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Changes in the urinary extracellular vesicle proteome are associated with nephronophthisis-related ciliopathies

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ABSTRACT

Nephronophthisis is one of the leading genetic causes of end-stage renal disease in childhood. Early diagnostics and prognostics for nephronophthisis are currently limited. We aimed to identify non-invasive protein biomarkers for nephronophthisis in urinary extracellular vesicles. Extracellular vesicles were isolated from urine of 12 patients with a nephronophthisis-related ciliopathy and 12 age- and gender-matched controls, followed by in-depth label-free LC-MS/MS proteomics analysis of gel fractionated extracellular vesicle proteins. Supervised cluster analysis of proteomic profiles separated patients from controls. We identified 156 differentially expressed proteins with fold change ≥4 in patients compared to controls ($P < .05$). Importantly, expression levels of discriminating proteins were correlated with chronic kidney disease stage, suggesting possible applications for urinary extracellular vesicle biomarkers in prognostics for nephronophthisis. Enrichment analysis of gene ontology terms revealed GO terms including signaling, actin cytoskeleton and endocytosis among the down-regulated proteins in patients, whereas terms related to response to wounding and extracellular matrix organization were enriched among upregulated proteins. Our findings represent the first step towards a non-invasive diagnostic test for nephronophthisis. Further research is needed to determine specificity of the candidate biomarkers. In conclusion, proteomic profiles of urinary extracellular vesicles differentiate nephronophthisis-related ciliopathy patients from healthy controls.

Significance: Nephronophthisis is an important cause of end-stage renal disease in children and is associated with an average diagnostic delay of 3.5 years. This is the first study investigating candidate biomarkers for nephronophthisis using global proteomics analysis of urinary extracellular vesicles in patients with nephronophthisis compared to control individuals. We show that measuring protein markers in urinary extracellular vesicles is a promising approach for non-invasive early diagnostics of nephronophthisis.

1. Introduction

Nephronophthisis is an important genetic cause of end-stage renal disease (ESRD) in children and young adults, accounting for 2.4% to 15% of pediatric ESRD cases [1–3]. Nephronophthisis is known to result from defective ciliary signaling and has been classified as a renal ciliopathy. It is characterized by chronic tubulointerstitial nephritis leading to loss of renal function and a reduced concentrating ability of the kidney. The diagnosis of nephronophthisis is currently based on clinical symptoms including polydipsia, polyuria, growth retardation and refractory anemia, chronic kidney disease (CKD) and a family history consistent with autosomal recessive disease [3, 4]. Renal ultrasound findings include hyperechogenic kidneys and corticomedullary cysts in approximately 50% of patients [5–7].

As a result of the variable and non-specific presenting symptoms, nephronophthisis is diagnosed on average 3.5 years after symptom onset, often in advanced stages of CKD when kidney damage is irreversible [7]. This diagnostic delay occurs especially in

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nephronophthisis patients without extra-renal manifestations (e.g. retinitis pigmentosa or neurological signs) that could be indicative of a ciliopathy and would usually prompt monitoring of renal function. In addition, DNA testing currently confirms the diagnosis in only 40–50% of nephronophthisis patients and genetic confirmation does not enable accurate prognostics yet, hindering disease management and genetic counseling [8–10]. Therefore, the discovery of early biomarkers for nephronophthisis-related ciliopathies (NPH-RC) is essential to permit early diagnostics and monitoring of disease progression. Early diagnostics will permit 'reverse phenotyping', i.e. the monitoring of other ciliopathy-related features such as retinal degeneration and liver fibrosis in apparently isolated nephronophthisis. Furthermore, early diagnostics will pave the way for timely supportive treatment, aimed at slowing down disease progression towards ESRD, and preparation for pre-emptive renal transplantation. Additionally, early detection of nephronophthisis may be indispensable for the success of future targeted therapies.

Biofluids contain extracellular vesicles (EVs) including exosomes that have emerged as a treasure trove of molecular markers [11]. Exosomes originate from 50 to 130 nm intraluminal vesicles of multivesicular bodies that are secreted into extracellular fluids [12]. Exosomes are important mediators of intercellular communication [13]. The content of exosomes has been shown to reflect the physiological state of the cells of origin, in the case of urine the cells lining the kidney and urinary tract [14]. Previous studies have shown that proteomic analysis of urinary exosomes offers unique insights into pathophysiological mechanisms that underlie kidney disease [15]. Thus, urinary exosomes are an excellent non-invasive source of biomarkers for kidney diseases [16].

Here, we present the first study identifying candidate biomarkers for nephronophthisis using global proteomics analysis of urinary EVs in 12 NPH-RC patients compared to 12 control individuals. The identification of biomarkers for nephronophthisis gives us opportunities to develop a non-invasive diagnostic test for children suspected of nephronophthisis and thereby improve the care for NPH-RC patients. When confirmed in larger patient series, we see promising applications in diagnostics and prognostics and, thereby, translation to clinical practice.

2. Materials and methods

2.1. Patients and urine collection

Twelve patients with NPH-RC and 12 age- and gender-matched control subjects were recruited at the Department of Pediatric Nephrology of the University Medical Center Utrecht, the Department of Genetics of the University Medical Center Utrecht, and the Department of Human Genetics of the Radboudumc Nijmegen. NPH-RC patients and controls were included in the AGORA (Aetiologic research into Genetic and Occupational/environmental Risk factors for congenital Anomalies) data- and biobank project, approved by the regional Committee on Research involving Human Subjects (CMO Arnhem-Nijmegen 2006/048 and METC UMC Utrecht 12–473). Signed informed consent was obtained for all subjects included in this study. Inclusion criteria for patients were a molecularly confirmed diagnosis of NPH-RC or a high suspicion thereof, based on clinical features including presence of polyuria, absence of recurrent urinary tract infections, CKD stage 1–5 [17], renal ultrasound showing parenchymal damage but absence of malformation of the kidneys and urinary tract, urinalysis showing absence of hematuria, erythrocyte casts and proteinuria in the nephrotic range, and exclusion of other causes of early-onset renal failure. Age and gender-matched control subjects were acquaintances of the researchers and showed no signs or symptoms of NPH-RC, no family history of pediatric-onset renal disease, no known congenital anomalies of the kidney and urinary tract, no consanguinity, and a protein/creatinine ratio within the normal range (i.e. < 20 mg/mmol in individuals over 2 years of age). Control urine samples were anonymized after collection and matching.

Table 1
Subject and urine characteristics.

Subject characteristics	Patients	Controls
Number (n)	12	12
Gender (M/F)	6/6	6/6
Age (years; mean ± SD)	14.8 (± 4.7)	14.7 (± 4.7)
Diagnosis	Isolated NPH (n = 6) BBS (−like) (n = 2) CED (n = 2) JBTS (n = 2)	NA
CKD stage	I (n = 1) II (n = 3) III (n = 4) IV (n = 3) V (n = 1)	NA
Urine characteristics	Patients	Controls
Amount (ml; mean ± SD)	113.3 (± 98.0)	90.0 (± 14.3)
Processing time (minutes; mean ± SD)	103.8 (± 79.0)	46.7 (± 41.1)
Storage time (days; mean ± SD)	119.6 (± 62.3)	95.0 (± 63.9)
Protein/creatinine ratio (mg/mmol mean ± SD)	58.0 (± 64.2)	11.6 (± 8.5)

NPH: nephronophthisis; BBS: Bardet-Biedl syndrome; CED: cranioectodermal dysplasia (Sensenbrenner syndrome); JBTS: Joubert syndrome; NA: not applicable.

Urine samples (40 to 400 ml) were collected from all subjects using sterile urine containers and kept on ice for a maximum of 5 h until processing. We did not use the first morning void to minimize the chance for an enrichment of EVs derived from the urinary bladder. A 4 µl aliquot of fresh urine was stored at −20 °C for determination of protein/creatinine ratio using the Bradford Protein Assay (Bio-Rad, Hercules, CA, USA) and the Creatinine PAP FS kit (DiaSys Diagnostic Systems, Holzheim, Germany) according to manufacturers' instructions. The remainder of the urine was centrifuged at 500 ×*g* for 10 min at 4 °C and at 2000 g for 20 min at 4 °C. Supernatants were stored at −80 °C until further use.

2.2. EV isolation

Supernatants were processed in three batches of eight subjects with equal numbers of patients and controls, male and female subjects and uniform mean subject age to preclude batch effects. Thawed supernatants were centrifuged at 2000 ×*g* for 20 min at 4 °C. 0.05% NP-40 (Sigma-Aldrich, Zwijndrecht, The Netherlands) was added to each supernatant as a detergent [18]. The samples were concentrated to 1 ml by centrifugation at 4000 ×*g* at room temperature using Amicon Ultracel 100 kDa cut-off centrifugal filters (Merck Millipore, Billerica, MA, USA), which removes proteins smaller than 100 kDa while retaining extracellular vesicles. Concentrated samples were diluted to 1.9 ml using PBS (BBraun, Melsungen, Germany). Subsequently, 100 µl cOmplete™, Mini, EDTA-free Protease Inhibitor Cocktail (PIC) (Roche, Basel, Switzerland) in 525 µl PBS was added to each sample. Urine was centrifuged for 16,000 ×*g* for 30 min at 4 °C to remove debris. Next, Vn96 peptide (ME-kit, New England Peptide, Gardner, MA, USA) was added to the supernatant to aggregate EVs including exosomes as previously described [19, 20]. The Vn96 peptide binds heat shock proteins present on the surface of EVs, allowing 'miniprep' isolation. Samples were incubated for 60 min at room temperature and centrifuged at 16,000 ×*g* for 15 min at 4 °C. The full pellets were washed with 5% cOmplete™ PIC in PBS, centrifuged at 16,000 ×*g* for 15 min at 4 °C and resuspended in 2 ml NuPAGE Sample Buffer (Life Technologies, Thermo Fisher Scientific, Carlsbad, CA, USA).

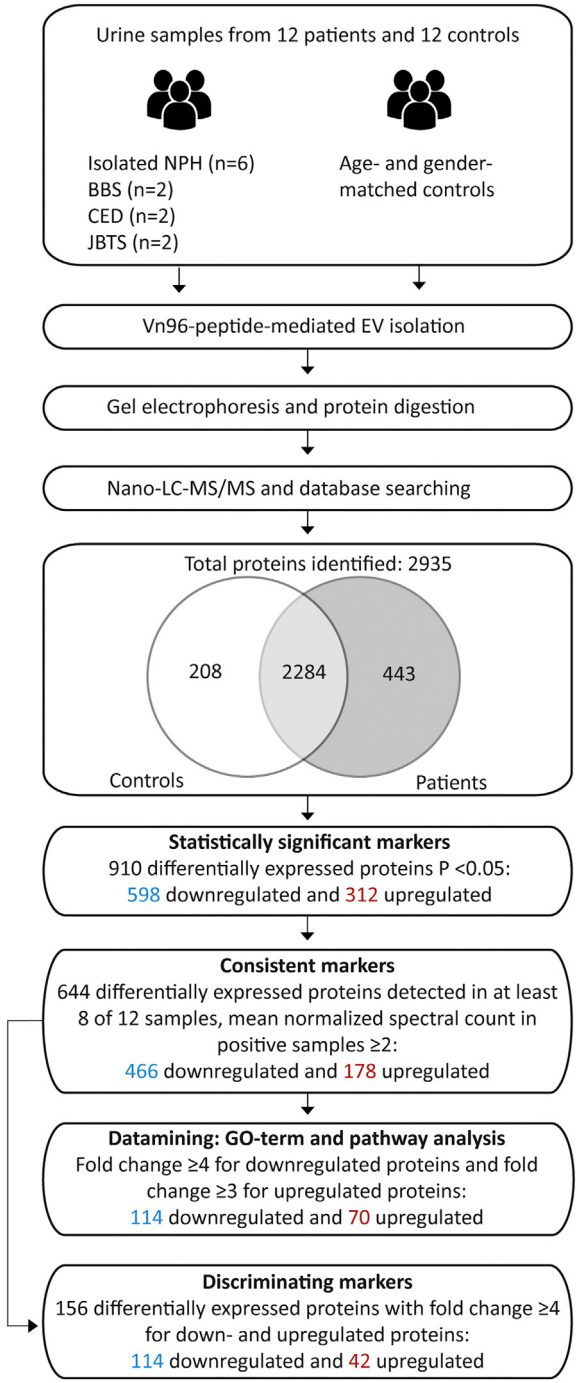

Fig. 1. Study methodology to identify differentially expressed proteins in nephronophthisis patients compared to age- and gender-matched controls. Venn diagram shows total number of proteins identified in controls and in patients based on raw spectral counts. NPH: isolated nephronophthisis; BBS: Bardet-Biedl syndrome; CED: cranioectodermal dysplasia (Sensenbrenner syndrome); JBTS: Joubert syndrome.

2.3. Western blot

Immunoblotting was performed in additional control urine samples to verify the isolation of EVs. Antibodies were directed against EV markers PDCD6IP (Alix, Mouse monoclonal; 1:1000; Cell signaling Technology, Danvers, MA, USA) and TSG101 (Rabbit polyclonal; 1:500; Atlas Antibodies, Bromma, Sweden) (Supplementary Fig. 1).

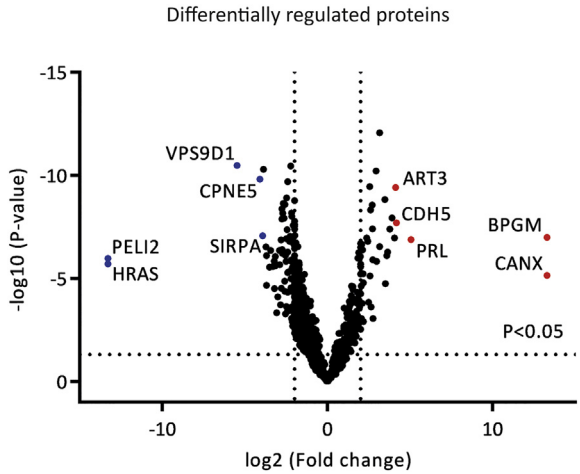

Fig. 2. Volcano plot of differentially regulated proteins in patients compared to controls. Dots represent individual proteins. Proteins were detected in at least 8 of 12 samples and had a mean normalized count in positive samples of ≥2. Vertical dashed lines mark fold change = 4 and horizontal dashed line marks the significance threshold $P < .05$. Top 5 most downregulated proteins (blue) and upregulated proteins (red) by fold change were highlighted and labeled with protein names. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.4. Gel electrophoresis and in-gel tryptic digestion

Sonified EV samples were size separated on NuPAGe Novex 4–12% Bis-Tris Protein Gels (Life Technologies, Thermo Fisher Scientific, Carlsbad, CA, USA) using one-dimensional gel electrophoresis. This eliminates contaminants and reduces sample complexity to improve peptide identification. Gels were processed with reducing and alkylating agents. Gels were cut prior to in-gel digestion with trypsin followed by peptide extraction as previously described [20, 21]. Extracts were stored at −20 °C until further use.

2.5. Nano-LC-MS/MS and database searching

Extracted peptides were concentrated in a vacuum centrifuge and the volume was adjusted using 4% acetonitrile in 0.5% trifluoro-acetic acid (TFA) acid to achieve equal protein loading based on gel images. Peptides were separated using nanoscale liquid chromatography and analyzed using tandem mass spectrometry (GelC-MS/MS) as previously described [22–24]. Briefly, peptides were separated by an Ultimate 3000 nanoLC-MS/MS system (Dionex LC-Packings, Amsterdam, The Netherlands) equipped with a 40 cm × 75 μm ID fused silica column custom packed with 1.9 μm 120 Å ReproSil Pur C18 aqua (Dr Maisch GMBH, Ammerbuch-Entringen, Germany). After injection, peptides were trapped at 6 μl/min on a 10 mm × 100 μm ID trap column packed with 5 μm 120 Å ReproSil Pur C18 aqua in 0.05% formic acid. Peptides were separated at 300 nl/min in a 10–40% gradient (buffer A: 0.5% acetic acid (Fischer Scientific), buffer B: 80% ACN (Biosolve), 0.5% acetic acid) in 60 min (90 min inject-to-inject) at 35 °C. Eluting peptides were ionized at a potential of +2 kVa into a Q Exactive mass spectrometer (Thermo Fisher, Bremen, Germany). Intact masses were measured at resolution 70.000 (at *m/z* 200) in the orbitrap using an AGC target value of 3E6 charges. The top 10 peptide signals (charge-states 2+ and higher) were submitted to MS/MS in the HCD (higher-energy collision) cell (1.6 amu isolation width, 25% normalized collision energy). MS/MS spectra were acquired at resolution 17.500 (at m/z 200) in the orbitrap using an AGC target value of 1E6 charges, a maxIT of 60 ms and an underfill ratio of 0.1%. Dynamic exclusion was applied with a repeat count of 1 and an exclusion time of 30 s. MS/MS spectra were searched against the Swissprot database (2015–09, 42,122

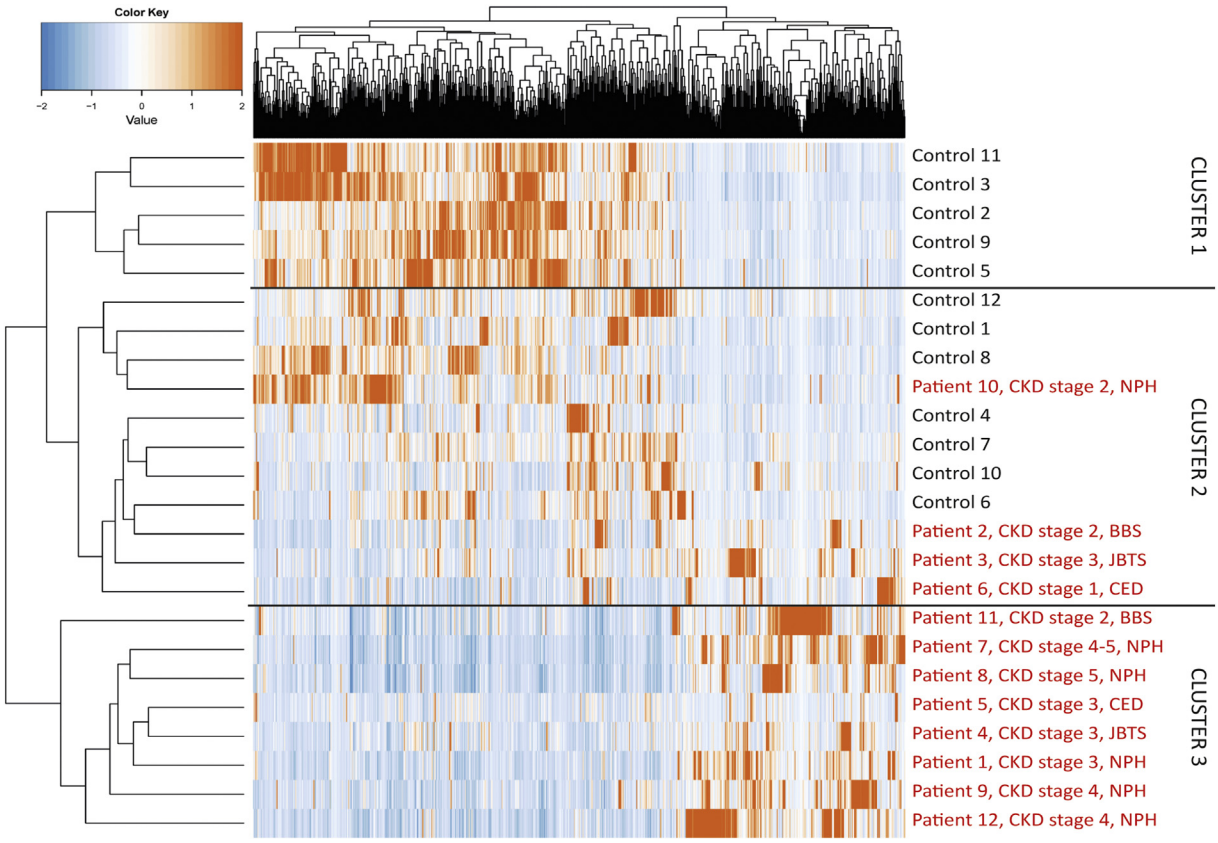

Fig. 3. Supervised hierarchical clustering of normalized protein counts (P < .05) separates NPH-RC patients from controls. Three separate clusters (marked 1,2 and 3) can be discerned. Blue represents downregulated proteins and orange represents upregulated proteins. NPH: isolated nephronophthisis; BBS: Bardet-Biedl syndrome; CED: cranioectodermal dysplasia (Sensenbrenner syndrome); JBTS: Joubert syndrome. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

entries) using MaxQuant software (version 1.5.2.8) [25], with a maximum allowed deviation of 4.5 ppm for the precursor mass and 20 ppm the for fragment masses. Cysteine carboxamidomethylation was treated as fixed modification and *N*-acetylation and methionine oxidation were treated as variable modifications. Identified peptides and proteins were filtered to a false discovery rate of 1% using the target-decoy method [26]. Raw spectral counts of identified protein groups were processed using R for further analysis.

2.6. Data mining and statistical analyses

Spectral counts were normalized using the average of the sum of raw counts across all samples as previously described [27]. Protein expression differences between patients and controls were calculated using the beta-binominal test for unpaired data [28, 29]. The P-values < .05 were considered statistically significant. R package gplots was used to perform hierarchical clustering [30].

Differentially expressed proteins were identified using stringent selection criteria: P < .05, fold change ≥4 for down- and ≥ 3 for upregulated proteins, detected in at least 8 of 12 samples and a mean normalized count in positive samples of ≥2. Human gene symbols corresponding to protein identifiers of differentially expressed proteins were imported into the STRING tool (version 10.0) to examine protein-protein interactions [31]. Interaction networks were annotated using Cytoscape (version 3.4.0) [32]. The PANTHER overrepresentation test in AmiGO 2 (version 2.4.24) was used for GO term enrichment analysis [33, 34]. Bonferroni-corrected P-values < .05 were considered statistically significant. ConsensusPathDB (version 31) was used to analyze enriched biological pathways per annotated gene cluster [35]. Interaction databases that contained > 50% drug-target and biochemical interactions were excluded from pathway enrichment analyses because they were considered irrelevant to the aims of the present study. Further statistical analyses, including correlation analyses of protein expression with CKD stage, were performed using Graph Pad Prism (Version 7.02). Finally, a PubMed literature search was performed for comparison of proteins that were previously associated with CKD with discriminating markers identified in the current study (Supplementary Methods). The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE [36] partner repository with the dataset identifier PXD009784.

3. Results

3.1. Differentially expressed proteins discriminate between NPH-RC patients and controls

Twelve patients with NPH-RC and 12 age-and gender-matched control subjects, age 6 to 25 years, were recruited for urine collection (Table 1). Patients' phenotypes included isolated nephronophthisis (NPH; n = 6), Bardet-Biedl syndrome (BBS; n = 2), Sensenbrenner syndrome (CED; n = 2), and Joubert syndrome (JBTS; n = 2). Urine protein/creatinine ratio was determined in each subject to confirm patient or control status with a normal range of < 20 mg/mmol in individuals over 2 years of age. The mean protein/creatinine ratio was 58.0 ± 64.2 mg/mmol in NPH-RC patients compared to 11.6 ± 8.5 mg/mmol in control individuals. Supplementary Table 1 contains an overview of the sample details.

Proteomic profiles of urinary EVs were determined in each patient

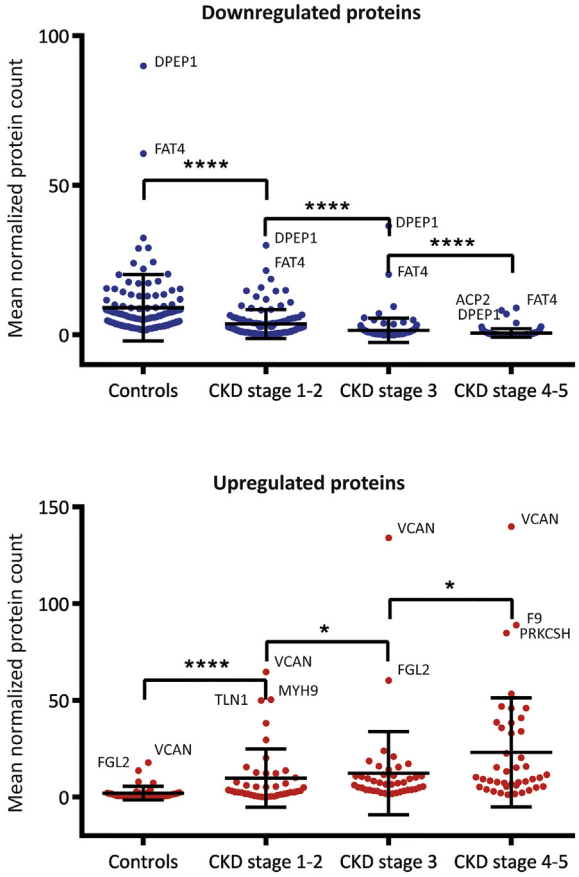

Fig. 4. Differentially expressed proteins relate to chronic kidney disease stage. Mean normalized protein count of downregulated proteins (blue) and upregulated proteins (red) is associated with stage of CKD in NPH-RC patients. Dots represent individual proteins. Lines and error bars show mean and standard deviation, respectively. The most highly abundant proteins are labeled with protein names. *P < .05, ****P < .0001, calculated using Mann-Whitney test. CKD: chronic kidney disease. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

and control individual by using EV protein fractionation by gel electrophoresis in conjunction with nanoLC-MS/MS. In total, 2935 unique EV proteins were identified, of which 2284 proteins were present in both patients and controls (Fig. 1, Supplementary Tables 2 and 3). Nine hundred ten proteins were differentially expressed in NPH-RC patients compared to controls (P < .05), of which 598 proteins were downregulated and 312 proteins were upregulated. Out of 910 statistically significant differentially expressed urinary EV proteins, 156 proteins met stringent criteria for potential biomarker selection (fold change ≥4, detected in at least 8 of 12 samples and a mean normalized count in positive samples of ≥2) (Fig. 2). Of the proteins that met these criteria, 114 proteins were downregulated and 42 proteins were upregulated (Supplementary Table 4).

Supervised hierarchical clustering of normalized protein counts showed a near-complete separation of patient and control samples (Fig. 3). Three separate clusters were discerned: 1) a cluster of 5 control samples, 2) a central cluster of 4 patients (patient 2, 3, 6 and 10) positioned among 7 controls, and 3) a cluster of 8 patient samples. The average CKD stage of patients in cluster 3 comprising 8 patient samples was higher compared to the mean CKD stage of patients in the central cluster (CKD stage 3–4 versus CKD stage 2, respectively), suggesting that proteomic profiles from patients with a more advanced CKD stage cluster together. Patient 10 with a tentative genetic diagnosis (a mutation and a variant of unknown significance (VUS) in *NPHP4*, Supplementary Table 5) clustered in the central cluster among controls. Patients without a molecular diagnosis (patients 1 and 8) clustered together with patients with a molecular diagnosis in the third cluster. In addition, protein-clustering analyses after stratification into different diagnostic subgroups (i.e. isolated nephronophthisis, Bardet-Biedl syndrome, Sensenbrenner syndrome, and Joubert syndrome) showed separation between patients and matched controls except in isolated nephronophthisis, in which patient 10 grouped among controls (Supplementary Fig. 2).

3.2. Expression levels of differentiating proteins are correlated with CKD stage

To assess the relationship between the expression level of discriminating proteins and CKD stage in NPH-RC patients, mean normalized counts of individual proteins were compared between controls and patients with CKD stage 1–2 (n = 4), CKD stage 3 (n = 4) and CKD stage 4–5 (n = 4), respectively. The mean normalized protein counts of down- and upregulated proteins were correlated with CKD stage in NPH-RC patients by showing increasing downregulation or upregulation with advancing disease stage (P < .05) (Fig. 4). The expression levels of the most highly abundant downregulated proteins (DPEP1 and FAT4) and upregulated protein (VCAN) clearly illustrated this correlation (Table 2). These findings indicate enhanced deregulation of proteins within urinary EVs of patients with advanced CKD stage and suggest a prognostic value for the discriminating proteins.

3.3. Differentially expressed proteins show enrichment for signaling pathways and response to tissue damage

Enrichment analysis for gene ontology (GO) terms related to 1) biological processes, 2) cellular components, and 3) molecular functions as well as pathway analysis was performed to gain insight into the functionality of 156 discriminating markers in NPH-RC patients. Sixty-eight GO terms related to biological processes were significantly enriched among 114 downregulated proteins in patients. Top enriched GO terms included multiple signaling pathways, 'regulation of actin cytoskeleton reorganization' and 'regulation of endocytosis' (Fig. 5A). Signaling pathways included 'insulin receptor signaling', 'FC-gamma receptor signaling' and 'small GTPase-mediated signal transduction'. Forty-two out of 156 proteins were upregulated. This number was too small for GO term and pathway analyses. To enable broader GO term and pathway analyses, a less stringent fold change of ≥3 was applied for upregulated proteins, resulting in 70 instead of 42 upregulated proteins. Among 70 upregulated proteins, 31 GO terms related to biological processes were significantly enriched, including 'positive regulation of cell-substrate adhesion', 'extracellular matrix organization' and 'response to wounding'. These results seem to reflect kidney damage in general, present in the NPH-RC patients, rather than ciliopathy-specific processes.

Overrepresented GO terms related to cellular components corresponding to both down- and upregulated proteins included 'extracellular exosome' and 'cytoplasmic vesicle', which are both related to EVs, 'adherens junction' and 'cortical cytoskeleton' (Fig. 5B). Of note, there was a two- to threefold decrease in mean normalized spectral counts of EV markers TSG101 and PDCD6IP in patients compared to controls (P = .0025, calculated using Mann-Whitney test), possibly accounting for the overrepresentation of EV-related GO terms among downregulated proteins. Enriched GO terms related to molecular function showed mainly terms related to enzymatic and binding activity (Fig. 5C).

Pathway analysis was performed using ConsensusPathDB to identify overrepresented pathways among differentially expressed proteins [35]. Two major clusters of highly connected downregulated proteins were identified based on GO term analyses (Supplementary Fig. 3A). Pathway analyses per cluster revealed an enrichment for pathways

Table 2
Most abundant proteins that correlate with stage of CKD.

Gene symbol	Protein name	Fold change	P-value	Protein function[a]	Relation to CKD and/or ciliopathies
FAT4	Protocadherin Fat 4	−4.0	2.9E-08	Role in maintenance of planar cell polarity and inhibition of neuroprogenitor cell proliferation and differentiation	- Disruption of *Fat4* causes cystic kidney disease in mice [51]. - Somatic mutations in *FAT4* have been demonstrated in renal tissue of patients with ADPKD [52].
ACP2	Lysosomal acid phosphatase	−4.0	8.7E-08	Phosphatase	NA
DPEP1	Dipeptidase 1	−4.2	2.6E-08	Involved in renal glutathione metabolism and expected role in regulation of leukotriene response	- Downregulated in urine proteome of FSGS patients compared to controls [50].
SLC26A4	Pendrin	−4.0	1.8E-05	Sodium-independent chloride/iodide transporter	- Increased expression in kidneys of thiazide-sensitive NaCl cotransporter (*Slc12a3*) knockout mice, possibly through a compensatory mechanism [53].
LYN	Tyrosine-protein kinase Lyn	−4.9	1.6E-07	Regulation of immune response and DNA damage reponse	- Disruption of *Lyn* causes Lupus-like kidney disease in mice [54].
VCAN	Versican core protein	5.9	3.4E-10	Expected role in intercellular signaling and cell-substrate adhesion	- Increased mRNA expression of VCAN isoform V0 and V1 in renal tissue is associated with progression of CKD [49]. - Upregulated in peripheral blood mononuclear cells of dialysis patients compared to healthy controls and CKD patients not receiving dialysis [55].
F9	Coagulation factor IX	8.9	8.3E-13	Part of intrinsic pathway of blood coagulation	NA
PRKCSH	Glucosidase 2 subunit beta	7.7	6.0E-11	Involved in glycan metabolism	- Mutations in PRKCSH cause isolated autosomal dominant polycystic liver disease [56], which can be classified as a ciliopathy. - PRKCSH depletion in HEK293T cells and H69 cholangiocytes results in defective ciliogenesis [57].
MYH9	Myosin-9	4.0	3.1E-07	Role in cytokinesis, cytoskeleton reorganization and focal contacts formation during cell spreading	- Common variants in MYH9 are associated with increased risk of CKD in various populations [58,59].
TNC	Tenascin	6.6	3.9E-08	Role in neuron migration and regeneration, and synaptic plasticity	NA

Top most abundant downregulated and upregulated proteins in patients with CKD stage 4–5. The abundance of these proteins is associated with stage of CKD in nephronophthisis patients. CKD: chronic kidney disease; ADPKD: autosomal dominant polycystic kidney disease; FSGS: focal segmental glomerulosclerosis.

[a] UniProt [60].

related to 'signaling', 'transport' and 'phagocytosis' (with overlapping genes involved), 'endocytosis' and 'adherens junctions' among downregulated proteins in both clusters (Supplementary Fig. 3B). In the subgroup of patients with isolated NPH (n = 6), pathways enriched among downregulated proteins included specific cilia-associated pathways such as 'cargo trafficking to the periciliary membrane' (P = .002) and the 'polycystic kidney disease pathway' (P = .004) (data not shown). Significantly enriched pathways among three clusters of upregulated proteins comprised 'signaling', 'extracellular matrix organization', and 'coagulation and complement cascades' (Supplementary Fig. 3C and D). These findings from pathway analyses, including overrepresentation of 'signaling' and 'endocytosis' pathways among downregulated proteins and 'extracellular matrix organization' pathways among upregulated proteins, were consistent with down- and upregulated GO terms.

3.4. Candidate biomarkers for nephronophthisis

The top five most discriminating downregulated proteins (PELI2, HRAS, VPS9D1, CPNE5, and SIRPA) and upregulated proteins (BPGM, CANX, PRL, CDH5, and ART3) based on fold change included two proteins (CANX and PRL) that have previously been reported to be upregulated in plasma and urine of patients with kidney diseases (Fig. 6, Table 3) [37, 38]. The other eight most discriminating proteins have not previously been described in the context of CKD and are potential novel biomarkers for ciliopathy-related CKD. NGAL, which has previously been described as a biomarker for kidney injury and was reported to be elevated in urine of ciliopathy patients [39], was moderately upregulated in patients (fold change 2.4, P < .05).

To assess the occurrence of cilia-associated proteins among potential biomarkers for NPH-RH, we used the SysCilia Gold Standard (Version 1) list of 303 confirmed ciliary genes (Supplementary Table 6) [40]. Five out of 156 differentially expressed proteins corresponded to known ciliary genes. Four proteins (PKD1, ARL13B, SMO, and RP2; Supplementary Fig. 4A-D) were downregulated (average fold changes 9.9, 6.8, 4.5, and 4.1 respectively; P < .05) and one protein (FLNA) was upregulated (fold change 4.0) in urinary EVs of NPH-RC patients (Supplementary Fig. 4E). Ciliopathy genes that were mutated in the NPH-RC patients (Supplementary Table 5) were not represented in the downregulated ciliary proteins, suggesting that gene mutations globally affect ciliary pathways and are not restricted to a narrow effect.

A PubMed literature search was performed to list proteins expressed in whole urine that were previously associated with CKD (Supplementary Methods and Supplementary Table 7). Comparison of 70 CKD-associated proteins acquired from the literature with the 156 discriminating markers in our NPH-RC cohort showed that 3/156 proteins correspond to known CKD-associated urinary proteins. Of these, two proteins (MMP7 and PLTP) were upregulated in NPH-RC patients described in the current study, corresponding to the direction of fold change described in literature [41, 42]. One downregulated protein in NPH-RC patients (ACE2) showed a different direction of fold change in urinary EVs compared to the direction of fold change described in literature [43]. This could be the result of the different compartment that was analyzed, namely urinary EVs instead of whole urine.

4. Discussion

Here we report that protein expression profiles in urinary EVs can distinguish NPH-RC patients (n = 12) from healthy controls (n = 12) that were analyzed as individual samples. By using an in-depth proteomics analysis using 140 nanoLC-MS/MS runs on a high resolution fast scanning tandem mass spectrometer, combined with statistical and quantitative filtering, we identified 156 promising candidate protein biomarkers. Importantly, expression levels of the most discriminating proteins were associated with CKD stage in patients, suggesting a relation to disease-state and a potential prognostic value. Thus, urinary

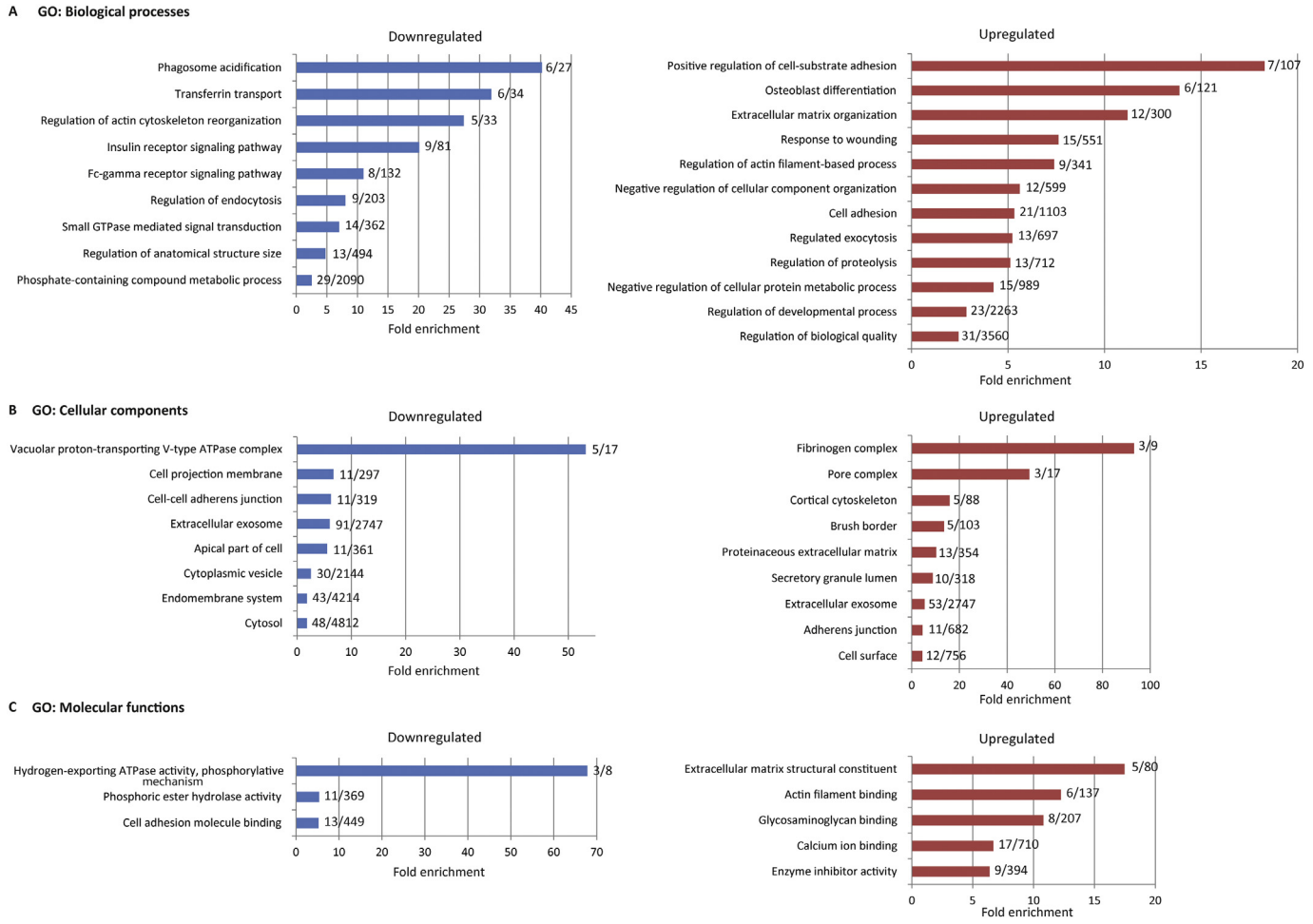

Fig. 5. Results from gene ontology (GO) term enrichment analysis of differentially expressed proteins in urinary extracellular vesicles (EVs) from NPH-RC patients. GO terms related to A) overrepresented biological processes, B) cellular components, and C) molecular functions were significantly associated with differentially expressed proteins in NPH-RC patients compared to controls by fold enrichment ($P < .05$). Fraction represents the number of genes detected/number of reference genes per GO term.

EVs form a promising non-invasive source of biomarkers for early detection and prognostics of nephronophthisis. Further research is needed to determine the specificity of these candidate biomarkers for NPH-RC.

Underscoring the validity of our systematic and unbiased interrogation of differentially expressed proteins in biofluid of NPH-RC patients, we found a significant upregulation of neutrophil gelatinase-associated lipocalin (NGAL) protein in NPH-RC patients. NGAL is the only biomarker for kidney injury in general that was previously identified in NPH-RC patients. An elevation of serum and whole urine NGAL protein levels has been reported in three patients with nephronophthisis-related Joubert syndrome [39]. This makes NGAL an interesting biomarker for further investigation.

GO terms and pathways enriched among proteins downregulated in NPH-RC patients were related to signaling, actin cytoskeleton reorganization and endocytosis. This could reflect a role for these proteins in cilia formation and in ciliary signaling pathways including the mTOR signaling pathway that was enriched among downregulated proteins. Alternatively, these GO terms and pathways could reflect a role in the (EV-mediated) response to kidney damage in general, similar to enriched GO terms and pathways among upregulated proteins, which were associated with extracellular matrix organization and response to wounding. Consequently, it is unclear whether the overrepresented pathways identified in this study point towards ciliopathy-specific disease mechanisms. Although cilia-associated pathways were overrepresented among downregulated proteins in patients with isolated nephronophthisis, including 'cargo trafficking to the periciliary membrane' and the 'polycystic kidney disease pathway', the limited number of patients per NPH-RC subgroup precludes firm conclusions about differences in pathogenesis of various NPH-RC syndromes.

The 114 discriminating downregulated proteins in urinary EVs of patients included cilia-associated proteins ARL13B, PKD1, RP2 and SMO. In addition, the top 20 downregulated proteins included the recently validated ciliary protein serine/threonine-protein kinase 11 (STK11) [44, 45]. Previous studies in polycystic kidney disease (PKD) patients have demonstrated significantly different expression levels of cilia-specific membrane proteins in urine EVs of patients compared to controls, supporting our finding that ciliary proteins are present in urine EVs and that their abundance can reflect disease state [46, 47]. Although the 156 discriminating markers could contain novel ciliary proteins, these proteins are also in part associated with CKD in general. As a result, the single cilia-associated proteins discussed above could be more specific biomarkers for nephronophthisis than the discriminating set of 156 proteins.

Two of the top five most discriminating upregulated proteins, calnexin (CANX) and prolactin (PRL), have previously been implicated in kidney diseases. CANX is a calcium-binding protein involved in the retention of incorrectly folded proteins within the endoplasmatic reticulum. CANX showed higher expression levels in plasma of renal cell carcinoma patients compared to controls [37]. A study in patients with diabetic nephropathy demonstrated increased levels of PRL in urine of

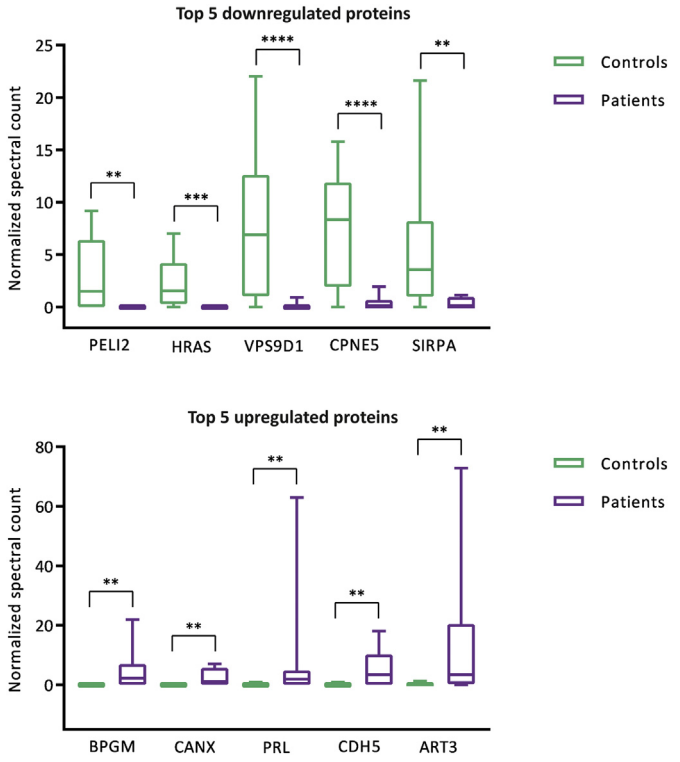

Fig. 6. Box plots of top 5 most differentially expressed proteins in patients and controls based on normalized spectral counts. Lines and whiskers show median, minimum and maximum values. **P < .01, ***P < .001, ****P < .0001, calculated using Mann-Whitney test.

patients compared to controls [38]. Our study corroborates that CANX and PRL are CKD-associated markers and identifies additional discriminating proteins including ART3 and CDH5 as candidate biomarkers for (ciliopathy-related) CKD. These highly differentially expressed proteins should be investigated further in larger NPH-RC patient cohorts.

The upregulated proteins that showed a strong correlation with CKD stage included Versican core protein (VCAN) (Fig. 4). VCAN is an extracellular matrix proteoglycan with normally low expression in kidneys [48]. Higher mRNA expression of VCAN in renal biopsies was reported to be correlated to histologic damage and progression of CKD [49]. By contrast, Dipeptidase 1 (DPEP1) and Protocadherin Fat 4 (FAT4) were decreased in urinary EVs of patients. DPEP1 is a kidney-specific protein that is involved in the metabolism of several proteins by dipeptide hydrolysis. In line with our findings, DPEP1 was found to be downregulated in the urine of focal segmental glomerulosclerosis (FSGS) patients compared to controls [50]. FAT4 is a planar cell polarity protein that localizes to cilia in kidney cells [51]. Importantly, loss of *Fat4* causes cystic kidney disease in mice [51]. The connection to cilia and cystic kidney disease makes FAT4 an interesting candidate biomarker for nephronophthisis. In addition, although FAT4 is not among the top five most discriminating markers based on fold change, its association with disease severity indicates potential prognostic value.

This study has several limitations. First, we did not investigate the specificity of the differentially expressed proteins. Therefore, comparison with a cohort of patients with early-onset CKD due to other causes is required to discern whether our top identified proteins are specific for NPH-RC or reflect CKD in general. Second, the identified markers need to be validated in larger patient cohorts in order to be able to stratify for disease subgroups. Third, longitudinal follow-up of individuals will establish whether urinary EV protein signatures can accurately predict

Table 3
Top differentially regulated proteins.

Gene symbol	Protein name	Fold change	P-value	Protein function[a]	Relation to CKD and/or ciliopathies
PELI2	E3 ubiquitin-protein ligase pellino homolog 2	∞	1.0E-06	Role in TLR and IL-1 signaling pathways	NA
HRAS	GTPase HRas	∞	1.9E-06	Activation of Ras signal transduction	NA
VPS9D1	VPS9 domain-containing protein 1	−44.3	3.2E-11	GTPase activation	NA
CPNE5	Copine-5	−17.1	1.5E-10	Expected role in calcium-mediated intracellular processes	NA
SIRPA	Tyrosine-protein phosphatase non-receptor type substrate 1	−15.2	8.4E-08	Immunoglobulin-like cell surface receptor for CD47, involved in neuron development and expected role in synaptic function	NA
BPGM	Bisphosphoglycerate mutase	∞	1.0E-07	Regulation of hemoglobin oxygen affinity	NA
CANX	Calnexin	∞	7.1E-06	Expected role in retention of incorrectly folded proteins in endoplasmatic reticulum	- Upregulated in plasma of patients with renal cell carcinoma compared to controls [37]. - Previousstudies focussed on role of CANX as a tumor biomarker in various types of cancer [61,62].
PRL	Prolactin	33.2	1.3E-07	Stimulates lactation	- Elevated in urine of patients with diabetes mellitus and impaired renal function compared to controls [38]. - Elevated in serum op patients with end-stage renal disease compared to controls [63].
CDH5	Cadherin-5	18.0	2.0E-08	Expected role in cohesion and intercellular junctions	NA
ART3	Ecto-ADP-ribosyltransferase 3	17.4	3.8E-10	Ribosyltransferase	NA

Top 5 downregulated proteins and top 5 upregulated proteins in urinary extracellular vesicles from patients with nephronophthisis-related ciliopathy compared to matched controls.
CKD: chronic kidney disease. ∞ Protein uniquely present in controls (blue) or patients (red).
[a] UniProt [60].

disease onset in individuals with biallelic NPH-mutations and serve as prognostic markers.

5. Conclusions

To our knowledge this is the first report on an in-depth clinical proteomics dataset of global urinary EVs proteomics in well-characterized NPH-RC patients. Our data show that measuring protein profiles in urinary EVs is a promising approach for non-invasive early diagnostics and prognostics in patients with NPH-RC. Since the current diagnostics and prognostics toolbox for nephronophthisis is far from complete, early biomarkers indicative of disease will be of great benefit to NPH-RC patients and their relatives.

Disclosure

All authors declared no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jprot.2018.07.008.

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