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In human nephrectomy specimens, the kidney level of tubular transport proteins does not correlate with their abundance in urinary extracellular vesicles.

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**Short title:** No correlation between kidney and uEVs

**Keywords:** Aquaporin, exosomes, K⁺ transporters, microvesicles, Na⁺ transporters

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Abstract

Human urinary extracellular vesicles (uEVs) contain proteins from all nephron segments. An assumption for years has been that uEVs might provide a non-invasive liquid biopsy that reflect physiological regulation of transporter protein expression in human. We hypothesized that protein abundance in human kidney tissue and uEV are directly related and tested this in paired collections of nephrectomy tissue and urine sample from 12 patients. Kidney tissue was fractioned into total kidney protein, crude membrane (plasma membrane and large intracellular vesicles) and intracellular vesicle enriched fractions, as well as sections for immunolabelling. uEVs were isolated from spot urine samples. Antibodies were used to quantify 6 segment-specific proteins (proximal tubular expressed Na/Phosphate cotransporter NaPi-2a, thick ascending limb expressed Tamm-Horsfall protein and renal-outer-medullary K⁺ channel ROMK, distal convoluted tubular expressed NaCl cotransporter NCC, intercalated cell expressed proton-pump subunit ATP6V1G3 and principal cell expressed aquaporin 2 (AQP2)) and 3 uEV markers (exosomal CD63, microvesicle marker VAMP3 and β-actin) in each fractions. By western blotting and immunofluorescence labelling, we found significant positive correlations between abundance of CD63, NCC, AQP2 and ATP6V1G3, respectively, within the different kidney-derived fractions. We detected all 9 proteins in uEVs, but their level did not correlate with kidney tissue protein abundance. The uEV protein levels showed higher inter-patient variability than the kidney-derived fractions, indicating that factors, besides kidney protein abundance, contribute to the uEV protein level. Our data suggest that, in a random sample of nephrectomy patients, uEV protein level is not a predictor of kidney protein abundance.
Introduction

The renal tubular epithelial cells are crucial for maintenance of whole-body water and electrolyte balance through controlled reabsorption and secretion of water and solutes. The epithelial transport is accomplished by specific transporter proteins located in the basolateral and apical membranes, which are tightly regulated by systemic and local factors. The regulation of transporter protein levels has been extensively studied in murine models revealing a complex interplay between different tubular segments and their transporters (13, 26, 31). Translation of the murine experimental findings to a human setting is difficult and has mostly been inferred using plasma and urinary electrolyte levels as a proxy for renal tubular transporter activity. Renal expressed transporter proteins from all tubular segments are excreted into the urine in extracellular vesicles (uEVs) (35). The uEVs therefore provide a non-invasive access to tubular epithelial cells that could potentially inform of physiological regulation of transporter activity in human kidneys. The correlation between kidney and uEV levels of transport proteins is, however, unknown.

EVs are heterogeneous populations of mainly two categories of nanosized membranous vesicle types: microvesicles and exosomes. Microvesicles are created by outward budding of the plasma membrane, while exosomes are of endosomal origin and released by fusion of the intracellular multivesicular bodies (MVB) with the plasma membrane (2). The molecular machinery involved in microvesicular and exosome cargo loading and secretion differs (39) and their protein content may therefore represent different sub-cellular compartments. Nonetheless, a direct correlation between aquaporin 2 (AQP2) abundance in cells and EVs isolated from cell conditioned medium has been demonstrated in collecting duct cell cultures (47). Similarly, uEV-associated Na\(^+\), K\(^+\), 2Cl\(^-\) co-transporter 2 (NKCC2) and NaCl co-transporter (NCC) abundance correlated with the kidney levels from rats on a low or high sodium diet (8), and uEVs from aldosterone-infused rats showed correlation between the level of phosphorylated NCC, but not the proteolytic activator of the
epithelial Na⁺ channel ENaC, prostasin (52). It is important to evaluate whether humans uEVs can be used to assess intrarenal electrolyte transporter protein levels in that it will allow for a detailed look at the molecular regulation of epithelial transporter regulation.

In humans, proteomic analyses of uEVs have been used in a number of studies to identify potential molecular biomarkers for a number of conditions including Bartter and Gitelman syndromes (3), nephrogenic diabetes insipidus (6, 15, 21), polycystic kidney disease (16), obstructive nephropathy (49), hyperaldosteronism (52), pre-eclampsia (18, 32) and essential hypertension (5). The EV level of transporter proteins is dynamic and uEV level of NCC and NKCC2 changes in response to pharmacological treatment with mineralocorticoid (57) and hydrochlorothiazide (34) in patients with primary aldosteronism and essential hypertension, respectively. On the other hand, uEV excretion of NKCC2 and NCC was not correlated with sodium reabsorption in hypertensive patients on high and low sodium diets (8). Thus, it is not known whether these responses in uEV protein abundance are a reflection of the human kidney expression level or if other factors are contributing.

The aim of the present study was to test the hypothesis that human uEV levels of kidney-specific proteins reflect their kidney level by using paired urine and kidney samples from patients undergoing nephrectomy.
Materials and Methods

Human samples

Paired urine and kidney samples from 12 consecutive patients undergoing nephrectomy were collected at the Department of Urology, Odense University Hospital, between February and May 2018 and obtained only after informed written consent and approval by the Ethics Committee (S-2014-0159). Patient characteristics are presented in Supplementary Table 1 (doi.org/10.6084/m9.figshare.7969667). Urine was collected immediately before nephrectomy and kidney and urine samples were stored at -80 °C. Protease inhibitor (Sigma Aldrich) and phosphatase inhibitor (Sigma Aldrich) were added to all samples.

Urine creatinine concentration

Creatinine concentration was determined with Creatinine 120 CP (ABX Pentra, Horiba ABX) on micro-lab 300 (Vital Scientific).

Homogenization of human kidneys

The kidney cortex was isolated and minced with a sterile scalpel. Cortex samples used for total proteins extraction were homogenized in RIPA Lysis Buffer (Millipore) supplemented with phosphatase inhibitors (Sigma Aldrich) and protease inhibitors (Sigma Aldrich) using a TissueLyser II (Qiagen). Cortex samples used for enrichment of crude membrane and subcellular vesicle protein were homogenized in isolation buffer (10 mM Tris pH 7.4, 1 mM EDTA, 0.5 mM EGTA and 140 mM NaCl) supplemented with phosphatase inhibitor and protease inhibitor in a TissueLyser II (Qiagen) and subjected to differential centrifugation as described (7). Briefly, the homogenized cortex samples were centrifuged at 2000g. The resulting supernatants were then centrifuged at 17,000g to pellet plasma membrane and various large intracellular vesicles. This fraction is termed
crude membranes. The supernatants were spun again at 200,000 g which separated small intracellular vesicles (pellets) from cytosolic proteins (supernatant). Protein concentrations were determined by Lowry Protein Assay (BioRad, Herlev, Denmark).

Precipitation of uEVs

Urine samples were thawed on ice, vortexed and centrifuged at 5,000 g for 10 min at 4 °C to clear the samples from cellular debris. The supernatant was collected and ExtraPEG (40) supplemented with phosphatase (Sigma Aldrich) and protease inhibitors (Sigma Aldrich) was added, mixed briefly and incubated at 4 °C. Following overnight incubation, the uEVs were pelleted by centrifugation at 5000 g for 10 min at 4 °C and resuspended in RIPA buffer supplemented with phosphatase and protease inhibitors. The loading amount of uEVs was normalized to urinary creatinine and all loading volume of resuspended uEVs corresponds to 3.5 μmol creatinine.

Development of monoclonal anti-NaPi-2a antibodies

Mouse monoclonal antibodies against NaPi-2a encoded by SLC34A1 were generated as described previously (24, 59) by immunizing NMRI mice twice with 14 days interval using peptide MLSYGERLGSPAV corresponding to amino acids 1-13 of the human SLC34A1) coupled to diphtheria toxoid via an added Cysteine and mixed with GERBU adjuvant. An intravenous booster injection of the peptide was administered after 2 weeks, and 3 days later lymphocytes were isolated from spleens and fused the SP2/O-AG14 cells using Polyethylene glycol (Sigma Aldrich). Positive hybridomas were selected by ELISA and cloned by limited dilution. Clones were characterized by western blotting of mock and Human SLC34A1 (Clone ID: 5186103, MHS6278-202800973, Dharmacon) transfected HEK293 cells and staining of human kidneys. Clone 4 was
selected for western blotting (Supplementary Figure 1 (doi.org/10.6084/m9.figshare.7969667)) and Clone 16 was selected for immunofluorescence labelling.

**SDS-Page and Western blotting**

Loading of uEVs were normalized to urinary creatinine and equalled 3.5 µmol/well and to protein content for kidney-derived fractions (15 µg protein/well). This loading amount produced western blot signals within the linear range (Supplementary Figure 2 (https://doi.org/10.6084/m9.figshare.7969667)). Samples were mixed with 4x Sample Buffer and 10x Reducing Agent (Thermo Fischer Scientific, Denmark) and incubated for 5 min at 95 °C. Samples for western blots of CD63 were not reduced, since it disrupted the epitope. Samples were subjected to SDS-PAGE on self-cast Tris-HCl polyacrylamide gels (8-12 % (Bio-Rad, Herlev, Denmark)) together with a size marker (Precision Plus Protein Dual Colour (Bio-Rad, Herlev, Denmark) in 1X Tris/Glycine/SDS Running buffer (Invitrogen). Proteins were blotted to a polyvinylidene difluoride (PVDF) membrane (Immobilon transfer membrane, Millipore, Denmark) by semidry blotting. Membrane was blocked for one hour in 3% dry skim milk in TBST (10 mM Tris/Base, 150 mM NaCl and 0.25% Tween-20). After additional washes with TBST, then probed with primary antibodies (Supplementary Table 2 (doi.org/10.6084/m9.figshare.7969667)) over-night and detected with horseradish peroxidase (HRP)-conjugated secondary antibodies (Supplementary Table 2 (doi.org/10.6084/m9.figshare.7969667)) diluted 1:2000 in TBST. Protein bands were detected with enhanced chemical luminescence (Merck Millipore) and visualized using ChemiDoc XRS+ system (Bio–Rad Laboratories). Densitometry of the western blots was performed using Image Lab (Biorad). All quantifications are normalised against a pool of patient samples, which were loaded together with all the individual patient samples.
Kidney samples were fixed in 4% paraformaldehyde overnight at room temperature and embedded in paraffin. Blocks were cut in sections, dewaxed and rehydrated through a series of Tissue-Clear (Sakura ProHosp) and ethanol (99%–70%). Antigen retrieval was performed with TEG-buffer (Merck) in microwave for 20 min. Sections were washed in TBST and blocked 30 min in 3% Bovine Serum Albumin/TBST, (Merck) washed and blocked 10 min in hydrogen-peroxide (Merck). The kidney sections were incubated overnight at 4 °C with primary antibodies (Supplementary Table 1), washed and incubated for 1 hour at room temperature with secondary AlexaFluor antibodies (Supplementary Table 1) diluted 1:500 in TBST. The sections were washed, nuclei were stained with 4′,6-Diamidine-2′-phenylindole dihydrochloride (DAPI, Sigma Aldrich) and mounted with fluorescence Mounting medium (Dako). Images were acquired using a 40× (UPlanFl, Olympus) objected on an Olympus BX51 microscope equipped with a DP26 camera controlled by cellSens software (Olympus). For each patient, images from 3 different regions were acquired. The expression level of the proteins of interest was quantified by automated image analysis using CellProfiler 3.1.5 (23) through a custom-made pipeline. Briefly, the images were converted to gray scale and antibody labelling was separated from background by global thresholding. The resulting binary image was used to define region of interest in the original images in which fluorescence intensity was measured. The automated image analysis could not be performed for anti-ROMK labeled sections due to lower signal-to-noise ratio and therefore the analysis was performed manually. Briefly, regions of interest were manually drawn around antibody-labeled tubular segments and fluorescence intensity was measured for each region using Fiji (ver. 2.0.0-rc-69/1.52i) (43).

EV track data
We have submitted all relevant data of our experiments to the EV-TRACK knowledgebase (EV-TRACK ID: EV190003) (53).

Statistics

All data were analyzed using R studio (48). Correlations were analyzed by linear regression and by calculation of Pearson correlation coefficients. Dark shaded areas indicate the 95% confidence interval. Statistical significance was accepted at $p < 0.05$. 
Results

To test the correlation between uEV and kidney protein levels, we obtained pre-surgery urine samples and kidney tissue from 12 patients undergoing nephrectomy (Supplemental table 1 (doi.org/10.6084/m9.figshare.7969667)). Each kidney sample was divided into 4 fractions (Figure 1): 1) a total protein fraction homogenized with detergent; 2) crude membrane protein enriched fraction (containing plasma membrane and large intracellular vesicles) homogenized without detergent and pelleted at 17,000g; 3) intracellular vesicle enriched fraction pelleted at 200,000g of the supernatant from the crude membrane protein enriched fraction; and 4) formalin fixed kidney section. uEVs were enriched from spot urine samples obtained before surgery and normalized to urinary creatinine concentration. To ensure that the protein levels measured in the uEVs originated from the kidney, we used mRNA expression data from the Human Protein Atlas (50) to identify genes with enriched expression in the kidney. We selected 6 genes encoding Na+/Phosphate cotransporter (NaPi-2a), Tamm-Horsfall protein (THP) renal-outer-medullary K⁺ channel (ROMK), NCC, V-type proton ATPase subunit G3 (ATP6V1G3) and AQP2, respectively, that were predominantly expressed in the kidney cortex as compared to other tissues and previously detected in uEVs (Table 1). To evaluate the enrichment of uEVs, we also investigated 3 general EV proteins: the exosomal marker CD63, the microvesicle marker vesicle-associated membrane protein 3 (VAMP3 (20)) and β-actin. Using this panel of antibodies on the 5 different protein fractions from each patient, allowed us to correlate kidney and uEV levels of proteins with segment-specific expression in proximal, thick ascending limb (TAL), distal convoluted tubule (DCT) and collecting duct principal and intercalated cells (Table 1). If proteins are incorporated passively into uEVs as a random sample of the cellular population, a direct linear positive correlation between uEVs and kidney levels is expected.
uEV markers are present in patient urine samples, but does not correlate with expression level in kidney

The 3 uEV markers CD63, VAMP3 and β-actin were detected in uEVs and kidney samples from patients (Figure 2a-c). CD63 was detected in uEV samples in 11 out of 12 patients (Figure 2a), while VAMP3 and β-actin uEV abundance showed higher inter-individual variance compared to the kidney samples and were detected at high levels in a subset of patients (Figure 2c and e). The uEVs samples had a heterogeneous composition and some samples had high CD63 but low VAMP3 and β-actin levels and vice versa (Figure 2a, c and e). The CD63 uEV level did not correlate with the total protein, crude membrane protein or intracellular vesicular protein kidney levels (Figure 2b). Similarly, VAMP3 and β-actin uEV levels were not correlated with their respective total protein, crude membrane protein or intracellular vesicular protein kidney levels (Figure 2d and f). A significant correlation between CD63 levels in intracellular vesicles and total protein (R = 0.83, p < 0.01) and crude membrane proteins (R = 0.64, p < 0.05), respectively, was detected.

Proximal tubular expressed NaPi-2a is detected in patient uEVs, but does not correlate with expression level in kidney

By immunofluorescence labelling of kidney sections, NaPi-2a was detected at the apical brush border membrane of proximal tubules (Figure 3a). uEV-associated NaPi-2a was detected in most patients and compared to the total protein, crude membrane protein and intracellular vesicle protein levels, the uEV abundance of NaPi-2a displayed a high degree of inter-individual variability (Figure 3b) and no significant correlations between any of the fractions were detected (Figure 3c).

Tamm-Horsfall protein (THP) and renal outer medullary K⁺ channel (ROMK) is present in uEVs but does not correlate with kidney expression
THP and ROMK were detected by immunofluorescence labelling of kidney sections in most patient samples (Figure 4a and b). THP was highly abundant in uEV samples from all patients and was immediately overexposed by western blotting. Since the THP western blot signal was directly correlated to the loaded amount of protein (Supplementary Figure 2i,j (doi.org/10.6084/m9.figshare.7969667)), we diluted uEV samples 100-fold, while the other kidney-derived fractions were run undiluted. THP was present as an abundant 90 kDa band in the uEV, total protein, crude membrane and intracellular vesicle enriched fractions from most patients (Figure 4c); however, no significant correlation between the uEV levels and the intrarenal expression level were detected (Figure 4e). ROMK has a predicted molecular mass of 45 kDa, but N- and C-terminal antibodies also detect a 90 kDa band, believed to be a dimer which is resistant to reduction and heating (32, 58). The 90 kDa form of ROMK was detected in uEVs and kidney samples from patients (Figure 4d). Densitometric analysis of the 90 kDa band in the uEVs, kidney samples and immunofluorescence labeled kidneys, revealed no significant correlation between the different fractions (Figure 4f).

NaCl co-transporter (NCC) is present in the DCT and uEVs, but its uEV abundance is not correlated to its expression in kidney

In kidney sections, NCC was expressed in DCT (Figure 5a) and NCC was also detected in uEVs from most patients (Figure 5b). Compared to the other markers, the uEV NCC level had a lower inter-individual variation. The uEV abundance of NCC was not significantly correlated with total protein, crude membrane, intracellular vesicle enriched fractions or immunofluorescence level, respectively (Figure 5b). A positive correlation was, however, detected between the NCC level detected by immunofluorescence labelling and total protein fraction (R = 0.69, p < 0.05) and crude membrane proteins (R = 0.85, p < 0.001), respectively. In addition, immunofluorescence level and
intracellular vesicle (R = 0.58, p < 0.10), and intracellular vesicles enriched, and total protein fractions tended to be positively correlated (R = 0.53, p < 0.10) (Fig. 5c).

Collecting system derived AQP2 and ATP6V1G3 is present in uEVs but not correlated with their abundance in kidney

AQP2 and ATP6V1G3 displayed strong expression in principal and intercalated cells of the collecting ducts, respectively, in kidney sections (Figure 6a). AQP2 was detected in uEVs from most patients (Figure 6b), while ATP6V1G3 was only present in uEV from some of the patients (Figure 6d). The uEV level of AQP2 and ATP6V1G3 did not correlate with their respective kidney levels (Figure 6c and e, respectively). For AQP2, significant positive correlations were found between the abundance in intracellular vesicle and crude membrane (R = 0.58, p < 0.05) and immunofluorescence level (R = 0.75, p < 0.01), respectively, as well as crude membrane and immunofluorescence levels (R = 0.63, p < 0.05). For ATP6V1G3, a significant positive correlation was detected between the total protein fraction and crude membrane enriched fraction levels (R = 0.69, p < 0.05).

uEVs are highly variable

We ranked the levels of the tested uEV proteins for each patient to test if the differences in uEVs were due to different amounts of uEV isolated. All patients, however, displayed low and high abundance of proteins (Figure 7), indicating that uEVs are a heterogeneous population of vesicle types and that the excretion of uEV associated proteins is a multifactorial process.
**Discussion**

Our study does not support the view that the uEV level of transporter proteins in a spot urine sample is a good predictor of the kidney expression level. The protein level of investigated transporters in uEV was highly variable between patients, indicating that additional individual factors, e.g. genetic and environmental, contribute to the uEV excretion of kidney expressed proteins. Thus, uEVs might not be a general non-invasive way to study the physiological regulation of kidney transporter proteins in humans.

We used the volume-excluding polymer polyethylene glycol (PEG) (40) to precipitate and enriched for uEVs. This method was primarily chosen due to the effective EV enrichment by PEG (4, 40) compared to other common EV isolation/enrichment methods such as differential ultracentrifugation, density gradient centrifugation, size-exclusion chromatography, ultrafiltration, and immune-capture (4, 53). The PEG method precipitates protein aggregates that are not EV associated (4). Since we performed antibody-based methods to analyse the samples this is, however, of minor importance, and by normalizing uEV input to urinary creatinine concentration, instead of e.g. uEV protein concentration, we obtained a relatively stable exosomal CD63 level in the uEVs fractions. This is in agreement with previous studies using differential ultracentrifugation (5, 8, 15, 32, 34) and immune-capture (42), and suggests that uEV levels and urinary creatinine/degree of urine concentration correlate. We confirmed that the 6 selected kidney- and segment-specific epithelial proteins were all present, but at variable levels, also in uEVs (1, 10, 12, 17, 22, 25, 28, 30, 35-38, 41, 46, 55, 60). Thus, our data indicate that while uEVs were isolated, the abundance of the kidney-specific proteins in uEVs was highly variable.

The high inter-patient variability of the uEV protein abundance did not reflect the level expressed in kidney and indicates that the abundance of these proteins in uEV is not solely determined by sorting of a constant fraction of the cellular protein content into EVs. The cellular abundance of a specific
protein is determined by the balance between its production rate and its rates of degradation and secretion. The uEV secretion rate of a protein is the product of EV loading and subsequent release to the urine, and our data indicate that these are highly regulated processes. In agreement with the present findings, the EV content of lipids and nucleic acid does not reflect the parental cells: the lipid composition of uEVs isolated from human urine samples is highly enriched in specific lipid species e.g. cholesterol(44) and differs from that of the parental cell (45), and the cellular and EV levels of RNA are also not correlated (51). Short sequence motifs have been identified which guide the exosomal loading of miRNA (54), resulting in exosomal miRNA levels that differs from the parental cell (14, 29, 33). A similar cue for sorting of proteins into EVs has not been identified, but protein oligomerization (9) and post-translational modification, e.g. ubiquitination (19), have been suggested to promote EV protein loading. In vitro, an increase in intracellular Ca\(^{2+}\) concentration has been shown to trigger EV release from cultured cells (27) and, together, indicate that the “cargo” loading and release of EV is an active and independently regulated process.

To our knowledge, this is the first study to perform a paired analysis of human kidney and uEVs, and only a limited number of studies have investigated the correlation between parental renal cells and their uEV protein content in rodent model and cultured cells. In medium from cultured collecting duct cells, the EV level of AQP2 was strongly correlated with the cellular level (47); however, we did not detect statistically significant correlations between the uEV and the renal expression level of any of the 9 tested proteins. Although CD63, VAMP3 and β-actin are expressed in the kidney (50), they are not tubular-specific markers and their ubiquitous expression could be an explanation for the lack of correlation in that other cell/tissue types could contribute to their uEV abundance. However, the 6 renal specific proteins were chosen due to their highly enriched kidney RNA expression among 37 different tissues (50), indicating a limiting contribution from other tissues. A limitation of the study is that the nephrectomy samples do not fully represent a healthy
kidney, even though samples are obtained from the “healthy” portion of the nephrectomised kidney.

Moreover, the urine samples represent the contribution from both kidneys and were collected prior to surgery, and anaesthetics, hemodynamic changes, intravenous fluids, or plasma electrolyte concentrations may have influenced the kidney protein abundance. However, we have previously shown that the human nephrectomy tissue specimens display physiological relevant changes in protein abundance, with e.g. higher γENaC expression level in nephrectomy samples from patients treated with diuretics (59) consistent with its regulation by extracellular volume contraction/aldosterone (11). Many factors are suspected to influence EV secretion such as age, gender and medication (56) and genetic and environmental factors could contribute to the high inter-patient EVs protein level variability through increased targeting of proteins to secretion in EVs. Thus, well-controlled studies, e.g. standardised conditions of water and food intake, activity level, and repeated samples from the same individual may serve as control, thereby limiting the individual differences and may allow for a better correlation between kidney and uEV levels.

In conclusion, our study confirms the uEV excretion of 9 proteins expressed in the human renal epithelial tubules; however, their level in uEV does not correlate with their abundance in kidneys from a random sample of nephrectomy patients. It does, however, not exclude the possibility that patient groups sharing the same pathology, kidney and uEV protein abundance could be correlated, especially if the condition is known to inhibit/stimulate specific transporters, e.g. primary aldosteronism. The EV abundance profile of the 9 tested proteins was highly variable between the patients and indicates that in addition to the renal expression level, unidentified environmental and genetic factors are contributing to the uEV protein excretion and further studies are needed to explore whether intra-patient changes in uEV protein level reflects the kidney expressed level.

Thus, our data indicate that the uEVs are an attractive source of segment-specific proteins originating from the renal epithelium, but at in a random sample of nephrectomy patients at
baseline, non-standardized conditions, the uEV protein abundance does not reflect the amount in kidney samples obtained from the same person.
Disclosures

The authors declare that there is no potential conflict of interest relevant to this article.

Author contributions

RS and PS designed the study; RS, KS, HD and PS carried out experiments; LG and LL provided materials; RS and PS analysed data; RS and PS interpreted results of experiments; RS and PS prepared figures; RS and PS drafted the manuscript; All authors edited and revised manuscript and approved final version of manuscript.

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# Kidney specificity calculated as the fold change of mRNA level in kidney compared to the tissue with the next highest expression (the data were obtained from https://www.proteinatlas.org (50)).

*TAL: Thick ascending limb; DCT: distal convoluted tubule; CNT: connecting tubule.
Figure legends

Figure 1: Urinary and kidney-derived fractions.
Paired urine and kidney samples were divided into a total of 5 fractions: A total protein fraction was produced by homogenizing kidney cortex with detergent. Crude membrane and intracellular vesicle enriched fractions were produced by homogenized kidney cortex tissue without detergent and subsequent pelleting at 17,000g and 200,000g, respectively. Immunofluorescence was performed on formalin fixed kidney tissue. uEVs was enriched from spot urine samples obtained immediately before surgery. * The crude membrane fraction contains plasma membranes and large intracellular vesicles.

Figure 2: uEV markers are present in patient samples but is not correlated with kidney levels.
Western blots against the exosome-marker (a) CD63, (c) microvesicle marker VAMP3 and (e) β-actin on uEV, total protein, crude membrane and intracellular vesicle samples. (b) The intracellular vesicles CD63 expression level was significantly correlated with CD63 expressing in the total protein fraction (Pearson correlation R = 0.83, \( p < 0.01 \)) and crude membrane fraction (Pearson correlation R = 0.64, \( p < 0.05 \)), respectively. No statistically significant correlations were detected between (d) VAMP3 and (f) β-actin level in uEV, total protein, crude membrane and intracellular vesicle fractions. The dark shaded areas indicate 95% confidence interval. * and ** indicate \( p < 0.05 \) and \( p < 0.01 \), respectively. “Correlation” indicates the Pearson correlation R.

Figure 3: Proximal tubular expressed NaPi-2a (SLC34A1) is not correlated with the uEV level.
(a) NaPi-2a (red) is expressed in the proximal tubular brush border membrane of the human kidney cortex. Blue: DAPI stained nuclei. Scale bar: 50 µm. (b) NaPi-2a is detected in uEV, total protein,
crude membrane and intracellular vesicle enriched fractions by western blotting. (c) The NaPi-2a levels in uEV, total protein, crude membrane and intracellular vesicle enriched fractions were not statistically significantly correlated; however, a borderline positive correlation between uEV and total protein fraction (Pearson correlation R = 0.50, \( p < 0.1 \)) and a borderline negative correlation between uEV enriched fraction and immunofluorescence (Pearson correlation R = -0.53, \( p < 0.1 \)) levels were detected. The dark shaded areas indicate 95% confidence interval. \# indicates \( p < 0.1 \). “Correlation” indicates the Pearson correlation R.

**Figure 4: Thick ascending limb expressed Tamm-Horsfall protein and ROMK is present in uEVs but are not correlated to kidney expression levels.**

(a) Tamm-Horsfall protein (THP, red) and (b) ROMK (red) is expressed luminal membrane of the thick ascending limb. Blue: DAPI stained nuclei. Scale bar: 50 \( \mu \)m. (c) THP and (d) ROMK are detected in uEV, total protein, crude membrane and intracellular vesicle enriched fractions by western blotting. The (e) THP and (f) ROMK expression levels, respectively, were not statistical significantly correlated between uEV, total protein, crude membrane and intracellular vesicle enriched fractions. The dark shaded areas indicate 95% confidence interval. “Correlation” indicates the Pearson correlation R.

**Figure 5: NCC is present in uEVs but is not correlated to kidney levels.**

(a) NCC (red) is expressed at the luminal membrane in the DCT. DAPI stained nuclei. Scale bar: 50 \( \mu \)m. (b) NCC is detected in uEV, total protein, crude membrane and intracellular vesicle enriched fractions by western blotting. (c) The NCC expression level detected by immunofluorescence labelling of kidney sections were statistical significantly correlated with the expression level in the crude membrane enriched (Pearson correlation R = 0.85, \( p < 0.001 \)) and total protein fraction.
(Pearson correlation R = 0.69, p < 0.05). The dark shaded areas indicate 95% confidence interval. *, ** and *** indicate p < 0.1, p < 0.05 and p < 0.001, respectively. “Correlation” indicates the Pearson correlation R.

Figure 6: AQP2 and ATP6V1G3 are present in uEVs but their abundances are not correlated to the kidney levels.

(a) AQP2 (red) and ATP6V1G3 (green) are expressed in the collecting duct of the human kidney. DAPI stained nuclei. Scale bar: 50 µm. (b) AQP2 is detected in uEV, total protein, crude membrane and intracellular membrane enriched fractions by western blotting. (c) AQP2 expression is statistically significant positive correlated between crude membrane enriched fraction and intracellular vesicle enriched fraction (Pearson correlation R = 0.58, p < 0.05) and immunofluorescence level (Pearson correlation R = 0.63, p < 0.05), respectively. The intracellular vesicle AQP2 expression level is correlated with the AQP2 immunofluorescence level (Pearson correlation R = 0.75, p < 0.01). (d) ATP6V1G3 is detected in uEV, total protein, crude membrane and intracellular membrane enriched fractions by western blotting. (e) The ATP6V1G3 expression level in the crude membrane enriched fractions is positively correlated with the expression level in the total protein fractions (Pearson correlation R = 0.69, p < 0.05). The dark shaded areas indicate 95% confidence interval. *, ** indicate p < 0.05 and p < 0.01, respectively. “Correlation” indicates the Pearson correlation R.

Figure 7: The protein content of the uEV is highly variable

Line graphs for each patient of the ranked uEV abundance of CD63, VAMP3, β-actin, SLC34A1, THP, ROMK, NCC, AQP2 and ATP6V1G3 show that level of the tested proteins is highly variable between patients.
Homogenize with detergent

Homogenize without detergent

200,000 g

17,000 g

Total protein fraction

Crude membrane enriched fraction

Intracellular vesicle enriched fraction

Immunofluorescence

Precipitate

Figure 1
Figure 2

a) CD63

- uEV
- Total protein
- Crude membrane
- Intracellular vesicles

b) VAMP3

- uEV
- Total protein
- Crude membrane
- Intracellular vesicles

c) β-actin

- uEV
- Total protein
- Crude membrane
- Intracellular vesicles
Figure 3

(a) DAPI staining of NaPi-2a expression in cells.

(b) Western blot analysis showing NaPi-2a expression levels across different patient samples.

(c) Graphs depicting the correlation between NaPi-2a expression (uEV), total protein, crude membrane, intracellular vesicles, and immunofluorescence intensity across different patient samples.
Figure 4

(a) DAPI

(b) DAPI

(c) THP

(d) ROMK

(e) THP

(f) ROMK
Figure 5

(a) Immunofluorescence of DAPI (blue) and NCC (red) in cells. Scale bar: 10 μm.

(b) Western blot analysis of NCC expression in different samples. Panel shows protein bands at 150 kDa. Samples are grouped by patient number.

(c) Correlation analysis of uEV vs. total protein, crude membrane, intracellular vesicles, and immuno-fluorescence. Correlation coefficients are indicated for each comparison.
Figure 6

(a) Immunofluorescence staining of AQP2, ATP6V1G3, and DAPI in the renal collecting ducts.

(b) Western blot analysis showing the expression of AQP2 in uEV, Total protein, Crude membrane, and Intracellular vesicles.

(c) Correlation analysis between uEV and AQP2 in different sample types.

(d) Correlation analysis between uEV and ATP6V1G3 in different sample types.

(e) Correlation analysis between uEV and AQP2 in different sample types.