

RESEARCH ARTICLE

Urinary extracellular vesicles as reservoirs of altered proteins during the pathogenesis of polycystic kidney disease

Gabriella Pocsfalvi¹, Delfin A. A. Raj¹, Immacolata Fiume¹, Annalisa Vilasi¹,
Francesco Trepiccione² and Giovambattista Capasso²

¹ Mass Spectrometry and Proteomics, Institute of Biosciences and BioResources, National Research Council of Italy, Naples, Italy

² Division of Nephrology, Department of Cardio-Vascular Sciences, Second University of Naples, Naples, Italy

Purpose: Recent findings indicate that urinary extracellular vesicles (EVs) might reflect the pathophysiological state of urinary system; and that EVs-induced ciliary signaling is a possible mechanism of intercellular communication within the tract. Here, we aimed to analyze the protein expression of urinary EVs during autosomal dominant polycystic kidney disease (ADPKD).

Experimental design: EVs were isolated from pooled urine samples of healthy control and ADPKD patients at two different stages of the disease and under tolvaptan treatment using the double-cushion ultracentrifugation method. Proteins were identified and quantified by iTRAQ and multidimensional protein identification technology (MudPIT)-based quantitative proteomics.

Results: Quantitative analyses identified 83 differentially expressed EV proteins. Many of these have apical membrane origin and are involved in signal transduction pathways of primary cilia, Ca²⁺-activated signaling, cell-cycle regulation, and cell differentiation.

Conclusions and clinical relevance: The reduced AQP-2 and the increased APO-A1 levels observed in all ADPKD-affected groups may reflect the impaired renal concentrating capability of these patients and correlated with estimated glomerular filtration rate decline. The levels of some upregulated proteins involved in Ca²⁺-activated signaling declined upon tolvaptan treatment. The results obtained suggest that the quantitative proteomics of urinary EVs might be useful to monitor proteins difficult to access noninvasively, and thus advance our understanding of urinary tract physiology and pathology.

Keywords:

Ciliary signaling pathway / Extracellular vesicles / Kidney physiology / Polycystic kidney disease / Quantitative proteomics



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Correspondence: Dr. Gabriella Pocsfalvi, Mass Spectrometry and Proteomics, Institute of Biosciences and BioResources, National Research Council of Italy, via P. Castellino 111, 80131 Napoli, Italy
E-mail: gabriella.pocsfalvi@ibbr.cnr.it

Abbreviations: ADPKD, autosomal dominant polycystic kidney disease; ALIX, apoptosis-linked gene-2 interacting protein X; APO-A1, apolipoprotein A1; AQP2, aquaporin-2; BASP1, brain acid soluble protein 1; BP, GO_Biological Process; CC, GO_Cellular Compartment; eGFR, estimated glomerular filtration rate; EM, electron microscopy; ERM, ezrin/radixin/moesin; EV,

extracellular vesicle; GO, Gene Ontology; iTRAQ, isobaric tags for relative and absolute quantitation; MF, GO_Molecular Function; MudPIT, multidimensional protein identification technology; NHE3, Na⁺/H⁺ exchanger 3; NKCC2, Na-K-Cl cotransporter isoform 2; NTA, nanoparticle tracking analysis; PC1, polycystin-1; PC2, polycystin-2; SCX, strong cation exchange; TEM, transmission electron microscopy; TSG101, tumor susceptibility gene 101; WB, Western blot

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Clinical Relevance

Extracellular vesicles (EVs) have been shown to be important mediators of intercellular communication and adaptive immune signaling. In ciliated cells EVs have been found in the vicinity of primary cilia suggesting that EV–cilia interaction could play a functional role in ciliary signaling. Because polycystic kidney disease is characterized by a compromised ciliary signaling, we were interested if the expression of EV proteins changes across the different stages of the disease.

The iTRAQ-based quantitative proteomics study shows that a number of ciliary proteins, and proteins involved in ciliary and planar cell polarity signaling

pathways are differentially expressed in urinary EVs of ADPKD patients with respect to healthy control. Most significantly, polycystin-1 and polycystin-2 expression, together with that of other Ca^{2+} -binding proteins (annexin A1, annexin A2, protein S100-A9, protein S100-A8, and retinoic acid induced protein 3), were found to be significantly altered.

The results from this study suggest that the quantitative proteomics of urinary EVs gives a unique opportunity to monitor expression changes of apical membrane proteins of the urinary track, which are difficult to access noninvasively, and supports the potential role of EVs in the kidney physiopathology.

1 Introduction

Extracellular vesicles (EVs) are a heterogeneous population of structures (i.e., microvesicles, apoptotic vesicles, and exosomes) surrounded by a membrane composed of a phospholipid bilayer. EVs have been shown to be important mediators of intercellular communication [1] and adaptive immune signaling [2]. EVs are secreted by both healthy and diseased cells, and the fact that they differ in their molecular, biochemical, and functional characteristics has generated considerable clinical interest [3–5]. Like plasma and other biological fluids, urine contains EVs (known as urinary EVs) *in vivo* that are released by the epithelial cells along the entire length of the urinary tract [3, 6]. The protein content of EVs largely reflects that of the parental epithelial cells they originate from and is enriched in certain proteins, such as cell-specific antigens, adhesion molecules, and proteins involved in membrane trafficking. A recent analysis identified more than a thousand proteins that are commonly present in the human urinary EV proteome, of which several have received considerable research interest for development of disease-related biomarkers due to their roles in kidney function [7].

Autosomal dominant polycystic kidney disease (ADPKD) is characterized by progressive cyst development and massive bilaterally enlarged kidneys, which results in kidney failure. Increased proliferation and apoptosis of renal tubular epithelial cells and altered intracellular Ca^{2+} homeostasis are the key features of cyst formation. The underlying mechanism of ADPKD remains unclear; however, it is generally accepted that functional loss caused by mutations in one of two large multiexon genes, *PKD1* and *PKD2*, account for cyst formation in almost all cases. *PKD1* and *PKD2* encode for the transmembrane proteins polycystin-1 (PC1) and polycystin-2 (PC2), respectively. In the normal kidney, PC1 and PC2 interact with each other and are expressed on the surface of primary cilia as a PC1–PC2 channel complex. Primary (nonmotile) cilia are an antenna-like plasma membrane sur-

rounded by an extension of the cellular cytoskeleton and can be found on almost every mammalian cell type. Cells appear to use primary cilia to generate context-dependent responses to extracellular signals [8]. In fact, the membranes of primary cilia are rich in receptors and ion channels. In a recent study, 195 proteins were identified in the mammalian primary cilia proteome [9], many of which are involved in ciliary signaling pathways. Electron microscopy (EM) studies showed that cilia promote cell–cell communication by interacting not only with other cilia but also with EVs [10, 11]. Biliary EVs, for example, have been found to accumulate in the proximity of cholangiocytes in ciliated bile ducts [11] and isolated urinary EVs appear to be in the vicinity of cholangiocyte cilia [10]. Furthermore, cell-to-cell transfer of functional aquaporin-2 (AQP2) mediated by urinary EVs has been confirmed in the kidney [12]. Dysfunction in primary cilia has been shown to alter renal tubular cell proliferation and differentiation and is associated with accelerated cyst formation [13]. Although the structure of kidney epithelial cilia may appear normal, sensing of fluid flow and calcium influx are altered in ADPKD. *In vivo* EV–cilia interactions have been shown in human and mouse ADPKD models, suggesting that these interactions play a functional role in ciliary signaling [10]. This led us to focus this study on differentially expressed urinary EV proteins isolated from healthy control and ADPKD patients at different stages of the disease. Proteins were isolated and identified using multiplex isobaric tags for relative and absolute quantitation (iTRAQ) and multidimensional protein identification technology (MudPIT).

2 Materials and methods

2.1 Sample collection and summary of patient data

ADPKD urine samples were from baseline visits of Tolvaptan Phase 3 Efficacy and Safety Study in ADPKD (TEMPO3/4,

Table 1. Details on samples used in this study

Groups		eGFR (mL/min × 1.73 m ²)	eGFR group	Number of samples	Age (range)	Age (average)	Patient treatment	EVs protein concentration (μg/mL)	iTRAQ label
I	Control	97.73 ± 05.97	>60	11	25–46	34	No	0.24	114
II	Late stage	30.65 ± 13.65	<60	24	33–66	49	No	0.23	115
III	Early stage	89.48 ± 21.12	>60	16	22–57	34	No	0.20	116
IV	Tolvaptan treated	97.00 ± 18.58	>60	6	32–44	35	Tolvaptan	0.27	117

Estimated glomerular filtration rate (eGFR) was calculated by the chronic kidney disease epidemiology collaboration (CKD-EPI) equation. Extracellular vesicles (EVs) protein concentrations are referred to the equivalent of 1 mL urine samples. Hundred micrograms of urinary EVs were used from each group for the quantitative proteomics analysis.

NCT00428948) [14]. Healthy control urine samples (Group I, Table 1) were from male volunteers that did not report any history of renal or chronic extra-renal diseases. Tolvaptan was administered for patients (Group IV, Table 1) over a 3-year period in the framework of the clinical trial. The study was ethically approved by the Ethical Committee of the Hospital Agency of the Second University of Naples as part of the tolvaptan trial consent process (N. 890 on September 7, 2012). Urine (both control and ADPKD samples) collection was at 36-month study visit of clinical trial timeline. Informed consent of all participating subjects was obtained. Second morning urine (100 mL per person) drawn after the first morning void urine were collected according to the National Institute of Diabetes and Digestive and Kidney Diseases Clinical Research Protocol 00-DK-0107 and stored at –80°C until analysis without any freeze/thaw cycles. Estimated glomerular filtration rate (eGFR) was determined by Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation (Table 1). Samples were thawed on ice immediately before analysis and 3.3 μL of 100 mM NaN₃, 500 μL of 100 mM PMSF, and 100 μL of 1 mM leupeptin were added to inhibit proteases. Individual samples from the same group were pooled together and 500 mL each of the pooled samples were used to isolate EVs. Combining individual biological samples in transcriptomic (microarray) and proteomic studies is fairly common. Pooling has its advantage and disadvantages, and both should be carefully considered during the proteomics study design [15]. The main disadvantage is that pooling does not allow for statistical comparison of changes between samples and can result in a loss of individual information. In this discovery work, the main aim was tough to find differences between patients with different eGFR values and those treated with tolvaptan. The benefit of pooling in this case is that the biological variation (interpersonal) should be reduced while the probability to detect study group related differences increased. Another considerable benefit of pooling is the reduced analysis cost, effort, and time.

2.2 Isolation of urinary EVs

Urinary nanosized vesicles were isolated by the double-cushion differential ultracentrifugation method [16] shown

in Fig. 1. The method allows the isolation and purification of two subpopulations of EVs with relatively well-defined size distributions. Briefly, the pooled sample was centrifuged at 400 × g, 800 × g, and 15 000 × g for 20 min at 4°C. The 15 000 × g supernatant was ultracentrifuged at 200 000 × g for 1 h at 4°C. The pellet (crude vesicle fraction) was solubilized in 20 mM Tris-HCl pH 8.6 at 4°C overnight. The sample was centrifuged at 15 000 × g and pellet containing mainly insolubilized uromodulin was discarded. The supernatant was underlaid with a double-cushion composed of 1 and 2 M sucrose in 20 mM Tris-HCl prepared in D₂O and ultracentrifuged at 110 000 × g for 3 h at 4°C. The 1 and 2 M fractions were collected, diluted 20 times of the original volume in 20 mM Tris-HCl pH 8.6, and ultracentrifuged two times at 110 000 × g for 90 min at 4°C. Pellets were resuspended in 50 μL 20 mM Tris-HCl pH 8.6. Protein concentrations were measured by BCA assay (Thermo Scientific, Rockford, IL, USA) using Nanodrop 2000 (Thermo Fisher Scientific Inc., Waltham, MA, USA). The 1 M fractions were used for further analysis.

2.3 SDS-PAGE and immunoblotting

Samples (20 μg protein) were electrophoretically separated under reducing conditions on a precast Novex 4–12% Bis-Tris NuPAGE gel using MOPS running buffer (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions and stained with colloidal Coomassie blue. Western blot (WB) analysis was performed on proteins separated by SDS-PAGE. Gel was destained in water, soaked in 1% SDS pH 8.5 for 30 min, washed twice in water and once using the NuPAGE Transfer Buffer for 10 min. Proteins were transferred onto PVDF membranes using a dry blotting system, iBlot (Life Technology, Carlsbad, CA, USA) according to the manufacturer's instruction. Transferred proteins were probed with mouse monoclonal antibodies. The blots were incubated with alkaline phosphatase conjugated secondary antibodies and the immune complexes were detected using a Western Light chemiluminescent detection system (Applied Biosystems, Bedford, MA) according to the manufacturer's protocol.

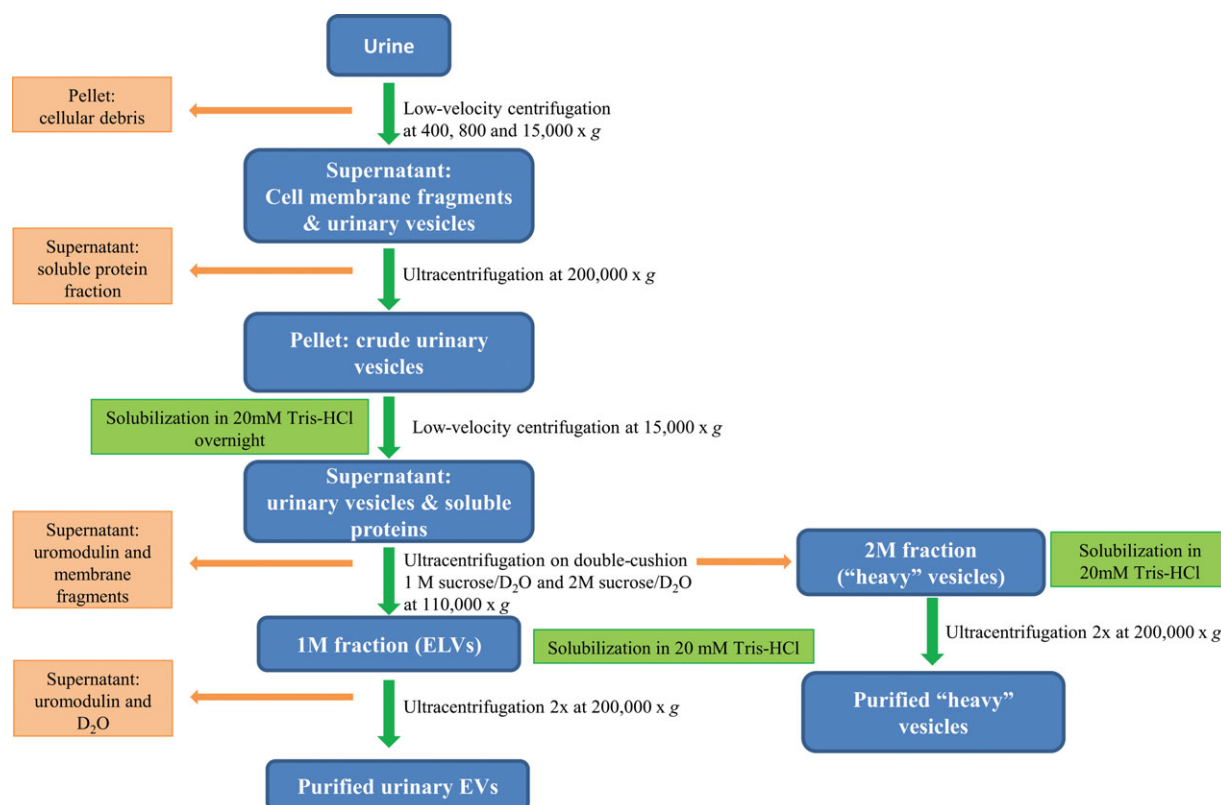


Figure 1. A schematic of the workflow used for the isolation and purification of urinary extracellular vesicles.

2.4 Analytical methods

Qualitative transmission electron microscopy (TEM) imaging analysis: Purified urinary EVs were absorbed onto the copper grid, stained with uranyl acetate dehydrate/methyl cellulose, and imaged using a JEM-1011 transmission electron microscope (JEOL, Japan) at 100 kV. Images were taken using the Morada cooled slow-scan CCD camera (3783 × 2672 pixels).

Nanoparticle tracking analysis (NTA): For particle size determination, NTA was performed with a NanoSight NS300 instrument (Malvern NanoSight) equipped with the NTA 2.3 analytical software. Experiments were carried out diluting the samples to 1 ng/mL protein concentration, leading to particle concentrations around $8 \times 10^8 \text{ mL}^{-1}$.

2.5 iTRAQ labeling, MudPIT, and shotgun analyses

Urinary EV samples (Table 1) were solubilized in 0.8% RapiGest SF (Waters Corp., Milford, MA, USA) and lysed by three freeze-thaw cycles in liquid nitrogen under sonication. Proteins (100 µg per samples) were reduced, alkylated, in-solution digested by trypsin, and labeled by 4-plex iTRAQ reagents (Applied Biosystems, Foster City, CA, USA) as described previously [17]. After labeling reactions, the four samples were combined and desalted using Oasis HLB 30 mg

columns (Waters Corp.) according to manufacturer's instruction. Sample was vacuum dried. Two independent iTRAQ analyses were performed. MudPIT was used to identify the iTRAQ-labeled proteins. The pooled sample (400 µg) was solubilized in strong cation exchange (SCX) solvent A (20% ACN, 0.1% formic acid) and offline fractionated by SCX chromatography using the Ultimate 3000 HPLC system (Dionex, Sunnyvale, CA, USA) and a PolySULFOETHYL A 100 × 2.1 mm, 5 µm, 200 Å pore column (PolyLC Inc, Columbia, MD). SCX chromatographic conditions were as follows: SCX solvent A for 10 min, SCX solvent B (20% ACN, 0.1% formic acid, 500 mM KCl) ramped up to 30% over 35 min, 60% over 5 min, 100% over 5 min, holding SCX solvent B at 100% for 15 min, all at a flow rate of 300 µL/min. Fifty fractions were collected from 10 to 60 min at 60 s intervals. After separation, the SCX fractions were vacuum dried and separated in the second dimension using RP nanoflow chromatography on the Ultimate 3000 HPLC system. Sample cleanup was performed online using an RP monolithic precolumn, 200 µm id × 5 mm length (LCPackings, Sunnyvale, CA, USA) at 25 µL/min flow rate. Peptides were separated at a flow rate of 300 nL/min on a PepSwift Monolithic column, 100 µm id × 5 cm length (LCPackings) using the following gradient: (RP solvent A: 2% ACN, 0.1% formic acid and RP solvent B: 98% ACN, 0.1% formic acid) 5–50% B in 90 min, 50–98% B in 6 s for 10 min. Eluted peptides were online analyzed in

information-dependent acquisition mode using the QSTAR Elite (Applied Biosystems) mass spectrometer equipped with a nanoflow electrospray ion source. Peptides were analyzed in positive-ion mode. Mass spectra were acquired between m/z 300 and 1500 for 1 s. The two most intense multiply charged peptides were selected for CID using rolling collision energy and dynamic acquisition time. One minute dynamic exclusion window was selected for the two CID experiments.

For the shotgun proteomics experiments, lysed vesicles samples (5 μ g) were *in-solution* digested according to [17] but without labeling reaction. Online nano-HPLC-ESI-MS/MS experiments were performed using a linear gradient of 5–50% B in 60 min.

2.6 Protein identification, quantitation, and statistical analysis

MS data were analyzed and MS/MS peak list was generated with Analyst QS 2.0 software (Applied Biosystems). Peak lists from the two independent iTRAQ experiments were merged. Mascot v.2.2 was used to search the peak list against SwissProt 2013_04 database (539 829 sequences) with human taxonomy (20 253) using trypsin with up to two possible missed cleavages. An analysis of false-positive rate of the protein identifications was performed by searching all tandem mass spectra from the nano-HPLC-ESI-MS/MS analyses against an in-house curated decoy UniProtKB human protein database containing forward and reversed sequences. In addition, contaminants such as human keratins, porcine trypsin, etc. were included in this database. The false-positive rate analysis resulted in an estimate, a percentage of false positives (1.87%) for the present peptide data set. For quantitative analysis, iTRAQ modification at lysine residue and at the N-termini of the peptide and carbamidomethylation of cysteines were set as fixed modifications. Oxidation of methionine and iTRAQ modification at tyrosine residues were set as variable modifications. Mass tolerance was set to 50 ppm for precursor and 0.1 Da for fragment ions. Mascot iTRAQ 4-plex quantification method was used for peptide and protein quantitation. The protein ratio was calculated as a weighted median ratio. Criteria for protein identification and quantitation were as follows: a minimum of two unique peptides and $p < 0.05$ significance threshold using MudPIT scoring. The fold-change cutoff was determined based on the two technical replicates of iTRAQ experiment according to Datta et al. [18]. Protein IDs were translated to GeneIDs using UniProt's ID Mapping (<http://www.uniprot.org/>) and the list of these IDs was compared with the three data sets: urinary human ExoCarta [19], Pistkun et al. [20], and Wang et al. [7].

Open-source DAVID 6.7 (<http://david.abcc.ncifcrf.gov/>) was used to obtain Gene Ontology (GO) terms associated with those proteins that showed significant expression differences across the samples. The list of gene IDs was loaded into the online tool clicking on functional annotation clustering and selecting gene ID as identifier and gene list as list type.

Three gene ontologies: molecular function, cellular component and biological process, and the KEGG pathways were analyzed.

2.7 Reagents

PBS, formic acid, ACN, and methanol (LC-MS grade) were obtained from Romil (Cambridge, GB). Sodium azide, PMSF, and leupeptin were from AppliChem (Darmstadt, Germany). Trizma base, minimum 99.9% titration and uranyl acetate were from Sigma-Aldrich (Steinheim, Germany). RapiGest was from Waters Corp.. Deuterium oxide, 99.8 Atom%D was purchased from Armar Chemicals (Dottlinger, Switzerland). Micro-BCA Protein Assay kit was from Thermo Scientific. Sequencing grade modified trypsin was from Promega (Madison, WI, USA). NuPAGE Bis-Tris precast gel 4–12%, MOPS SDS buffer and reagent kits including the SeeBlue Plus2 Pre-Stained Standard and the electrophoretic cell for SDS-PAGE were acquired from Life Technologies. Monoclonal antibodies PC1 (7E12), PC2 (D3), CD9, tumor susceptibility gene 101 (TSG101, Y16), Na-K-Cl cotransporter isoform 2 (NKCC2, H-110) apoptosis-linked gene-2 interacting protein X (ALIX), β -actin (β -ACTIN), and Na^+/H^+ exchanger 3 (NHE3) rabbit polyclonal antibody were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Polyclonal AQP2 antibody was a gift from Prof. Christensen B. M., at Aarhus University.

3 Results

The study involved 46 previously diagnosed male ADPKD patients (Table 1). Because linear regression analysis shows a significant inverse relationship between the rate of renal volume increase and the rate of decline in the GFR [21], study participants were grouped according to eGFR values as control (group I), late stage (group II), early stage (group III), or tolvaptan treated (group IV). Tolvaptan is a vasopressin V2-receptor antagonist that modulates the serum sodium concentration by increasing renal solute-free water excretion. The use of tolvaptan in ADPKD patient management has been encouraged by results obtained in cellular studies [22], animal models [23], and a recent clinical trial [14]. Clinical studies showed that tolvaptan effectively slows down the decline in kidney function by reducing the rate of cyst growth [23, 24]. Therefore, in addition to study the protein expression profiles of urinary EVs isolated from control and ADPKD patients at different stages, we also included in the analysis a group of tolvaptan-treated patients (Table 1).

3.1 Purification of urinary EVs

High-quality preparation of urinary EVs for the downstream proteomics pipeline is fundamental since a variable level of contamination by nonvesicle-derived proteins has a

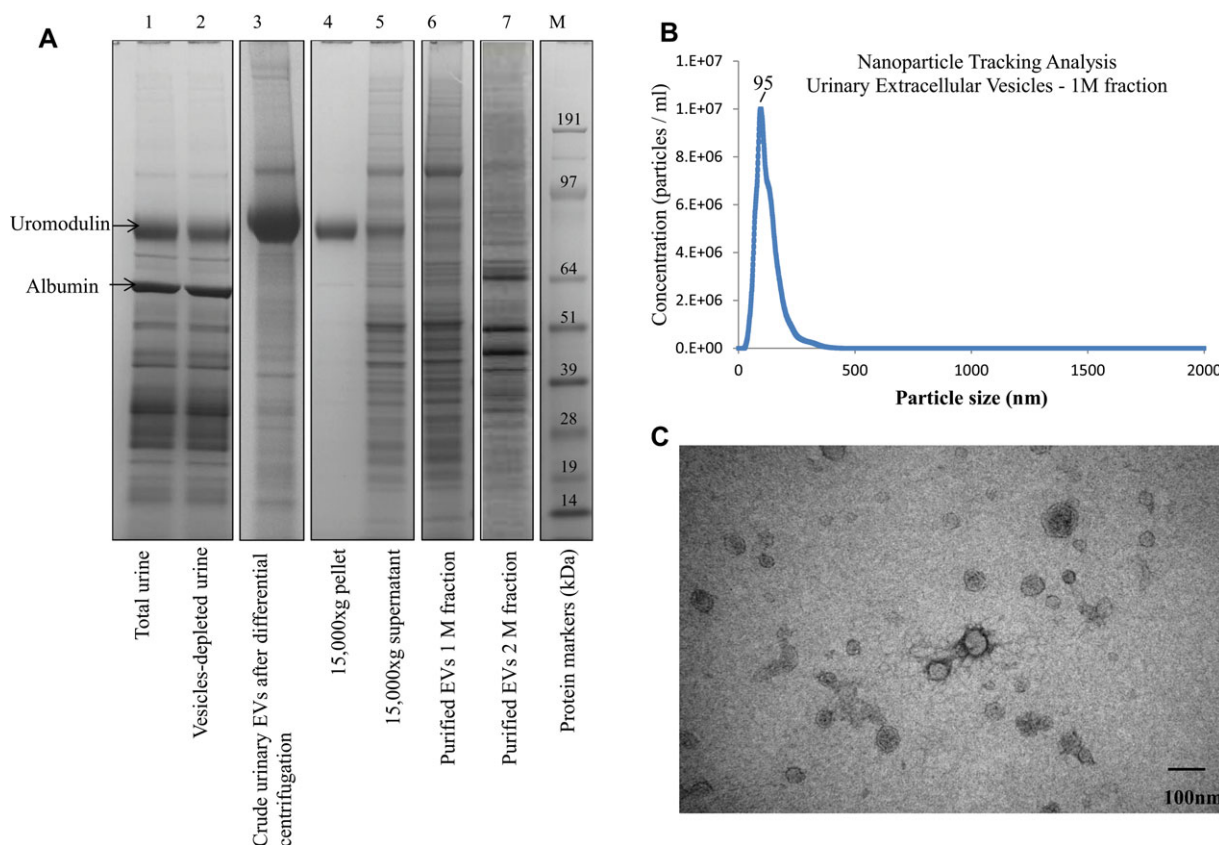


Figure 2. Physicochemical characterization of urinary extracellular vesicles (EVs). (A) Images of the SDS-PAGE protein profiles at the different stages of the isolation of EVs using the double-sucrose cushion ultracentrifugation method. (B) Particle size distribution measured by nanoparticle tracking analysis (Nanosight) (mean: 127 nm, mode: 95 nm, SD: 53 nm). (C) Transmission electron microscopy image of urinary EVs isolated from the pooled control healthy sample.

negative effect on the quantitative analysis. Samples prepared by the conventional differential centrifugation and the filtration methods result in a raw urinary EVs preparation characterized by a high level of vesicle heterogeneity, a high level of matrix contamination (mainly uromodulin, albumin, keratin, etc.) and by a low level of purity grade [6, 17]. EVs were purified from pooled urine samples from control and ADPKD patients according to our previously developed protocol [16] shown in Fig. 1. Briefly, the raw urinary EVs fraction obtained by differential centrifugation was resolubilized using 10 mM Tris-HCl at pH 7.8, centrifuged at $15\,000 \times g$, and further purified by ultracentrifugation on a 1 and 2 M sucrose/ D_2O double-layer cushion. This preparation has the advantage to separate the larger vesicles into the 2 M fraction and to remove uromodulin aggregates by solubilization. SDS-PAGE was performed to profile the proteins at the different steps of the isolation/purification process: that is, pooled total urine, EVs-depleted urine, crude urinary vesicles fraction, $15\,000 \times g$ pellet and supernatant after the resolubilization, and the final samples purified from the crude EVs by the sucrose double-cushion ultracentrifugation. SDS-PAGE images (Fig. 2A) show the progressive removal of the two major soluble urinary proteins, that is, uromodulin (centered at

approximately 85 kD) and albumin (centered at approximately 60 kD) from the samples. Moreover, the purified urinary EVs show a characteristic SDS-PAGE profile similar to those reported [10]. Size and morphology of the vesicles have been characterized by means of NTA and TEM. NTA showed particles with an average particle size 127 nm, mode 95 nm, and standard deviation 53 nm in the 1 M sucrose fraction (Fig. 2B). TEM analysis resulted in smaller (30–100 nm) average particle diameter (Fig. 2C), which was explained by the known dehydration of the vesicles upon analysis. The purity level of the urinary EVs was further assessed by shotgun proteomics because it can rapidly identify the most abundant and easily accessible proteins in complex biological samples [25]. Proteins identified in the 1 M sucrose fraction isolated from the urine of healthy donors (Table 1, group I) are listed in Supporting Information Table 1. All the identified proteins was found to be present in the EVpedia vesicle protein database [26] since had previously been identified in urinary exosomes' studies. They also have high identification counts (between 13 and 128), which indicates that they are conserved vesicle proteins. Furthermore, proteins known to contaminate urinary EVs were not identified among the first 25 top-ranking proteins (i.e.,

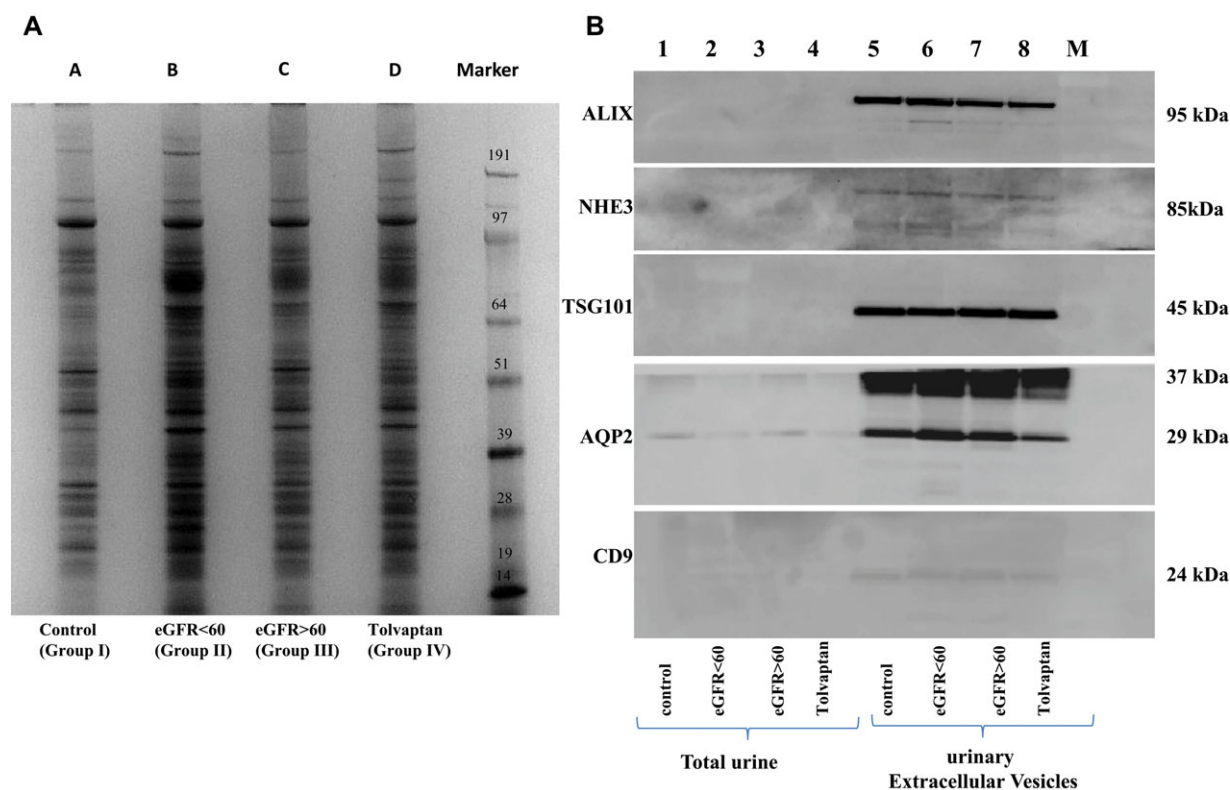


Figure 3. (A) SDS-PAGE image of urinary extracellular vesicles isolated from healthy control (lane A), ADPKD patients with eGFR < 60 mL/min/1.73 m² (lane B), ADPKD patients with eGFR > 60 mL/min/1.73 m² (lane C), and ADPKD patients with eGFR > 60 mL/min/1.73 m² treated with tolvaptan (lane D). (B) Western blots of EV marker proteins ALIX, NHE3, TSG101, AQP2, and CD9.

albumin could not be identified at all, and uromodulin is 44 in the ranking list of this particular data set). Thus, the results prove that the samples contained nanosized vesicles and the contamination from the soluble urinary proteome was expected to be low.

As ADPKD is characterized by a high rate of cellular proliferation, an elevated level of EV secretion was expected in the ADPKD-related samples. The total protein yields determined from the 1 M sucrose fractions though were very similar (Table 1), which suggested that the urinary EV secretion rate for normal subjects and ADPKD patients could be similar. A qualitative analysis of the SDS-PAGE image of urinary EV proteins isolated from ADPKD patients and healthy donors (Fig. 3A) revealed very similar profiles with only slight differences in the intensities of some of the bands. WB analyses showed that several urinary EV-specific proteins (ALIX, NHE3, TSG101, and CD9) were enriched in all the 1 M samples (lanes 5–8, Fig. 4B) but not in total urine (lanes 1–4, Fig. 3B), confirming that this fraction contained the specific vesicles. WBs were performed without normalization, which may explain that they are odds with the proteomic data in the case of AQP2. It should be noted that because of the lack of adequate and widely accepted “housekeeping” urinary EVs protein as a reference signal for rigorous normalization, quantitation with WB or other antibody-based analysis in EVs

in general, and in disease-related EVs in particular, is very challenging.

3.2 Quantitative proteomic analysis of urinary EVs isolated from ADPKD patients

To obtain insights into the alterations in protein expression in urinary EVs isolated from ADPKD patients, samples were analyzed by quantitative proteomics using an isobaric-tag labeling strategy. By 2D nano-LC-MS/MS analysis, we identified 172 iTRAQ-labeled proteins (Supporting Information Tables 2 and 3) common to the four groups. Comparing our data to those of the human urinary data set contained in the ExoCarta database (<http://www.exocarta.org/>) [19] and to the recently published urinary EV proteome sets of Wang et al. [7] and Raj et al. [17], we found that 20 (over 1%) of our MS-identified proteins were not yet listed and may be novel entries into these databases. An unexpectedly high percentage (68%) of the 126 quantified proteins (Supporting Information Table 4) showed a significant fold-change with respect to the control samples (Supporting Information Table 5, Table 2, and Fig. 4). A total of 50 proteins showed abundance increases of more than 1.7-fold (Table 2A), and 40 proteins showed abundance decreases of more than 0.6-fold (Table 2B)

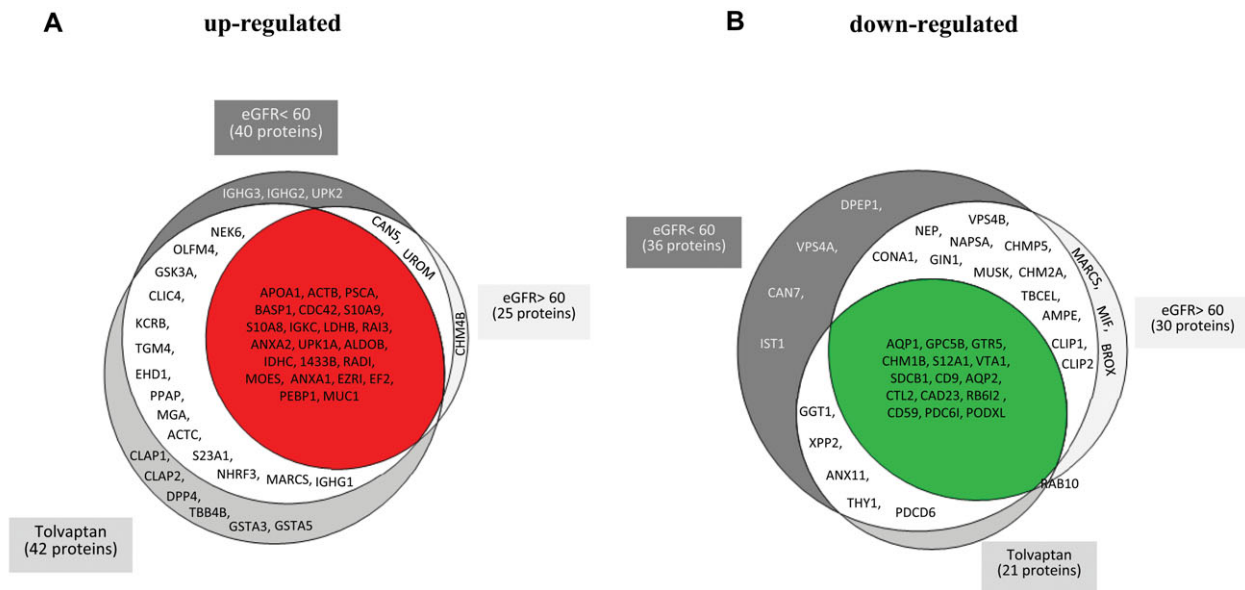


Figure 4. Comparison of (A) upregulated and (B) downregulated urinary extracellular vesicle proteins in the different ADPKD patient groups studied: eGFR < 60 mL/min/1.73 m², eGFR > 60 mL/min/1.73 m², and eGFR > 60 mL/min/1.73 m² treated with tolvaptan with respect to the healthy control sample at fold-change higher than 1.7 (upregulated) and 0.6 (downregulated).

in at least one of the groups. In particular, we identified 22 upregulated proteins (Fig. 4A) and 15 downregulated proteins (Fig. 4B) in samples from all three ADPKD groups. In addition, the eGFR < 60 mL/min/1.73 m² ADPKD patient group showed a significantly higher number of differentially expressed proteins (76) than did the eGFR > 60 mL/min/1.73 m² group (55) relative to the normal group. The average relative abundance of the upregulated proteins was found to be higher in the eGFR < 60 mL/min/1.73 m² ADPKD patient group (Supporting Information Table 4). These data suggest that as the disease progresses, the alterations in the urinary EV proteome become more pronounced.

In order to highlight changes in biological processes, the molecular functions and pathways associated with the differentially expressed proteins were mapped using the DAVID GO functional classifications tool. We obtained 45, 16, and 37 enriched GO_Biological Process (BP), GO_Molecular Function (MF), and GO_Cellular Compartment (CC) terms linked with the significantly differentially expressed proteins, respectively (Supporting Information Table 6). Analyses of the enriched GO_BP and GO_MF proteins suggested that there are significant alterations in cytoskeletal arrangement, cell polarity, cell division and motion, Ca²⁺ binding, and endocytotic pathways in ADPKD patients (Supporting Information Tables 7 and 8).

3.3 PC1 and PC2 in urinary EVs of ADPKD patients

First, we sought to determine the expression profiles of the PKD-related proteins (PC1 and PC2) in urinary EVs of ADPKD patients. PC1 primarily localizes in the cilia but is

also expressed at the lateral domain of the plasma membrane and adhesion complexes in polarized epithelial cells. PC2 appears to reside primarily in the ER and Golgi, but some PC2 forms a complex with PC1 in the ciliary membrane. In a recent work, a subfraction of EVs isolated from urine samples of healthy humans was shown to be positive for both PC1 and PC2 [10]. Because these proteins were not directly detected in our iTRAQ experiment, we performed WB analyses in order to specifically detect the expression of these proteins in urinary EVs from ADPKD patients. Immunoblotting experiments performed with an antibody raised against recombinant wide-type PC1 showed an abundant high molecular mass band at 450–460 kDa; however, this band was detected only in the control sample (Fig. 5). This result was in accord with the reported loss of plasma membrane localized PC1, which leads to the disruption of the multiprotein complex composed of PC1, PC2, E-cadherin, and β -catenin in PKD-affected cells [27]. In fact, WB analysis of PC2 using a mAb raised against 689–968 amino acid fragment of PC2 showed a faint and diffuse band at 130–150 kDa in the control sample that was largely absent in samples from the ADPKD patient groups (Fig. 5). Interestingly, loss of the genes encoding PC1 or PC2 has been shown to result in the upregulation of a group of genes related to apolipoprotein synthesis, transport, and expression in murine tissues [28]. In agreement with these results, we found that expression of apolipoprotein A1 (APO-A1), the major protein component of high-density lipoprotein in plasma, was heavily upregulated in the ADPKD patient groups in general (Table 2). In particular, APO-A1 was upregulated more than tenfold in the eGFR < 60 mL/min/1.73 m² group.

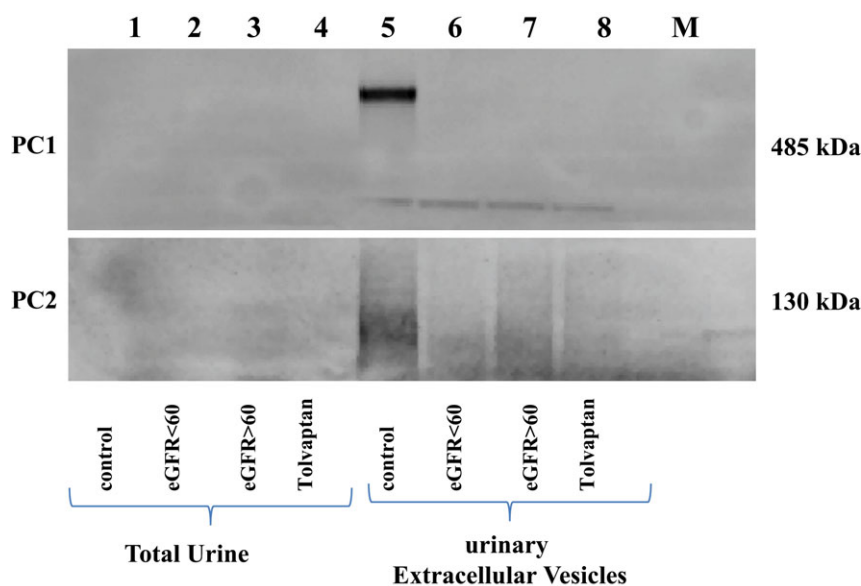


Figure 5. Western blot images of polycystin-1 (PC1) and polycystin-2 (PC2) in total urine and urinary extracellular vesicles isolated from healthy control and ADPKD patient groups II (late stage, eGFR < 60), III (early stage, eGFR > 60), and IV (tolvaptan treated).

3.4 Reduction in the AQP2 level and induction of cytoskeleton rearrangement in ADPKD

AQP2 is a vasopressin-regulated water channel protein expressed both in the apical plasma membrane of renal collecting duct cells and in small intracellular vesicles [29]. Up- and downregulation of the AQP2 water channels is controlled by AQP2 translocation from the storage vesicles to the apical membrane. Defects in AQP2 trafficking have a significant effect on the urine-concentrating capability of the kidney in general, and consequently, these defects are important in ADPKD pathogenesis. It is clinically acknowledged that ADPKD patients have an impaired maximum urine concentrating capacity that is present even during the early stage of the disease. AQP2 is excreted in the urine by exocytosis of AQP2-containing intracellular vesicles [20], but urinary EV-mediated AQP2 secretion has also been demonstrated [12]. Here, we found that the level of AQP2 was 0.4-fold lower in urinary EVs isolated from the different ADPKD patient groups than in control samples (Table 2B). This was associated with observed downregulation of several isoforms of charged multivesicular body proteins (CHMP5, CHM1B, CHM2A, and CHM4B) and vacuolar protein sorting associated proteins (VPS4A and VPS4B) involved in AQP2 trafficking. During EV biogenesis, the vesicles retain a membrane topology similar to that of the plasma membrane due to the two membrane inversion events. Therefore, the reduced AQP2 level in the urinary EVs indicates a lower expression of aquaporin water channels in the apical plasma membrane and thus correlates with the reduced renal concentrating capability of ADPKD patients. Importantly, these results also imply that urinary EVs may harbor proteins indicative of the state of the urinary tract (i.e. collecting duct) that are difficult or impossible to study noninvasively.

By searching for common GO functions among the up- and downregulated urinary EV proteins in ADPKD patients (Table 2A and B), we found that expression of proteins of the evolutionarily conserved ezrin/radixin/moesin (ERM) family was significantly upregulated in all ADPKD groups (Fig. 3A). ERM family proteins are known to be involved in actin cytoskeleton organization, cell adhesion, proliferation, and migration. During cystogenesis, cyst-lining epithelial cells undergo extensive changes in cellular composition, location, and shape, highlighting the important roles of membrane-cytoskeleton interactions. In addition to ERM family proteins, annexin A2, a ubiquitous endothelial cell surface protein that is also involved in cytoskeletal organization, was also upregulated. Annexin A2 forms a heterotetramer with its primary ligand, S100A10, which connects two membrane surfaces. Similarly, podocytes-related brain acid soluble protein 1 (BASP1) known to be involved in cytoskeletal and lipid raft dynamics was overexpressed in all ADPKD groups too (Table 2A). Evidence suggests that dynamic changes in the actin cytoskeleton play a critical role in the intracellular trafficking of AQP2 [30]. Depolymerization of actin filaments has been shown to result in an increase in AQP2 translocation to the cell surface, whereas highly polymerized actin networks trap AQP2-containing vesicles. Accordingly, upregulation of cytoskeleton-linking proteins (ERM family, and annexin A2) may induce polymerization of the actin cytoskeleton and thus reduce the rate of AQP2 translocation to the plasma membrane.

3.5 Alterations in Ca²⁺-binding protein expression

In addition to compromised cytoarchitecture, ADPKD is also characterized by aberrant ion currents in cyst-lining epithelial

Table 2. List of proteins significantly (A) upregulated and (B) downregulated with respect to healthy control in urinary extracellular vesicles (1 M fraction) isolated from ADPKD patients groups: eGFR < 60 mL/min/1.73 m² (late stage, iTRAQ isotope ratios 115/114), eGFR > 60 mL/min/1.73 m² (early stage, iTRAQ isotope ratio 116/114), and eGFR > 60 mL/min/1.73 m² treated with tolvaptan (tolvaptan, iTRAQ isotope ratio 117/114), and their fold-change measured by MuDPIT-iTRAQ

UniProt Entry name (Human)	UniProt protein name	Score ^{a)}	Mass ^{b)}	Matches ^{c)}	Coverage ^{d)}	Late stage/control		Early stage/control		Tolvaptan/control	
						115/114	SD (geo) ^{e)}	116/114	SD (geo) ^{e)}	117/114	SD (geo) ^{e)}
(A) Upregulated	Apolipoprotein A1	52	34 073	19	37.5	10.7	1.104	2.4	1.112	2.1	1.6
	Actin, cytoplasmic 1	489	44 934	114	49.9	5.1	1.684	3.6	1.491	4.4	NN
	Calpain-5	43	79 712	11	15.5	4.6	1.384	2.0	1.245	1.5	1.299
	Prostate stem cell antigen	38	14 338	36	8.1	4.4	NN	2.5	1.328	2.5	NN
	Brain acid soluble protein 1	193	27 435	20	65.6	4.4	1.192	2.6	1.360	3.5	1.320
	Cell division control protein 42 homolog	54	24 181	6	26.2	3.9	NN	4.6	2.562	3.6	2.133
	Serine/threonine protein kinase Nek6	30	39 575	11	8.6	3.8	1.219	1.4	1.188	2.5	1.168
	Protein S100-A9	39	15 020	13	44.7	3.8	1.281	6.3	1.407	4.6	1.323
	Ig gamma-1 chain C region	47	40 775	19	22.4	3.7	1.231	1.0	1.335	1.7	NN
	Ig gamma-3 chain C region	29	46 322	16	24.1	3.7	1.186	1.0	1.167	1.6	NN
	Protein S100-A8	34	12 758	29	46.2	3.5	1.510	7.4	2.146	4.2	1.611
	Olfactomedin-4	49	61 564	26	12.9	3.5	1.425	1.5	1.217	2.1	NN
	Uromodulin	211	74 901	73	27	3.4	NN	2.0	NN	1.6	NN
	Glycogen synthase kinase-3 alpha	38	54 720	7	2.7	3.2	1.199	1.2	1.172	2.1	1.190
	Ig kappa chain C region	116	13 070	24	48.1	3.2	1.331	1.9	1.284	1.7	1.418
	Ig gamma-2 chain C region	25	40 108	6	10.7	3.1	1.250	0.9	1.537	1.7	1.216
	L-lactate dehydrogenase B chain	25	40 791	23	22.5	3.0	1.165	1.8	1.330	1.8	1.270
	Retinoic acid induced protein 3	125	43 074	13	13.4	3.0	1.411	2.2	1.119	2.2	1.270
	Annexin A2	213	43 563	49	49.6	3.0	1.345	1.9	NN	2.6	1.410
	Chloride intracellular channel protein 4	30	32 728	6	14.2	2.7	1.402	1.2	2.069	4.6	1.345
	Uroplakin-1a	37	30 150	12	22.5	2.7	1.947	2.2	2.240	1.8	1.208
	Fructose-bisphosphate aldolase B	39	43 564	7	10.2	2.5	2.208	2.1	1.365	2.4	1.709
	Isocitrate dehydrogenase [NADP] cytoplasmic	28	52 390	17	19.1	2.5	1.825	2.1	1.104	2.5	1.533
	14-3-3 protein beta/alpha	44	31 205	31	34.6	2.2	1.310	2.0	1.380	2.1	1.293
	Creatine kinase B-type	32	46 073	13	27	2.2	1.212	1.5	2.166	2.1	1.573
	Protein-glutamine gamma-glutamyltransferase 4	74	84 436	24	25.9	2.1	NN	1.3	2.403	4.5	NN
EH domain-containing protein 1	38	67 130	14	27.3	2.1	1.357	1.6	1.181	2.0	1.461	
Radixin	112	78 434	65	17	2.1	2.188	2.1	1.618	2.8	NN	
Prostatic acid phosphatase	81	47 762	22	23.1	2.1	1.240	1.5	1.126	2.3	1.144	
Moesin	91	76 970	70	24.3	2.1	1.281	2.1	1.675	2.8	1.607	
Annexin A1	202	43 673	37	33.8	2.0	1.652	3.5	1.567	2.1	1.690	

Table 2. continued

UniProt Entry name (Human)	UniProt protein name	Score ^{a)}	Mass ^{b)}	Matches ^{c)}	Coverage ^{d)}	Late stage/control		Early stage/control		Tolvaptan/control	
						115/114	SD (geo) ^{e)}	116/114	SD (geo) ^{e)}	117/114	SD (geo) ^{e)}
MGA	Maltase-glucoamylase, intestinal	109	220 398	41	12.2	2.0	1.311	0.8	1.412	2.6	1.209
EZRI	Ezrin	334	77 554	113	38.7	1.9	NN	1.9	NN	2.4	NN
ACTC	Actin, alpha cardiac muscle 1	172	45 216	63	27.3	1.9	1.343	1.2	1.226	1.8	NN
EF2	Elongation factor 2	29	105 757	6	7.8	1.9	2.687	2.1	1.587	2.3	1.164
S23A1	Solute carrier family 23 member 1	66	68 094	10	11.4	1.8	2.380	1.2	1.855	2.1	2.093
PEBP1	Phosphatidylethanolamine-binding protein 1	47	23 463	11	31	1.7	2.722	2.1	NN	1.8	1.260
NHRF3	Na(+)/H(+) exchange regulatory cofactor NHE-RF3	28	63 864	29	17.7	1.7	1.467	1.2	1.588	2.9	1.243
UPK2	Uroplakin-2	39	20 404	15	33.7	1.7	1.301	1.3	1.418	1.2	1.234
MUC1	Mucin-1	210	123 785	44	6.1	1.7	1.355	1.9	1.373	2.1	1.421
MARCS	Myristoylated alanine-rich C-kinase sub.	52	35 886	4	11.1	1.7	1.121	0.4	1.392	1.7	1.150
CHM4B	Charged multivesicular body protein 4b	48	28 825	11	23.7	1.5	1.326	2.8	1.801	0.9	1.146
DPP4	Dipeptidyl peptidase 4	139	94 815	72	33.2	1.1	1.492	1.0	1.402	1.9	NN
TBB4B	Tubulin beta-4B chain	25	52 561	9	22	1.0	1.640	1.5	1.079	1.7	1.161
GSTA3	GST A3	32	28 657	23	15.3	0.8	1.089	1.2	1.097	1.8	1.216
GSTA5	GST A5	32	29 077	29	14.4	0.8	1.089	1.2	1.097	1.8	1.216
NAPSA	Napsin-A	34	47 429	12	11.4	0.4	1.241	0.4	1.418	1.7	1.279
CLAP1	CLIP-associating protein 1	28	183 342	12	4	—	—	—	—	2.2	1.982
CLAP2	CLIP-associating protein 2	28	152 651	15	4.1	—	—	—	—	2.2	1.982
(B) Downregulated											
GPC5B	G-protein coupled receptor family C group 5 member B	63	47 152	16	11.2	0.2	1.491	0.4	1.203	0.5	1.279
DPEP1	Dipeptidase 1	432	47 686	81	21.7	0.3	1.389	0.7	1.245	0.9	NN
PDCD6	Programmed cell death protein 6	49	22 921	30	29.8	0.3	1.363	0.7	1.334	0.3	1.258
GTR5	Solute carrier family 2, facilitated gluc	35	57 844	19	14.4	0.3	1.677	0.5	1.588	0.5	1.365
CHM1B	Charged multivesicular body protein 1b	69	25 034	68	37.2	0.3	1.095	0.4	1.057	0.4	NN
S12A1	Solute carrier family 12 member 1	92	132 138	22	8.6	0.4	1.224	0.5	1.183	0.4	1.258
VTA1	Vacuolar protein sorting associated protein VTA1 homolog	45	37 025	15	26.4	0.4	1.941	0.5	1.900	0.6	1.989
THY1	Thy-1 membrane glycoprotein	49	19 448	8	24.8	0.4	2.003	0.9	1.002	0.6	1.750
NAPSA	Napsin-A	34	47 429	12	11.4	0.4	1.241	0.4	1.418	1.7	1.279

Table 2. continued

UniProt Entry name (Human)	UniProt protein name	Score ^{a)}	Mass ^{b)}	Matches ^{c)}	Coverage ^{d)}	Late stage/control		Early stage/control		Tolvaptan/control	
						115/114	SD (geo) ^{e)}	116/114	SD (geo) ^{e)}	117/114	SD (geo) ^{e)}
CHMP5	Charged multivesicular body protein 5	152	28 215	19	31.5	0.4	1.626	0.6	1.538	0.8	1.153
SDCB1	Syntenin-1	47	35 189	45	31.5	0.4	1.091	0.5	1.226	0.3	NN
CD9	CD9 antigen	43	28 419	14	11	0.4	1.473	0.5	1.586	0.6	1.491
AQP2	Aquaporin-2	65	29 768	27	18.5	0.4	NN	0.4	1.301	0.4	1.107
GGT1	Gamma-glutamyl/trans-peptidase 1	232	64 884	92	20.9	0.5	1.545	0.7	1.596	0.3	1.673
XPP2	Xaa-Pro aminopeptidase 2	54	79 837	11	13.5	0.5	1.243	0.7	1.132	0.6	1.3181
CTL2	Choline transporter-like protein 2	93	86 798	20	13.6	0.5	1.726	0.4	1.629	0.5	1.337
PDC6l	Programmed cell death 6-interacting protein	187	105 813	89	36.8	0.5	1.363	0.6	1.334	0.6	1.258
NEP	Neprilysin	99	94 070	113	34	0.5	1.756	0.6	1.678	0.9	NN
CONA1	Collagen alpha-1(XXIII) chain	25	57 442	17	9.8	0.5	1.167	0.5	1.523	0.8	1.261
GIN1	Gypsy retrotransp	25	66 544	12	4	0.5	1.167	0.5	1.523	0.8	1.261
MUSK	Muscle, skeletal receptor tyr	25	105 332	14	2.3	0.5	1.167	0.5	1.523	0.8	1.261
CAD23	Cadherin-23	63	381 592	29	3.9	0.5	1.041	0.3	1.041	0.5	1.026
VPS4B	Vacuolar protein sorting associated protein 4B	123	55 639	67	41.9	0.5	1.439	0.6	1.433	0.7	1.264
ANX11	Annexin A11	209	58 300	71	42	0.5	1.652	0.9	1.567	0.2	1.690
CAN7	Calpain-7	38	101 837	17	15.9	0.5	1.580	0.7	1.088	0.7	1.667
RB612	ELKS/Rab6-interacting/CAST family member 1	27	144 663	20	6.4	0.5	1.137	0.3	1.136	0.5	1.077
CHM2A	Charged multivesicular body protein 2a	60	27 970	43	24.8	0.5	NN	0.5	1.236	0.8	1.173
PODXL	Podocalyxin	26	62 946	16	9.5	0.6	1.495	0.4	1.366	0.6	1.276
TBCEL	Tubulin-specific chaperone cofactor E-like protein	39	52 280	18	8.3	0.6	1.181	0.4	1.108	0.7	NN
VPS4A	Vacuolar protein sorting associated protein 4A	151	55 060	59	42.8	0.6	NN	0.9	NN	0.9	1.246
CD59	CD59 glycoprotein	37	15 948	10	23.4	0.6	1.068	0.6	1.100	0.6	1.155
IST1	IST1 homolog	342	43 643	133	35.2	0.6	NN	0.8	1.386	0.8	NN
RAB10	Ras-related protein Rab-10	36	25 925	10	15.5	0.7	1.062	0.6	1.187	0.5	1.934
MIF	Macrophage migration inhibitory factor	30	13 216	9	17.4	0.8	1.447	—	—	1.2	1.426
AQP1	Aquaporin-1	203	30 033	18	22.3	—	—	0.1	2.173	0.3	1.436

Proteins showing a fold-change ≥ 1.7 in expression level compared to control are highlighted in red, and fold-change ≤ 0.6 in expression level are highlighted in green.

a) This column indicates the protein mascot scores.

b) In this column, the calculated molecular masses of the proteins are reported.

c) In this column, the number of spectrum matches to the identified proteins at 95% confidence is reported.

d) The percentage of protein sequence coverage.

e) These columns indicate the fold-changes and the standard geometric deviations (SD (geo)). SD (geo) factor values are provided for protein identities obtained from at least three peptide fragmentations with expect score < 0.05 . For example, for a fold-change of 1.5 and SD (geo) of 1.3 the 95% confidence interval is obtained by $1.5 \times 1.3 = 1.95$ and $1.5/1.3 = 1.154$. Information on individual spectra are provided in Supporting Information Table 2; NN, not determined.

cells. ADPKD cyst cells with mutations in the *PKD1* and/or *PKD2* genes lack flow-sensitive Ca^{2+} signaling and consequently show a reduced cytoplasmic Ca^{2+} concentration. In addition to changes in PC1 and PC2 expression, in our study, the expression of ten Ca^{2+} -binding proteins (GO:0005509, Supporting Information Table 5) was altered in urinary EVs of ADPKD patients compared with normal subjects. In particular, we found increases in the expression of annexin A1 and annexin A2 and two proteins belonging to the S100 family, S100A8 and S100A9. Annexins (12 members) and S100 proteins (25 members) represent two large but distinct calcium-binding protein families. Annexins A1 and A2 localize apically in ADPKD epithelia and show Ca^{2+} -dependent binding to phospholipids and the cytoskeletal protein F-actin [31]. Their presence in our samples further confirms that urinary vesicles could provide important information regarding the in vivo regulation of apical membrane proteins of polarized renal epithelial cells.

Moreover, our data revealed a decrease in the expression of annexin A11 in the urinary EVs from the patient groups compared with those from healthy subjects. Annexin A11 is a mid-body protein known to be involved in the terminal phase of cytokinesis, when abscission occurs and the two daughter cells separate. It has been shown that cells lacking normal cilia structure and/or function undergo abnormal cell division [32] and vesicle trafficking [33]. Thus, the observed reduction in the annexin 11 level in the urinary EVs of ADPKD patients may be indicative of defective cytokinesis and vesicle transport.

3.6 Alterations in Wnt signaling pathways

Several studies have revealed an unforeseen connection between deregulation of Wnt/ β -catenin signaling and polycystic kidney disease [34]. Besides proteins involved in cytoskeletal organization, here we found that some proteins involved in regulating Wnt signaling pathways are differentially expressed in urinary EVs isolated from ADPKD patients. In particular, reduced expressions of one of the six members of the glypican family, GPC5B, a positive regulator of Wnt signaling pathway was observed in all ADPKD-related groups (0.2, 0.4, and 0.5 fold-changes in patient groups II, III, and IV, respectively). Glypicans enhance Wnt signaling by stabilizing the interaction between Wnt proteins and frizzled receptors expression of glypican-5 is developmentally regulated in the kidney and it is frequently deregulated in cancer. CD9 tetraspanin, a candidate upstream negative regulator of the Wnt signaling pathway, exhibited reduced expression levels too. This result is interesting in light of emerging data suggesting a novel role for tetraspanins in the regulation of Wnt/ β -catenin signaling through exosomal-released β -catenin [35]. The noncanonical Wnt and planar cell polarity signaling pathways are overlapping but distinct pathways that play roles in controlling cilia orientation and cell motility. CDC42, one of the core components of the planar cell polarity

pathway, has been shown to be significantly upregulated in cystic kidneys [36], and here we found that it is also upregulated in urinary EVs. In addition, PEBP1 and UPK1A, which are proteins involved in the establishment/maintenance of cell polarity, were also significantly upregulated (Table 2).

3.7 Effect of tolvaptan treatment on the urinary EV proteome profile

To evaluate the effect of tolvaptan on the urinary EV proteome of ADPKD patients, patients with an eGFR > 60 mL/min/1.73 m² were divided into two groups (treated (group IV) and not treated (group III)). The 117/116 iTRAQ reporter ion ratios were calculated (Table 3 and Supporting Information Table 5). Analysis of the relative ratios showed that overall 25 proteins exhibited a significant fold-change. The expression of 18 proteins was upregulated and that of seven proteins was downregulated. The upregulated group included the immunoreactive proteins IGHG3, IGHG1, IGHG2, and IGKC. Similarly, increases in the levels of Nek6 serine/threonine kinase (1.8-fold) and kinase substrate MARCS (3.8-fold) were detected. Upregulation of these proteins could be related to VPV2 antagonist-mediated inhibition of Ras/mitogen-activated protein kinase signaling (demonstrated in polycystic kidney models). Furthermore, the expression levels of three proteins related to Ca^{2+} -activated signaling (S10A8, annexin A1, and annexin A11) were found to diminish upon tolvaptan treatment.

4 Discussion

Research on urinary EVs is rapidly expanding in clinical nephrology. Over the past 10 years, EVs have been isolated from urine by different methods and studied in relation to a number of different nephropathies with the specific aim of discovering novel biomarkers [7]. Recently developed isolation and purification methods now allow not only qualitative but also quantitative analyses of the urinary EV proteome, the latter of which is fundamental for comparing protein expression profiles of samples obtained from different sources [17]. To the best of our knowledge, this is the first report describing the multiplex quantitative proteomic analysis of urinary EVs in the study of patients with ADPKD.

Our study showed that the expression of a high percentage of proteins is significantly altered in urinary EVs isolated from ADPKD patients relative to urinary EVs isolated from healthy controls (Table 2 and Fig. 3). GO term enrichment analysis (Supporting Information 7 and 8) highlights that upregulated proteins were enriched in membrane to membrane docking, establishment or maintenance of apical/basal cell polarity, and microtubule polymerization GO terms (Fig. 6). This is in agreement with the cystogenesis characterized by compromised cytoarchitecture and membrane protein polarity defects in ADPKD [37] and shows the potential of

Table 3. List of proteins significantly (A) upregulated and (B) downregulated with respect to the eGFR > 60 mL/min/1.73 m² ADPKD patient group (early-stage ADPKD) in urinary extracellular vesicles isolated from patient with similar eGFR values but treated with tolvaptan (iTRAQ isotope ratio 117/116) and their fold-change measured by MuDPIT-iTRAQ

UniProt ID (Human)	UniProt protein name	Score ^{a)}	Mass ^{b)}	Matches ^{c)}	Coverage ^{d)}	Tolvaptan/early stage	
						117/116	SD (geo) ^{e)}
(A) Upregulated							
NAPSA	Napsin-A	34	47 429	12	11.4	4.8	1.805
CLIC4	Chloride intracellular channel protein 4	30	32 728	6	14.2	3.8	1.539
MARCS	Myristoylated alanine-rich C-kinase substrate	52	35 886	4	11.1	3.8	NN
TGM4	Protein-glutamine gamma-glutamyltransferase 4	74	84 436	24	25.9	3.4	1.364
MGA	Maltase-glucoamylase, intestinal	109	220 398	41	12.2	3.3	NN
IGKC	Ig kappa chain C region	116	13 070	24	48.1	2.7	2.236
AQP1	Aquaporin-1	203	30 033	18	22.3	2.5	1.543
NHRF3	Na(+)/H(+) exchange regulatory cofactor NHE-RF3	28	63 864	29	17.7	2.4	1.354
IGHG2	Ig gamma-2 chain C region	25	40 108	6	10.7	1.9	1.869
DPP4	Dipeptidyl peptidase 4	139	94 815	72	33.2	1.9	1.319
GSK3A	Glycogen synthase kinase-3 alpha	38	54 720	7	2.7	1.8	1.027
IGHG1	Ig gamma-1 chain C region	47	40 775	19	22.4	1.8	1.246
NEK6	Serine/threonine protein kinase Nek6	30	39 575	11	8.6	1.8	1.017
TBCEL	Tubulin-specific chaperone cofactor E-like protein	39	52 280	18	8.3	1.8	1.012
S23A1	Solute carrier family 23 member 1	66	68 094	10	11.4	1.8	1.128
IGHG3	Ig gamma-3 chain C region	29	46 322	16	24.1	1.7	1.259
NHRF1	Na(+)/H(+) exchange regulatory cofactor NHE-RF1	134	42 444	32	40.5	1.7	NN
GNAS1	Guanine nucleotide-binding protein G(s) subunit α isoforms XLas	48	117 317	29	5.8	1.7	1.197
(B) Downregulated							
CHMP4B	Charged multivesicular body protein 4b	48	28 825	11	23.7	0.3	2.064
GGT1	Gamma-glutamyltranspeptidase 1	232	64 884	92	20.9	0.4	NN
ANX11	Annexin A11	209	58 300	71	42	0.4	1.259
PDCD6	Programmed cell death protein 6	49	22 921	30	29.8	0.5	NN
S10A8	Protein S100-A8	34	12 758	29	46.2	0.6	1.412
THY1	Thy-1 membrane glycoprotein	49	19 448	8	24.8	0.6	1.439
ANXA1	Annexin A1	202	43 673	37	33.8	0.6	1.259

a) This column indicates the protein mascot scores.

b) In this column, the calculated molecular masses of the proteins are reported.

c) In this column, the number of spectrum matches to the identified proteins at 95% confidence is reported.

d) The percentage of protein sequence coverage.

e) This column indicates the fold-changes and the standard geometric deviations (SD (geo)). SD (geo) factor values are provided for protein identities obtained from at least three peptide fragmentations with expect score < 0.05. For example, for a fold-change of 1.5 and SD (geo) of 1.3 the 95% confidence interval is obtained by $1.5 \times 1.3 = 1.95$ and $1.5/1.3 = 1.154$. Information on individual spectra are provided in Supporting Information Table 2; NN, not determined.

unbiased, hypothesis-free quantitative proteomics analysis of urinary EVs. Our data showed that expression of proteins involved in cytoskeletal organization (ERM family and annexin 2) by linking the actin cytoskeleton to the plasma membrane is significantly upregulated in EVs of ADPKD patients. Cytoskeletal rearrangement plays critical roles in cyst formation and the intracellular trafficking of AQP2. The observed reduction in the AQP2 level in ADPKD patients is consistent with

the impaired urinary concentrating ability of these patients. However, because all patients in the study were encouraged to drink water in order to keep the circulating vasopressin level low, we cannot exclude the possibility that the low AQP2 level was due to water load.

In addition, most of the proteins showing altered expression could be related to the cilium and the ciliary pathways. This is interesting, as primary cilia are considered to be key

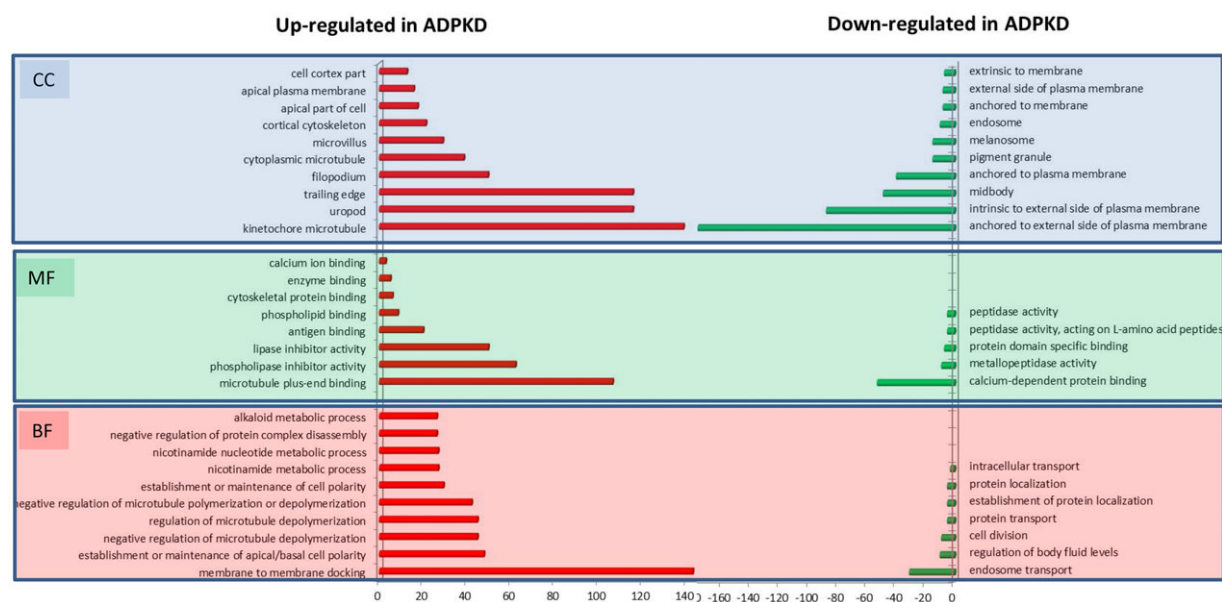


Figure 6. Enriched Go terms (i.e. cellular compartment (CC), molecular function (MF), and biological function (BF)) of differentially expressed proteins in urinary extracellular vesicles (EVs). Blue bars (left side): upregulated proteins (fold enrichment ≥ 3.0); red bars (right side): downregulated proteins (fold enrichment ≥ 3). Negative value means that the GO term was downregulated in ADPKD-related urinary EVs. $p < 0.05$ was considered as statistical significance.

regulators in the development of polycystic kidneys. Most significantly, PC1 and PC2 expression, together with that of other Ca^{2+} -binding proteins (annexin A1, annexin A2, S100-A9, S100-A8, retinoic acid induced protein 3) was found to be significantly altered in urinary EVs isolated from ADPKD patients. Some of these proteins showed a reduced expression level upon tolvaptan treatment with respect to ADPKD $\text{eGFR} > 60 \text{ mL/min/1.73m}^2$ patient group. Recent findings outlining interactions between EVs and cilia are intriguing. Reports of altered expression of surface tetraspanins and immune regulatory proteins, including APO-A1, may further support this theory. Several proteins highly expressed in the different sections of the urinary system showed significant alterations, like 4.4 fold-change of prostate stem cell antigen expressed in urinary bladder, 4.4 fold-change of BASP1 highly expressed in podocytes, 0.3 fold-change of dipeptidase 1 highly expressed in bladder and kidney (Table 2); 4.8 fold-change of napsin A and 3.8 fold-change of chloride intracellular channel 4 proteins highly expressed in the apical domain of proximal tubule cells (Table 3). These proteins have been frequently identified in urinary EVs preparations (EVPedia community database [26]), and thus there altered expression might indicate dysregulation of complex biological processes characteristic of the specific part(s) of the urinary tract.

Finally, the results of this study show that the quantitative analysis of urinary EV proteome provides a unique opportunity to investigate the expressions of proteins that are difficult to isolate noninvasively. In particular, since the pattern of membrane protein expression in urinary EVs is highly similar to that of the plasma membrane surface of the epithelial cells

the EVs originate from, suggests that urinary EVs will likely become a useful tool for the in vivo study of kidney-related disease processes. This is particularly important, as monitoring of patients with kidney diseases will turn out to be more important with the development of therapeutic interventions.

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