentration was measured using a spectrophotometer (Thermo Scientific Nanodrop Lite). A uEV standard pool containing resuspension of mixed uEVs isolated from a large amount of urine collected from healthy normotensive volunteers between 9-11AM on multiple days was used as a universal control for normalisation of performance errors across all blots. uEVs were treated with 5x Laemmli sample buffer (1:4, v:v) and incubated at 60°C for 10 min before sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Twenty µg of each sample were loaded and separated on 8% poly-acrylamide mini gels, and were transferred to polyvinyl difluoride membranes (Bio-Rad) under 1.3A and 21V for 26 mins on a Bio-rad Turbo transferring system. Each blot was duplicated for proteins with similar size. Blots were then blocked with 5% BSA (A3858, Sigma) in Tris-Buffered saline (TBS), followed by overnight incubation with primary antibodies including: anti-AQP2 (1/1000; SPC-503, StressMarq Biosciences), anti-NCC (1/1000; AB3553, Merck Millipore), antipNCC (pT53/pT58; 1/1000), anti-ALIX (1/2000; ABC40, Merck Millipore), and anti-TSG101 (1/2000; MASBC649, Merck Millipore). AQP2 was measured by bands detected at 35-50 kDa (glycosylated) and at 29 kDa (unglycosylated protein); NCC and pNCC were measured by the dominant band around150 kDa despite the observation of dimerisation; uEV markers ALIX and TSG101 were measured by the dominant bands at 96 kDa and 45 kDa respectively. HRP conjugated goat anti-rabbit IgG antibody (12-348, Merck Millipore) was used as the secondary antibody at 1/20,000 for luminol-based enhanced chemiluminescence (1705061, Bio-rad), respectively before image capture using a BioRad ChemiDoc XRS+ Imager on configure signal accumulation mode with Image Lab software.

• Bioinformatic analyses

Calculations were processed with R. Overlap analyses were performed using Vesiclepedia and ExoCarta protein databases to compare the MS dataset with other human urine studies and to identify EV-enriched proteins. A rat renal transporter protein database (https://hpcwebapps.cit.nih.gov/ESBL/Database/NephronRNAseq/Transporters\_and\_Cha nnels.html) was used to identify renal transmembrane proteins. Gene ontology analysis was performed by ClueGO plugin (v2.5.5) in the Cytoscape environment (v3.7.2), and gene lists corresponding to 878 DEPs were used as input. GO terms were updated on 11<sup>th</sup> June 2020. Evidence level was set at "All without IEA (Inferred from Electronic Annotation)". GO term fusion was enabled, and only pathways with  $p\leq0.01$  are shown. All other parameters were left at default. Figure S1. Flow diagram of report numbers of individuals at each stage of study.

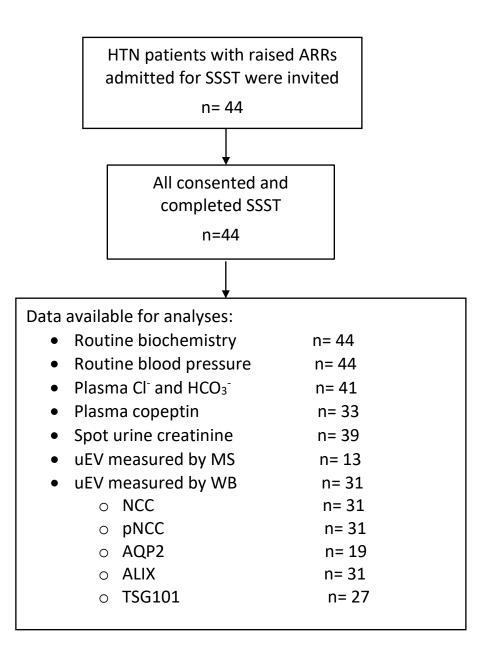


Figure S2.A heat map clustering of 294 differentially expressed proteins during SSST between PA and LRH subjects.

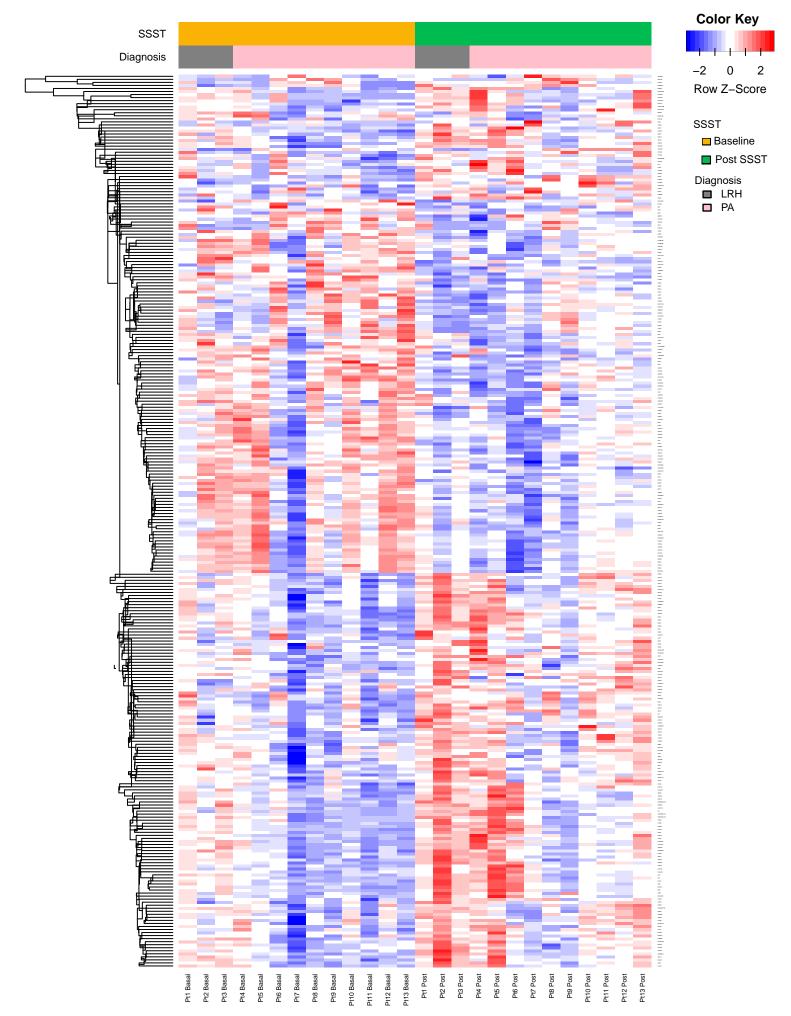
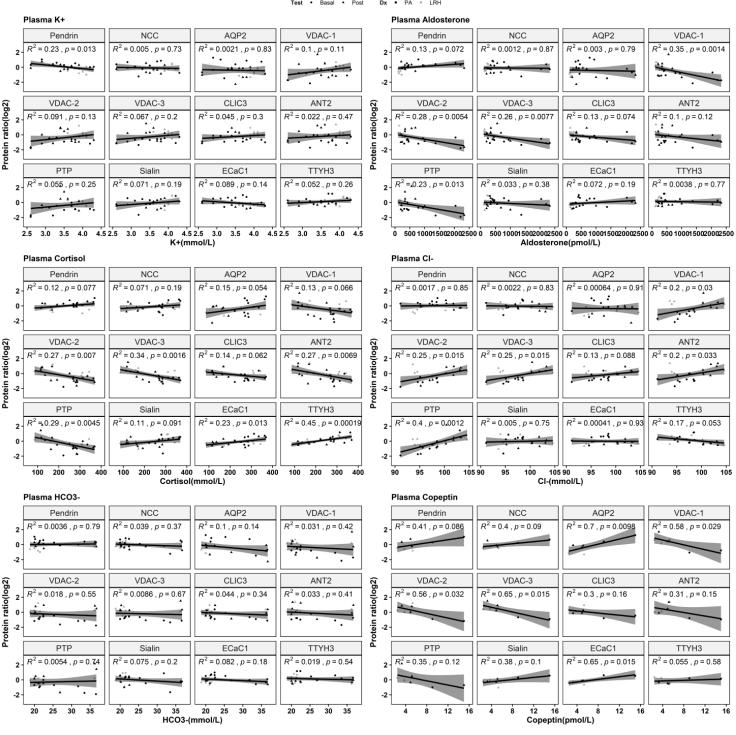


Figure S3. Functional enrichment analyses of the 878 DEPs.

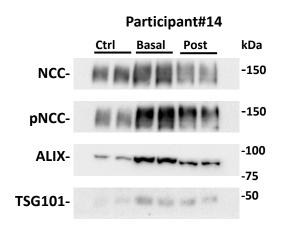
	• 50
United Reserved	
Unit of the second of	
141900000000000000000000000000000000000	
Intersection in a contract of	
Ubtrace         6.50           Ubtrace         6.30           Ubtrace         6.30           Ubtrace         6.31           Ubtrace         6.34           Ubtrace         6.30           Ubtrace         6.30           Ubtrace         6.30           Ubtrace         6.30 <td></td>	
isynchronization	
Note set 100         • 0 201           Note set 100         • 204           Note set 1000         • 104           Note set 1000         • 1000	
1         0         2.24           Summary 1000         2.25           Summary 1000         2.25           Summary 1000         2.50           Summary 1000         <	
Lagrando         • 2.24           Norma Nucl.         • 2.24           Lagrando         • 2.24           Norma Nucl.         • 3.24           Norma Nucl.         • 1.24           Norma Nucl. </td <td></td>	
Joseph 100         • 2.34           Note interview 10000000         • 2.34           Joseph 1000000000000000000000000000000000000	
Start In Vol.         • 2.24           No.         • 2.24           Start In Vol.         • 2.23           Start In Vol.         • 2.23           Start In Vol.         • 2.23           Start In Vol.         • 3.36           Start In Vol.         • 3.36           Start In Vol.         • 13.26           Start In Vol. </td <td></td>	
Billioning         •• 2.20         •• 13.46           Microsoft Amology of the state of the st	
Contention 5 and corps         •• 2.2.3         •• 13.46           Weinstreich 5 and corps         •• 13.46         •• 13.46           Weinstreich 5 and corps         •• 13.46         •• 13.46           Weinstreich 5 and 5 and 5         •• 13.46         •• 13.46           Weinstreich 5 and 5         •• 13.47         •• 13.46           Weinstreich 5 and 5         •• 13.46         •• 13.46           Weinstreich 5 and 5         •• 13.47         •• 13.46           Weinstreich 5 and 5         •• 13.46         •• 13.46           Weinstreich 5 and 5 and 5         •• 13.46         •• 13.46           Wein 10 and 10	
umanitolio         ••••••••••••••••••••••••••••••••••••	
With Name         • 13.6           Service Stress         • 11.52           Service Stress         • 11.52           Service Stress         • 6.47           Operating Stress         • 6.47           Service Stress         • 6.57           Service Stress         • 6.57           Service Stress         • 6.58           Service Stress         • 6.57           Service Stress         • 6.57           Service Stress         • 6.58           Service Stress         • 7.55           Service Stress         • 7.55 <td< td=""><td></td></td<>	
elsa hava hava hava hava hava hava hava ha	
unions a face         0.0.7           course trains         0.0.47           course trains         0.0.77           course trains         0.0.90           course trains	
Organisation6.47Sin Minko Quan6.847Sin Minko Quan6.847Sin Minko Quan6.847Sin Minko Quan6.847Sin Minko Quan6.577Sin Minko Quan6.577Sin Minko Quan6.508Core Quants Fino Quants Nam6.508Sin Minko Quan6.508Sin Minko Quan6.357Sin Minko Quan6.508Sin Minko Quan6.508Sin Minko Quan6.508Sin Minko Quan6.508Sin Minko Quan6.357Sin Minko Quan6.357Sin Minko Quan6.357Sin Minko Quan6.357Sin Minko Quan6.357Sin Minko Quan6.358Sin Minko Quan6.357Sin Minko Quan6.357Sin Minko Quan6.357Sin Minko Quan6.357Sin Minko Quan6.169Sin Minko Quan6.169Sin Minko Quan6.169Sin Minko Mi	
was         • 0.47           bas O Werkes         • 0.87           issuest was         • 0.577           issuest was         • 0.507	
Ensite Your              • 8.47             • 6.58             • • 6.59             • • 6.9             • 1.89             • • 6.6             • 1.89             • • 1.89             • • 1.89             • • 1.99             • • 1.9             • 1.99             • • 1.9             • • 1.9             • • 1.9             • • 1.9             • • 1.9             • • 1.9             • • 1.9             • • • 1.9             • • • 1.9             • • • 1.9             • • • • • 1.9             • • • • • • • • • • • • •	
Bis Sold Sold Sold Sold Sold Sold Sold Sold	
Autom Juniol         0         5.08           CON-Case IRF Tooly Imposed         0         5.08           Marcine Instruction Imposed         0         3.35           Instruction Imposed         0         1.50           Instruction Imposed         0         0.50           Instruction Imposed         0         0.50           Instruction Imposed         0         0.50           Instruction Imposed         0         0.50           Instructin Imposed         0.50         0.50 <td></td>	
COPI-Canad The Day Provide values         - 0 5.08           Martines Woods Days Mark         - 0 5.08           Second 26 Testore         - 0 3.39           Control Transport         - 1 60           South Ediptions: Hotorian         - 1 60           South Ediption: Hotorian         - 1 60           South Ediption: Hotorian         - 6 7.3           Controls: Instance         - 6 7.3           Controls: Instance Hotorian Hotorian	
Martanian Sanota Gogana              •             5.08            Martanian Sanota Gogana              •             5.08            Name Sanota Gogana              •             3.39            Constantiana              •             1.69            Constantiana              1.69            Constantiana              1.69            Constantiana              1.69            Sanota Constante              1.69            Constantiana              1.69            Marchanal Muscana               1.69            Marchanal Muscana               1.69            Marchanal Muscana               1.6	
Image: Second	
Aus-based of Presents	
Entrosyste Value Lamb         • • 3.39           Name Cell Optimie         • • 3.39           Control Entropie         • • 1.69           Entropie Entropie         • • 1.69           Control Entropie         • • 1.69           Contropie Entropie         • • 1.69           Mutchood Market         • • 1.69           Mutchood Market Market         • • 1.59           Mutchood Market Market         • • 1.59           Mutchood Market Marke	
Contract Torus;	
by max         •• 1.69           Charlyto Bay 2 Syntems         •• 1.69           Charlyto Bay 2 Syntems         •• 1.89           Stock Edizations Fronts         •• 1.89           Stock Edizations Fronts         •• 1.69           Stock Edizations Fronts         •• 1.69           Mathematic Bay         •• 1.69           Stock Edizations Fronts         •• 1.69           Stock Edizations Fronts         •• 1.69           Calcoms         •• 1.69           Stock Edizations Fronts         •• 1.69           Calcoms         •• 1.69           Stock Edizations Fronts         •• 1.59           Wite-Schleich Proses         •• 1.61           Calcoms Mathema Proses         •• 1.59           Wite-Schleich Proses         •• 1.59           Calcoms Mathema Proses         •• 1.73           Calcoms Proses         •• 6.51           Proses Biolysch Proses         •• 6.61	
Casayse Says 2 Spirocova         - • 1 69           Booth Technologiane Returnal         • 1 69           Booth Technologiane Returnal         • 1 69           Booth Technologiane Returnal         • • 1 69           Booth Technologiane Returnal         • • 1 69           Booth Technologiane Returnal         • • • 1 69           Michologiane Returnal         • • • • • • • • • • • • • • • • • • •	
Smach Edaguani: Rational	
Cal Cana Matcharden Nadeas Service N p2 Establishment Of Localization In Cal Lakappa Ashtelon Generation Of Pecaron Matolish Add Energy Vasis- Module Theorem Organomispin Composit Matolish Compares Regulation Of Calling Compares Matolish Regulation Of Calling Compares Matolish Regulation Of Calling Compares Matolish Contraction Cantogic Acti Matolish Process Organomispin Compare Compares Matolish Regulation Of Calling Process Organomispin Compares Matolish Process Regulation Of Calling Process Organomispin Compares Matolish Process Organomispin Compares Compares Matolish Process Organomispin Compares Matolish Process Organomispin Compares Matolish Process Organomispin Compares Matolish Process Organomispin Compares Process Organomispin Compares Matolish Process Organomispin Compares	
Mitochonikal Nackoda    1.69       Besterio PC Calculation In Coll    25.32       Ladoopie Activator    18.33       Generation Of Precurs Matholice Nacional	
Estatistation ID Localization ID Call       25.32         Generation Of Procurs Mutabolies And Enrop Weads-Mattalin Strapped       11.59         Vision-Mattalin Strapped       9.44         Regulation Of Compound Mutabolies Process Pacified Biosynthes Process       9.44         Regulation Of Callular Compound Mutabolies Process Pacified Biosynthes Process       9.44         Comfication Comfication Comfication Constraints       7.3         Cartoxylic Add Mutabolie Process Regulation Of Certification Hesses Booghnesis Process Regulation Of Certification Passines Regulation Of Certification Posterine Regulation Of Certification Regulation Of Certification Posterine Regulation Of Certification Posterine Regulation Of Certification Posterine Regulation Of Certification Regulation Of Certification Posterine Regulation Of Certification Regulation Of Certification Posterine Regulation Of Certification Regulation Of Certification R	
Lakkoyle Atditation Generation Of Precursor Metadata Transport Veside-Mediata Transport Organositions Compand Matabia Frozess Regulation Of Califuar Component Motions Regulation Of Califuar Component Matabia Frozess Compand Peptide Biosyntheir Process Contraction Contra	
Generation Of Precursor Metabolites And Energy     11.59       Vesicle-Mediati Transport     9.44       Organoninogen Compoont Metabolis Process     9.44       Regulation Of Califura Compoont Metabolis Process     8.58       Contraction     7.3       Contraction     7.3       Contraction     6.01       Organoninogen Compoont Metabolis Process     6.01       Contraction     7.3       Contraction     5       Regulation Of Centractione Duplication     4.17       Methorano Duplication     4.17       Proteine Biologin Dration     4.33       Viral Budding Of Transport     3.43       Viral Budding Of Centractione     3.43       Viral Budding Of Centractione     3.43       Viral Budding Of Protein Binding Involved in Bundio Of Protein     2.5       Regulation Of Cell Communication     2.5       Regulation Of Protein Metabolic Process     2.5       Regulation Of Protein Metabolic Process     2.15	
Organonitrogen Compound Matabolic Proces       9.44         Regulation Of Cellular Component Movement       8.58         Puptide Biosynthetic Proces       7.3         Commitation       7.3         Carboxyle Acid Matabolic Proces       7.3         Carboxyle Acid Matabolic Proces       6.01         Chondrolin Sultate Catabolic Proces       5         Organonitrogen Compond Catabolic Proces       5         Organonitrogen Compond Catabolic Process       5         Regulation Of Centrosome Duplication       5         Membrance Granization       4.17         Membrance Granization       4.17         Programmed Cell Death       3.43         Viral Budding Via Host ESRCT Complex       3.33         Organation Of Protein Matabolic Proces       3.33         Negativer Regulation Of Protein Matabolic Proces       3.33         Organization Of Centomization       3.43	
Regulation Of Callular Component Movement       8,58         Papide Biosynthetic Process       7,3         Comfication       7,3         Carboxylic Acid Metabolic Process       6,01         Chondrotin Sulfate Catabolic Process       5         Organoatringen Compond Catabolic Process       5         Organoatringen Compond Catabolic Process       5         Membrane Organization       4,17         Hecose Biosynthetic Process       4,17         Virial Budding Via Host ESRCT Complex       3,43         Organetile Organization       3,43         Negative Regulation Of Chrosense       3,33         Organetile Organization       3,33         Organetile Organization       2,5         Negative Regulation Of Protein Metabolic Process       2,15         Intlar Adhesive Protein Binding Involved In Bundie Of His Cell-Purkinje Mycoyte Communication       1,72	
Conflication       7.3         Carboxylic Acid Matabolic Process       6.01         Chondrollin Sulfate Catabolic Process       5         Organonitregen Compond Catabolic Process       5         Regulation Of Centrosome Duplicaton       5         Hexose Biosynthetic Process       4.17         Membrane Organization       4.17         Programmed Cell Death       4.17         Viral Budding Via Not ESRNC Complex       3.43         Organelle Organization       3.33         Organelle Organization       3.33         Regulation Of Chromunication       2.5         Regulation Of Chromunication       3.33         Organelle Organization       2.5         Negative Regulation Of Ortex Matabolic Process       2.5	
Carboxylic Acid Metabolic Process          • 6.01          Chondroitin Sulfate Catabolic Process          • 5          Organonitrogen Compond Catabolic Process          • 5          Regulation Of Centrosome Duplication          • 5          Hexoses Biosynthetic Process          • 4.17          Membrane Organization          • 4.17          Positive Regulation Of Transport          • 4.17          Programmed Cell Death          • 3.43          Viral Buiding Vir Abust ESRCT Complex          • 3.43          Organelio Organization          • 3.43          Negative Regulation Of Cell Communication          • 2.15          atluar Adhesive Protein Binding Involved In Bundie Of His Cell-Purkinje Myocyte Communication          • 1.72	
Chodrolin Sultate Catabolic Process Organonitrogen Compond Catabolic Process Regulation Of Centrosome Duplication Hexose Biosynthetic Process Membrane Organization Positive Regulation Of Transport Pogrammed Cell Death Viral Budding Via Host ESRCT Complex Organelle Organization Regulation Of Protein Metabolic Process allular Adhesive Protein Binding Involved In Bundle Of His Cell-Purkinje Myocyte Communication in 1.72-	
Regulation Of Centrosome Duplication       5         Hexose Biosynthetic Process       4.17         Membrane Organization       4.17         Programmed Cell Death       4.17         Viral Budding Via Host ESRCT Complex       3.43         Organelle Oragnization       3.33         Organelle Oragnization       3.33         Negative Regulation Of Cell Communication       2.5         Regulation Of Protein Binding Involved In Bundle Of His Cell-Purkinje Myocyte Communication       1.72	
Hexose Biosynthetic Process       4.17         Membrane Organization       4.17         Positive Regulation Of Transport       4.17         Programmed Cell Death       4.17         Viral Budding Via Host ESRCT Complex       3.43         Organelle Oragnization       3.33         Organelle Oragnization       3.33         Negative Regulation Of Cell Communication       2.5         Regulation Of Protein Binding Involved In Bundle Of His Cell-Purkinje Myocyte Communication       1.72	
Membrane Organization       4.17         Positive Regulation Of Transport       4.17         Programmed Cell Death       3.43         Viral Budding Via Host ESRCT Complex       3.33         Organelle Oragnization       3.33         Organelle Oragnization       3.33         Negative Regulation Of Cell Communication       2.5         Regulation Of Protein Metabolic Process       -2.15         Athesive Protein Binding Involved In Bundle Of His Cell-Purkinje Myocyte Communication       -1.72	
Programmed Cell Death	
Viral Budding Via Host ESRCT Complex Organelle Oragnization Negalive Regulation Of Cell Communication Regulation Of Protein Metabolic Process cellular Adhesive Protein Binding Involved In Bundle Of His Cell-Purkinje Myocyte Communication	
Organelle Oragnization <ul> <li></li></ul>	
Negative Regulation Of Cell Communication Regulation Of Protein Metabolic Process2.5 Cellular Adhesive Protein Binding Involved In Bundle Of His Cell-Purkinje Myocyte Communication	
cellular Adhesive Protein Binding Involved In Bundle Of His Cell-Purkinje Myocyte Communication	
Oxoacid Metabolic Process	
Glomerular Visceral Epithelial Cell Differentiation	
Vesicle Organization	
Response To Endoplasmic Reticulum Stress	
De Novo' Posttranslational Protein Folding	
Antigen Processing And Presentation Of Exogenous Peptide Antigen	
Cellular Lipid Metabolic Process	
Cellular Iron Ion Homeostasis	
Vitamin Metabolic Process 0.83	
Protein Tetremerization 0.43	
Immune Response To Tumor Cell 0.43	
Platelet Formation	
Regulation Of Phagocytosis	
Establishement Of Skin Barrier 0.43	
Structural Constituent Of Cytoskeleton	
Cell-Cell Adhesion	
Regulation Of Cell Growth	
Osteoblast Differentiation	
Homeostasis Process	
0 10 20 30 40 50 0 10 20 30 40 Percentage	

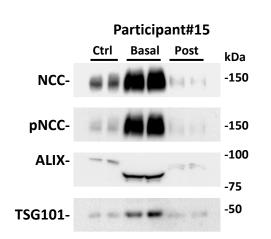
Figure S4. Correlations between biochemical parameters and the 12 differentially expressed renal transmembrane proteins.

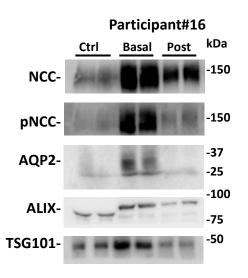


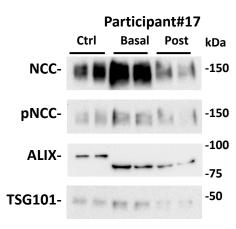
Test • Basal ▲ Post Dx • PA ◎ LRH

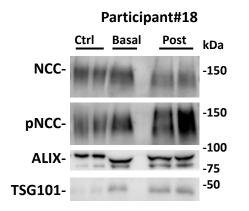
Figure S5. Immunoblots of analysed proteins.

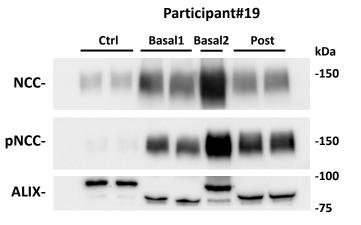


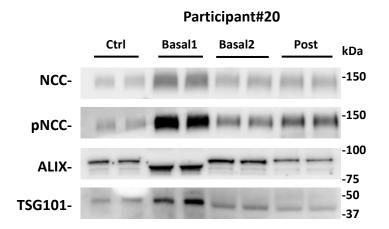


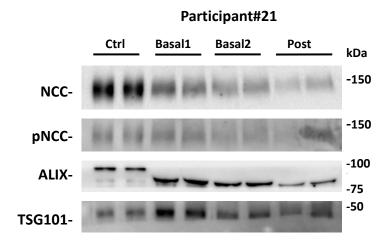












Participant#23 Basal Post

kDa

-150

-150

-37 -25

-100

-75

-50

Ctrl

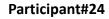
NCC-

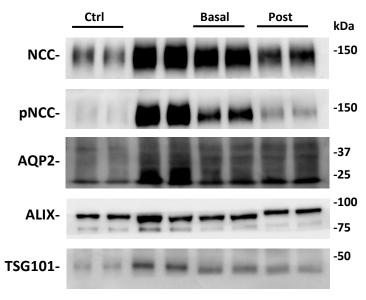
pNCC-

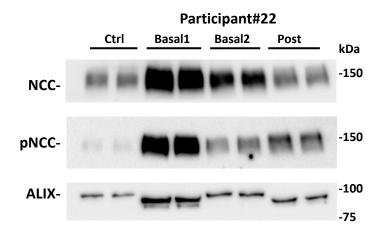
AQP2-

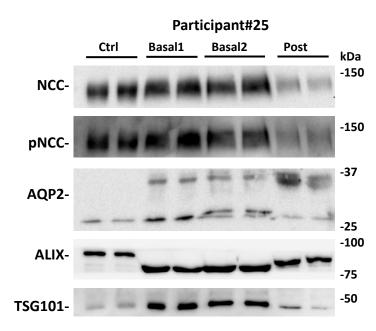
ALIX-

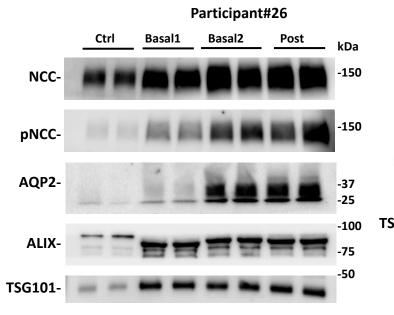
TSG101-

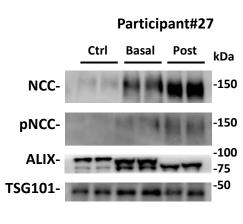


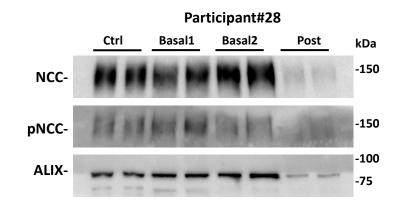


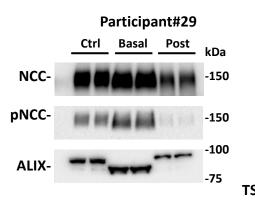


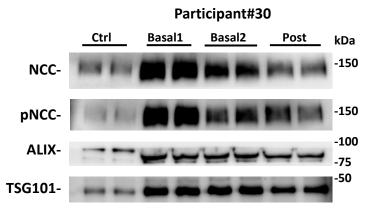


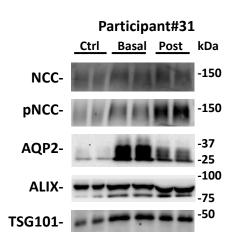


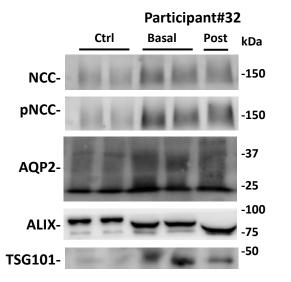


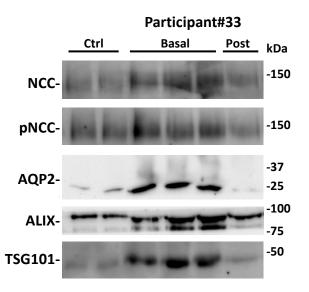


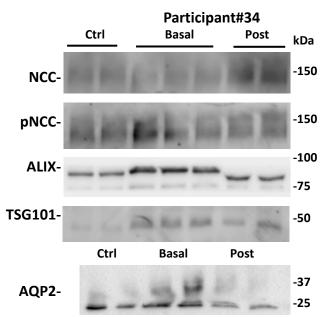


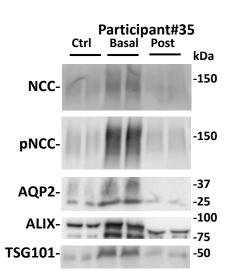


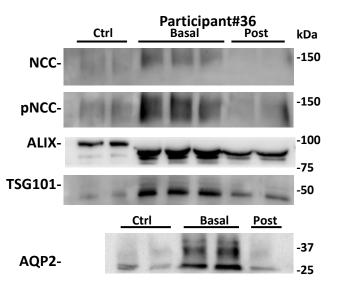


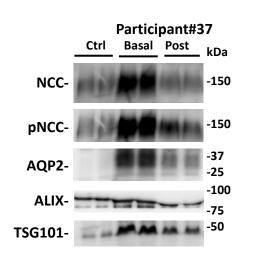


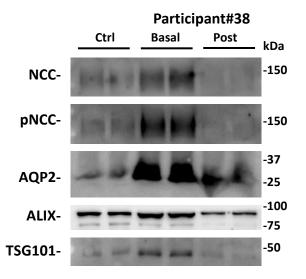


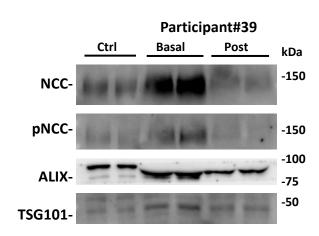


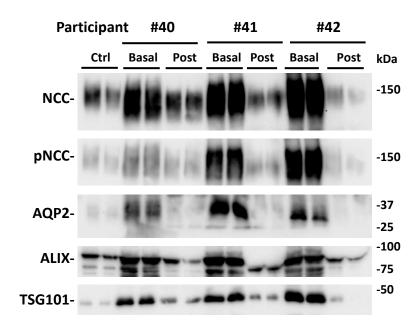


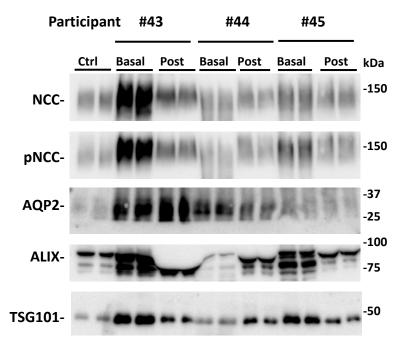


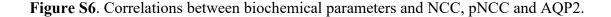


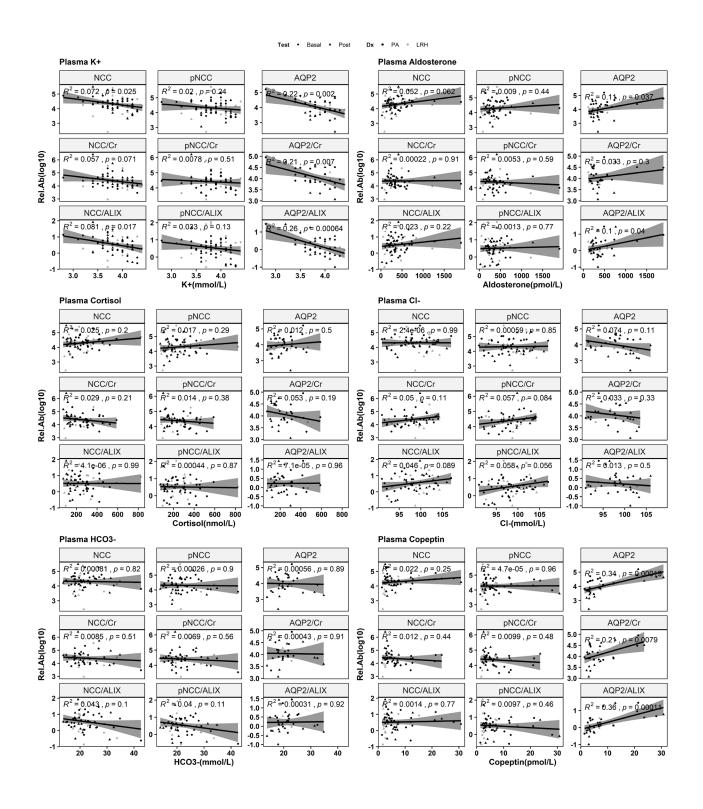




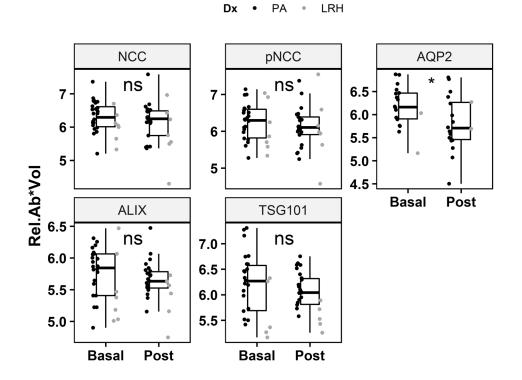




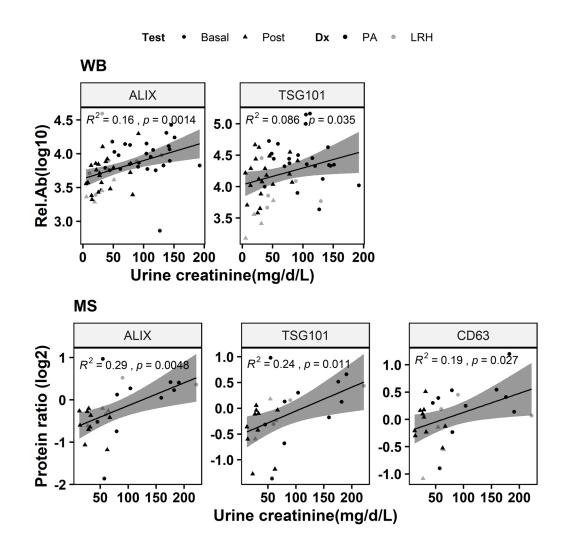




**Figure S7.** Boxplots of changes in the total contents in the urine during SSST. PA, primary aldosteronism; LRH, low renin essential hypertension; Rel. Ab\*Vol, protein relative abundance multiplying total urine volume.



**Figure S8**. Correlations between EV markers (quantified by immunoblotting [WB] and mass spectrometry [MS]) and spot urine creatinine.



Datiant		Age	BMI		SBP/DBP			Plasma	Baseline	Baseline	Anti-	HTN dru R/	gs with AS (daily		ecting
Patient No.	Sex	at SSST	(kg/m <sup>2</sup> )	Dx	(mmHg/mmHg)	ADX	(ml/min)		plasma [K <sup>+</sup> ] (mmol/L)	ARR	No. of drugs	Moxo (mcg)	Praz (mg)	Vera (mg)	Hydra (mg)
Norm	nal ran	ge	18.5 - 24.9		<140/90 in adults		>60	45/90	3.5-5.5	2-75					
1	F	41.6	42.2	LRH	143/93	Non	>90	56	3.6	69	1	-	-	90	-
2	F	54.9	29.7	LRH	160/110	Non	>90	57	4.1	61	1	-	-	120	-
3	F	46.2	31.1	LRH	118/82	Non	>90	63	4.1	78	1	-	-	240	-
4	F	59.2	29.9	PA	159/79	Non	>90	49	3.3	1810	3	200	-	240	50
5	F	56.9	23.5	PA	150/88	Non	>90	61	3.1	401	0	-	-	-	-
6	F	45.0	47.0	PA	138/95	Non	>90	52	4.3	129	1	-	-	90	-
7	F	39.8	43.5	PA	145/115	Non	>90	73	3.2	178	1	-	-	180	-
8	М	50.5	28.7	PA	163/103	Non	83	92	4.2	51	2	-	1	90	-
9	М	58.3	51.4	PA	155/87	Non	81	90	2.6	275	2	-	-	240	50
10	М	37.5	30.1	PA	138/105	Non	82	101	3.8	336	2	-	-	240	25
11	М	42.2	26.2	PA	147/103	Non	83	97	3.7	232	3	200	-	120	50
12	М	31.8	37.4	PA	180/111	Non	>90	69	2.9	211	3	-	1	120	50
13	F	65.2	28.5	PA	149/100	Non	74	74	3.5	352	2	-	-	120	12.5
14	М	48.9	28.2	PA	118/70	Non	>90	78	4.3	65	1	-	-	240	-
15	F	69.9	27.9	LRH	146/69	Non	90	60	3.7	276	1	-	1	-	-
16	М	50.7	32.1	PA	160/89	Non	>90	76	3.4	121	3	-	10	240	100
17	F	39.3	27.7	LRH	158/72	Non	>90	43	3.2	54	2	600	-	240	-
18	М	50.7	31.5	PA	138/68	Non	75	100	3.6	166	1	-	-	90	-
19	F	36.0	30.2	LRH	154/100	Non	>90	53	3.8	47	0	-	-	-	-
20	F	57.2	26.9	PA	140/102	Non	>90	62	3.5	109	1	-	-	120	-
21	F	67.8	29.6	LRH	163/85	Non	>90	45	4.0	212	2	-	-	120	50
22	F	51.4	24.9	PA	125/68	Non	>90	59	3.9	95	2	-	-	240	50

**Table S1**. Participants' clinical features and anti-hypertensive drugs at baseline.

23	F	51.6	33.4	LRH	149/83	Non	>90	56	4.4	41	2	-	-	240	100
24	F	35.7	32.8	LRH	132/80	Non	>90	59	4.3	32	1	-	-	180	-
25	F	62.5	30.0	PA	110/65	Non	90	64	4.3	128	1	-	-	180	-
26	М	38.6	39.3	PA	144/100	Non	>90	69	3.4	335	2	200	-	120	-
27	F	57.5	24.1	PA	140/80	Non	>90	62	3.8	301	0	-	-	-	-
28	F	42.8	23.1	LRH	125/70	Non	84	76	4.1	411	1	-	-	180	-
29	F	69.5	31.8	PA	170/88	Non	>90	52	3.9	144	2	400	-	240	-
30	М	44.0	28.5	PA	156/106	Non	>90	73	3.8	134	2	-	-	240	37.5
31	М	64.5	35.8	PA	138/74	Non	>90	78	3.5	609	4	400	4.5	480	150
32	F	64.7	28.0	PA	168/80	Non	>90	55	3.7	49	2	-	-	240	50
33	М	65.4	31.6	PA	166/102	Non	>90	73	3.9	130	3	-	1.0	240	75
34	F	55.3	31.3	PA	163/101	Non	>90	46	3.9	88	0	-	-	-	-
35	F	52.9	29.4	PA	122/80	Non	>90	52	3.8	243	1	-	-	360	-
36	М	68.1	34.0	PA	142/92	Non	87	80	3.7	136	2	-	-	240	50
37	М	66.2	26.0	PA	161/58	Non	>90	68	2.8	143	3	200	4.0	240	-
38	F	43.6	41.8	PA	118/82	Non	>90	57	4.1	51	1	-	-	240	-
39	F	53.6	27.3	PA	164/92	Non	>90	61	3.6	281	2	-	-	180	25
40	F	45.1	38.1	PA	164/71	Non	>90	57	4.2	36	2	400	-	360	-
41	F	39.3	25.6	PA	134/84	Non	84	77	4.0	98	0	-	-	-	-
42	F	51.7	25.6	PA	100/60	Non	>90	55	3.6	246	0	-	-	-	-
43	М	38.5	36.8	PA	170/98	Non	75	108	3.7	48	4	600	4	360	75
44	F	70.4	39.0	PA	154/69	Non	77	69	3.9	122	4	600	4	60	100

No., number; SSST, seated saline suppression testing; BMI, body mass index; Dx, diagnosis of primary aldosteronism; SBP, systolic blood pressure; DBP, diastolic blood pressure; ADX, unilateral adrenalectomy; eGFR, estimated glomerular filtration rate; plasma [K<sup>+</sup>], plasma potassium concentration; ARR, aldosterone-to-renin ratio; anti-HTN, anti-hypertensive; RAS, renin angiotensin ii system; Moxo, Moxonidine; Praz, Prazosine; Vera, Verapamil; Hydra, Hydralazine;F, female; M, male; PA, primary aldosteronism; LRH, low renin essential hypertension.

Sample No.	Dilution	Particle	Size (nm)	Participle concentration	Deutieles (fueres	
	Dilution -	Mean	Mode	(*10 <sup>9</sup> particle/mL)	Particles/frame	
1	1:750	218.9 ± 1.6	142.8 ± 7.2	1.55 ± 0.074	78.6 ± 3.7	
2	1:750	266.0 ± 3.1	171.4 ± 13.9	1.65 ± 0.022	83.7 ± 1.1	
3	1:500	222.8 ± 11.4	139.9 ± 7.0	$0.99 \pm 0.11$	50.2 ± 5.6	
4	1:500	230.6 ± 5.5	146.1 ± 2.5	$1.38 \pm 0.028$	70.3 ± 1.4	
5	1:250	227.9 ± 6.0	143.8 ± 4.8	$1.29 \pm 0.026$	65.5 ± 1.3	
6	1:250	341.3 ± 5.7	178.5 ± 3.6	1.37 ± 0.040	69.4 ± 2.0	
7	1:750	341.7 ± 5.9	194.9 ± 6.7	1.37 ± 0.089	69.4 ±4.5	
8	1:750	306.9 ± 8.6	179.6 ± 8.1	1.76 ± 0.063	89.2 ± 3.2	
9	1:150	271.9 ± 4.1	163.3 ± 11.5	1.93 ± 0.053	98.2 ± 2.7	

<b>Table S2</b> . NTA measures of p	particle size and	concentration of	f nine uEV samp	les.
-------------------------------------	-------------------	------------------	-----------------	------

Abbreviations	Description
ALIX	Apoptosis-Linked Gene 2-Interacting Protein X
AQP2	Aquaporin 2
ARR	Aldosterone-to-Renin Ratio
CD9	Tetraspanin CD9
Cl-	Chloride
DEP	Differentially Expressed Protein
ESCRT	The Endosomal Sorting Complexes Required for Transport
EV	Extracellular Vesicles
FC	Fold Change
FDR	False Discovery Rate
GO	Gene Ontology
HCO3-	Bicarbonate
K+	Potassium
KCI	Potassium Chloride
LC-MS/MS	Liquid Chromatography with Tandem Mass Spectrometry
LRH	Low Renin Essential Hypertension
Na+	Sodium
NaCl	Sodium Chloride
NCC	Sodium Chloride Cotransporter
NTA	Nanoparticle Tracking Analysis
PA	Primary Aldosteronism
pNCC	Phosphorylated NCC
RAAS	Renin Angiotensin Aldosterone System
SSST	Seated Saline Suppression Testing
TMT	Tandem Mass Tag
TSG101	Tumour Susceptibility Gene 101
uEVs	Urinary Extracellular Vesicles

 Table S3. Abbreviation list.