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URINARY EXTRACELLULAR VESICLES: BIOMARKERS AND BEYOND

Urine extracellulaire vesikels: biomarkers en meer

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CONTENTS

ABBREVIATIONS

ADPKD	Autosomal dominant polycystic kidney disease
AKI	Acute kidney injury
AQP2	Aquaporin-2
CKD	Chronic kidney disease
CS	Cushing's syndrome
eGFR	Estimated glomerular filtration rate
ELISA	Enzyme-linked immunosorbent assay
ENaC	Epithelial sodium channel
EV	Extracellular vesicle
HtTKV	Height adjusted total kidney volume
KIM-1	Kidney injury molecule 1
NCC	Sodium chloride cotransporter
NGAL	Neutrophil gelatinase-associated lipocalin
NHE3	Sodium hydrogen exchanger type 3
NKCC2	Sodium potassium chloride cotransporter
pNCC	phosphorylated sodium chloride cotransporter
RAAS	Renin-angiotensin-aldosterone system
uEVs	Urinary extracellular vesicles

CHAPTER

01

GENERAL INTRODUCTION AND AIMS OF THE THESIS
INTRODUCTION

Extracellular vesicles

Extracellular vesicles (EVs) are nanosized vesicles released by all cells. EVs can be further classified in exosomes, microvesicles and apoptotic bodies (**Figure 1**) (1, 2). Although EVs have been discovered more than three decades ago (3), EVs were initially considered cellular waste (4, 5). Only recently their diverse roles in biology have been uncovered. One of these functions is to transfer information from one cell to another, thereby altering recipient cell behavior (1, 4). This information transfer is mediated by lipids, proteins, nucleic acids and sugars contained in EVs (1).

Among EVs, there has been special interest in exosomes, because they are thought to be specifically packaged with cytosolic proteins and RNA (6). Commonly used exosome markers include proteins involved in their formation, such as tetraspanins (CD9, CD63, CD81), intracellular membrane transporters (flotillin, annexin) and multivesicular body proteins (ALIX, TSG-101) (7). Pivotal biological roles have now been ascribed to EVs, including in immunity, reproductive system, bone calcification and kidney injury repair (1).

Urinary extracellular vesicles

For centuries, the analysis of urine has been an essential part of the diagnostic evaluation in clinical medicine and changes in urinary composition can frequently be linked to disease. Urine has therefore been extensively studied as a source of biomarkers for renal and urological disease. This search has been facilitated by technological progress in protein mass spectrometry and RNA-sequencing (8, 9). Urine, however, is a complex biofluid, because it contains proteins, such as Tamm-Horsfall protein, which reduce the detection sensitivity for low-abundant proteins present in urinary EVs (uEVs) (9, 10). The characterization of uEVs has provided a novel approach to urinary biomarker discovery, because uEVs appear to reflect renal epithelial cell status and contain various disease-associated proteins and RNA (11-13).

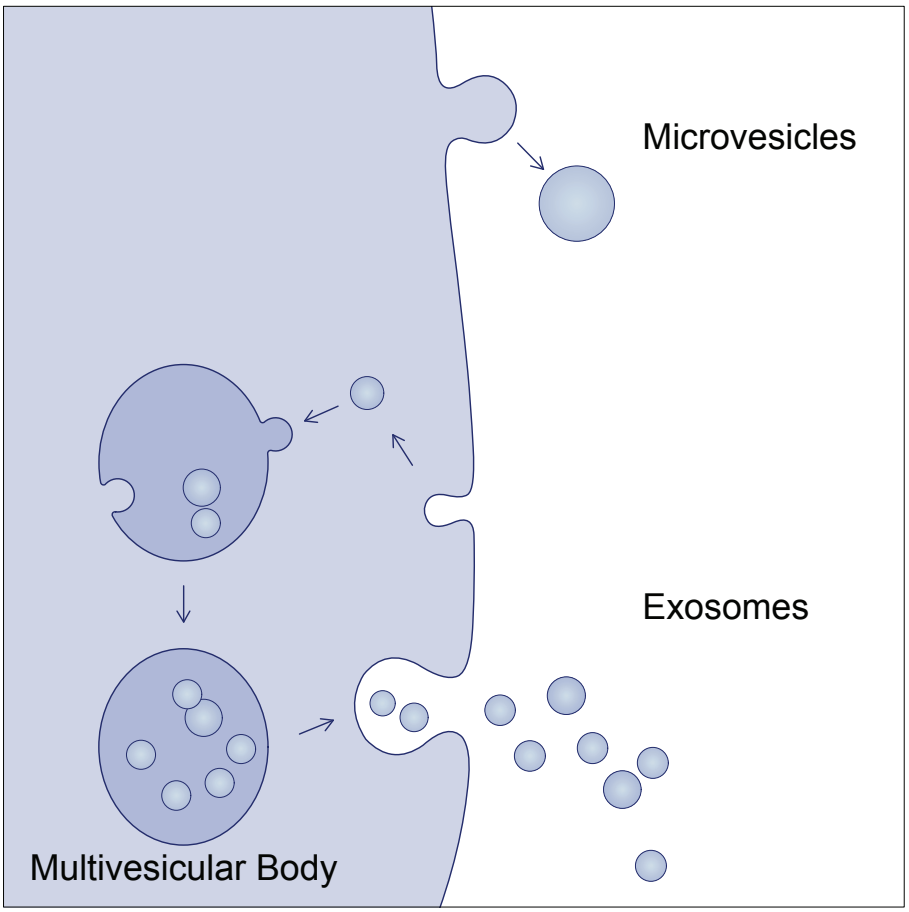

Figure 1: Formation of microvesicles and exosomes.
A cell is depicted, with microvesicles being formed by outward budding from the plasma membrane. Exosomes are formed by endocytosis, fusion with and formation within multivesicular bodies, and finally release of multivesicular body content into extracellular space.

Content, function and isolation of uEVs

It is estimated that in healthy subjects, approximately 3% of total urinary protein is contained in uEVs (7). Proteomic analysis of uEVs has shown that they contain more than 1600 membrane bound and cytosolic proteins from all segments of the kidney and the urinary tract (14). Although most studies have focused on the use of uEVs as biomarkers, other functions include paracrine effects between nephron segments, and a defense mechanism against urinary pathogens (15-17). Several techniques have been used to isolate uEVs,

including immuno-isolation, filtration, precipitation and ultracentrifugation (11). This latter technique remains the gold standard, but ultracentrifugation is a time-consuming and laborious technique that also requires a large sample volume. Potential biomarkers in uEVs have been identified for different renal disorders (18, 19). These include acute kidney injury, glomerular disease, renal tubular disorders, salt-sensitive hypertension, and autosomal dominant polycystic kidney disease (ADPKD). In this thesis, we studied salt-sensitive hypertension and ADPKD because urinary markers for both conditions are needed.

Salt-sensitive hypertension

Dietary sodium intake is linked to blood pressure, with a pivotal regulatory role of sodium excretion by the kidney (20-22). Many hypertensive disorders are due to increased renal sodium reabsorption (23). The factors contributing to primary hypertension include age, body weight and salt intake, whereas secondary forms of hypertension are caused by a specific etiology (24). These include activation of renal sodium transporters due to single gene mutations or through hormone-excess, as occurs in primary aldosteronism or Cushing's syndrome (CS) (21). Clinical and biochemical clues may indicate increased activity of renal sodium transporters. However, analyzing them directly was so far not possible in humans without a kidney biopsy. With the discovery of renal sodium transporter in uEVs, measuring their abundances and post-translational modifications is now possible and being investigated to further differentiate the cause of hypertension and possibly personalize treatment (12, 14, 25-28).

Autosomal dominant polycystic kidney disease

Autosomal dominant polycystic kidney disease (ADPKD) is the most common inherited kidney disease, affecting approximately 3 per 10.000 subjects in the general population (29). In the majority of cases it is caused by a mutation in one of the genes encoding for polycystin 1 or 2 (*PKD1* or *PKD2*), resulting in progressive bilateral cyst formation, disruption of normal renal parenchyma, and ultimately renal failure in approximately 70% of patients (30-34). With

emerging treatment options for ADPKD, such as the recent approval of the vasopressin V2 receptor antagonist tolvaptan, identification of patients at high risk for rapid disease progression has become increasingly important (35, 36). **Table 1** shows the markers that are associated with disease progression in ADPKD. Among other factors, the causative gene mutation is the major determinant of the rate of progression, with a median age of onset of renal failure of 54 years for *PKD1* and 74 years for *PKD2* (34, 37, 38). However, family members who share the same mutation have variable courses of disease progression, possibly related to somatic mosaicism (34, 39). The potential of uEVs as a source of biomarkers for ADPKD has recently been demonstrated, as they are enriched for polycystins and also interact with primary cilia, which are involved in the pathophysiology of ADPKD (40, 41). The ratio of polycystin proteins to transmembrane protein 2 in uEVs correlated with total kidney volume, and could thus be used to monitor ADPKD (40).

Category	Marker	Reference
Imaging	High total kidney volume*	(42)
	Low renal blood flow	(43, 44)
Genetic	*PKD1* disease, in particular *PKD1* truncating mutations*	(34, 45)
Clinical	Early onset of hypertension*	(46, 47)
	Gross hematuria*	(46, 48)
	Early decrease in GFR*	(42, 49)
Biomarker	Serum: copeptin, uric acid	(50-52)
	Urine: NGAL, KIM-1, overt proteinuria, uEVs	(40, 53-55)

Table 1: *Markers of disease progression in ADPKD (adapted from (36, 56)). *Best validated markers. KIM-1, kidney injury molecule 1; NGAL, neutrophil gelatinase-associated lipocalin; uEVs, urinary extracellular vesicles*

Disease progression is also influenced by total kidney volume, proteinuria and hypertension (46, 48, 57). Early development of hypertension in ADPKD is common and the mechanism likely multifactorial (58). Although salt-sensitivity suggests activation of the circulating renin-angiotensin system, no evidence has been provided in support of this hypothesis (59-61). Locally produced or

filtered renin-angiotensin components may activate the intra-renal renin-angiotensin system, thereby contributing to hypertension (62). Indeed, previous studies show a higher urinary angiotensinogen level in ADPKD (63, 64). Targeting the intrarenal renin-angiotensin system reduced cyst growth in an animal model of ADPKD (65). Taken together, the intra-renal renin-angiotensin system in ADPKD may have a prominent contributing role in hypertension and disease progression.

AIMS OF THE THESIS

1. To explore the role of uEVs in renal physiology and pathophysiology, and their potential for biomarker discovery
2. To develop a novel technique to isolate, quantify, and characterize uEV proteins
3. To test the hypothesis that renal sodium transporters in uEVs correlate with salt-sensitive hypertensive disorders
4. To identify novel biomarkers for ADPKD in uEVs using proteomics
5. To test the hypothesis that increased urinary excretion of components of the renin-angiotensin system is a unique characteristic of ADPKD

This thesis is divided into three sections with uEVs as the common denominator.

Section 1 provides insight into the function of uEVs, their isolation, and characterization. In *Chapter 2*, the different roles of uEVs in nephrology are reviewed. This chapter also deals with the methodological issues of isolating and characterizing uEVs. In *Chapter 3* a novel method to isolate, quantify, and characterize uEVs is presented.

Section 2 assesses the role of uEVs as markers for salt-sensitive hypertension. *Chapter 4* provides an overview of the use of uEVs as an approach to assess renal sodium transport. In *Chapter 5-7*, uEVs are studied in salt-sensitive hypertensive disorders, including CS (*Chapter 5*), primary aldosteronism (*Chapter 6*) and Liddle syndrome (*Chapter 7*). Furthermore, a novel disease-causing mutation of Liddle syndrome is presented in *Chapter 7*.

In ***Section 3***, markers for ADPKD, including uEVs are studied. In *Chapter 8* we set out to discover novel biomarkers in uEVs of patients with ADPKD, using proteomics. In *Chapter 9*, we compared urinary markers of the renin-angiotensin system in ADPKD and chronic kidney disease.

The *appendix* includes the methods of the DIPAK1 study, a cohort used for the studies in ***Section 3***.

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SECTION

01

URINARY EXTRACELLULAR VESICLES: POTENTIAL FOR NEPHROLOGY & NOVEL ISOLATION METHOD

CHAPTER

02

URINARY EXTRACELLULAR VESICLES IN THE KIDNEY: BIOMARKERS AND BEYOND

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Am J Physiol Renal Physiol. 2014; 306 (11): F1251-F1259

ABSTRACT

Extracellular vesicles have been isolated in various body fluids including urine. The cargo of urinary extracellular vesicles (uEVs) is composed of proteins and nucleic acids reflecting the physiological and possibly the pathophysiological state of cells lining the nephron. Because urine is a non-invasive and readily available biofluid, the discovery of uEVs has opened a new field of biomarker research. Their potential use as diagnostic, prognostic, or therapeutic biomarkers for various kidney diseases, including glomerulonephritis, acute kidney injury, tubular disorders and polycystic kidney disease is currently being explored. Some challenges, however, remain. These challenges include the need to standardize isolation methods, normalization between samples, and validation of candidate biomarkers. Also, the development of a high-throughput platform to isolate and analyze uEVs, for example an enzyme-linked immunosorbent assay, is desirable. Here, we review recent studies on uEVs dealing with kidney physiology and pathophysiology. Furthermore, we discuss new and exciting developments regarding vesicles, including their role in cell-to-cell communication and the possibility to use vesicles as therapy for kidney disorders.

INTRODUCTION

Urine contains vesicles derived from the kidney and the urinary tract. Vesicles may be released into the pre-urine by direct shedding or budding from the plasma membrane, or through the fusion of intracellular multivesicular bodieswith the plasma membrane (1). The latter resembles a process of exocytosis, and the vesicles formed in this way are often called "exosomes" (2). Exosomes are vesicles with a lipid bilayer membrane that are 30 to 120 nanometer in size and flote at a density of 1.15 to 1.19 g/ml in continuous sucrose gradient (3). Although these characteristics are ascribed to exosomes, other vesicles are usually co-purified. This was illustrated by one study that used immune-electron microscopy to demonstrate that podocalyxin-positive vesicles differ from exosomes in their biogenesis and may be used as a biomarker for podocyte injury (4). Because strict separation between the different vesicle populations in urine has not been established, we will use the term urinary extracellular vesicles (uEVs) throughout this review (5-7). **Figure 1** shows uEVs as visualized by electron microscopy. Proteomic analysis has shown that uEVs contain proteins from glomerular, tubular, prostate, and bladder cells (2, 8). Two online databases showing the proteins that have thus far been identified in uEVs are publically accessible at dir.nhlbi.nih/gov/papers/lkem/exosome and exocarta.org. Commonly identified proteins in uEVs are tetraspanins, ALIX, and TSG-101, which are now commonly used as uEV markers (9, 10). In addition to proteins, uEVs also contain nucleic acids (11). Although mRNA profiles from whole cells in urine are being studied as disease markers (12), the use of mRNA in uEVs may have specific advantages. Namely, the RNA integrity profile in uEVs was similar to that of kidney tissue and was better preserved than RNA in whole urine, because the membrane protects against RNase (11, 13). Of interest, uEVs also contain miRNA, which are small non-coding RNAs that regulate mRNA processing (13). It appears that small RNAs including miRNAs make up the majority of RNA species contained in uEVs (13). In addition to analyzing proteins, mRNA or miRNA in uEVs, analysis of post-translational modification

of plasma membrane proteins in uEVs may be another method for biomarker discovery, as was recently illustrated for polycystic kidney disease (5). Our aim in this review is to summarize the techniques used to isolate and characterize uEVs and to discuss the kidney disorders in which uEVs have been used for biomarker discovery.

ISOLATION OF URINARY EXTRACELLULAR VESICLES

Methods used to isolate EVs from biofluids and cell culture media include ultracentrifugation, filtration, precipitation and immunoisolation (**Table 1**) (6, 14). Because storage and the use of reducing agents and detergents are related to the isolation procedure, we start this section with a brief discussion on these topics.

Storage

For large biomarker studies, optimal storage conditions of urine samples are essential to prevent proteolysis. The addition of protease inhibitors has been shown to prevent degradation of uEVs (10, 15). In addition, storage at -80 ºC, compared to +4 or -20 ºC seemed better to prevent degradation, although freshly processed urine is most favorable (15). Importantly, it seems that degradation of uEVs could occur within 2 hours of urine collection (15). It is therefore recommended that urine samples are stored immediately after addition of a protease inhibitor. uEVs can be recovered from urine up to 7 months after freezing (10). In our hands, uEVs could be recovered from urine samples that had been stored even longer, although it is not known if more degradation had occurred in these samples (unpublished observation).

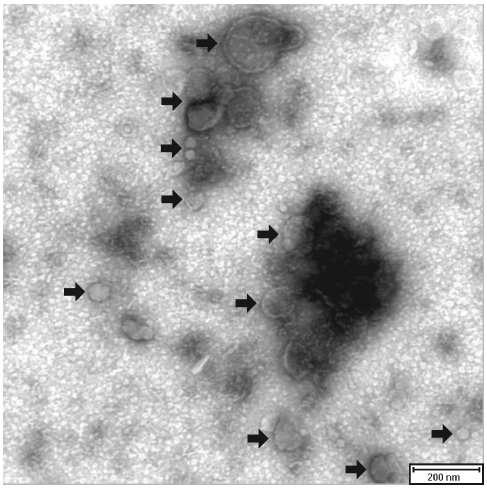

Figure 1: Electron Microscopy Image of uEVs From Rat Urine Isolated With Ultracentrifugation*. 5 µL of uEVs were spotted onto a Formvar-coated grid (200 mesh) and negatively stained using 4% uranylacetate. Samples were examined by a Philips CM100 electron microscope at 80 kV. Arrows indicate uEVs, ranging in size between 30 and 200 nm.*

Use of Reducing Agents and Detergents

Urine frozen at -20 or -70°C forms calcium-containing precipitates after thawing, mainly composed of calcium oxalate and amorphous calcium crystals, giving it a cloudy appearance (16). This precipitate co-sediments proteins such as Tamm Horsfall protein (THP, also called uromodulin) at low-speed centrifugation (16). THP is a high-molecular-weight polymeric glycoprotein secreted by the epithelial cells lining the thick ascending limb of the loop of Henle. Under physiological conditions, THP is the most abundant protein in urine, excreted at a rate of about 20 to 100 mg/day (17). THP is capable of entrapping uEVs by forming polymers that precipitate at low-speed centrifugation (relative centrifugal force 17,000 x g), leading to a reduced yield of isolated uEVs in the final ultracentrifugation pellet (9). Because THP is released by the thick ascending limb of the loop of Henle, uEVs from this nephron segment may be more likely to be entrapped. Vigorous vortexing after a complete thaw of samples can redissolve the calcium-precipitates (16), thereby reducing EV entrapment in THP (10). Further increase of the uEV yield can be achieved by adding dithiothreitol (DTT), a strong reducing agent capable of

disrupting the zona pellucida disulphide bonds of THP (9). DTT will release entrapped uEVs from the low-speed pellet (17,000 x g) into the high-speed pellet (200,000 x g). Of note, CD9 positive uEVs seem to escape the low-speed entrapment (9).

CHAPS (cholamidopropyldimethylammoniopropanesulphonate) is a detergent that can solubilize THP aggregates, but its use is more time-consuming. A possible advantage of using CHAPS instead of DTT is the preservation of uEV associated protease activities such as dipeptidyl peptidase IV (18), a potential biomarker for diabetes mellitus (19).

Ultracentrifugation

Ultracentrifugation is still the most commonly used method to isolate EVs from biofluids. Prior to actual ultracentrifugation, most protocols use one or more centrifugation steps to eliminate whole cells, large vesicles, and debris. Ultracentrifugation is then performed, usually at 100,000 to 200,000 x g for at least one hour (3, 14). Of note, ultracentrifugation does not isolate all uEVs, because 40% of uEVs could still be isolated from the supernatant using ammonium sulphate precipitation (20). This is in line with earlier reports from cell lines, in which ultracentrifugation recovered only 5 to 25% of total EVs (21). Although the loss of up to 40% of uEVs during ultracentrifugation seems problematic, it has yet to be shown that unique biomarkers are present in this unisolated fraction (22). Because the buoyancy of vesicles depends on the density of urine, adjusting this density may increase the uEV yield with ultracentrifugation.

Sucrose Gradient Ultracentrifugation

While DTT and CHAPS increase the uEV yield, THP fragments remain present in the final pellet, leading to a reduced purity of isolated vesicles. To increase this purity, several flotation methods have been proposed, using single (3), double (23) and gradient (24-27) sucrose in heavy water giving rise to different populations of uEVs. One study used sucrose gradient ultracentrifugation and identified three fractions of different densities characterized by the presence of

podocin and podocalyxin (glomerular origin), polycystin proteins and aquaporin-1 (from renal tubular epithelia esp. proximal tubules), and aquaporin-2 (collecting duct origin) (26). While these methods enrich the final pellet of uEVs, they are time consuming (up to 24 hours) and require larger starting volumes of about 25 mL, compared to 5 mL of urine for other ultracentrifugation protocols (**Table 1**). This may result in a lower yield of uEVs, even less than 1% compared to the crude pellets (24).

Ultracentrifugation With Size Exclusion Chromatography

Special handling is required for isolation of EVs from urine with heavy or nephrotic-range proteinuria. Albumin and other proteins tend to be retained in the final pellet, thereby limiting the detection of uEVs. Ultracentrifugation followed by size exclusion chromatography results in a high (>670 kDa) and low (10-670 kDa) molecular weight fraction, containing uEVs and high abundant interfering proteins, respectively (29). This method resulted in higher EV purity compared to nanomembrane ultrafiltration or standard ultracentrifugation methods, with and without DTT (29). Although ultracentrifugation with size exclusion chromatography is considered the technique of choice for proteinuric urine, it was recently shown that the sucrose gradient ultracentrifugation method also reliably and reproducibly removed interfering proteins (27).

Filtration

Attempts to isolate uEVs using filtration-based methods, omitting ultracentrifugation, have shown discrepant results. This may be due to the fact that protein quickly accumulates on the filters blocking further flow. One example of a commercially available filter is a nanomembrane concentrator with 13 nm pore size, which requires only 0.5 mL of urine as starting volume. While some proteins, such as annexin V, podocalyxin and neuron specific enolase do not adhere to the membrane and are easily recovered, other proteins are more adherent, such as aquaporin-2 and TSG-101 (31). Compared to other isolation methods, the purity of recovered protein in uEVs remains low (25)

and this method therefore seems less suitable for nephrotic urine (29) and to isolate RNA from uEVs (25). Hydrophilized polyvinylidene difluoride microfilters, which have a 100 nm pore size, have been successfully used to isolate uEVs (30). A limiting factor for high-throughput application of these microfilters is the need for a stirred cell apparatus (a positive pressure filtration based concentrating device). Incorporation of the microfilter into a commercial spin filtration device may alleviate this limitation.

Isolation principle	Technique	Minimum starting volume (mL)	Isolation time (hours)	Suitable for RNA	Advantage	Disadvantage	Ref
Ultracentrifugation	UC	5	1 – 2	Yes	High yield	THP entrapment	*(2, 3, 10, 28)*
	UC with reducing agent or detergent	5	1.5 – 2.5	Yes	Very high yield	Loss of functionality	*(9, 18, 25)*
	Sucrose gradient UC	25	2.5 – 24	Yes	High purity	Low yield, time consuming	*(3, 23-27)*
	UC with size exclusion chromatography	45	Unknown	Unknown	Suitable for proteinuria	Need for HPLC	*(29)*
Filtration	Microfiltration	12.5	Unknown	Unknown	No need for UC	Low-throughput	*(30)*
	Nanofiltration	0.5	5	No	No need for UC	Unsuitable for proteinuria	*(25, 29, 31)*
Precipitation	ExoQuick TC	5	12*	No	No need for UC	Not suitable for urine	*(25)*
	Modified ExoQuick TC	5	13*	Yes	No need for UC	Unknown reagent	*(25)*
Immunoisolation	ELISA	0.05	5.5	No	High throughput	Selection of subpopulation	*(19, 32)*

Table 1: Methods for Isolation of uEVs.
*ELISA, enzyme-linked immunosorbent assay; HPLC, high-performance liquid chromatography; THP, Tamm-Horsfall protein; UC, ultracentrifugation; * including overnight incubation*

Precipitation

ExoQuick™ is a commercially available precipitation kit to isolate microvesicles (System Biosciences, Mountain View, CA, USA). For urine, its current protocol results in a very low yield of both protein and RNA (25). Modification of the sample work-up resulted in the highest quantities of mRNA and miRNA and an acceptable protein yield compared to the ultracentrifugation methods (25). This method is relatively easy, omits the need for ultracentrifugation and uses less sample material (5 mL urine). However, the overnight incubation step limits its use for immediate diagnostic use, making it less suitable for disorders such as acute kidney injury.

Immuno-isolation

For clinical applications, it is desirable to be able to directly isolate vesicles from urine without the need for ultracentrifugation. To this end, immuno-isolation methods may be especially attractive, as has been successfully performed for the water channel aquaporin-2 (33). An enzyme linked immunosorbent assay (ELISA) or a protein microarray, in which uEV cargo can be detected in a specific and high-throughput manner, is desirable. At present, two studies used ELISA with different capture antibodies, including AD-1 (a potential uEV marker) (19) and the sodium chloride cotransporter (32). In the first study whole urine was directly added to the wells of an AD-1 coated plate, which was possible because AD-1 is present at the extracellular surface of uEVs (19). In the second study, however, urine was first ultracentrifuged, the pellet was then lysed, and finally added to a plate coated with an antibody against the sodium chloride cotransporter (NCC) (32). This lysis step was necessary because the antibody targets the intracellular domain of NCC, which is located inside the uEVs. Although promising, two limitations must be considered. First, proteins in EVs have the same orientation as the cell they originate from. Therefore, antibodies need to be directed against the extracellular domain of the protein of interest, unless EVs are lysed. While the latter approach would increase specificity of the identified protein, it includes an additional lysis step, lacks specificity for EV origin and therefore still requires an ultracentrifugation

or precipitation step to concentrate uEVs (32). The second limitation is that capture antibodies should be selected carefully to ensure that these marker proteins (e.g., CD9) are indeed expressed by the uEVs of interest.

CHARACTERIZATION OF UEVS

uEVs can be further characterized for size distribution, to normalize between samples, and, ultimately, to analyze differential expression of proteins of interest. Several techniques are available to characterize uEVs (**Table 2**). Normalization between samples remains the "holy grail" in the field of uEVs (22). Normalization methods that have been used include urinary creatinine, timed urine collection, THP, use of 'household' uEV markers (e.g., CD9), and estimating the actual number of uEVs (1, 9, 34). Urinary creatinine has been used most often and has the advantage that it allows for the use of spot urines (10, 32, 35). A potential limitation, however, is that it assumes that the number of uEVs correlates with the concentration of the urine sample. Although timed urine has the advantage that all uEVs are collected within the defined time period and that it accounts for intra-day variability, it is notoriously incomplete (1). It is also unknown if nephron loss in chronic kidney disease results in the formation of less uEVs. CD9, CD63, CD81, TSG-101, and ALIX have been proposed as uEV markers and have been used for uEV normalization (immunoblot) or isolation (ELISA or immunobeads coupled to flowcytometry) (25, 34). This approach assumes that these markers remain unchanged during different physiological and disease states. In addition, although this method accounts for uEV quantity, only a subpopulation of uEVs expressing the marker are quantified. To overcome these shortcomings, the ability to count the actual number of uEVs would be desirable. Several methods have become available in recent years (6, 36, 37). Currently, resistive pulse sensing and nanoparticle tracking analysis hold promise, as other techniques are being developed. Resistive pulse sensing (qNano, Izon, New Zealand) uses a

stretchable nanopore on a polyurethane membrane, allowing real-time manipulation of the pore size (38). EVs travel across the membrane pore, thereby altering the current, which is a measure for EV volume. This technique provides both the size distribution and the concentration of EVs. Protein content, however, cannot be measured by this technique. Nanoparticle tracking analysis (Nanosight Ltd, United Kingdom) measures Brownian motion of EVs in solution by illuminating them with a laser beam. Size is calculated through the Stokes-Einstein equation, whereas concentration is determined by particle counting. Recently, nanoparticle tracking analysis was combined with fluorescently labeled antibodies directed against CD24 and aquaporin-2, to directly quantify this subpopulation without sample processing (15). The upregulation of aquaporin-2 in mice after treatment of desmopressin was readily detectable. This method also reduced the intra-assay variability due to interference of larger particles, one of the limiting factors for nanoparticle tracking analysis in unprocessed samples (15). The approach of using two labeling antibodies (one against the protein of interest and one against an unchanged protein) has been proposed previously and may solve the quantification issue especially if the outcome is dichotomous (39).

USE OF UEVS IN NEPHROLOGY

uEVs have been used for biomarker discovery and therapeutic purposes in various kidney disorders, including acute kidney injury, glomerular disease, renal tubular disorders, and polycystic kidney disease (**Table 3, Figure 2**).

Acute Kidney Injury

Acute kidney injury (AKI) is characterized by a sudden deterioration in kidney function. It is common in hospitalized patients and associated with high morbidity and mortality (41). Because serum creatinine concentration starts to increase only when glomerular filtration rate has decreased by more than 50%,

earlier biomarkers of reduced kidney function are being sought (41). In one study, the increased abundance of the sodium hydrogen exchanger type 3 (NHE3) in uEVs was able to differentiate between prerenal azotemia, acute tubular necrosis and other causes of renal failure in sixty-eight patients admitted to the intensive care unit (42). NHE3 in uEVs was more specific than the fractional excretion of sodium, a routinely used parameter to assess the cause of AKI. Fetuin-A, a negative acute-phase response protein synthesized by the liver, is another potential biomarker in AKI. It was found to be increased in uEVs using a rat model of cisplatin-induced AKI.

The increase in fetuin A was detectable two days before the increase in serum creatinine. This biomarker was subsequently tested in rat model of ischemia-reperfusion and in intensive care patients with AKI; the diagnostic utility of fetuin A in uEVs was also confirmed in these settings (43). The same group also studied activated transcription factor 3 in experimental and clinical AKI (44). While transcription factors are undetectable in whole urine and uEVs from healthy subjects – even when using proteomics (8) – they are readily detectable in uEVs during AKI, thereby representing a new class of biomarkers in kidney disease. The water channel aquaporin-1 in uEVs was found to be decreased after ischemia-reperfusion injury in rats and patients undergoing kidney transplantation (45). Although NHE3, fetuin-A, and aquaporin-1 were not compared directly, aquaporin-1 appears to have some advantages. That is, aquaporin-1 was detectable 6 to 96 hours after renal ischemia-reperfusion, while NHE3 was undetectable after 48 hours. In addition, fetuin-A was also increased in uEVs of the kidney donor, indicating a nonspecific rise in response to surgery or changes in hemodynamics. Besides their use as potential biomarkers in AKI, EVs may also have therapeutic potential. Mesenchymal stem cells (MSCs) have shown to contribute to the recovery of kidney injury (46-49). Possibly, MSCs exert their function in a paracrine fashion, releasing trophic growth factors, cytokines and chemokines, as they are only transiently present within the injured organs (50, 51). There is evidence to suggest that EVs play a pivotal role in this paracrine effect (52-57). Bruno *et al.* isolated EVs

derived from human bone marrow MSCs and used these EVs to test their effect in tubular epithelial cells and immunodeficient mice with glycerol-induced AKI. After labeling EVs with a PKH26 dye, uptake by TECs was visualized. Furthermore, blocking or trypsinizing the surface markers CD29 and CD44 inhibited the uptake of EVs by TECs. When taken up by TECs, EVs inhibited apoptosis that was induced by serum deprivation, vincristine or cisplatinum. *In vivo*, treatment with either MSCs or MSC-derived EVs showed similar renoprotective effects, which was not observed when fibroblast-derived EVs were used. After infusion of fluorescently labeled EVs, an accumulation of these EVs in kidney tubular cells was observed only in kidneys of mice with AKI, but not in the control mice. Interestingly, RNase treatment abrogated the protective effects of MSC EVs in all studies, suggesting an RNA-dependent mechanism (52-56). Indeed, mRNA and miRNA seem to be specifically incorporated into EVs, suggesting a horizontal transfer of MSC EVs to target cells (58, 59). Tomasoni *et al.* showed that mRNA of the receptor for insulin-like growth factor 1 (IGF-1R) but not the mRNA of the growth factor itself was present in EVs excreted by MSCs (59). When fibroblast cell lines derived from mice lacking the IGF-1R were incubated with MSC-derived EVs, functional IGF-1R was transcribed. Furthermore, incubation of these cells with the EVs led to an increase in proliferation in cisplatin-treated proximal tubular epithelial cells. This effect was enhanced when IGF-1 was co-incubated. This also suggests that MSC-derived EVs contribute to the repair of damaged cells in an RNA-dependent fashion.

Platform	Principle of technique	Detection limit	Normalization between samples	Estimate of number uEVs	Size distribution	Need for EV isolation	Allowing protein analysis	Ref
Western blot	Protein separation followed by antibody based detection	Depends on antibody affinity	Yes	No	No	Yes	Yes	(3)
ELISA	Antibody based capture and detection	Depends on antibody affinity	Yes	No	No	Sometimes	Yes	(19, 25, 32)
Flow cytometry	Light scattering or fluorescence	300 nm	Yes	No	No	Yes	In combination with immunolabelling	(28)
Nanoparticle tracking analysis	Brownian motion or light scattering	20 nm	Yes	Yes	Yes	No	In combination with immunolabeling	(15)
Resistive pulse sensing	Physical passage of particles through micropore	50 nm	Yes	Yes	Yes	No	No	(38, 40)
Transmission electron microscopy	Electron scattering	1 nm	No	No	Yes	Yes	In combination with immunolabeling	(3)

Table 2: Commonly Used Techniques to Characterize Urinary EV's

Glomerular Diseases

Urinary biomarkers may ultimately replace the need for a kidney biopsy in glomerular disease. As podocyte injury is a common feature of glomerular disease, the presence of podocytes, podocyte-derived RNA or podocyte-derived EVs have been postulated as potential biomarkers (60-63). A number of specific biomarkers for glomerular disease in uEVs have been identified, including Wilm's Tumor 1, podocalyxin, α-1 antitrypsin, aminopeptidase N, vasorin precursor, ceruloplasmin, and miR-145 (**Table 3**). In animal models of focal segmental glomerulosclerosis and collapsing glomerulopathy, Wilm's Tumor-1 (WT-1) protein in uEVs, a transcription factor required for kidney development, increased one week earlier than urinary albumin (44) and also predicted the effect of angiotensin receptor blocker treatment (62). One study

found that WT-1 in uEVs was increased in patients with FSGS, but not in those with AKI or in healthy volunteers (44, 62); this finding, however, was not confirmed by others (64). The increase in uEV WT-1 was also associated with a decline in kidney function in diabetic nephropathy, suggesting early podocyte damage (65). Another podocyte injury marker is podocalyxin, which, unlike WT-1, was found in shedding vesicles (no expression of CD24 and CD63) (4, 66). In addition to diabetic nephropathy, podocalyxin was also increased in uEVs isolated from patients with IgA nephropathy (67, 68). In practice, IgA nephropathy should often be differentiated from thin membrane disease, as both disorders present with glomerular erythrocyturia. Therefore, a urinary biomarker that could differentiate IgA nephropathy from thin membrane disease could potentially omit the need for a kidney biopsy. Using a label-free quantitative proteomic approach, four proteins in uEVs were found to be differentially expressed in IgA nephropathy, including α-1 antitrypsin, aminopeptidase N, vasorin precursor and ceruloplasmin (69). In type 1 diabetics with diabetic nepropathy, miRNA expression profiling revealed differential expression of 22 out of 226 miRNAs isolated from uEVs (70). Increase in miR-145 was further explored in streptozotocin induced diabetic mice where it was found that both glomeruli and uEVs contained more miR-145. The finding that mesangial cells exposed to high glucose had an increase in whole cell and exosomal miR-145 content, suggested that higher local glucose concentrations caused this increase. While all of these findings are promising, they need to be validated in larger studies.

Renal Tubular Disorders

The identification of most kidney solute and water transporters in uEVs raised the possibility that these proteins may be used as biomarkers for renal tubular disorders (1, 2, 8). Less clear is if the abundance of transporters in uEVs correlates with their transport activity in the kidney, although a number of studies suggest this may be so (34, 75). In 1995, Kanno *et al.* were the first to identify aquaporin-2 in urine (33). Aquaporin-2 is a vasopressin-sensitive water channel located in the principal cells of the collecting duct. Immunogold

labeling showed that aquaporin-2 was present in uEVs, but the nature of these vesicles and how they had been released in urine, was not known. The abundance of aquaporin-2 in urine correlated with vasopressin activity, because it increased after water restriction and desmopressin infusion, but was not increased in patients with nephrogenic diabetes insipidus. In another study, the abundance of aquaporin-2 in uEVs highly correlated with its abundance in murine kidney collecting duct cells after desmopressin stimulation (34). Interestingly, the transfer of uEVs from desmopressin-treated cells to untreated cells resulted in an increase of functional aquaporin-2 expression in the untreated cells (**Figure 1**). Similar effects were seen in rats after demopressin treatment, because the excretion of aquaporin-2 in uEVs increased (34). The good correlation between desmopressin stimulation and aquaporin-2 excretion in uEVs was confirmed by others (75). In addition to plasma vasopressin, aquaporin-2 in uEVs was also influenced by alkalization of the urine (75). Because sodium bicarbonate did not increase plasma vasopressin, urinary alkalization likely increased the sensitivity of the vasopressin type 2 receptor for vasopressin. The diagnostic utility of uEVs in renal tubular disorders was also illustrated by the absence of the sodium-potassium-chloride cotransporter (NKCC2) in urinary exosomes from patients with Bartter syndrome type 1, a rare disease caused by mutations in the gene encoding for NKCC2 (8). Similarly, the sodium-chloride cotransporter (NCC) was absent in urinary exosomes of patients with Gitelman syndrome, who have an inactivating mutation in the gene encoding for NCC (71). The opposite was also true, because patients with familial hyperkalemic hypertension, which is characterized by NCC overactivity, had increased NCC in urinary exosomes (32, 72). Because NCC is sensitive to aldosterone, we tested whether NCC showed the same response in uEVs as in kidney tissue from animal models of aldosteronism (35). Indeed, feeding rats a low sodium diet or infusing aldosterone increased total and phosphorylated NCC in kidney tissue and uEVs (**Figure 3**). Furthermore, the abundance of phosphorylated NCC in uEVs was higher in patients with primary aldosteronism than in matched patients with essential hypertension. Phosphorylated NCC also performed better than

prostasin, a serine protease known to regulate the epithelial sodium channel, which is also sensitive to aldosterone. Another disorder in which kidney transporters in uEVs were analyzed, was American cutaneous leishmaniasis (76). American cutaneous leishmaniasis may be complicated by defects in urinary concentration and acidification. The urinary concentration defect was associated with a reduced abundance of aquaporin-2 in uEVs, while NKCC2 was upregulated, possibly as a compensatory response. The urinary acidification defect may have been explained by the increase of pendrin in uEVs, while increases in NHE3, and H^+ ATPase may again have been compensatory.

Polycystic Kidney Disease

Polycystic kidney disease (PKD) is the most common inherited kidney disease and is caused by dysfunction of cilia (77). EVs may be important in cilia biology and as biomarkers for PKD (**Figure 3**). Primary cilia are flow-sensing organelles, expressed in nearly all renal epithelial cells except for the intercalated cells of the collecting duct (78-80). Mutations in genes encoding for proteins involved in the function of primary cilia lead to PKD, including polycystin-1, polycystin-2, and fibrocystin/polyductin (81). The proteins polycystin-1 and polycystin-2 form a complex that is involved in mechano-sensing by cilia. Changes in luminal flow induce a calcium influx through the polycystin-2 channel, which is a non-selective cation channel (82). How are cilia and EVs related? Left-right asymmetry in the embryonic stage depends on ciliary leftward fluid flow. So called nodal vesicular particles (300-500 nm) contain sonic hedgehog and retinoic acid, which are both proteins involved in the symmetry breaking process (83). These vesicles are secreted by cells as a result of fibroblast growth factor and are subsequently carried to the left by ciliary motion. Before reaching the left wall of the ventral node, they are fragmented by cilia and then taken up, thereby creating left-specific intracellular calcium elevation in the cells of the left ventral node. Mutations in polycystin-2 hamper this increase in intracellular calcium, disturbing the left-right breaking process. This in turn could lead to situs inversus, a congenital condition in

which the major organs are reversed (84). Furthermore, so-called PKD-positive vesicles seem to specifically interact with primary cilia of kidney and biliary epithelial cells, as observed by transmission electron microscopy images (24, 74). Biliary derived EVs attach to cholangiocyte cilia and alter ERK signaling, miR-15A expression and cholangiocyte proliferation *in vitro*. Pharmacological removal of cilia by chloral hydrate abolished these effects (73). Interestingly, the proteins involved in the pathogenesis of PDK, polycystin-1, polycystin-2 and fibrocystin/polyductin, have also been found in uEVs (2, 8). Characterization of uEVs in which polycystin-1, polycystin-2, and fibrocystin/polyductin were present, identified two additional proteins previously known to be involved in polycystic kidney disease, namely cystin and ADP-ribosylation factor-like 6 (24). Recently, surface glycosylation profiles in uEVs were compared between seven patients with PKD and seven matched healthy volunteers. Significant differences in surface glycosylation were identified in 6 out of 43 lectins studied, illustrating the potential use as biomarker. Our group is currently searching for potential biomarkers in uEVs for PKD using quantitative proteomics (85).

Disorder	**Potential biomarkers in uEVs**
Acute kidney injury	Sodium hydrogen exchanger type 3, activated transcription factor 3, fetuin-A, aquaporin-1
Focal segmental glomerulosclerosis	Wilm's Tumor 1
IgA nephropathy	α-1 antitrypsin, aminopeptidase N, vasorin precursor and ceruloplasmin, Podocalyxin
Diabetic nephropathy	Podocalyxin, dipeptidyl peptidase IV, miR-145, Wilm's Tumor 1
Nephrogenic diabetes insipidus	Aquaporin-2
Bartter syndrome	Sodium potassium chloride cotransporter type 2
Gitelman syndrome	Sodium chloride cotransporter
Familial hyperkalemic hypertension	Sodium chloride cotransporter
Primary aldosteronism	Sodium chloride cotransporter, prostasin
Polycystic kidney disease	Polycystin-1, polycystin-2, fibrocystin/polyductin, surface glycosylation profiles

Table 3: Potential biomarkers in uEVs for different kidney disorders

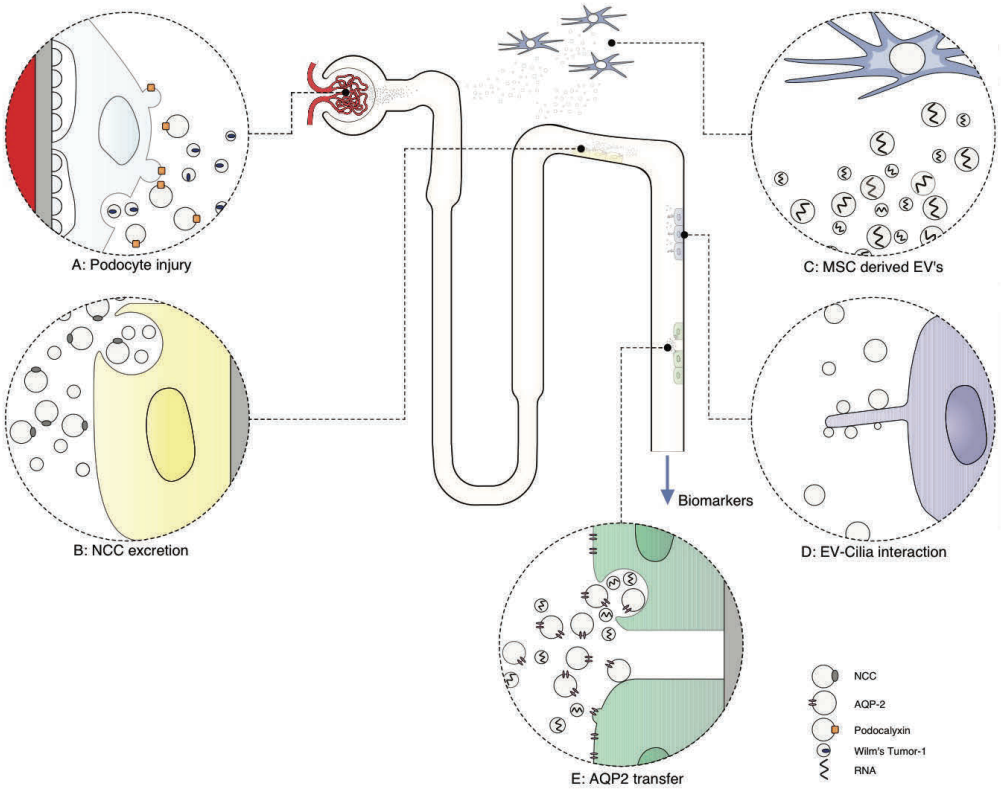

Figure 2: Illustration of the Many Functions of uEVs in Kidney Disorders. *The figure shows a schematic drawing of the nephron with the glomerulus and the kidney tubule. Five magnifications illustrate local processes in which uEVs are involved along the nephron.* ***Panel A*** *shows uEVs containing podocalyxin and Wilm's Tumor-1 protein, which are excreted after podocyte injury (4, 44, 62, 65-68).* ***Panel B*** *shows uEVs containing the sodium chloride cotransporter (NCC). These uEVs may be used in primary or secondary aldosteronism (**Figure 3**), Gitelman syndrome, or familial hyperkalemic hypertension (**Table 2**) (32, 35, 71, 72).* ***Panel C*** *shows that EVs derived from mesenchymal stem cells (MSC) may contribute to the recovery of acute kidney injury, most likely in an RNA dependent fashion (see text for details, (52-57)).* ***Panel D*** *shows how uEVs may interact with primary cilia and possibly exert a downstream effect, for example in polycystic kidney diseases (24, 73, 74).* ***Panel E*** *shows thepossibility that uEVs transfer functional aquaporin-2 (AQP2) between kidney collectingduct cells (based on in vitro findings,*

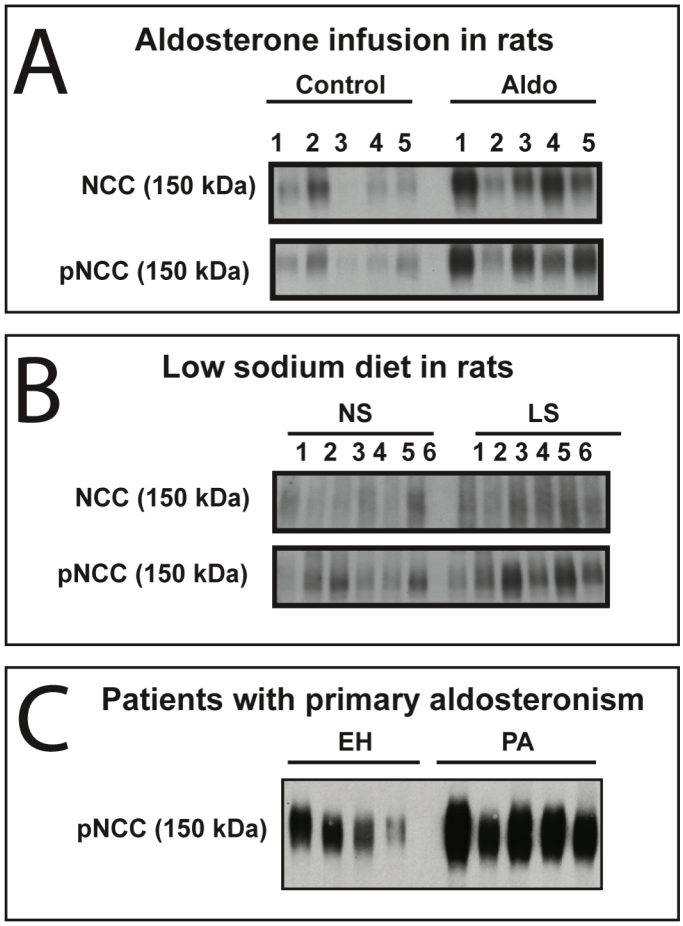

Figure 3: Aldosteronism Results in Increased Abundance of the Sodium Chloride Cotransporter in uEVs.

The figure shows three panels of immunoblots performed in uEVs. ***Panel A*** *shows the increase in the abundance of the sodium chloride cotransporter (NCC) and its phosphorylated form (pNCC, threonine 58 phosphorylation site) in uEVs after a two-day aldosterone (Aldo) infusion in rats.* ***Panel B*** *shows that secondary aldosteronism as induced by a low sodium diet in rats also increases NCC and pNCC abundance in uEVs.* ***Panel C*** *shows that the abundance of pNCC is higher in uEVs of patients with primary aldosteronism compared to patients with the same degree of essential hypertension. Figure adapted from (35), with kind permission.*

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CHAPTER

03

AN IMMUNOASSAY FOR URINARY EXTRACELLULAR VESICLES

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ABSTRACT

Although nanosized urinary extracellular vesicles (uEVs) are increasingly used for biomarker discovery, their isolation currently relies on time-consuming techniques hindering high-throughput application. To navigate this problem, we designed an immunoassay to isolate, quantify and normalize uEV-proteins. The uEV-immunoassay consists of a biotinylated CD9 antibody to isolate uEVs, an antibody against the protein of interest, and two conjugated antibodies to quantify the protein of interest and CD9. As a proof of principle, the immunoassay was developed to analyze the water channel aquaporin-2 (AQP2) and the sodium chloride cotransporter (NCC). CD9 was used as a capture antibody because immunoprecipitation showed that anti-CD9 antibody, but not anti-CD63 antibody, isolated AQP2 and NCC. CD9 correlated strongly with urine creatinine, allowing CD9 to be used for normalization of spot urines. The uEV-immunoassay detected AQP2 and NCC with high sensitivity, low coefficients of variance and stability in dilution series. After water loading in healthy subjects, the uEV-immunoassay detected decreases in AQP2 and NCC equally well as the traditional method using ultracentrifugation and immunoblot. The uEV-immunoassay also reliably detected lower and higher AQP2 or NCC levels in uEVs from patients with pathological water or salt reabsorption, respectively. In summary, we report a novel approach to analyze uEVs that circumvents existing isolation and normalization issues, requires small volumes of urine and detects anticipated changes in physiological responses and clinical disorders.

INTRODUCTION

Extracellular vesicles (EVs), including exosomes, are nanosized lipid membrane vesicles released by cells (1). They have been identified in all biofluids, including urine (2). EVs contain proteins and nucleic acids, which may reflect the physiological and pathophysiological state of the cell from which they were released. Therefore, EVs have sparked interest with regard to biomarker discovery (1, 3). Urinary extracellular vesicles (uEVs) have the advantage that they can be obtained non-invasively and may be of special interest for disorders of the kidney or urinary tract (4, 5). Indeed, several disease biomarkers have recently been reported in uEVs (6-8). Ultracentrifugation followed by immunoblotting is currently the most widely used method to isolate uEVs and quantify proteins of interest (9). Other techniques to isolate uEVs rely on filtration, precipitation, or size exclusion chromatography, and also require immunoblotting for protein quantification (4, 5). All of these techniques are time-consuming and require large starting volumes of urine, hampering high-throughput clinical application. Here, we report a novel approach using an enzyme-linked immunosorbent assay (ELISA) combined with a time-resolved fluorescence immunoassay to isolate uEVs, requiring small volumes of whole urine and allowing quantification and normalization of uEV proteins of interest. For proof-of-concept of the immunoassay, we assessed the regulation of the water channel aquaporin-2 (AQP2) and the sodium chloride cotransporter (NCC), because these two proteins have previously been identified in uEVs and are relevant in physiological and pathophysiological settings (2, 7).

MATERIALS AND METHODS

Urine samples

All urine samples were collected with protease inhibitors (Roche cOmplete, Woerden, The Netherlands) and centrifuged (3000 x g for 20 minutes) to remove cells and cell debris and were stored at -80°C. Urine samples were then used for the uEV-immunoassay (100 µl/well in duplicates or triplicates) or isolation of uEVs using high-speed and ultracentrifugation (40 ml/sample), as described previously (7). uEVs isolated by ultracentrifugation were solubilized and prepared for immunoblotting. For immunoprecipitation, we used the Dynabeads protein G immunoprecipitation kit according to the manufacturer's instructions (Thermo Fisher Scientific, Waltham, USA). The following antibodies were used for immunoprecipitation: mouse anti-CD9 (R&D systems, Minneapolis, USA, clone 209306), anti-CD63 (BD Pharmingen, San Jose, USA, clone H5C6), rabbit anti-ALIX (Sigma-Aldrich, St. Louis, USA, clone HPA011905), and rabbit anti-AQP2 and anti-NCC (see below). Urine creatinine and osmolality were measured by our clinical chemistry department using CREP2 Cobas (Roche) and the Arkray Osmo Station (Menarini Diagnostics, Florence, Italy).

uEV-immunoassay

Biotin labeled mouse anti-human CD9 antibodies (eBioscience, San Diego, USA, clone SN4 C3-3A2) in Red Buffer containing 0.01% Tween 40 (Kaivogen, Turku, Finland) were added to neutravidin coated plates (Thermo Fisher) and incubated overnight at 4°C with gentle shaking. After incubating 100 µl of urine sample in the wells for one hour, captured uEVs were lysed by 0.01% SDS (10 minute incubation with gentle shaking). Subsequently, rabbit anti-human AQP-2 or NCC, both generated by one of the investigators, was added to the wells (1 hour incubation). Anti-AQP2 antibody was described before (10) and anti-NCC antibody was raised in rabbit targeting the peptide RRDCPWKISDEEINKNR. The antibody was confirmed to be specific for NCC using immunohistochemistry, immunoblotting of kidney lysates and NCC-

transfected/non-transfected cells, or immunoprecipitation followed by protein mass spectrometry (data not shown). Finally, the donkey anti-rabbit HRP-conjugated antibody (Biolegend, San Diego, USA, clone poly4064) followed by the Europium-conjugated anti-CD9 antibody (R&D Systems, labeled as described before (11)) were added to the wells (each for 1 hour). Each incubation step (urine and antibodies) was followed by six washes with wash buffer (Kaivogen). Addition of ECL followed by enhancement solution (Perkin-Elmer, Turku, Finland) allowed detection of signals at 445 nm (1 ms) and 615 nm (time-resolved) by the Wallac Victor 2 multilabel counter (Perkin-Elmer). For all immunoassay-results, we report the remaining signal after subtracting PBS-signal.

Healthy subjects and patients

The medical ethics committee approved the water loading experiment and urine collections in patients (MEC-2015-204). Healthy subjects (no medical history, no medication) underwent thirsting (10 p.m. – noon), and then drank 20 ml/kg water within 30 minutes. Spot urine samples were obtained at five time points (10 a.m., noon, 2 p.m., 3 p.m., 5 p.m.). Nine patients were recruited from our outpatient clinic and included patients with central diabetes insipidus (one patient due to *AVP-NPII* mutation, one patient due to hypophysectomy), nephrogenic diabetes insipidus (one patient due to *AVPR2* mutation, one patient with clinical phenotype and family history consistent with nephrogenic diabetes insipidus, mutation analysis pending), syndrome of inappropriate antidiuresis (one patient due to pituitary adenoma, one patient due to subdural hematoma), Gitelman syndrome (two patients with *SLC12A3* mutation), and familial hyperkalemic hypertension (one patient due to *KLHL3* mutation).

RESULTS

Development and validation of uEV-immunoassay

CD9 and CD63 are commonly used as uEV-markers and are thought to play a role in the biogenesis of uEVs (12). We first isolated uEVs from human urine by immunoprecipitation using antibody coated magnetic beads to analyze if CD9+ and CD63+ uEVs express AQP2 or NCC (**Figure 1A**). AQP2 and NCC were present in CD9+ but not in CD63+ uEVs. Analysis of ALIX, another uEV marker protein, suggested a lower concentration of CD63+ uEVs. This is in agreement with tissue distribution, as CD63 is low in abundance in the kidney but highly expressed in bladder and prostate (11, 13). Therefore, we selected CD9 as our capture antibody for uEVs. **Figure 1B** shows the design of our sandwich uEV-immunoassay. The uEV-immunoassay included four antibodies: (1) a biotinylated anti-CD9 antibody (capture antibody), (2) an antibody directed against the protein of interest (anti-AQP2 or anti-NCC), (3) a horseradish-peroxidase (HRP) conjugated antibody against the anti-AQP2 or anti-NCC antibody, and (4) a Europium conjugated anti-CD9 antibody. We used two conjugated antibodies to allow quantification of both the protein of interest and CD9 (as uEV-marker). Because our anti-AQP2 and anti-NCC antibodies are directed against an intracellular epitope, our uEV-immunoassay required the use of a detergent.

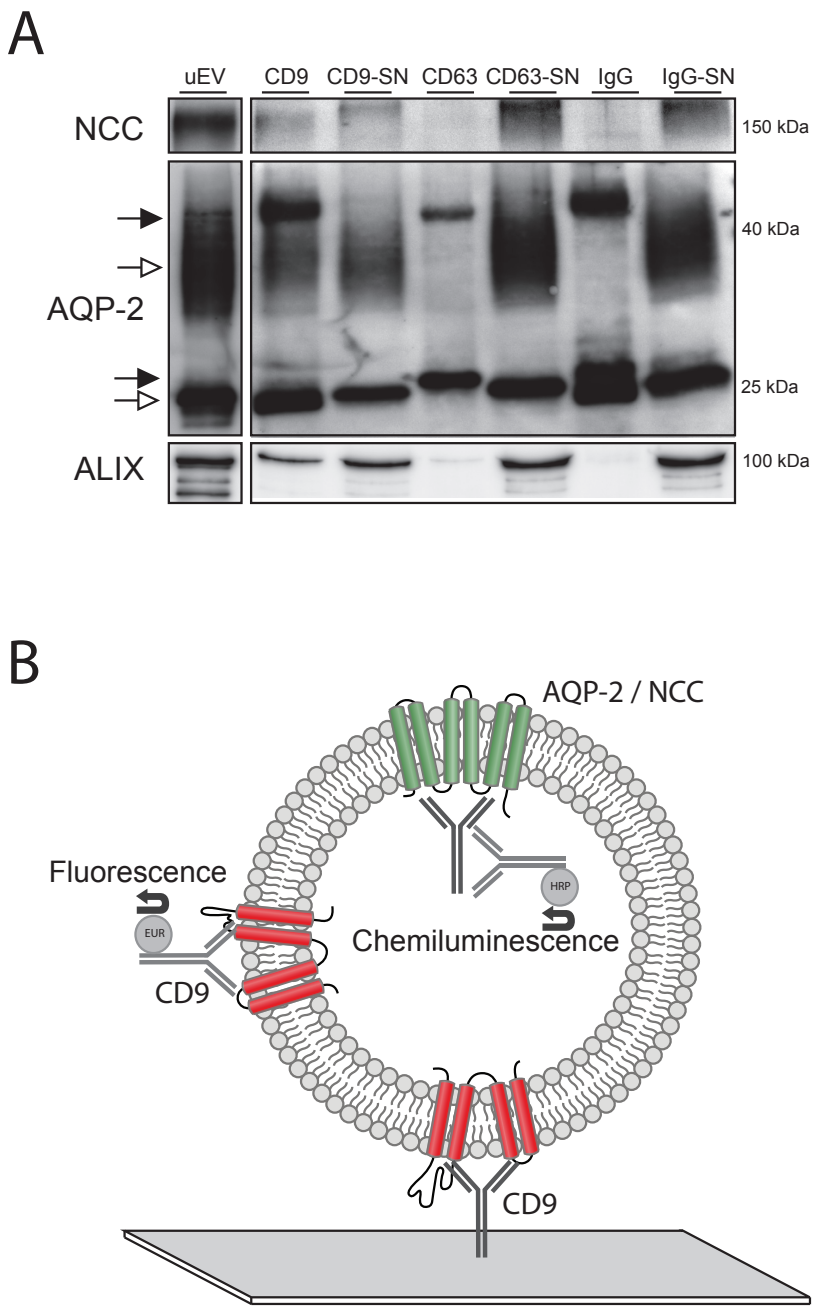

Figure 1: Set-up of an immunoassay for urinary extracellular vesicles

*(**A**) Immunoprecipitation study showing that CD9+ but not CD63+ uEVs express NCC and AQP2. Analysis of ALIX, another uEV-marker, suggested lower concentration of CD63+ uEVs. uEVs isolated with ultracentrifugation ('uEV') were used as positive controls. CD9-SN and CD63-SN refer to the supernatant that remained after immunobead isolation. To exclude non-specific binding, IgG was used as negative control. Black arrows show heavy (55 kDa) and light (25 kDa) chains of the antibodies. White arrows show glycosylated (40 kDa) and non-glycosylated (25 kDa) AQP2. The latter overlaps with the light chain of the antibodies.*

*(**B**) uEV-immunoassay set-up showing the neutravidin coated plate and the four antibodies, including the biotinylated anti-CD9 (capture) antibody, anti-AQP2 or anti-NCC antibody (protein of interest) targeting an intracellular domain, and the two luminescent antibodies to quantify the number of uEVs (Europium-conjugated anti-CD9 antibody) and the protein of interest (horseradish-peroxide-conjugated antibody).*

We tested various detergents in different concentrations and with varying incubation times, of which 0.01% SDS for 10 minutes resulted in the most optimal signal to noise ratio (**Figure 2A**). A recent study that determined the lysis sensitivity in EV subpopulations, also showed that low concentrations of SDS (0.01%) or Triton X-100 (0.025%) were most effective (14). Subsequently, we validated our uEV-immunoassay by selectively removing one of the antibodies which resulted in loss of signal (**Figure 2B**). In this set of experiments we also confirmed the requirement of the detergent, because the signals for AQP2 and NCC were lost when the detergent was not added. The CD9 signal, however, remained intact, because the anti-CD9 antibody is directed against an extracellular domain of the protein. No loss of CD9 signal occurred when the detergent was added to the uEV-immunoassay, suggesting the detergent did not result in disintegration of bound uEVs and loss of membrane fragments during subsequent washing steps. To be practical clinically, it should be possible to use the uEV-immunoassay on spot urines instead of 24-hour urine. Clinically, spot urines are routinely used for diagnostic purposes, for example by using the protein-to-creatinine ratio, which shows excellent correlation with 24h urine protein (15). At present, urine creatinine is the most commonly used parameter to normalize uEV-protein abundances, assuming that the concentration of the urine sample correlates with uEV number. To test this assumption, we compared urine creatinine concentration with CD9 signal and observed an excellent correlation (**Figure 2C**). This also implies that with the current uEV-immunoassay set-up it is no longer necessary to measure urine creatinine separately. It remains to be studied whether urine creatinine and CD9 truly correlate with uEV-number, for example by nanoparticle tracking analysis (16). The signals for AQP2, NCC, and CD9 remained stable in serial dilutions (**Figure 2D**), suggesting AQP2/CD9 and NCC/CD9 ratios can be used as proxy for the abundance of the protein of interest normalized for uEV number. The coefficients of variance for the uEV-immunoassay were 5.6% for AQP2 and 3.3% for NCC up to 8-fold dilutions. All these experiments were also performed with uEVs isolated by ultracentrifugation, which gave similar results (data not shown).

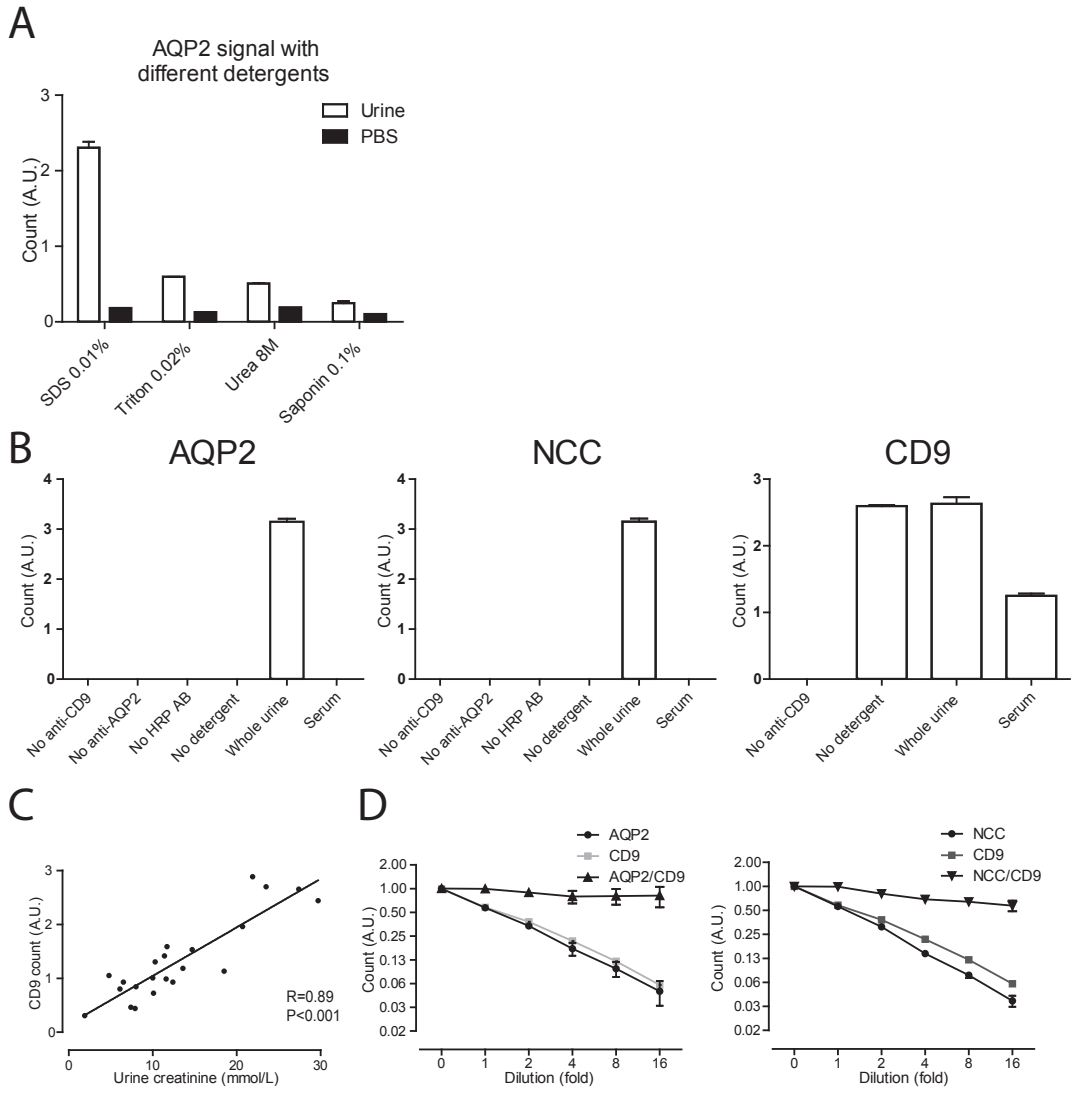

Figure 2: validation of the uEV-immunoassay

*(**A**) Testing of different detergents showed that SDS 0.01% resulted in the most optimal signal for AQP2. Detergent was used to lyse uEVs because our anti-AQP2/NCC antibodies target an intracellular domain. Incubation time for all detergents was 10 min. Results are shown as means ± SD (experiments performed in duplicates).*

*(**B**) Validation of uEV-immunoassay showing that selectively removing one of the antibodies results in loss of signal. CD9-signal did not require detergent, because our anti-CD9 antibody targets an extracellular domain. No loss of CD9-signal occurred when detergent was used. As expected, CD9 but not AQP2 and NCC were detected in serum. Results are shown as means ± SD (experiments performed in triplicates).*

*(**C**) Correlation between urine creatinine and CD9. 4-5 random spot urines from four healthy volunteers were used.*

*(**D**) Serial dilutions showing stable AQP2, NCC and CD9 signals. AQP2/CD9 and NCC/CD9 ratios remained close to 1. Results are shown as means ± SD (experiments performed in triplicates). All experiments were performed with whole urine from healthy volunteers. A.U., arbitrary units.*

Application of uEV-immunoassay in physiological and pathophysiological settings

To test whether the uEV-immunoassay was capable of detecting physiological and pathophysiological changes in AQP2 and NCC, we tested it in normal subjects and patients (**Figures 3 and 4**). First, we performed a water loading experiment in healthy subjects after overnight thirsting. As expected, urine osmolality reflected maximally concentrated urine during thirsting and maximally dilute urine after water loading (**Figure 3A**). Urine osmolality is determined by the degree of water reabsorption in the collecting duct through AQP2 water channels, which are stimulated by vasopressin (17). More recently, vasopressin was also shown to activate NCC (18). We first analyzed AQP2 and NCC expression in uEVs during the water loading experiment with the conventional method using immunoblotting of uEVs isolated with ultracentrifugation and normalized by urine creatinine (**Figure 3B**). Even after normalization by urine creatinine, which is another measure of urinary concentration, the decreased abundances of AQP2 and NCC after water loading were clearly visible, and likely represented a decrease in vasopressin levels in response to hypotonicity (17). We also tested aquaporin-3, which is also regulated by vasopressin, but did not detect it in uEVs (data not shown), possibly because it is present in the basolateral plasma membrane**.** We analyzed the same urine samples (without ultracentrifugation) with our uEV-immunoassay using AQP2/CD9 and NCC/CD9 ratios, again showing the same pattern (**Figure 3C**). The uEV-immunoassay correlated with the conventional method for both AQP2 and NCC (**Figure 3D**). The correlation for AQP2 was stronger than for NCC, possibly because a few data points were near the detection limit (dilute urine) or because of differences in the quality of the antibody. To test whether the uEV-immunoassay could detect pathophysiological changes in AQP2 or NCC we tested it in patients. We selected patients with inherited or acquired disease in which AQP2 or NCC activity is disturbed, including nephrogenic or central diabetes insipidus, syndrome of inappropriate antidiuresis (SIAD), Gitelman syndrome, and familial hyperkalemic hypertension (FHHt, also called pseudohypoaldosteronism type 2 or Gordon syndrome). For most of these

disorders, lower or higher abundances of AQP2 or NCC have been shown by immunoblot in uEVs isolated with ultracentrifugation (19-21). We confirmed lower and higher uEV-AQP2 abundances in diabetes insipidus and SIAD (**Figure 4A**). Similarly, we confirmed lower and higher uEV-NCC abundances in Gitelman syndrome and FHHt.

We then tested whether the same results could be obtained with our uEV-immunoassay using whole urine and CD9 normalization. Indeed, the uEV-immunoassay reliably recapitulated the expected changes in AQP2 and NCC in uEVs in these disorders (**Figure 4B**).

DISCUSSION

We report a novel approach to isolate and analyze uEVs using an immunoassay. Although immunoassays have been used previously for uEVs, these approaches usually concerned the quantification of a single protein (9, 11, 22). Alvarez *et al.* used the commercially available ExoELISA to quantify CD9 as measure of uEV-number (9). Duijvesz *et al.* developed a time-resolved fluorescence immunoassay to quantify the number of CD9+ or CD63+ uEVs as biomarker for prostate cancer (11). The fact that CD9+ uEVs also derive from prostate may be a limiting factor for our uEV-immunoassay as it may skew the AQP2/CD9 or NCC/CD9 ratio and may yield different results in men. However, in the study by Duijvesz *et al.*, the number of CD9+ uEVs only increased after digital rectal examination (11), suggesting this limitation is minimal. Isobe *et al.* developed an ELISA to quantify both total and phosphorylated NCC in uEVs (22). However, because they did not use a capture antibody to isolate uEVs, their approach still required ultracentrifugation. Oosthuyzen *et al.* used a different approach by adding a fluorescently labeled AQP2 antibody to whole urine and then using a nanoparticle tracking device to quantify AQP2+ uEVs (16). By doing so, they confirmed an increase in AQP2+ uEVs after desmopressin in normal mice and

patients with central diabetes insipidus. The studies by Isobe *et al.* and Oosthuyzen *et al.* still required normalization by urine creatinine, because no uEV-marker was quantified (16, 22). We believe our uEV-immunoassay may be used as template for other proteins of interest. This requires the confirmation that the protein of interest is indeed present in CD9+ uEVs. Previous immunohistochemical studies in human kidneys demonstrated high CD9 but low CD63 expression (13). In our study CD9+ uEVs contained both AQP2 and NCC (Figure 1A), suggesting CD9 must be expressed in the distal convoluted tubule and collecting duct. If anti-CD9 will be used as capture antibody in future studies, it would be important to know which tubule segments express CD9.

Alternatively, an antibody against AQP2 or NCC may be used as capture antibody to isolate tubule-specific uEVs, but this would require the development of antibodies targeting the extracellular domains of these proteins. Another consideration is that the pathophysiological setting by itself may change the number of CD9+ uEVs (11). Although there may be a subpopulation of CD9- vesicles that contains AQP2 or NCC, our uEV-immunoassay results correlated with the ultracentrifugation method which isolates all uEVs (**Figure 3D**). We believe our method is most suitable for plasma membrane associated proteins, as cytosol-derived proteins within uEVs are likely lost after lysis, although this remains to be tested. In summary, we report a novel approach to analyze uEVs that circumvents existing isolation and normalization issues and that detects anticipated changes in physiological responses and clinical disorders.

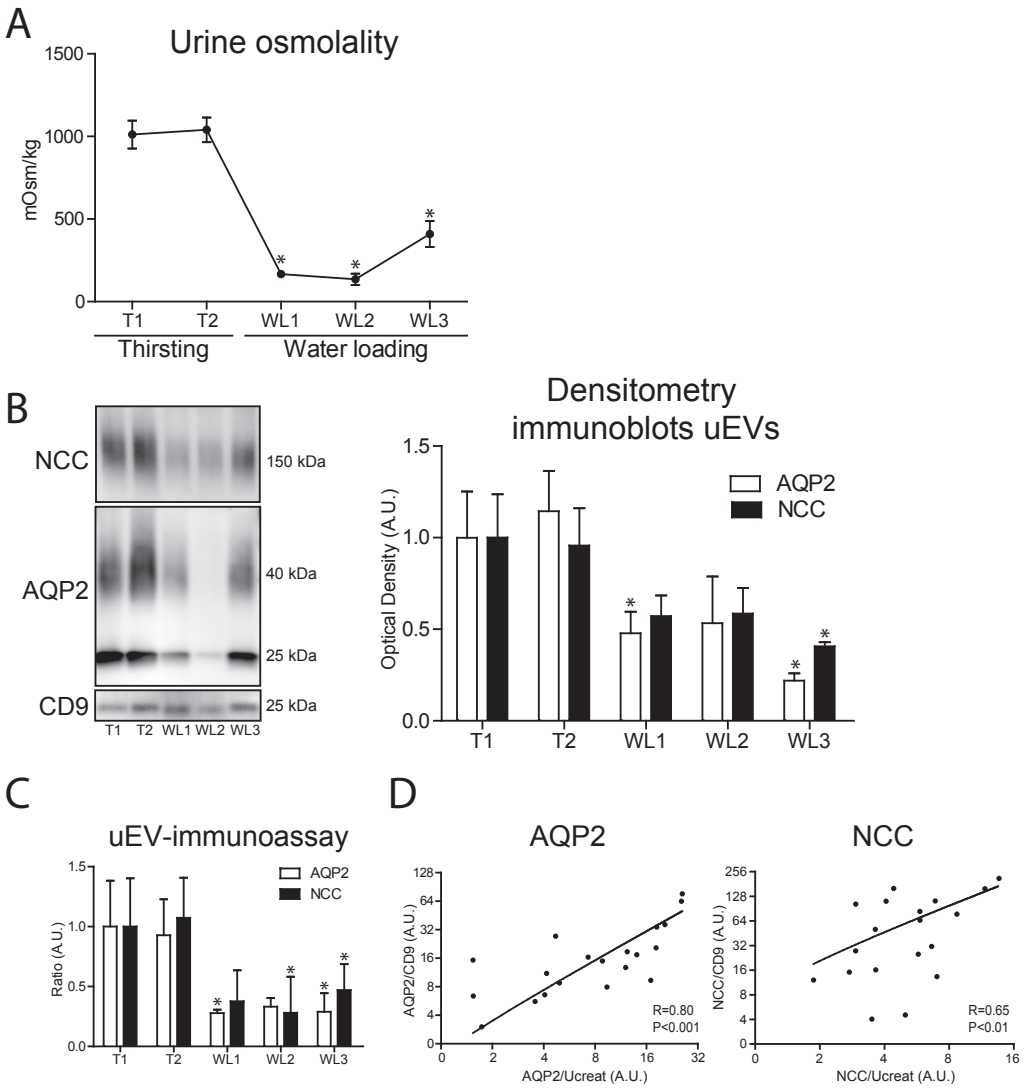

Figure 3: Application of uEV-immunoassay in physiological AQP2 and NCC regulation

*(**A**) Urine osmolality (means ± SEM) during thirsting and water loading in four healthy subjects. Spot urine samples were measured in four subjects at five time points: 12h and 14h after thirsting (T1 and T2), and 2h, 3h, and 5h after water loading (WL1-3). * P < 0.05 by paired T-test.*

*(**B**) Representative immunoblot from one of the participants of AQP2 and NCC in uEVs isolated by ultracentrifugation. In addition, average densitometry of immunoblots from all participants (n = 4) is shown at the different time-points (means ± SEM). * P < 0.05 by paired T-test compared to T2 after setting all individual T2-values to 1.*

*(**C**) AQP2/CD9 and NCC/CD9 ratios obtained with the uEV-immunoassay from urine samples of the water loading experiments (means ± SEM). * P < 0.05 by paired T-test compared to T2 after setting all individual T2-values to 1.*

*(**D**) Correlations of AQP2/U_{Creat} vs. AQP/CD9 and NCC/U_{Creat} vs. NCC/CD9 were plotted to compare immunoblotting of uEVs isolated with ultracentrifugation and results from uEV-immunoassay.*

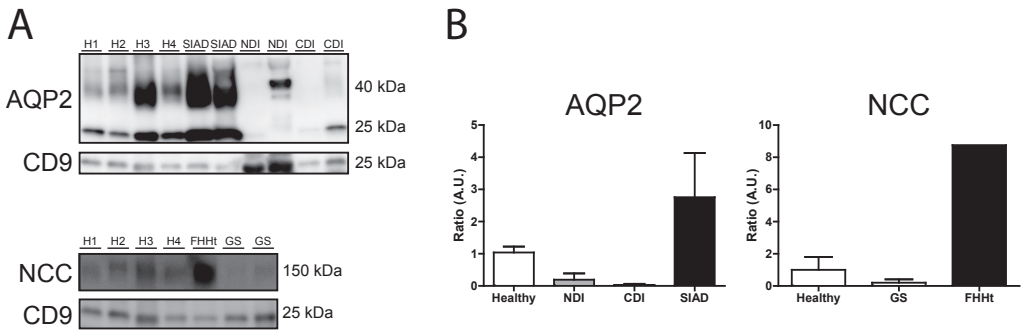

Figure 4: Application of the uEV-immunoassay in disorders with disturbed AQP2 or NCC regulation

*(**A**) AQP2 and NCC were immunoblotted in uEVs isolated with ultracentrifugation. Urine samples were collected from healthy subjects (n = 4) and patients with central (CDI, n = 2) or nephrogenic (NDI, n = 2) diabetes insipidus, syndrome of inappropriate antidiuresis (SIAD, n = 2), Gitelman syndrome (GS, n = 2), and familial hyperkalemic hypertension (FHHt, n = 1). See Methods for details. The second NDI patient did have a visible glycosylated AQP2-band that ran at a higher molecular weight, which may be due to the type of the mutation (mutation analysis pending).*

*(**B**) AQP2/CD9 and NCC/CD9 signals obtained with uEV-immunoassay using the urine samples from the healthy subjects and patients.*

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SECTION

02

URINARY EXTRACELLULAR VESICLES: MARKERS FOR SALT-SENSITIVE HYPERTENSION

CHAPTER

04

URINARY EXTRACELLULAR VESICLES AS MARKERS TO ASSESS KIDNEY SODIUM TRANSPORT

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ABSTRACT

Purpose of review: To summarize studies that have analyzed sodium transporters in urinary extracellular vesicles (uEVs) in relation to hypertension.

Recent findings: The majority of kidney sodium transporters are detectable in uEVs. Patients with loss or gain of function mutations in sodium transporter genes have concomitant changes in the abundances of their corresponding proteins in uEVs. The expected effects of aldosterone in the kidney, including activation of the sodium-chloride cotransporter (NCC) and epithelial sodium channel (ENaC), are transferred to uEVs as increases in phosphorylated NCC and the γ-subunit of ENaC. Specific forms of hypertension, including aldosteronism and pseudohypoaldosteronism, are characterized by higher abundances of total or phosphorylated NCC in uEVs. The proteolytic processing of ENaC by urinary proteases is detectable in uEVs as cleaved γ-ENaC, as demonstrated in hypertensive patients with diabetic nephropathy. Analysis of uEVs from patients with essential or salt-sensitive hypertension identified potential candidates for uEV-markers of hypertension, including retinoic acid-induced gene 2 protein and hsa-miR-4516.

Summary: Analysis of sodium transporters in uEVs is a promising approach to study renal epithelial transport processes non-invasively in human hypertension.

INTRODUCTION

Extracellular vesicles (EVs) are nanosized vesicles released by all cells. Based on their biogenesis and size, they can be classified into exosomes, microvesicles, and apoptotic bodies (1). Due to their overlap in size and marker proteins, they are collectively referred to as EVs (which is the term we will use throughout this review) (2). Exosomes may closely reflect regulatory processes in the cell, because they are indirectly derived from the endosomal pathway, and are formed after fusion of multivesicular bodies with the plasma membrane (3). EVs have been identified and characterized in virtually all biofluids, including urine, and have been shown to contain protein, mRNA, miRNA, and DNA (3, 4). EVs can be isolated using ultracentrifugation, filtration, or precipitation, allowing subsequent analysis with mass spectrometry, immunoblotting, or RNA sequencing (5). Novel methods including resistive pulse indexing and nanoparticle tracking analysis also allow characterization, counting and quantification of EVs (6). Transmission electron microscopy, however, is still the main technique used to verify that the sizes of isolated vesicles is indeed consistent with EVs (30 – 1000 nanometer). Protein mass spectrometry-based analysis of urinary extracellular vesicles (uEVs) has identified proteins derived from podocytes, all kidney tubule segments, the urinary tract, and the bladder (3, 7). In addition to biomarker discovery, analysis of uEVs may serve as non-invasive tool to assess renal epithelial cell function in humans. In this context, uEVs are increasingly used for profiling of kidney sodium transporters (8, 9). Indeed, most sodium transport proteins on the apical plasma membrane have been identified in uEVs, including the sodium hydrogen exchanger (NHE3), sodium potassium chloride co-transporter (NKCC2), and sodium chloride co-transporter (NCC) (3, 7). The epithelial sodium channel (ENaC) was more difficult to detect in uEVs until recently (9-11). Given the link between disturbed renal sodium handling and hypertension, the analysis of sodium transporters in uEVs may offer opportunities for pathophysiological or biomarker studies in human hypertension (4, 12, 13). This review will

summarize and discuss recent studies analyzing renal sodium transporters in uEVs.

Key points

- Extracellular vesicles are nanosized vesicles released by all cells and classified into exosomes, microvesicles, and apoptotic bodies.
- The majority of renal sodium transporters have been identified in uEVs and their abundance in uEVs correlates with their kidney expression as influenced by hormonal regulation or disease states.
- Aldosterone increases the phosphorylated form of the sodium-chloride cotransporter (NCC) and the γ-subunit of the epithelial sodium channel in human urinary extracellular vesicles (uEVs).
- Several hypertensive disorders exhibit increased abundance of total or phosphorylated NCC in uEVs, including primary aldosteronism, familial hyperkalemic hypertension, and calcineurin-inhibitor induced hypertension.
- In addition to the use of uEVs as biomarkers, uEVs may also play a biological role in paracrine signaling, innate immunity, and kidney injury repair.

TEXT OF REVIEW

Sodium transporters in uEVs

Initial proteomic analysis of uEVs identified several renal sodium transporters (3, 7), raising a number of questions that have been partly addressed by more recent studies. For example, is lower or higher sodium transporter activity in the kidney always reflected by lower or higher abundance of this transporter in uEVs? Are post-translational modifications detectable in uEV-proteins? Does gender or circadian rhythm affect uEV-protein excretion? Is the hormonal control of sodium transporters reflected in uEVs?

uEVs in genetic disorders of renal sodium transport

The question whether the altered activity of a transporter is also observed in uEVs was studied in monogenetic disorders of renal sodium transport. These studies were performed in patients with Bartter syndrome type 1 or Gitelman syndrome, which are caused by loss-of-function mutations in the genes encoding NKCC2 and NCC, respectively (3, 7). Corbetta *et al.* studied the genetic and biochemical characteristics of patients with Bartter type 1 or Gitelman syndrome and correlated this with NKCC2 and NCC abundances in uEVs (14). NCC abundance in isolated uEVs differentiated between patients with Gitelman syndrome from those with Bartter syndrome or controls, reaching a sensitivity and specificity >80%. A more severe genetic mutation (frameshift or large deletions) resulted in complete absence of NCC in uEVs, whereas less severe mutations (missense mutations) caused NCC abundance to be detectable at lower levels. Similarly, patients with Bartter syndrome type 1 due to a frameshift mutation had a complete absence of NKCC2 in uEVs.

Effects of hormones on sodium transporters in uEVs

Genetic disorders are homogenous. Therefore, a next question was whether uEVs can also be used in more heterogeneous settings, for example hormone-induced changes in protein levels. A logical focus in this regard is the renin-angiotensin-aldosterone system (RAAS). Both NCC and ENaC are aldosterone and angiotensin II -sensitive proteins (15-17). We recently addressed the question whether an increase in aldosterone would translate to increased abundances of NCC and ENaC in uEVs (8) (**Figure 1**). We navigated the previous difficulties to detect ENaC in uEVs by analyzing the accessory protein prostasin. Prostasin is a serine protease that activates ENaC by cleaving the inhibitory tract of γ-ENaC (18, 19). In rats, we infused aldosterone via osmotic minipump and also increased it endogenously by feeding the animals a low sodium diet. Both maneuvers increased NCC and prostasin in kidney homogenates and in uEVs isolated from 24h urine. Aldosterone especially increased the phosphorylated (active) form of NCC, illustrating the ability to detect post-translational modifications in uEVs. This was recently also shown in

healthy subjects, in whom a low potassium diet increased phosphorylated NCC in uEVs, as it did in mouse kidney (20). In addition to animal models of aldosteronism, we also studied phosphorylated NCC and prostasin in uEVs isolated from patients with primary aldosteronism (8). Indeed, both phosphorylated NCC and prostasin were increased in uEVs from these patients. Olivieri *et al.* analyzed prostasin in whole urine and in uEVs (21). Prostasin correlated positively with the aldosterone–renin-ratio and with urinary sodium levels up to 200 mmol/l. Prostasin decreased when urine sodium exceeded 200 mmol/l, possibly reflecting suppression of aldosterone by a high sodium diet. No gender differences were identified. In a subsequent study, the same group identified a circadian rhythm in NCC and prostasin excretion, which decreased from early morning to noon, but then increased towards midnight (22). This pattern resembled the circadian rhythm of vasopressin and urinary aquaporin-2 excretion, but not plasma renin, aldosterone, cortisol, or ACTH. This led the authors to conclude that NCC and prostasin exhibit diurnal variation, which may be determined by vasopressin, which can regulate both NCC and ENaC (23) (**Figure 1**). Qi *et al.* studied the effects of a low sodium diet and aldosterone infusion in healthy subjects and analyzed uEVs by mass spectrometry after these interventions (9). NCC, all ENaC-subunits, NKCC1, and the chloride-bicarbonate exchanger pendrin increased after the low sodium diet, but only γ-ENaC and pendrin remained significant after correction for multiple testing. The phosphorylated form of NCC was not detectable with the mass spectrometry methods used in this study. Of interest, the low sodium diet also increased a number of proteins and proteases involved in ENaC-activation, including furin, kallikrein, and elastase. Surprisingly, however, the abundance of prostasin in uEVs was unchanged. In addition to the low sodium diet, aldosterone infusion also increased γ-ENaC, or, more specifically, the γ-$ENaC_{[112\text{-}122]}$ peptide (**Figure 1**). The increased excretion of γ-$ENaC_{[112\text{-}122]}$ after low sodium diet or aldosterone infusion correlated with both the plasma aldosterone concentration and the urine sodium to potassium ratio (a urine parameter reflecting aldosterone's action on the kidney).

uEVs in hypertensive disorders

uEVs have been analyzed in various hypertensive disorders, including familial hyperkalemic hypertension (FHHt), calcineurin inhibitor (CNI) induced hypertension, and primary aldosteronism, and mostly focused on NCC (10, 11, 24-26) (**Figure 1**). The focus on NCC was rational when uEVs were analyzed in patients with familial hyperkalemic hypertension (FHHt, also called pseudohypoaldosteronism type 2 or Gordon syndrome) (24). FHHt is caused by mutations in kinases or ligase accessory proteins that ultimately regulate NCC activity, including WNK1, WNK4, kelch-like 3, and cullin 3 (27). NCC overactivity and a brisk antihypertensive effect of thiazide diuretics are considered hallmarks of FHHt (28, 29). The expected overactivity of NCC was reflected in uEVs, as total and phosphorylated NCC levels were increased in uEVs from patients with FHHt due to a WNK4 mutation (24, 30). Whether treatment with thiazide diuretics reduced the abundances of NCC was not studied. FHHt shares many features with calcineurin inhibitor (CNI) induced hypertension. CNIs are immunosuppressive drugs that are used after most transplantations to prevent rejection. The occurrence of hypertension after kidney transplantation is associated with poorer graft and recipient survival, illustrating the clinical importance of CNI-induced hypertension. Although CNIs increase vasoconstriction or impair vasodilation, more recent studies have indicated that NCC activation also contributes to CNI-induced hypertension (31, 32). In addition to hypertension, CNIs can also cause the additional tubular disorders seen in FHHt, including hyperkalemia, metabolic acidosis, and hypercalciuria (33). Rojas-Vega *et al.* studied 52 patients six months after kidney transplantation who were using the CNI tacrolimus (25). They found that male participants developed hypertension more frequently than female participants (49 vs. 18%), although this may have been due to included women being younger. In uEVs of hypertensive kidney transplant recipients both total and phosphorylated NCC abundances were increased 1.5- to 2-fold. In contrast, Esteva-Font *et al.* failed to identify higher NCC abundance in 39 kidney transplant recipients taking cyclosporine when comparing them to 8 patients who used a non-CNI immunosuppressive regimen after transplantation

(26). Of interest, a higher abundance of NKCC2 was observed in uEVs of these patients. NKCC2 but also NCC abundance in uEVs correlated with the trough levels of cyclosporine. The effect of cyclosporine on NKCC2 is in agreement with a recent study showing that cyclosporine increased phosphorylation of NKCC2 (34). Although both tacrolimus and cyclosporine inhibit calcineurin, they have different binding proteins and also differ in terms of side-effects, including nephrotoxicity, and diabetogenic effects (35). uEVs were also studied in hypertensive disorders characterized by increased sodium reabsorption through ENaC (10, 11). As proteolytic processing of γ-ENaC increases its open probability, it was postulated that this cleavage would be detectable in uEVs. Indeed, full length and cleaved γ-ENaC are detectable in uEVs (10, 11). In fact, in uEVs from healthy subjects, the fully cleaved form of γ-ENaC (37 kDa) is relatively more abundant compared to uncleaved γ-ENaC (11). The opposite pattern is seen in human kidney tissue (i.e., more uncleaved γ-ENaC). Salt-sensitive hypertension in diabetics has been postulated to result from aberrantly filtered plasminogen, which is converted to plasmin in pre-urine. Plasmin may activate ENaC by proteolytic processing either directly or through prostasin. The phenomenon of ENaC activation by urinary proteases was studied in type 1 diabetics with or without diabetic nephropathy (10). Patients with diabetic nephropathy had increased urinary excretion of plasmin, prostasin, and urokinase, which correlated with the degree of albuminuria. When cortical collecting duct cells were exposed to urine from these patients, this evoked an inward potential, suggesting electrogenic transport through ENaC. Proteolytically cleaved γ-ENaC was present in uEVs isolated from the diabetic nephropathy group but not from the control group, again suggesting ENaC-activation.

uEV biomarkers of hypertension

Protein and miRNA biomarkers have also been studied in uEVs (36, 37). Damkjaer *et al.* compared the uEV-proteome of 11 male patients with essential hypertension to that of 12 matched healthy control subjects (36). They identified two proteins with lower abundances in uEVs isolated from the

patients with essential hypertension, including retinoic acid-induced gene 2 protein (RAIG-2) and syntenin. It is unclear if these proteins directly relate to hypertension. Retinoic acid has been implicated in nephron endowment (38), which may determine predisposition to hypertension, whereas syntenin appears to be primarily involved in the biogenesis of uEVs (36). Gildea *et al.* studied miRNAs by microarray analysis in uEVs isolated from patients with salt-sensitive, salt-resistant, or inverse salt-sensitive hypertension (37). 194 miRNAs were identified in the uEVs of these patients, 45 of which were significantly different between the salt-sensitive and salt-resistant individuals, or between the inverse salt-sensitive and salt-resistant subjects. However, hsa-miR-4516 was the only miRNA that could differentiate salt-sensitive from inverse salt-sensitive hypertension. Several of the identified miRNAs pointed in the direction of hypertensive pathways, including PPAR-γ, EGFR, TGF-β1, and PTEN/PI3K. EGFR regulates PPAR-γ-mediated sodium and water reabsorption via upregulation of NHE3 and aquaporin-1 (39). Furthermore, PPAR-γ has been implicated in the regulation of ENaC via SGK1 (40). TGF-β1 inhibits prostasin expression and may thereby reduce the open probability of ENaC (41, 42). Oxidative stress (H_2O_2) increases activation of ENaC by increasing PI3K and reducing PTEN (43). Similarly, PI3K is involved in the insulin-induced activation of NCC (44), while PPAR-γ decreases NCC activity in obese pre-diabetic rats (45).

Role of EVs beyond biomarkers

An emerging field is the biological role of EVs in paracrine signaling, innate immunity, and kidney injury repair (46-49). For example, Street *et al.* studied whether a vasopressin-induced increase in the water channel aquaporin-2 could be transferred to other cells via EVs (49). When desmopressin-unexposed cells were incubated with EVs from desmopressin-exposed cells, water transport and AQP2 increased, suggesting that the signal to produce more AQP2 was transferred via EVs. Another question is if EVs may mediate inter-nephron crosstalk. Gildea *et al.* addressed this question *in vitro* by using cell lines from different tubular segments (47). Fluorescently labeled EVs derived from

proximal tubule cells were taken up by proximal tubule, distal tubule and collecting duct cells (47). When proximal tubule cells were exposed to fenoldopam (a dopamine receptor agonist), EV production increased and transfer of these EVs to distal tubule and collecting duct cells reduced the basal reactive oxygen species production in these recipient cells (47), again suggesting transfer of EV-content. Another recent finding is the potential role of EVs in the innate immune response to pathogens in the urinary tract (48). Proteomic profiling of uEVs identified several proteins related to the innate immune system, including mucin-1, myeloperoxidase, lysozyme C and dermcidin. uEVs were then studied in a lysis assay with *Escherichia Coli*, the organism most commonly responsible for urinary tract infection. In this assay, uEVs were shown to be capable of inducing lysis of *Escherichia Coli* in a dose-dependent fashion. Finally, EVs derived from mesenchymal stem cells may enhance the morphological and functional recovery from acute kidney injury, possibly through miRNAs (46).

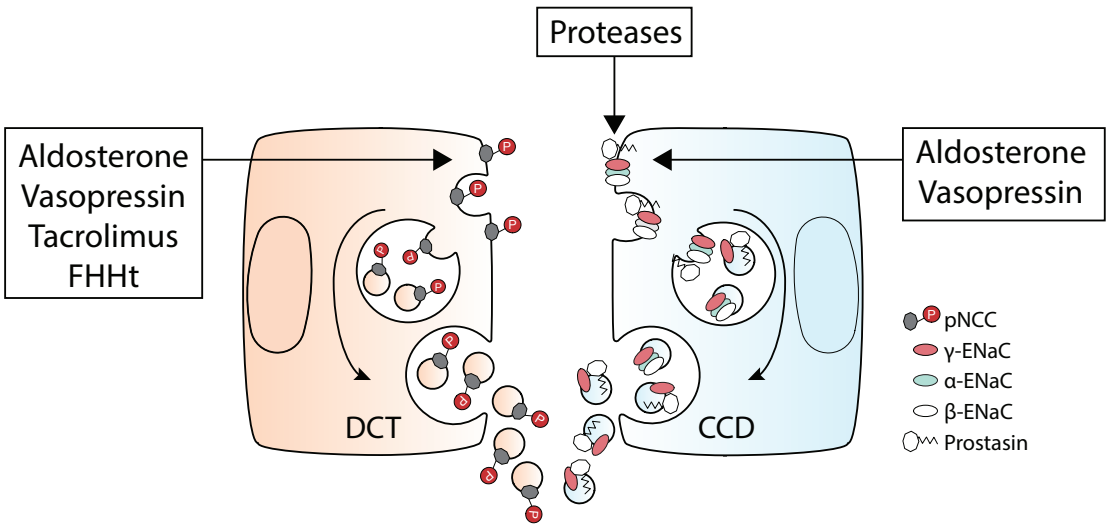

Figure 1: The formation of urinary extracellular vesicles in cells from the distal convoluted tubule (DCT) and cortical collecting duct (CCD). *As shown, the process resembles the formation of one group of vesicles, namely exosomes. Urinary exosomes are formed via endocytosis of (membrane) proteins and subsequent fusion of multivesicular bodies with the plasma membrane (here indicated by arched arrow). The figure focuses on the activation of the sodium chloride cotransporter (NCC) and the epithelial sodium channel (ENaC) through phosphorylation and proteolytic cleavage by prostasin, respectively. The boxes show factors that increase phosphorylated NCC and cleaved γ-ENaC both in kidney and in uEVs.*

CONCLUSION

Analysis of sodium transport proteins in uEVs allows a read-out of renal epithelial sodium transport. In the studies reviewed, the direction of change in sodium transporter abundance in uEVs was identical to the expected change in the kidney, although few studies directly correlated kidney and uEV proteins. Aldosterone-induced post-translational modifications in sodium transporters, including phosphorylation and proteolytic processing, are detectable in uEVs. Sodium transporter analysis in uEVs has provided disease correlates for monogenetic disorders of sodium transport, aldosteronism, and CNI-induced hypertension. In addition to their role as potential biomarkers, uEVs may also be biologically active in paracrine signaling, the innate immune response, and kidney injury repair.

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CHAPTER

05

CUSHING'S SYNDROME INCREASES RENAL SODIUM TRANSPORTERS IN URINARY EXTRACELLULAR VESICLES

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Submitted

ABSTRACT

Context: Increased renal sodium reabsorption contributes to hypertension in Cushing's syndrome (CS). Renal sodium transporters can be analyzed non-invasively in urinary extracellular vesicles (uEVs) and correlate with their activity in kidney.

Objective: To analyze renal sodium transporters in uEVs of patients with CS.

Design: Observational study in outpatients with newly diagnosed CS.

Setting: University hospital.

Patients and Interventions: uEVs were isolated by ultracentrifugation and analyzed by immunoblotting in 10 CS patients and 7 age-matched healthy subjects. In 3 CS patients uEVs were analyzed before and after treatment.

Results: The 10 patients with CS were hypertensive, and were divided in those with suppressed and non-suppressed renin-angiotensin-aldosterone system (RAAS, n = 5/group). CS patients with suppressed RAAS had similar blood pressure but significantly lower serum potassium than CS patients with non-suppressed RAAS. Compared to healthy subjects, all CS patients had increased abundance of the Na^+/H^+ exchanger type 3 (NHE3) in uEVs. In contrast, only those with suppressed RAAS had higher phosphorylated Na^+-K^+-Cl^- cotransporter type 2 (pNKCC2) and higher total and phosphorylated Na^+-Cl^- cotransporter (NCC) in uEVs. Serum potassium but not urinary free cortisol correlated with pNKCC2, pNCC, and NCC in uEVs. Treatment of CS reduced pNKCC2, pNCC, and NCC abundances in parallel with serum potassium.

Conclusions: CS increases renal sodium transporter abundance in uEVs especially in patients with suppressed RAAS. Potassium has recently been identified as an important driver of NCC activity. Therefore, in addition to excess glucocorticoids, low serum potassium may also contribute to increased renal sodium reabsorption and hypertension in CS.

INTRODUCTION

A common clinical feature of Cushing's syndrome (CS) is hypertension, which occurs in approximately 75% of patients (1,2). Due to the pleiotropic effects of glucocorticoids, the pathogenesis of hypertension in CS is believed to be multifactorial (3-8).

In the kidneys, excess glucocorticoids increase renal blood flow and increase renal tubular sodium reabsorption (3). In fact, glucocorticoids have been shown to be capable of activating all of the major sodium transport proteins along the nephron (9-12). This includes the Na^+/H^+ exchanger type 3 (NHE3) in the proximal tubule (9), the Na^+-K^+-Cl^- cotransporter type 2 (NKCC2) in the thick ascending limb of the loop of Henle (13), the Na^+-Cl^- cotransporter (NCC) in the distal convoluted tubule (10), and the epithelial sodium channel (ENaC) in the collecting duct (3,11). In addition to these sodium transporters expressed at the apical plasma membrane, glucocorticoids can also increase activity of the basolateral Na^+-K^+-ATPase (14). Activation of these transport proteins may be mediated through activation of the glucocorticoid receptor, which is expressed throughout the nephron (3,15). In addition, supraphysiological concentrations of cortisol can overwhelm the capacity of 11β-hydroxysteroid dehydrogenase type 2 to inactivate cortisol to cortisone. This enables activation of the mineralocorticoid receptor with subsequent activation of NCC and ENaC. Because ENaC is electrochemically coupled to potassium secretion, CS may cause hypokalemic hypertension, similar to primary aldosteronism (16,17). However, mineralocorticoid receptor antagonists do not fully prevent hypertension in CS, suggesting that this mechanism does not fully explain hypertension in CS (16,18).

The effects of glucocorticoids on renal sodium transport have mainly been studied *in vitro* and in experimental animals (9-11,13,14). Urinary extracellular vesicles (uEVs) are nanosized vesicles that can be isolated from human urine and contain most renal transport proteins (19). Thus, isolation of uEVs in

healthy subjects and patients now allows for a non-invasive analysis of renal sodium transport. Previously we confirmed both in rats and humans that aldosterone activation of sodium transporters in the kidney results in corresponding changes of these transporters in uEVs (20). In the present study, we analyze several of the renal sodium transport proteins in uEVs isolated from patients with CS and hypertension. In addition to total abundances, we also analyze the phosphorylated forms of NCC and NKCC2, which are considered the active forms of these transporters (21). Because ENaC is difficult to analyze in uEVs, we instead analyzed one of its activating serine proteases, prostasin, as has been done previously (20,22). Finally, we analyzed the pleiotropic kinase Rac1, because it has been considered a measure of mineralocorticoid receptor activity, and is detectable in uEVs (23,24).

MATERIALS AND METHODS

Patients

Our Medical Ethics Committee approved this study (MEC-2007-048). Consecutive patients who were newly diagnosed with CS in our center were considered for inclusion during the period 2012–2015. To analyze renal sodium transporters in uEVs, we excluded patients who were using interfering medication (renin-angiotensin inhibitors or diuretics). Although beta-blockers may increase plasma renin, this effect is considered less strong, and these drugs were therefore allowed. Using these criteria, we included 10 out of the 18 patients who were newly diagnosed with CS. Patients with CS were further subdivided in those with or without suppressed plasma renin-angiotensin aldosterone system (RAAS). The rationale for this was that patients with suppressed RAAS are more likely to have increased renal sodium reabsorption, which may be reflected in uEV transporter status. In 3 of the 10 patients uEVs were analyzed before and after treatment. Seven patients were excluded from further analysis, including four who were started on interfering medication

(renin-angiotensin inhibitors or diuretics), and three in whom no remission was reached at the end of the study period. Healthy subjects were recruited and matched by age (age 44 ± 3 years; 3 men, 4 women); they did not use any drugs and had no hypertension or history of endocrine disorders.

Measurements

Blood pressure was measured in supine position using an automatic oscillometric device for at least 15 minutes; the average of the last three measurements was used for analysis. Hypertension was defined as systolic blood pressure of ≥ 135 mmHg and/or diastolic blood pressure of ≥ 85 mmHg (25). Both 24-hour urine and spot urines were collected. Plasma and urine electrolytes were measured using an ion-selective electrode (ISE indirect, Cobas, Roche Diagnostics, Mannheim, Germany). Creatinine was measured using an enzymatic colirometric method (Crep2, Cobas, Roche Diagnostics). Renin in plasma was measured using an immunoradiometric kit (Cisbio Bioassays, Codolet, France). Aldosterone was measured by solid-phase radioimmunoassay (Diagnostic Products Corporation, Los Angeles, California, USA) and cortisol was measured using liquid chromatography coupled to mass spectrometry. Urinary free cortisol is expressed as the fold elevation above the upper limit of normal.

Isolation and immunoblotting of uEVs

Spot urines were treated with a protease inhibitor (Roche Complete, Woerden, The Netherlands) and centrifuged (3000 x g for 5 minutes) to remove cells and cell debris before storage at -80 °C. uEVs were isolated using high-speed and ultracentrifugation, as described previously (26). Briefly, urine was first centrifuged at 17,000 x g for 15 minutes to pellet high-density particles, separating the supernatant (supernatant 1). Dithiothreitol was then used to disrupt the Tamm-Horsfall polymers, after which the samples were diluted in isolation buffer and centrifuged at 17,000 x g (supernatant 2). The two supernatants were then ultracentrifuged at 200,000 x g for 2 hours. The pellets were suspended in Laemmli buffer for immunoblot analysis, and subsequently

heated at 60 °C for 15 minutes. uEVs of CS patients and healthy control subjects were isolated simultaneously. Urine creatinine was used for normalization of spot samples, as done previously. SDS-PAGE was carried our on a gradient gel (4-20%) after which the gel was transferred to trans-blot turbo system (Bio-Rad, Hercules, CA, USA). The membranes were blocked in 5% milk and incubated overnight at 4 °C. Antibodies against the following proteins were used: NHE3 (1:1000, Stressmarq; (27)), NCC (1:1000, Stressmarq; (28)), pNCC (1:500, kindly provided by Dr. Fenton; (29)), NKCC2 (1:1000, kindly provided by Dr. Knepper; (30)), (1:2000, kindly provided by Dr. Mutig; (31)), Rac1 (1:500, Millipore; product # 05-389, (32)), (1:1000, AQP2, Stressmarq; (33)), Na^+/K^+-ATPase (1:250, Abcam; product # ab7671), CD9 (1:500, Santa Cruz Biotechnology; product # sc-13118). Secondary antibodies were peroxidase conjugated goat anti-rabbit or mouse (1:3000, Sigma-Aldrich).

Statistical analysis

Results are expressed as mean and standard deviation or median and range, as appropriate. Data were logarithmically transformed before analysis in case of non-normal distribution. Student's t-test or analysis of variance (ANOVA) was used for group comparison. A paired *t*-test was used to analyze effects in uEVs before and after treatment. A P-value < 0.05 was considered statistically significant. Statistical analyses were performed with SPSS (version 21, IBM).

RESULTS

Patient characteristics

Ten patients with CS were recruited of whom the majority had an ACTH-producing pituitary adenoma (1 macroadenoma, 7 microadenoma) (**Table 1**). The average blood pressure was elevated (systolic blood pressure 144 ± 14

mmHg, diastolic blood pressure 92 ± 17 mmHg). Two patients used metoprolol. The ten patients with CS were further divided in those with suppressed RAAS and those with non-suppressed RAAS (**Table 1**). CS patients with suppressed RAAS had significantly lower body mass index, higher estimated glomerular filtration rate, and lower serum potassium levels. No differences in blood pressure were observed between the two groups.

Cushing's syndrome increases renal sodium transporters in uEVs

Compared to healthy subjects, patients with CS expressed more NHE3 in uEVs regardless of whether RAAS was suppressed or not (**Figure 1**). The abundance of NHE3 was approximately two-fold higher in uEVs of patients with CS. In contrast, total NCC and the phosphorylated forms of NCC and NKCC2 were only more abundant in uEVs of patients with CS and a suppressed RAAS. In these patients, the uEV abundances of NCC and NKCC2 were increased three- to four-fold. None of the other analyzed proteins in uEVs showed significant differences in abundance, including aquaporin-2, Rac1, and prostasin.

Serum potassium determines sodium cotransporter status in uEVs

Although still in the normal range, serum potassium was significantly lower in patients with CS and suppressed RAAS (**Table 1**). Serum potassium has recently been recognized as an important driver of NCC activity (34,35). Therefore, we analyzed the correlation between serum potassium and aldosterone and serum potassium and the NKCC2 and NCC, which were upregulated in uEVs of patients with CS and a suppressed RAAS (**Figure 1**). Plasma aldosterone correlated with serum potassium (**Figure 2**). However, plasma aldosterone levels were suppressed despite serum potassium concentrations in the normal range. This suggests that cortisol exerted a mineralocorticoid effect, thereby both lowering plasma aldosterone and serum potassium. Furthermore, serum potassium negatively correlated with NCC, pNCC, and pNKCC2 abundances in uEVs. No correlation was observed between urinary cortisol levels, NCC, pNCC and pNKCC2.

Parameters	All patients (n = 10)	Suppressed RAAS (n = 5)	Non-suppressed RAAS (n = 5)	P-value
Clinical				
Age, years	43.2 ± 7.8	40.8 ± 10.9	45.6 ± 2.3	0.4
Male gender, n (%)	2 (20)	2 (20)	3 (43)	0.6
BMI, kg/m^2	29 ± 8	24 ± 3	34 ± 8	0.02
Cause of Cushing				
• Pituitary adenoma, n (%)	8 (80)	3 (60)	5 (80)	0.5
• Adrenocortical adenoma, n (%)	2 (20)	2 (40)	0	0.2
Systolic BP, mmHg	144 ± 14	141 ± 18	147 ± 11	0.5
Diastolic BP, mmHg	92 ± 17	89 ± 23	96 ± 11	0.5
Blood				
Sodium, mmol/L	141 ± 3	142 ± 3	140 ± 2	0.2
Potassium, mmol/L	4.2 ± 0.4	3.9 ± 0.2	4.4 ± 0.3	0.04
eGFR, mL/min/1.73 m^2	94 ± 18	107 ± 10	80 ± 12	< 0.01
Renin, uU/mL	18.5 (7.1–89.3)	9.7 (7.1–16.6)	41.5 (16.9–89.3)	N.T.
Aldosterone, pg/mL	40.8 (10.0–187.0)	23.5 (10.0–45.0)	81.3 (13.0–187.0)	N.T.
Urine				
Sodium, mmol/day	196 (138 – 273)	196 (138 – 273)	196 (173 - 247)	0.1
Free cortisol, x ULN*	1.9 (0.3 – 6.1)	2.0 (0.3 – 6.1)	1.6 (1.0 – 2.3)	0.4

Table 1: *Clinical characteristics, blood, and urinary measurements of patients with Cushing's syndrome with or without suppressed renin-angiotensin-aldosterone system (RAAS). BMI, body mass index; BP, blood pressure; eGFR, estimated glomerular filtration rate; N.T., not tested; ULN, upper limit of normal. *Average of two measurements.*

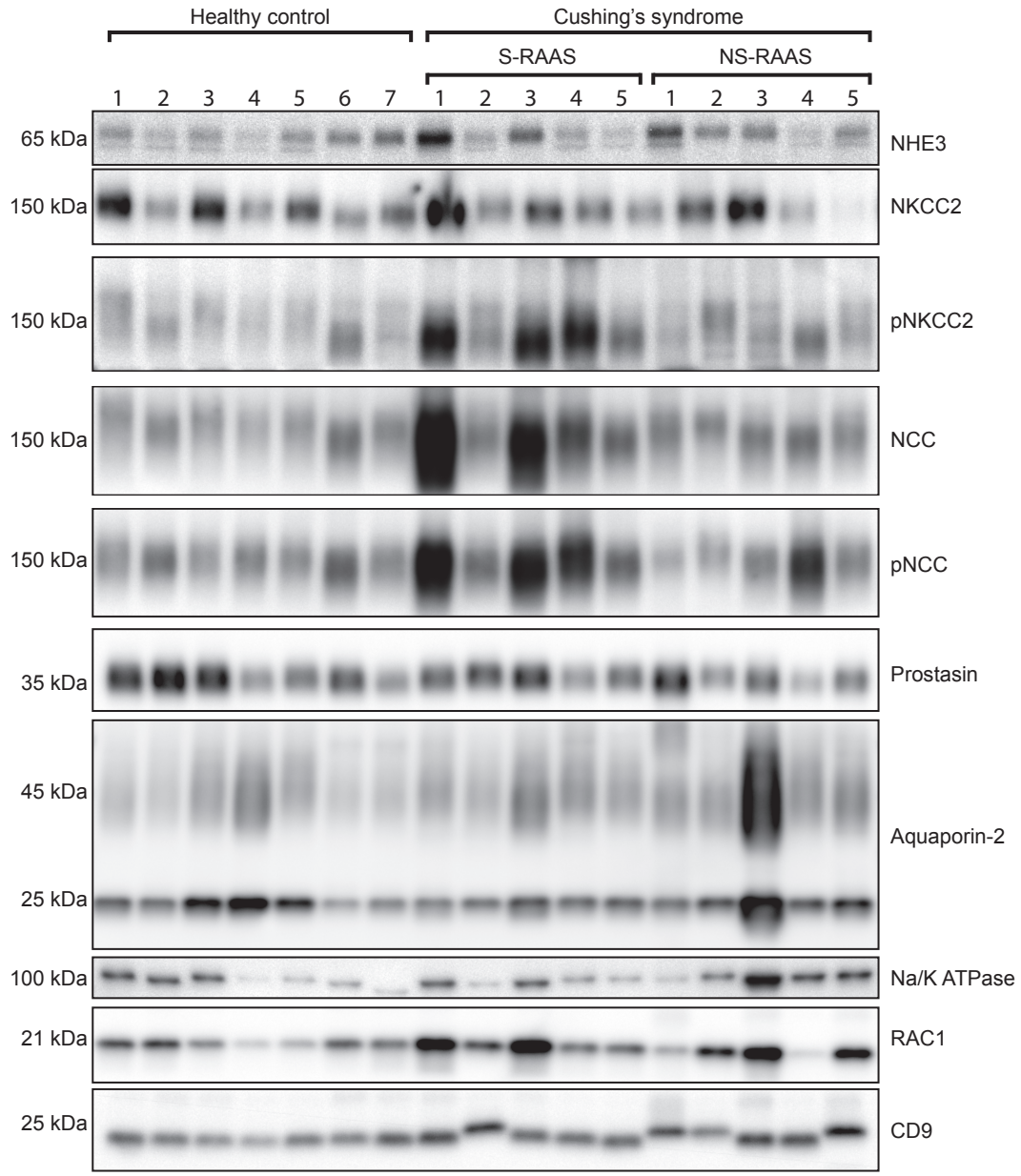

Figure 1A: Immunoblot analysis of renal sodium transporters and related proteins in urinary extracellular vesicles from patients with Cushing's syndrome (CS) and healthy subjects. *Patients with CS were divided based on a suppressed (S) or non-suppressed (NS) renin-angiotensin-aldosterone system (RAAS).*

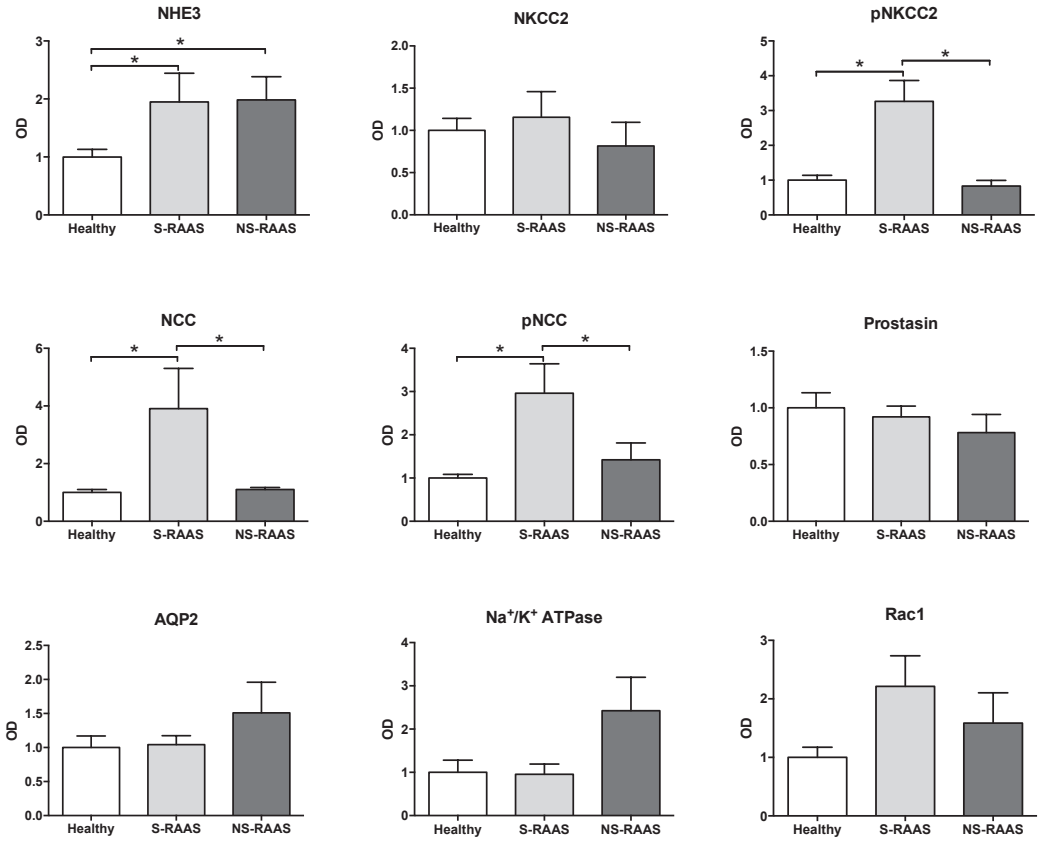

Figure 1B: *Group comparisons of immunoblot densitometry were performed with analysis of variance using log-transformed data of optical densitometries (OD). * P < 0.01.*

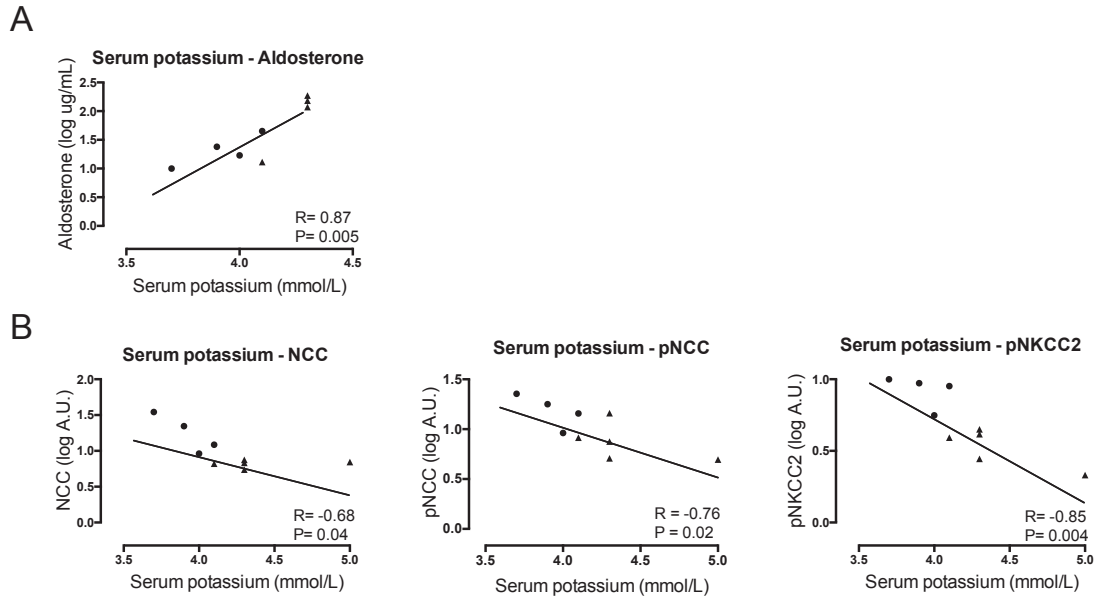

Figure 2: *Correlations between the serum potassium concentration and aldosterone (**panel A**) and serum potassium, pNKCC2, pNCC, and NCC (**panel B**) in uEVs of patients with Cushing's syndrome.*

Effect of treatment

Three patients were successfully treated during the study period and in these patients uEVs were analyzed before and after treatment. Patient 1 (non-suppressed RAAS) was treated by unilateral adrenalectomy, while patients 2 and 3 (both suppressed RAAS) were treated by ketoconazole. In all patients, serum potassium increased after treatment from 4.0 ± 0.3 to 4.4 ± 0.06 mmol/l. In uEVs, the abundances of pNKCC2, NCC, pNCC and Rac1 decreased significantly after treatment (2.0 to 2.6-fold, all $P < 0.05$, **Figure 3**).

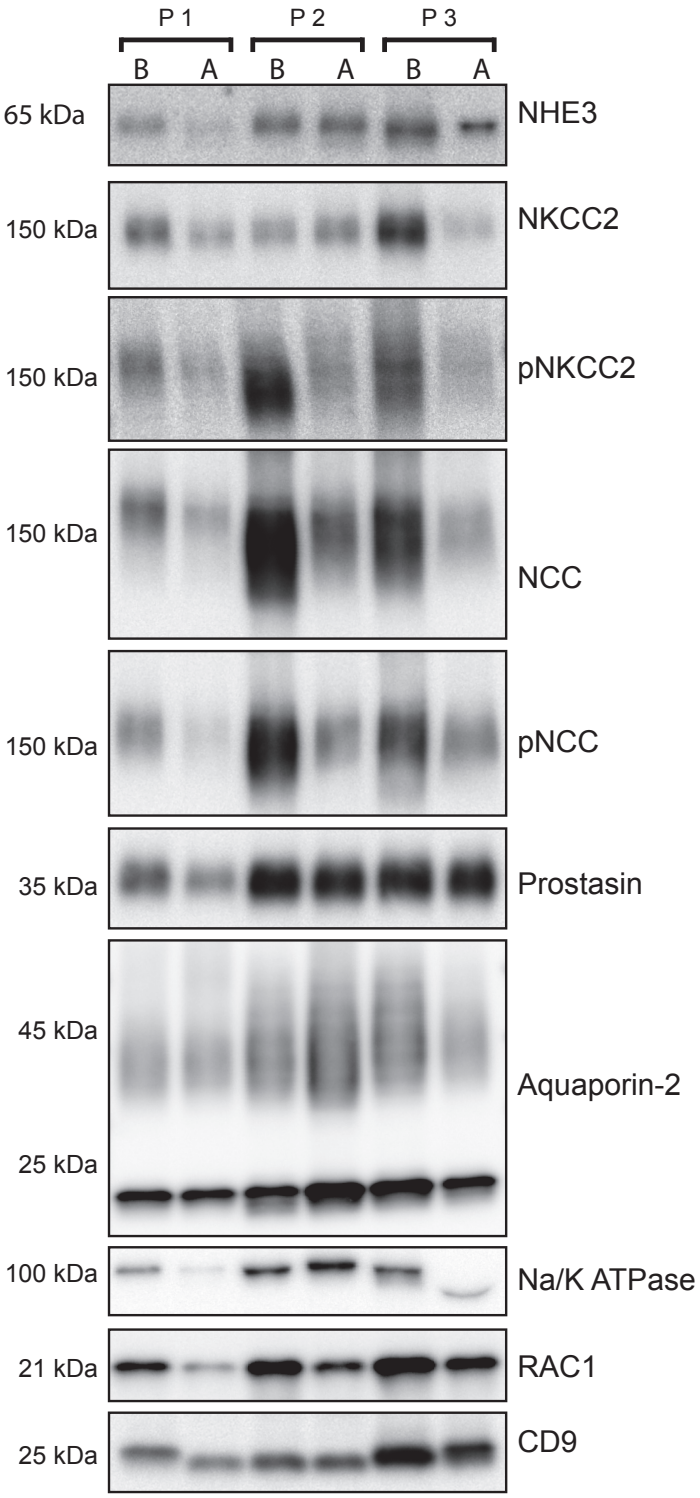

Figure 3A: Immunoblot analysis of renal sodium transporters and related proteins in uEVs from patients with Cushing's syndrome before (B) and after (A) treatment.

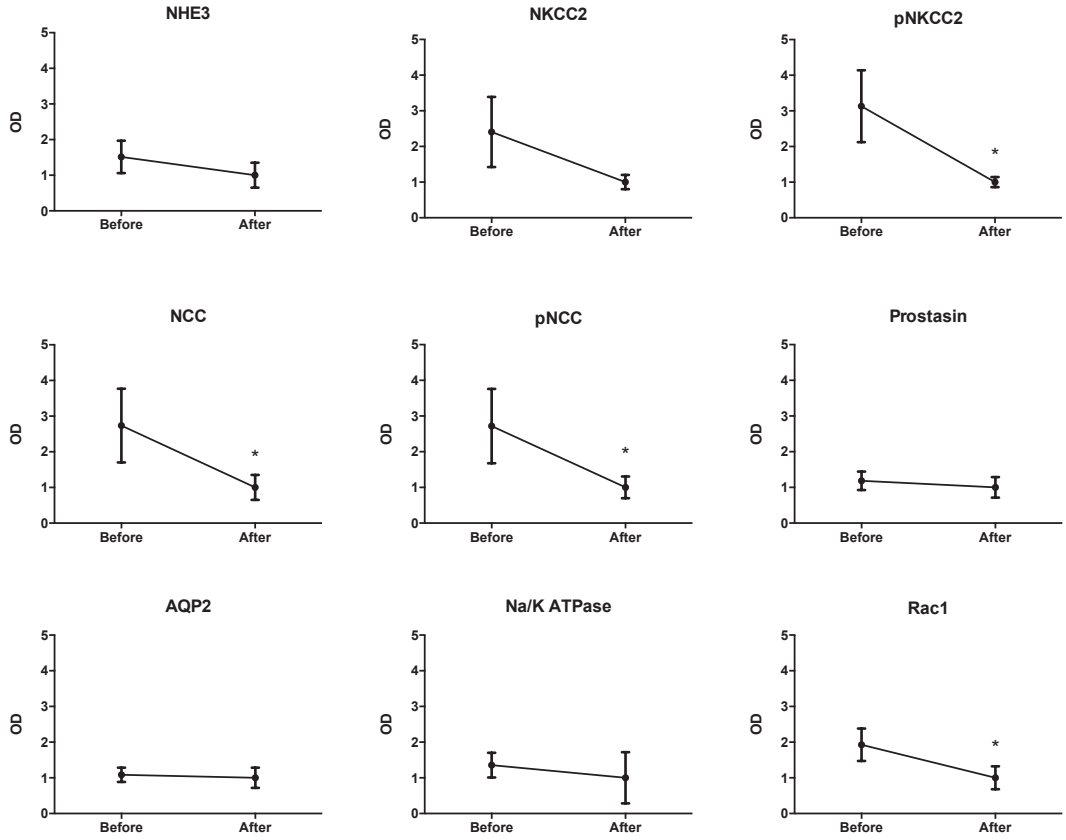

Figure 3B: *The log-transformed optical densitometries (OD) were analyzed using a paired t-test in which the post-treatment (after) abundances were set at 1. * P < 0.05.*

DISCUSSION

In this study we isolated and analyzed urinary extracellular vesicles (uEVs) in patients with Cushing's syndrome (CS) and hypertension to analyze renal sodium transport. uEVs are nanosized vesicles that contain all of the major renal sodium transport proteins and reflect their activity in kidney (19,20,36,37). Using this approach, we found that all of the CS patients included in this study had a higher abundance of NHE3 in uEVs compared to healthy subjects. In contrast, only CS patients with a suppressed RAAS had increased abundances of NKCC2 and NCC. Of interest, this activation of NKCC2 and NCC appears not to reflect the mineralocorticoid effect of

hypercortisolism, but rather an indirect effect induced by reduced serum potassium concentration. This is supported by the observation that these transporter abundances reduced after treatment in parallel with an increase in serum potassium. Indeed, potassium has recently been identified as a major driver of NCC activity (34,35,38,39). Our results are in perfect agreement with recent experimental and clinical studies showing a linear relationship between the serum potassium concentration and NCC expression in kidney and uEVs (38,40). This effect of potassium appears to be specific for NCC and it is therefore unclear if the observed relation between serum potassium and pNKCC2 can be explained by similar mechanisms (34).

Together, this proof-of-principle study provides insight in renal sodium handling during CS and the pathogenesis of hypertension in CS (summarized in **Figure 4**). Previous studies on hypertension in CS also showed that some patients with CS have a suppressed RAAS, whereas others do not (41-45). Indeed, several mechanisms contribute to hypertension in CS. In addition to effects on renal sodium reabsorption, hypercortisolism can also increase the peripheral vascular sensitivity to adrenergic agonists (46), and the production of angiotensinogen by the liver (44). The increased uEV abundances of NHE3, pNKCC2, pNCC, and NCC in CS observed in this study merit further discussion. The stimulatory effect of glucocorticoids on NHE3 has long been recognized (47), and is likely mediated through serum and glucocorticoid inducible kinase 1 and the scaffold protein NHERF2 (9,48-50). Glucocorticoid-mediated NHE3 activation may contribute to hypertension, because it limits pressure natriuresis (51). Based on our data, increased NHE3 activity appears a rather universal finding in patients with CS. Tubuloglomerular feedback can compensate for increased sodium reabsorption through NHE3 in the proximal tubule, which may explain why increased NHE3 uEV-abundance was also observed in patients without suppressed RAAS. The more selective increase of pNKCC2, pNCC, and NCC in uEVs of patients with CS and a suppressed RAAS, suggests that increased sodium reabsorption through these transporters contributed to an increase in extracellular fluid volume (and therefore

suppression of the RAAS). This may also explain the higher estimated glomerular filtration rate. The glucocorticoid activation of NCC is well described. In adrenalectomized rats, Velazquez *et al.* showed that both aldosterone and dexamethasone increased NCC-mediated sodium reabsorption fivefold (10). More recently, Ivy *et al.* showed that glucocorticoids cause nondipping of blood pressure via NCC activation (52). Glucocorticoid and mineralocorticoid receptors are expressed in the distal convoluted tubule, and activation of these receptors could therefore explain NCC activation (3). However, recent data indicate that NCC activation may occur indirectly via potassium. For example, Veiras *et al.* showed that angiotensin II stimulates sodium-potassium exchange through ENaC and the potassium channel ROMK (53). This results in potassium deficiency and thereby NCC activation (53). Wolley *et al.* confirmed this mechanism clinically in patients who were screened for primary aldosteronism using the fludrocortisone test (40). Similar to our data, they also showed that serum potassium was strongly and negatively correlated with pNCC and NCC in uEVs (40). Similarly, glucocorticoids may have activated ENaC and ROMK with increased potassium secretion and a decrease in serum potassium (11,13,54). Unfortunately, ENaC is difficult to analyze in uEVs (55). We used prostasin as surrogate marker for ENaC, but found no consistent effect of glucocorticoids. However, ENaC activation is not universally accompanied by an increase of prostasin in uEVs, as recently shown by Qi *et al* (56). Finally, it is unclear how to explain the increase in pNKCC2. In adrenalectomized rats, Stanton did not observe that glucocorticoids increased sodium reabsorption in the thick ascending limb (57). Frindt and Palmer, however, did show an increase in NKCC2 in dexamethasone treated rats (11).

A number of limitations should be mentioned. First, this was a proof-of-principle study in a small number of patients. Because this was an observational study we could not discontinue anti-hypertensive drugs and therefore excluded patients already on renin-angiotensin inhibitors or diuretics. However, one could argue that the effects in these patients may have been even stronger. Second, although several groups have shown that transporter analysis in uEVs

correlates with their activity in the kidney (19,20,36,37), this still remains an indirect measure of true transporter activity. We tried to address this by analyzing the phosphoproteins of the transporters, which are generally considered the active forms (21).

In conclusion, CS increases renal sodium transporter abundance in uEVs especially in patients with suppressed RAAS. In addition to excess glucocorticoids, low serum potassium may also contribute to increased renal sodium reabsorption and hypertension in CS.

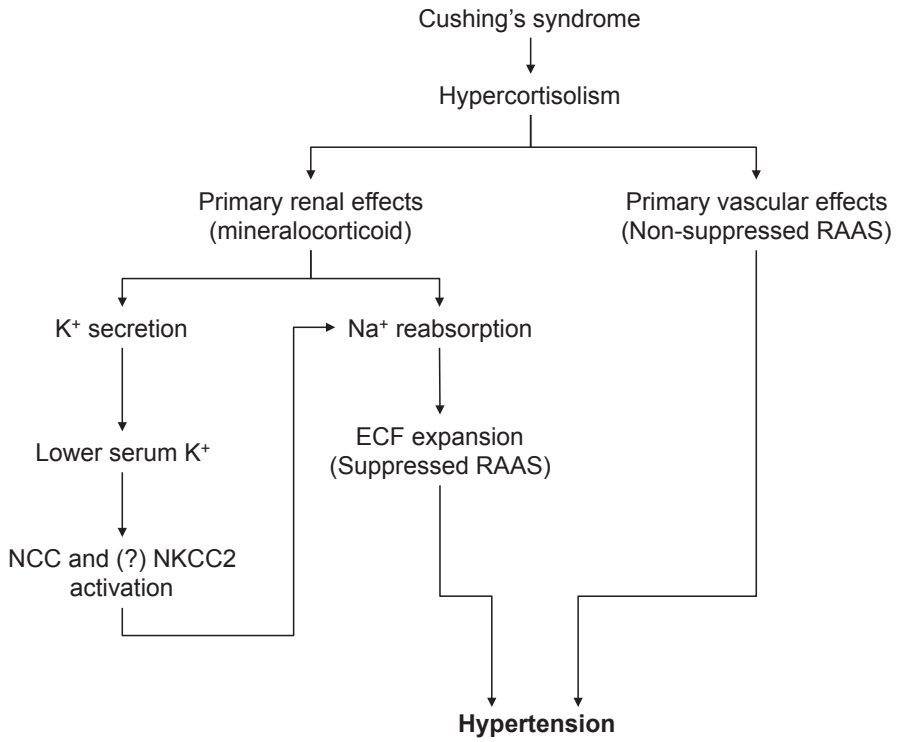

Figure 4: Proposed model for the pathogenesis of hypertension in Cushing's syndrome including vascular and renal effects. *The contribution of activation of renal sodium transport is partly based on the results in this study.*

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CHAPTER

06

THE PHOSPHORYLATED SODIUM CHLORIDE COTRANSPORTER IN URINARY EXOSOMES IS SUPERIOR TO PROSTASIN AS A MARKER FOR ALDOSTERONISM

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ABSTRACT

Urinary exosomes are vesicles derived from renal tubular epithelial cells. Exosomes often contain several disease-associated proteins, and are thus useful targets for identifying biomarkers of disease. Here, we hypothesized that the phosphorylated (active) form of the sodium chloride cotransporter (pNCC) or prostasin could serve as biomarkers for aldosteronism. We test this in two animal models of aldosteronism (aldosterone infusion or low sodium diet) and in patients with primary aldosteronism. Urinary exosomes were isolated from 24-hour urine or spot urine using ultracentrifugation. In rats, a normal or a high dose of aldosterone for two, three or eight days increased pNCC 3-fold in urinary exosomes ($p < 0.05$ for all). A low sodium diet also increased pNCC in urinary exosomes approximately 1.5-fold after four and after eight days of treatment. The effects of these maneuvers on prostasin in urinary exosomes were less clear, showing a significant 1.5-fold increase only after two and three days of high aldosterone infusion. In urinary exosomes of patients with primary aldosteronism, pNCC was 2.6-fold higher ($p < 0.05$) while prostasin was 1.5-fold higher ($p = 0.07$) than in patients with essential hypertension. Urinary exosomal pNCC and, to a lesser extent, prostasin are promising markers for aldosteronism in experimental animals and patients. These markers may be used to assess the biological activity of aldosterone and potentially as clinical biomarkers for primary aldosteronism.

INTRODUCTION

Urinary proteins originate from various sources. They may be derived from glomerular filtration, tubular secretion, shedding, glycosylphosphatidyl inositol anchored protein detachment (e.g., Tamm-Horsfall protein), or exosome secretion (1). Exosomes are low-density membrane vesicles that originate from multivesicular bodies. Urinary exosomes have sparked interest as potential biomarkers for human disease (1-3). The presence of urinary exosomes and a reproducible method for their isolation was reported in 2004 by Pisitkun and colleagues (4). Proteomic analysis of these exosomes showed that they contain many disease related proteins (4, 5). However, the question remained whether the presence of a given protein in urinary exosomes could provide information on physiological or disease processes in the kidney. Studies addressing this question analyzed urinary exosomes in patients with monogenetic diseases resulting in inactivity or overactivity of renal sodium transport proteins. For example, in Bartter and Gitelman syndrome, in which the sodium potassium chloride cotransporter (NKCC2) and the sodium chloride cotransporter (NCC) are genetically inactivated, these proteins were also found to be absent or reduced in urinary exosomes (5, 6). Conversely, Mayan *et al.* found the abundance of the sodium chloride cotransporter (NCC) to be increased in patients with familial hyperkalemic hypertension, in which mutations in NCC-regulating kinases cause overactivity of this cotransporter (7). Thus, in these homogeneous groups the expression of sodium transport proteins in urinary exosomes correlated with what one would expect from their renal expression. The next step in assessing the potential of exosomes as urinary biomarkers is to analyze their performance in acquired disease. Therefore, in this study, we asked whether various forms of aldosteronism resulted in increased expression of aldosterone-sensitive proteins in urinary exosomes. To address this question, we used animal models of primary and secondary aldosteronism and also studied patients with primary aldosteronism. In the kidney, the two main sodium transporters activated by aldosterone are NCC and the epithelial sodium channel (ENaC) (8, 9). It therefore appears logical to study NCC and

ENaC in urinary exosomes in different forms of aldosteronism. Esteva-Font *et al.*, however, found no difference in the abundance of NCC in urinary exosomes of patients with salt-sensitive hypertension (10). Recently, it has become clear that the phosphorylated form of NCC (pNCC) represents the active form of NCC and that trafficking and phosphorylation of NCC can be regulated independently (11). Therefore, we propose that pNCC is a better reflection of NCC's biological activity. ENaC is difficult to study in urinary exosomes because it is present in very low quantities (4). Instead, prostasin has emerged as an interesting surrogate marker of ENaC activity (12). Prostasin is a serine protease that can increase the activity of ENaC and is also sensitive to aldosterone (13). Here, we test the hypothesis that pNCC and prostasin in urinary exosomes are markers for aldosteronism.

METHODS

Animal studies

All animal protocols were approved by the Animal Care Committee of the Erasmus University Medical Center Rotterdam (EUR 127-11-01 and EUR 127-10-11). Three studies were conducted. In the *first* study, ten 15-week old male Sprague-Dawley rats (Charles River, Sulzfeld, Germany) were adrenalectomized via a bilateral lumbodorsal incision and randomized to receive either high-dose aldosterone (100µg/kg/day) or vehicle via osmotic minipump (subcutaneous insertion, Alzet, Cupertino CA, USA). All rats also received glucocorticoid replacement (dexamethasone, 5µg/kg/day) and an angiotensin receptor blocker (losartan, 10 mg/kg/day) to inhibit the effects of angiotensin II on pNCC and prostasin (14, 15). The *second* study was similar to the first one, but now included a third group of five rats that received a normal dose of aldosterone (50 µg/kg/day) (16). In addition, this study lasted for eight days instead of three days. In the second study, we also harvested the right kidney for immunoblotting analysis. In the *third* study twelve rats were randomly assigned

to receive a normal (0.5%) or low (0.001-0.002%) sodium chloride diet for eight days (Harlan diets, Harlan Laboratories, Venray, The Netherlands). In all three experiments, animals were housed in metabolic cages to collect 24-hour urine for isolation of urinary exosomes. Urinary exosomes were isolated at various time-points in the three studies (days 1, 2, and 3 in the first study, day 8 in the second study, and days 0, 4, and 8 in the third study). Finally, at the end of each experiment, plasma renin and aldosterone as well as urine sodium were measured.

Studies in patients

Five patients with primary aldosteronism and four patients with essential hypertension were randomly selected from an ongoing study on primary aldosteronism (17). Briefly, patients were eligible to participate in this study when they had uncontrolled hypertension despite the use of at least two antihypertensive drugs. All patients were subjected to volume expansion (two litres of 0.9% NaCl during four hours) to analyze whether aldosterone was suppressible. Primary aldosteronism was defined as unsuppressible plasma aldosterone (> 235 pmol/L) after volume expansion. The patients whose post-test aldosterone was below 235 pmol/L were considered to have essential hypertension. In all patients, spot urine was collected under controlled circumstances for isolation of urinary exosomes. Urine sodium, potassium, and creatinine concentrations were measured as well.

Isolation and immunoblot analysis of urinary exosomes

Urinary exosomes were isolated as reported previously (4, 18, 19); a more detailed protocol is provided as Online Supplement. Briefly, all urine samples were treated with a protease inhibitor prior to storage at –80°C; no phosphatase inhibitors were used. Urinary exosomes were isolated using a two-step centrifugation process. First, urine was centrifuged at 17,000 x g for 15 minutes at 37°C to remove whole cell membranes and other high density particles. Subsequently, the samples were subjected to ultracentrifugation at 200,000 x g for 105 minutes at 25°C. The pellet that formed during

ultracentrifugation was suspended in isolation buffer and dithiothreitol to disrupt the Tamm-Horsfall polymeric network (18). Finally, the suspended pellets were solubilized in Laemmli buffer for immunoblot analysis. Immunoblotting of the urinary exosomes and kidney samples was performed as described previously (see also Online Supplement) (8). The antibody against NCC phosphorylated at threonine 58 was generated by one of the inverstigators (RAF) and has been characterized previously (20). All other antibodies were obtained: prostasin (BD biosciences, Breda, The Netherlands) and NCC (Stessmarq biosciences, Victoria, Canada). For the animal studies, the complete volume of the 24-hour urine was used to isolate urinary exosomes, and therefore no normalization was used in the analysis. Coomassie blue staining was used to confirm that there were no differences in total protein contents. Conversely, for the patient study, the amount of sample loaded during immunoblotting was normalized by the urinary creatinine concentration (1).

Statistics

All data are expressed as mean ± standard error of the mean. Group comparisons were made using the unpaired Student's T-test or analysis of variances with a post-hoc test, as appropriate. For analysis, the natural logarithm of the plasma aldosterone concentration was used to yield a normal distribution. $P \leq 0.05$ was considered statistically significant.

RESULTS

Three-day infusion of aldosterone increased pNCC and prostasin in urinary exosomes

Adrenalectomized rats were infused with high-dose aldosterone or vehicle for three days. The differences in plasma aldosterone and urinary sodium between the two groups confirmed that both the adrenalectomy and the infusion of aldosterone were successful (**Figure 1A**). The infusion of aldosterone

significantly increased pNCC, NCC, and prostasin in urinary exosomes on days 2 and 3 (**Figure 1B**). The abundance of pNCC in urinary exosomes increased 2.9 ± 0.4 fold on day 2 and 3.2 ± 0.3 fold on day 3 ($p < 0.05$ for day 2 and $p < 0.01$ for day 3). The abundance of NCC in urinary exosomes also increased 2.3 ± 0.4 fold on day 2 and 1.8 ± 0.4 fold on day 3 ($p < 0.05$ for both). The abundance of prostasin in urinary exosomes increased 1.8 ± 0.2 fold on days 2 and day 3 ($p < 0.05$ and $p < 0.01$, respectively).

Effects of an eight-day infusion of aldosterone on pNCC and prostasin in kidney and urinary exosomes

Adrenalectomized rats were infused with vehicle, a normal or a high dose of aldosterone for eight days. The plasma aldosterone concentrations were significantly different among the three groups (**Figure 2A**). Both the normal and high aldosterone dose increased the abundance of pNCC and NCC in urinary exosomes (**Figure 2B**). pNCC increased 3.0 ± 0.4 fold with the normal dose and 2.5 ± 0.5 fold with the high dose ($p < 0.01$ and $p < 0.05$, respectively); NCC increased only with the normal dose (1.5 ± 0.2 fold, $p < 0.05$). Although pNCC and NCC in exosomes showed similar responses to aldosterone compared with the abundances of these proteins in the kidney[23], no direct correlation was observed (data not shown). In contrast to the three-day infusion (**Figure 1C**), the eight-day infusion of aldosterone did not increase prostasin in urinary exosomes. In kidney, only the high aldosterone dose increased prostasin significantly (1.5 ± 0.1 fold, $p < 0.05$).

A low salt diet increased pNCC but not prostasin in urinary exosomes

To induce a physiological increase in plasma aldosterone, two groups of rats were fed a normal sodium diet, after which one group was switched to a low sodium diet. The low sodium diet caused a higher plasma renin activity and higher plasma aldosterone after eight days (**Figure 3A**). When both groups were on the normal sodium diet, the abundance of pNCC and NCC in urinary exosomes was similar (**Figure 3B**). The low sodium diet increased pNCC in urinary exosomes on day 4 (1.7 ± 0.2 fold) and day 8 (1.4 ± 0.1 fold, $p <0.05$

for both). The low sodium diet increased NCC in urinary exosomes on day 4 (1.5 ± 0.1 fold) and day 8 (2.0 ± 0.3 fold, $p < 0.05$ for both). In contrast, it did not cause significant changes in the abundance of prostasin in urinary exosomes (**Figure 3C**).

pNCC and prostasin are increased in urinary exosomes of patients with primary aldosteronism

The characteristics of five patients with primary aldosteronism and four patients with essential hypertension are shown in **Figure 4**. Both groups had a similar degree of hypertension, but had significant differences regarding the plasma aldosterone to renin ratio and the urine sodium to potassium ratio. The abundance of pNCC in urinary exosomes of patients with primary aldosteronism was higher in patients with essential hypertension (2.6 ± 0.3 fold, $p < 0.05$, **Figure 4**). The abundance of prostasin in urinary exosomes showed a trend towards being higher in the patients with primary aldosteronism (1.5 ± 0.3 fold, $p = 0.07$).

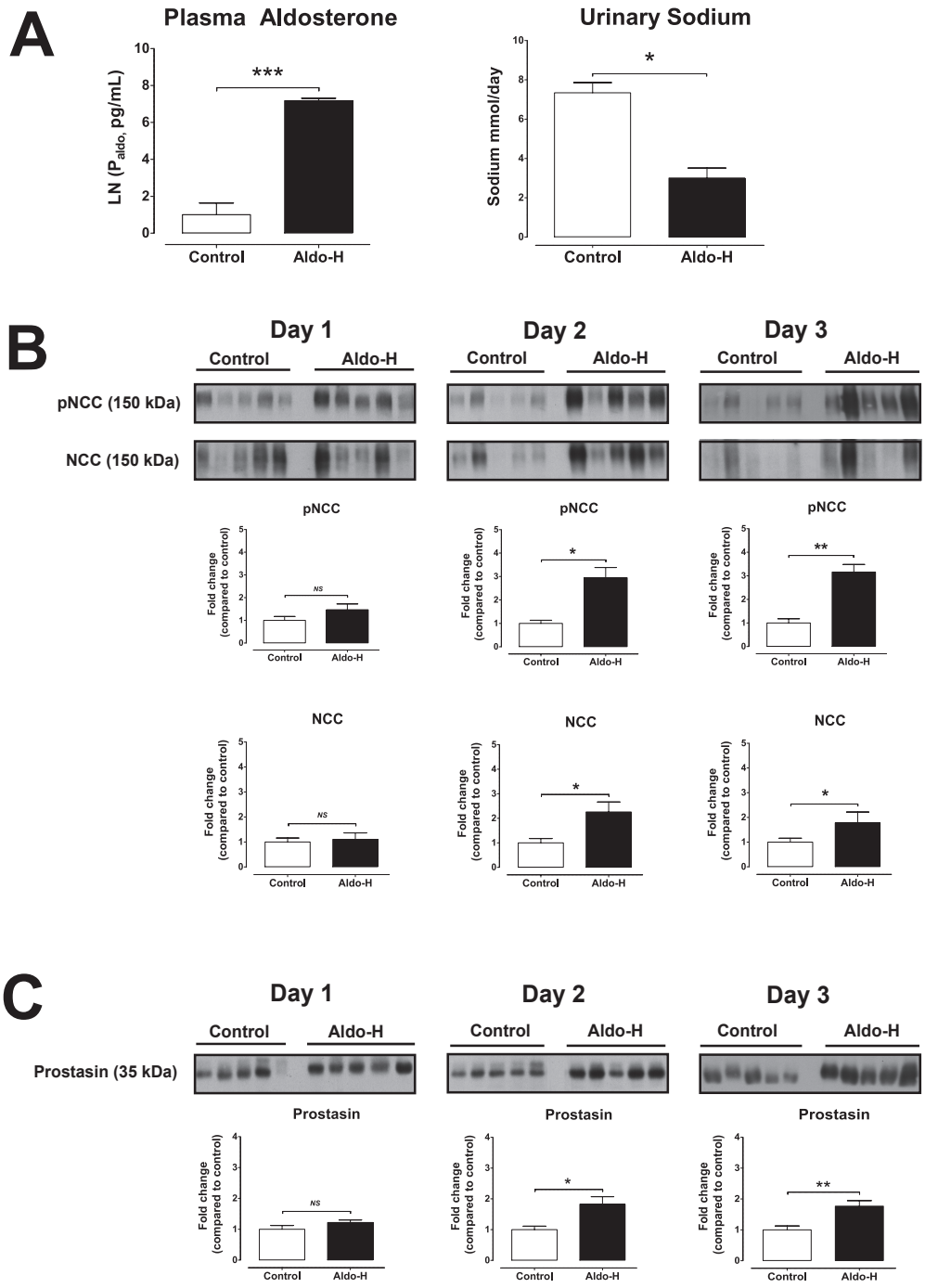

Figure 1: Effects of chronic aldosterone infusion in rats on the abundances of pNCC and ***prostasin in urinary exosomes.*** *Adrenalectomized rats were treated with high-dose aldosterone (Aldo-H, 100 µg/kg/day) or vehicle (control) for three days.* ***A:*** *plasma aldosterone and urine sodium.* ***B:*** *pNCC and NCC in urinary exosomes.* ***C:*** *prostasin in urinary exosomes*
** p < 0.05, ** p < 0.01, *** p < 0.001 by Student's T-test*

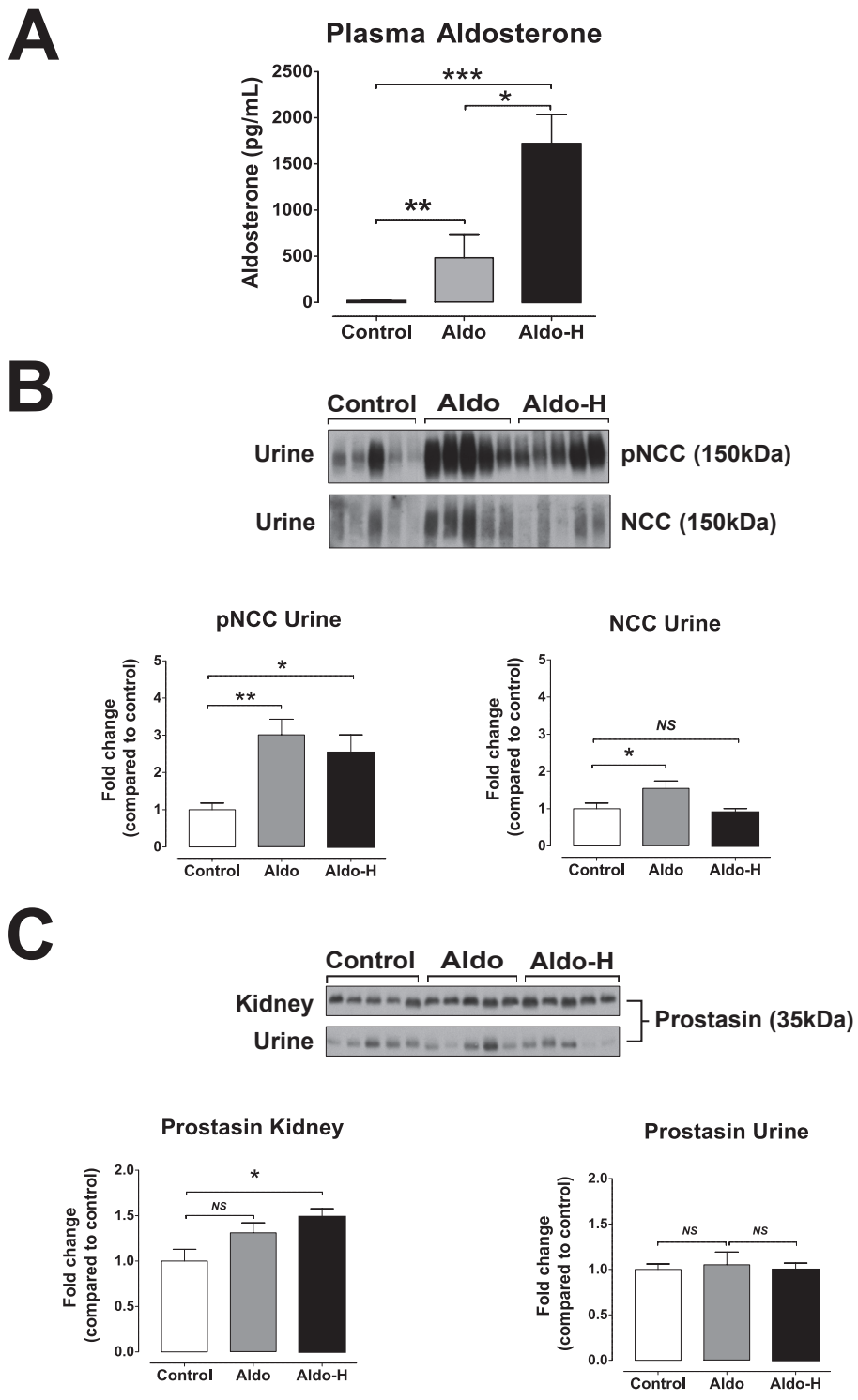

Figure 2: Effects of normal and high aldosterone infusion in rats for eight days on plasma aldosterone, pNCC and prostasin in kidney and urinary exosomes. *Adrenalectomized rats were infused with vehicle (control), normal (Aldo, 50 µg/kg/day) or high-dose (Aldo-H, 100 µg/kg/day) aldosterone for 8 days.* ***A:*** *plasma aldosterone concentrations.* ***B:*** *pNCC and NCC in urinary exosomes.* ***C:*** *prostasin abundance in kidney and urinary exosomes. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ by analysis of variance and post-hoc test*

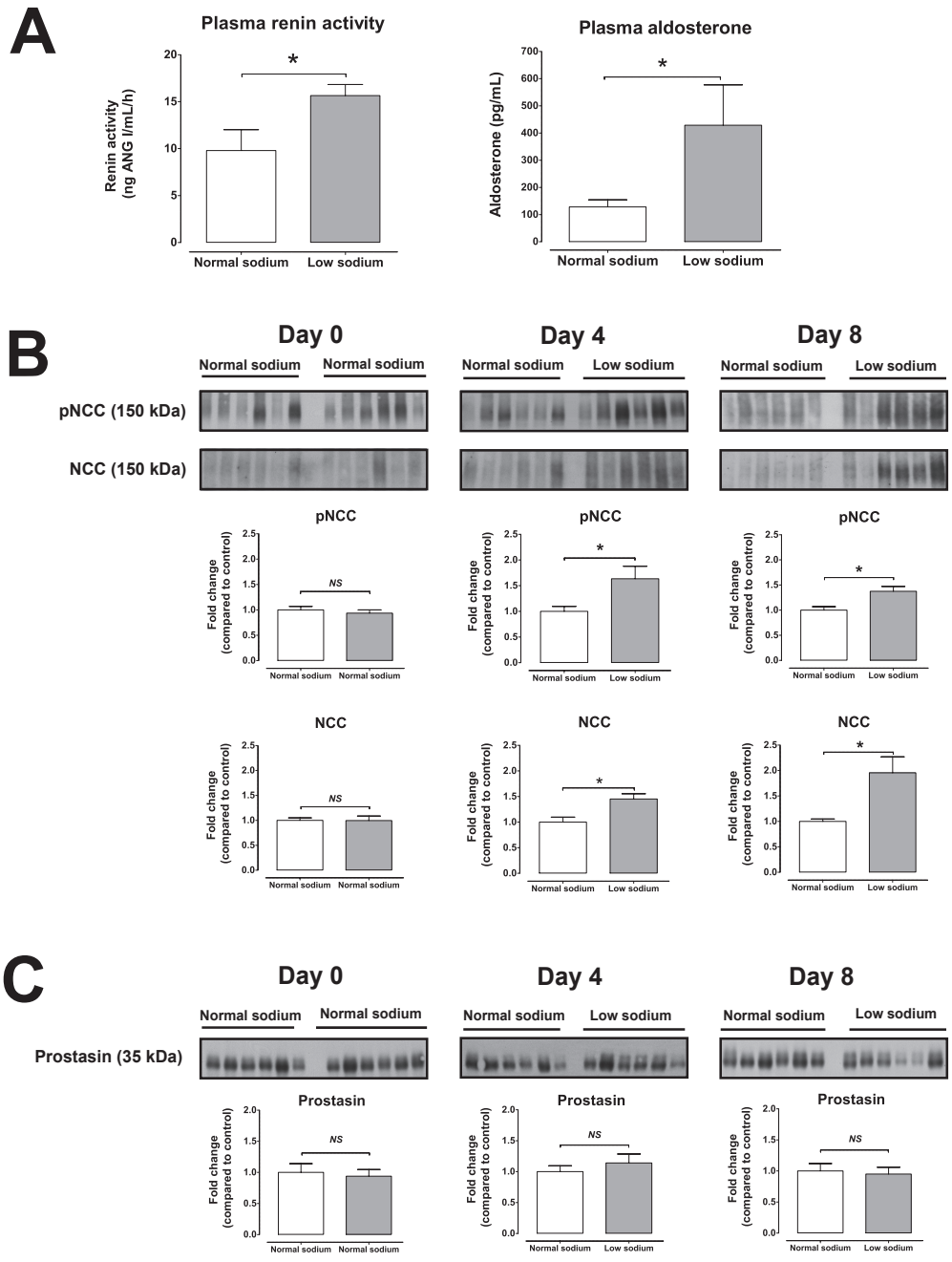

*Figure 3: A low salt diet increased pNCC but not prostasin in urinary exosomes of rats. A: plasma renin activity and plasma aldosterone. B: pNCC and NCC in urinary exosomes. C: prostasin in urinary exosomes. * $p < 0.05$, by Student's T-test*

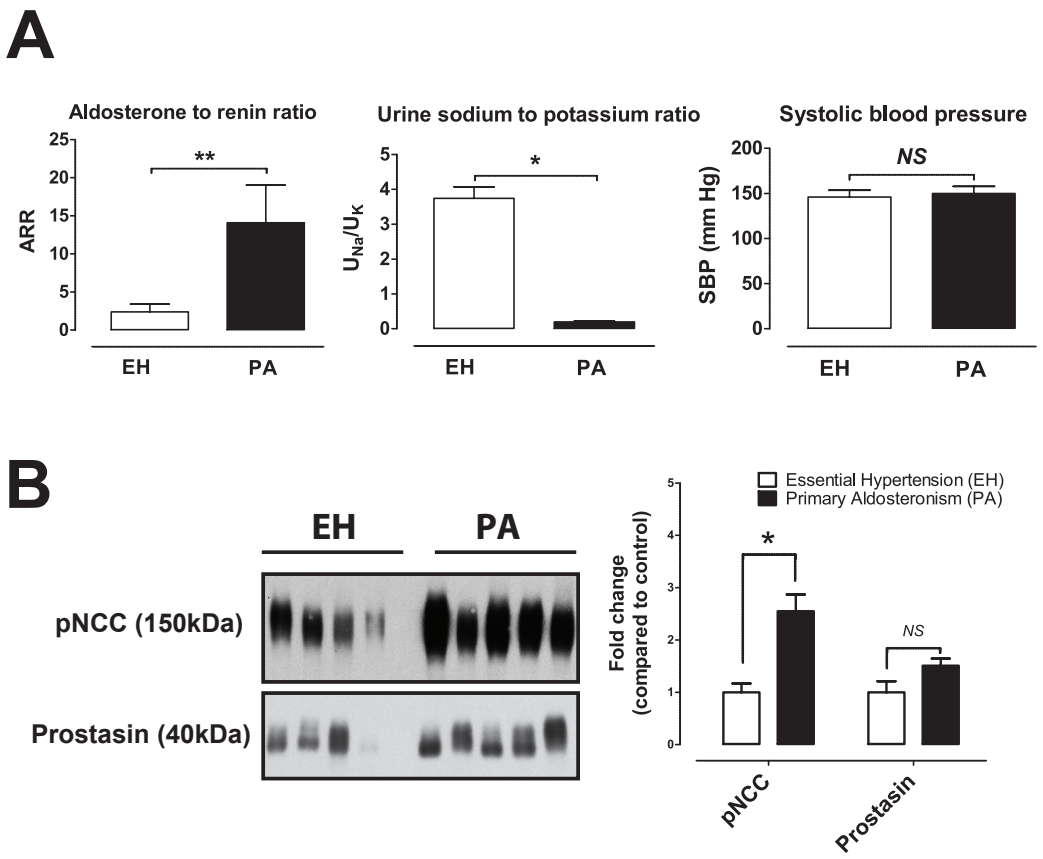

Figure 4: Patients with primary aldosteronism show increased abundance of pNCC and prostasin in urinary exosomes. A: *Characteristics of patients with essential hypertension (EH) or primary aldosteronism (PA).* ***B:*** *Patients with primary aldosteronism show increased abundance of pNCC and prostasin in urinary exosomes*
** $p < 0.05$ by Student's T-test /or Mann-Whitney*

DISCUSSION

In this study we asked whether NCC and prostasin in urinary exosomes can be used as markers for primary and secondary aldosteronism. In our hands, both total and phosphorylated NCC were superior to prostasin as a marker for aldosteronism . In fact, the abundance of prostasin in urinary exosomes only increased during high-dose, short-term treatment with aldosterone (**Figure 1C**), whereas the abundance of pNCC in urinary exosomes was higher during aldosteronism regardless of the duration, dose or stimulus (**Figure 5**). Because

the changes in pNCC were more pronounced than for NCC, pNCC appears the better marker. In addition, pNCC was increased significantly in urinary exosomes of patients with primary aldosteronism compared to patients with essential hypertension (**Figure 4**). In contrast, the increase in the abundance of prostasin in urinary exosomes of patients with primary aldosteronism was of borderline significance.

Several observations regarding the dynamics of pNCC and prostasin excretion in urinary exosomes during aldosteronism observed in this study merit discussion. For example, the results of the short-term infusion of aldosterone in rats suggest that it takes at least one day for both pNCC and prostasin to increase in urinary exosomes (**Figure 1**). This may be related to the time it takes aldosterone to increase transcription or post-translational modification of these proteins (21). The long-term infusion of aldosterone in rats showed that a lower ("physiological") dose of aldosterone was already sufficient to increase pNCC (**Figure 2B**). In fact, pNCC expression in urinary exosomes was slightly lower with the high dose. This suggests that the stimulatory effect of aldosterone on pNCC in urinary exosomes either saturates, or that aldosterone escape has occurred, a defense mechanism known to reduce NCC in the kidney (22). Although the induction of secondary aldosteronism by a low sodium diet also increased pNCC in urinary exosomes (**Figure 3B**), the magnitude of this effect was less than with aldosterone infusion (**Figure 5**). One could have predicted even higher pNCC in urinary exosomes during a low sodium diet, because this maneuver is likely to increase both plasma angiotensin II and aldosterone. We recently showed that angiotensin II can increase renal pNCC independently of aldosterone, and that the combination of angiotensin II and aldosterone leads to an additive effect (15, 23). Apparently other factors limit pNCC excretion in urinary exosomes during a low sodium diet. Furthermore, differences in the plasma aldosterone concentrations in the control groups should be taken into consideration (virtually absent in adrenalectomized rats vs. ~100 pg/ml in rats on a normal sodium diet, **Figure 1A and 3A**). Of interest, the increase in NCC in urinary exosomes we observed in this study with a low sodium diet was

similar to the increase Esteva-Font *et al.* found for NCC in a separate study (10).

Our study suggests that pNCC was better than prostasin as a marker of aldosteronism, which could be explained by several factors. First, although prostasin is sensitive to aldosterone and can activate ENaC, this does not necessarily render it a direct marker of ENaC activity and as such it may not be a direct marker of distal sodium reabsoption (12, 13). Prostasin is only one of the proteins present in the complex signaling cascade that regulates ENaC (24). Second, because prostasin is also present in prostate epithelial cells, urinary exosomes may also contain prostasin from this source (25). This may have limited the specificity of prostasin as a marker for aldosterone actions in the kidney, especially in males. Third, by using two-dimensional electrophoresis, Olivieri and colleagues have previously shown that several subunits of prostasin exist, only some of which are aldosterone sensitive (12). Our prostasin results differ from those reported by Narikiyo and coworkers (13). They found that rats continued to increase urinary prostasin during seven days of aldosterone infusion (from 1.5 to 4-fold), whereas we were unable to detect increased prostasin in urinary exosomes after eight days of treatment (**Figure 2**). This difference may be explained by the use of a three-fold higher aldosterone dose in the previous study and that their analysis of prostasin was performed on whole urine instead of urinary exosomes.

In recent years, the potential to use proteins in urinary exosomes as markers of diseases affecting the kidney has attracted much interest (1-3, 26). Progress has been somewhat hindered by technical and normalization issues, but the proposal of uniform protocols has been a step in the right direction (18, 27). We believe that the strength of this study was to combine a controlled experimental setting with a clinical setting for the analysis of urinary exosomes in aldosteronism. Because of the well-characterized actions of aldosterone on distal tubular sodium transport, aldosteronism appears especially suitable for analysis with urinary exosomes. Clinically, primary aldosteronism is important

because recent studies suggest it to be a common condition among patients with resistant hypertension that is often difficult to diagnose (28). We do emphasize, however, that the primary aim of this study was to provide proof of principle that the abundance of aldosterone-sensitive proteins in urinary exosomes is increased during aldosteronism. The question whether pNCC in urinary exosomes has diagnostic potential in patients with primary aldosteronism remains to be determined. This will require larger and well-characterized groups of patients to be tested against a golden standard. In addition, the overall differences in NCC and pNCC between conditions were relatively mild even under controlled experimental conditions. Even if the sensitivity and specificity of pNCC in urinary exosomes would outweigh existing tests such as the ARR, the current method of exosome isolation is not suitable for clinical use (29). Instead, development of an enzyme-linked immunoassay for pNCC would be an attractive alternative.

This study suggests several directions for future research. One obvious next step will be to evaluate how pNCC and prostasin in patients with primary aldosteronism respond to treatment with either mineralocorticoid receptor antagonists or adrenalectomy. Narikiyo *et al.* showed that urinary prostasin decreased in three patients with primary aldosteronism who had undergone adrenalectomy (13). Similarly, Olivieri *et al.* showed that prostasin decreased in normotensive subjects with aldosteronism due to a low sodium diet who were subsequently treated with spironolactone (12). It would also be informative to know whether other commonly used antihypertensive drugs such as diuretics, angiotensin converting enzyme inhibitors or angiotensin receptor blockers affect urinary exosome excretion. Because total NCC also increased, it would be important to known whether NCC mRNA is also present in urinary exosomes and if it behaves similarly. If so, it could be a more sensitive marker because the signal can be amplified. In kidney, however, previous studies found that the protein and mRNA abundances of NCC did not always correlate.[22,23] Finally, given the recent insights into the effects of angiotensin II on distal tubular sodium transport (14, 15), it would be important to compare urinary exosomal pNCC and prostasin in patients with primary and secondary aldosteronism.

In conclusion, in urinary exosomes of animals and patients, pNCC was superior to prostasin as a marker of aldosteronism. These results justify further evaluation of the applicability of urinary exosomes as a diagnostic tool in primary aldosteronism and, possibly, other forms of hypertension. Furthermore, pNCC and, to a lesser extent, prostasin, may be used experimentally as a non-invasive method to analyze the biological action of aldosterone in the kidney.

Perspectives

In hypertension, urinary exosomes could be applied as a diagnostic test for primary aldosteronism, to evaluate salt-sensitivity or the response to antihypertensive drugs acting on the kidney. Our study can be regarded as proof of principle that analysis of urinary exosomes can be applied to aldosteronism. Subsequent validation studies will be necessary to define their utility in experimental or clinical settings (30).

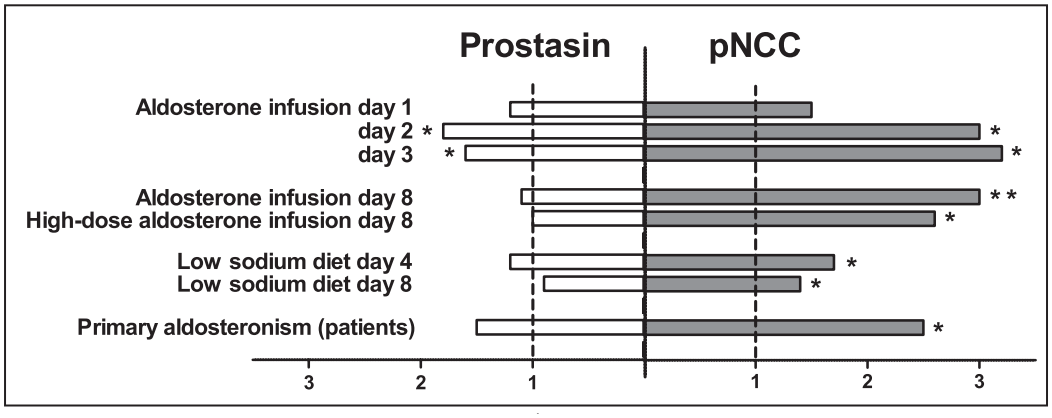

Figure 5: Summary of the performance of urinary exosomal prostasin and pNCC as markers of aldosteronism. *The densitometry values of the immunoblot analyses are shown. A value of 1 represents no difference compared to the control group. * $p < 0.05$, ** $p < 0.01$*

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CHAPTER

07

A MISSENSE MUTATION IN THE EXTRACELLULAR DOMAIN OF αENAC CAUSES LIDDLE SYNDROME

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ABSTRACT

Liddle syndrome is an autosomal dominant form of hypokalemic hypertension due to mutations in the β- or γ-subunit of the epithelial sodium channel (ENaC). For the first time, we describe a family with Liddle syndrome due to a mutation in αENaC. The proband was referred because of resistant hypokalemic hypertension, suppressed renin and aldosterone, and no mutations in the genes encoding β- or γENaC. Exome sequencing revealed a heterozygous, non-conservative T>C single nucleotide mutation in αENaC that substituted Cys479 to Arg (C479R). C479 is a highly conserved residue in the extracellular domain of ENaC and is likely involved in a disulfide bridge with the partner cysteine C394. In oocytes, the C479R and C394S mutations resulted in a similar ~2-fold fold increase in amiloride-sensitive ENaC-current. Quantification of mature cleaved αENaC in membrane fractions showed that this gain of function was not due to a higher number of channels. Trypsin, which increases open probability of the channel by proteolytic cleavage, resulted in significantly higher currents in wild-type than in C479R or C394S mutants. In summary, a mutation in the extracellular domain of αENaC causes Liddle syndrome by increasing intrinsic channel activity. This mechanism differs from the β- and γ-mutations that result in an increase in channel density at the cell surface. This mutation may explain other cases of resistant hypertension and also provides novel insight in ENaC activation, relevant for kidney sodium reabsorption and salt-sensitive hypertension.

INTRODUCTION

Hypertension is one of the most common non-communicable disorders worldwide and a major risk factor for stroke, myocardial infarction, heart failure, and end-stage renal disease (1). Primary or essential hypertension is a complex genetic trait that is also influenced by other risk factors such as dietary sodium and potassium intake, obesity, and diabetes (2, 3). In contrast, monogenic forms of hypertension are very rare, but have been instrumental in revealing the molecular pathways contributing to primary hypertension (4). The majority of these pathways point towards a role for increased sodium reabsorption by the kidneys, especially in the aldosterone-sensitive distal nephron (5). Indeed, several monogenic forms of hypertension are caused by mutations increasing sodium reabsorption in this segment through the sodium chloride cotransporter or the epithelial sodium channel (ENaC) (6). In 1963 G. Liddle and colleagues reported a 'familial renal disorder simulating primary aldosteronism but with negligible aldosterone secretion' (7). Liddle syndrome or pseudoaldosteronism (OMIM# 177200) is now known as an autosomal dominant form of salt-sensitive hypertension that is further characterized by suppressed plasma renin and aldosterone, hypokalemia, and metabolic alkalosis (8). The syndrome was linked to mutations in the *SCNN1B* or *SCNN1G* gene, encoding the β- or γ-subunit of ENaC (9, 10). Mutations in *SCNN1B* or *SCNN1G* delete or modify the intracellular PY motifs in ENaC in such a way that Nedd4-2 fails to ubiquitylate the channel leading to a retention of active ENaC at the cell surface (11, 12). Here, we report a family with Liddle syndrome due to a gain of function mutation in the extracellular domain of the α-subunit of ENaC (*SCNN1A*) that predominantly increases channel open probability but not channel surface density.

RESULTS

Clinical and genetic characteristics of a novel ENaC mutation

The proband was referred because of resistant hypertension, hypokalemia, metabolic alkalosis, and suppressed levels of plasma renin and aldosterone. Despite a positive family history for hypertension (**Figure 1A**), no mutations in *SCNN1B* or *SCNN1G* were identified. Diagnostic exome sequencing revealed a novel heterozygous, non-conservative T>C single nucleotide mutation that results in the substitution of cysteine 479 to arginine (C479R) in αENaC (c.1435T>C(p.(Cys479Arg), **Figure 1B**). The mutation is reported at a very low frequency in a large database collecting >60,000 exomes as proxy for variant allele frequencies in the general population (seven times heterozygously in >100,000 alleles; Exome Aggregation Consortium). The ENaC-blocker triamterene normalized blood pressure and serum potassium in the proband. Genotyping of the five siblings also identified the novel C479R mutation in subject II-4. The mutation segregated with suppressed plasma and aldosterone, but not with hypertension (**Figure 1C**). Whole-exome sequencing in the proband did not identify additional mutations to explain the hypertensive trait in this family. Subject II-4 had mild hypertension (average ambulatory blood pressure 138/88 mmHg) that was sensitive to sodium chloride supplementation (145/91 mmHg) and also improved with triamterene (121/71 mmHg). In a standardized diuretic test, the natriuretic response to triamterene in the proband and II-4 was in the high range or increased compared to the response in healthy volunteers (**Figure 1D**) (13). Thus, two siblings (the proband and II-4) show a clinical picture compatible with Liddle syndrome and carry the C479R missense mutation.

C479 is located in the extracellular domain of ENaC

The ultimate proof of Liddle syndrome, however, is the demonstration that the mutation results in a gain of function of ENaC. The DNA variant that encodes the C479R mutant has so far never been described. The C479 is a highly conserved Cys residue that belongs to the second cysteine-rich domain (CRD2)

of the extracellular domain of ENaC that is likely involved in disulfide bridges (14). The hαENaC C479 is conserved not only among the ENaC subunits and among ENaC homologs but also among the Acid-Sensing Ion Channel 1 (ASIC1) orthologs (**Figure 2A**). The crystal structure of chicken ASIC1 reveals that the cASIC1 C366 forms a disulfide bond with another highly conserved cysteine, C291 in CRD2, that corresponds in αhENaC to a disulfide bond between C479 and the C394 (**Figure 2B**) (15). Therefore, we analyzed not only the consequences of the C479R mutation on hENaC function in *Xenopus laevis* oocytes, but also the functional effects of the mutation of the partner Cys C394S involved in the disulfide bond. In addition, since C479 is a highly conserved Cys, we performed a similar functional analysis of the corresponding Cys mutations C507S and C422S in rat αENaC.

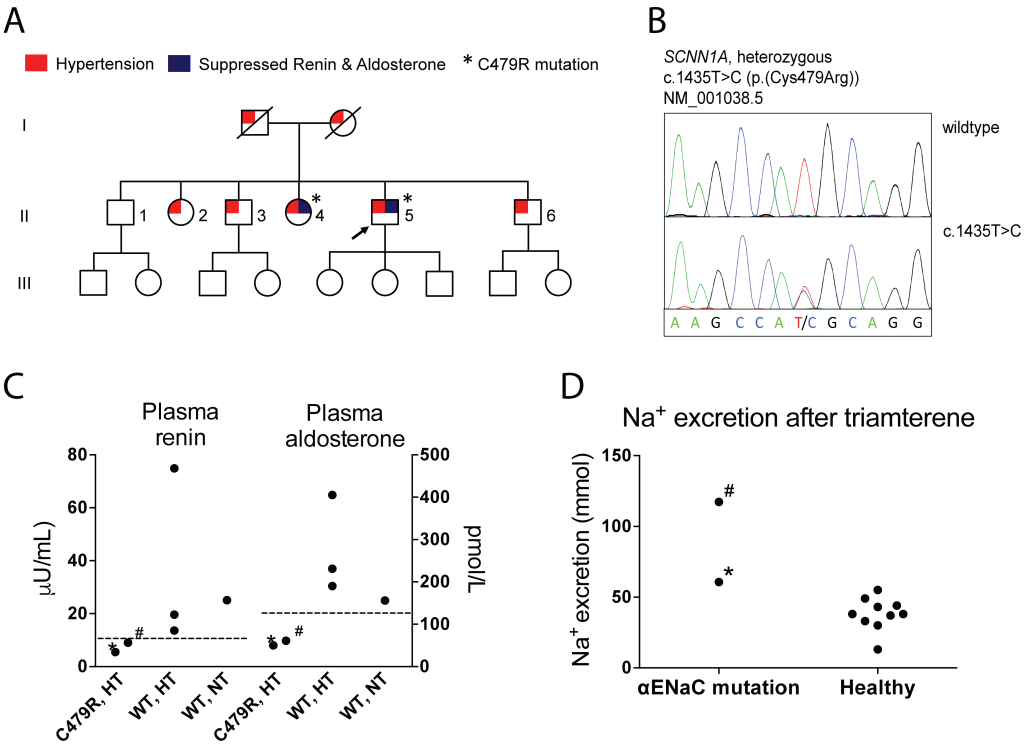


Figure 1. Clinical, genetic, and structural characteristics of the αENaC mutation. *(A) Pedigree showing three generations of the family with Liddle syndrome. Generation II was analyzed by genotyping and biochemical profiling. Arrow indicates the proband. (B) Sequence chromatogram. (C) The C479R mutation segregated with suppressed plasma renin and aldosterone but not with hypertension. Renin and aldosterone were measured in the absence of interfering drugs. Dashed lines represent lower limits of normal. Footnotes: * proband; # subject II-4. Abbreviations: HT, hypertension; NT, normotension; WT, wild-type. (D) Results of a standardized diuretic test showing the natriuretic response to a single dose of the ENaC-blocker triamterene in the proband (*) and subject II-4 (#) in comparison to healthy volunteers (13).*

C479R increases ENaC current in oocytes

Both C479R and C394S result in a similar ~2-fold increase in amiloride-sensitive ENaC-current (**Figure 3A**). These results strongly suggest that the channel gain of function is due to the disruption of the disulfide bond between the two Cys. Since the proband is heterozygous for the C479R mutation, we replicated this condition *in vitro* by co-injecting ENaC wild-type and C479R in a 1:1 ratio and observed still a significant increase in ENaC-current, but this effect was reduced by half compared to C479R expressed alone (**Figure 3B**). To provide further evidence for a gain of function due to the disruption of the disulfide bridge we tested the corresponding mutations in rαENaC and found that the C507S and C422S mutations have comparable stimulatory effects on ENaC activity as C479R and C394S in hαENaC (**Figure 3C**). These results are consistent with previous observations that disruption of particular disulfide bonds in the CRD2 of the extracellular domain of rENaC results in a channel gain of function (14). Furthermore both C479Arg and C507Ser substitutions in hαENaC and rαENaC have comparable stimulatory effects on ENaC current indicating that the effect does not depend on the substituting amino acid. Together these observations support the idea that the disruption of the C479-C394 disulfide bridge by the C479R substitution is likely the primary cause of the observed gain of function in ENaC.

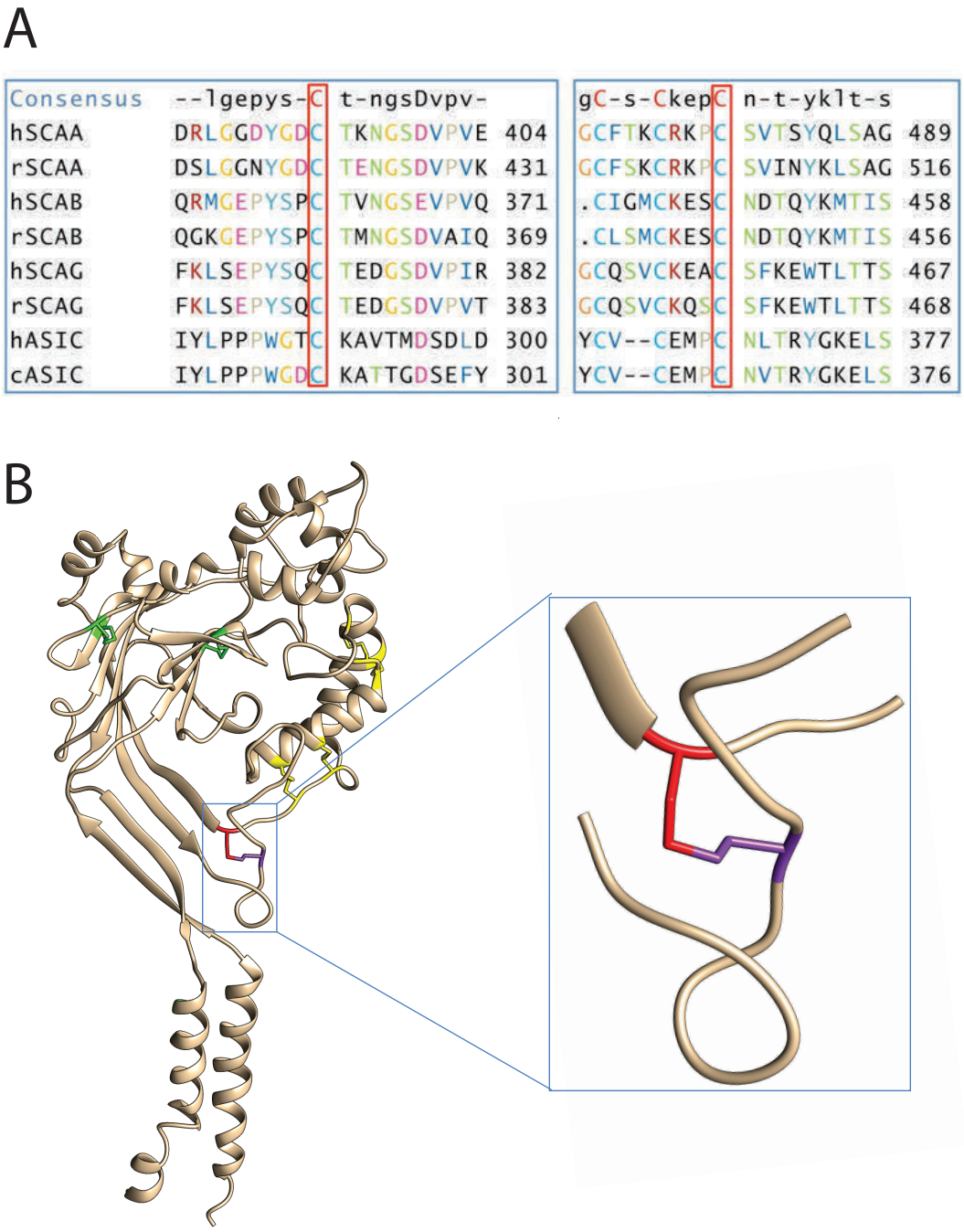


Figure 2. Sequence comparison and subunit structure of an ENaC homologue. *(A) Sequence comparison of human (h) and rat (r) αENaC (SCAA), βENaC (SCAB), and γENaC (SCAG) subunit isoforms with human hASIC1 and chicken cASIC1. (B) Crystal structure of a cASIC1 subunit with the disulfide bonds in the extracellular domain labelled in green for the first cysteine-rich domain (CRD1) and in yellow for CRD2. The Cys366 (red) corresponding to Cys479 in the human αENaC (hSCAA) makes a disulfide bond with Cys291 (purple) corresponding to C394 in hSCAA (inset).*

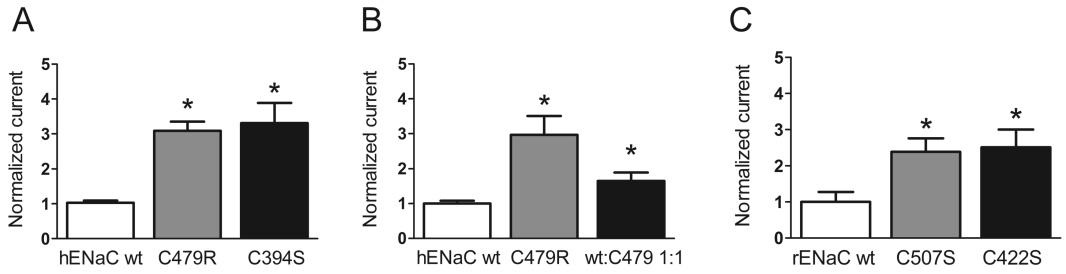


Figure 3. αENaC C479R is a gain of function mutation. *(**A**) Amiloride-sensitive current increase of αC479R ENaC mutant and of the Cys partner, αC394S, mutant. Current values were normalized for the average INa of the wild-type (wt) control obtained in oocytes of each independent batch (n ≥ 4). Bars represent mean ± SD for hαENaC wt (n = 225), hαENaC C479R (n = 231), and hαENaC C394S (n = 18). (**B**) Normalized amiloride-sensitive current values as in A with hαENaC wt (n = 14), hαENaC C479R expressed alone (n = 13) or together with hαENaC wt at a cRNA weight ratio of 1:1 (n = 13). (**C**) Normalized amiloride-sensitive current values for rat αENaC wt (n = 12), C507S (n = 12) and C422S (n = 12), corresponding respectively to C479R and C394S in the human αENaC sequence.*

Surface density of C479R channel mutant

The increase in ENaC activity due to the C479R mutation can result from an increase in channel open probability, in single channel conductance, or in the number of channels at the cell surface. To test the latter possibility we analyzed on Western blot the cleaved (CL) forms of wild-type and C479R α and γENaC subunits (**Figure 4A and B**), expressed in the whole oocyte, at the cell surface (**Figure 4C and D, Supplemental Figure 1**), and analyzed urinary exosomes (**Supplemental Figure 2**) (16). It is now well established that the CL-forms of α and γ represent the mature ENaC subunits that are incorporated in the functional channel complex present at the cell surface (17). The full length (FL) of αENaC (93 KDa) was detected in oocytes expressing α subunit alone or αβγENaC wt and C479R mutant (**Figure 4A**). The CL form of αENaC (69kDa) was detected only for the αβγENaC wt and the αC479Rβγ mutant. The CL form of γ subunit (76 kDa) was detected for both αβγENaC wt and αC479Rβγ mutant. Quantification of the intensities of the CL forms of α and γ subunits from the αC479Rβγ ENaC complex relative to those of α and γ in the αβγENaC complex did not show any significant difference. Subsequently, cell surface

expression of ENaC wt and mutant was assessed by biotinylation of surface proteins and followed by affinity purification on Neutravidin-agarose beads. The representative immunoblot in **Figure 4C** shows that in oocytes expressing comparable amounts of αENaC wt and mutant under their FL and CL forms (left panel: CL 69 kDa, FL 97 kDa), the CL band is the main detected form at the cell surface, at similar amounts for both channel types (right panel). We quantified the FL+CL band intensities in total membrane and in cell surface fractions of wt and C479R mutant ENaC expressing oocytes (**Figure 4D**). The data obtained from four independent experiments show that, for a comparable expression of αENaC wt and C479R mutant, the amount of wt and C479R mutant ENaC functional channels at the cell surface is similar. Similarly, the αENaC C479R mutation did increase γENaC levels at the cell surface of injected oocytes (**Supplemental Figure 1**). Based on these data, we can conclude that the ~2-fold higher ENaC current measured for the $h\alpha_{C479R}$ mutant is *not* correlated with an increase in the mature channel density at the cell surface. Consistently, no differences in the abundance of cleaved αENaC in urinary extracellular vesicles were detected in the two subjects carrying the mutation (**Supplemental Figure 2**) (16).

Intrinsic activity of C479R mutant

To test the possibility of a gain of function C479R mutation due to an increase in channel open probability (P_o) we used trypsin, that proteolytically cleaves ENaC and activates the channel by increasing the P_o (18). We reasoned that, if the C479R channel mutant has a higher P_o than the ENaC wild-type, then it should show a higher current but a lower sensitivity to activation by trypsin. The magnitude of the currents in the absence of trypsin was significantly higher for the C479R and C394S mutants as compared to wild-type (respectively 2.1 and 2.4-fold increase). After trypsin treatment, the currents expressed by the mutant forms were no longer different from that of ENaC wt although a trend towards higher currents was observed for the mutants (**Figure 5A** and **Supplemental Figure 3** for original traces). Shown in **Figure 5B** for individual oocytes, the wild-type hENaC has a significantly lower current than either of

the mutants, but the trypsin-induced increase in current is higher than that for the C479R and C394S mutants. This smaller effect of trypsin shown for the C479R and C394S gain of function mutants is consistent with a higher basal P_o. It is interesting to note that even at high ENaC baseline currents as those observed for the mutants, the trypsin effect plateaued at a 2-fold increase in INa. This suggests that the baseline P_o of C479R and C394S is below or equal to 0.5, whereas the baseline P_o of wild-type hENaC is below or equal to 0.27.
An alternative, but less likely explanation is that the C479R and the C394S mutations decrease the efficiency of ENaC cleavage by trypsin, despite a gain of function effect of the mutation. An alternative explanation for the lower sensitivity to trypsin of the C479R and C394S mutants is an apparent saturable expression of the ENaC-current because of the limited capacity of the oocyte to face large inward Na^+ currents (19). The latter possibility was, however, excluded in experiments testing the effect of trypsin on wild-type, C479R, and C394S hENaC at different levels of current expression by injecting increasing amounts of cRNAs ranging from 0.1 to 10 ng/oocyte (**Figure 5C).** The slope of this linear relation was approximately 2-fold lower for the ENaC mutants than for wild-type. This lower response to trypsin for the gain of function ENaC mutants is consistent with a higher intrinsic activity of the channel with a higher open probability. We then verified these observations with the corresponding Cys mutations, C507S and C422S, of rat ENaC, and also included the rβENaC Liddle mutant Y618A (**Figure 6**). Y618A disrupts the PY motif and increases the number of active channels at the cell surface (20). Wild-type ENaC and the Y618A mutant show a significantly higher response to trypsin compared to the C507S and C422S mutants, in spite of the fact that the Y618A baseline current was significantly higher than that of wild-type and of the Cys mutants (**Figure 6A**). The linear relationship between currents with or without trypsin was similar for wild-type and Y618C rENaC, but approximately 2-fold smaller for the C507S and C422S rENaC mutants (**Figure 6B**). Taken together these results suggest that the ENaC gain of function mutations C479R and C507S are essentially due to an increased intrinsic channel activity.

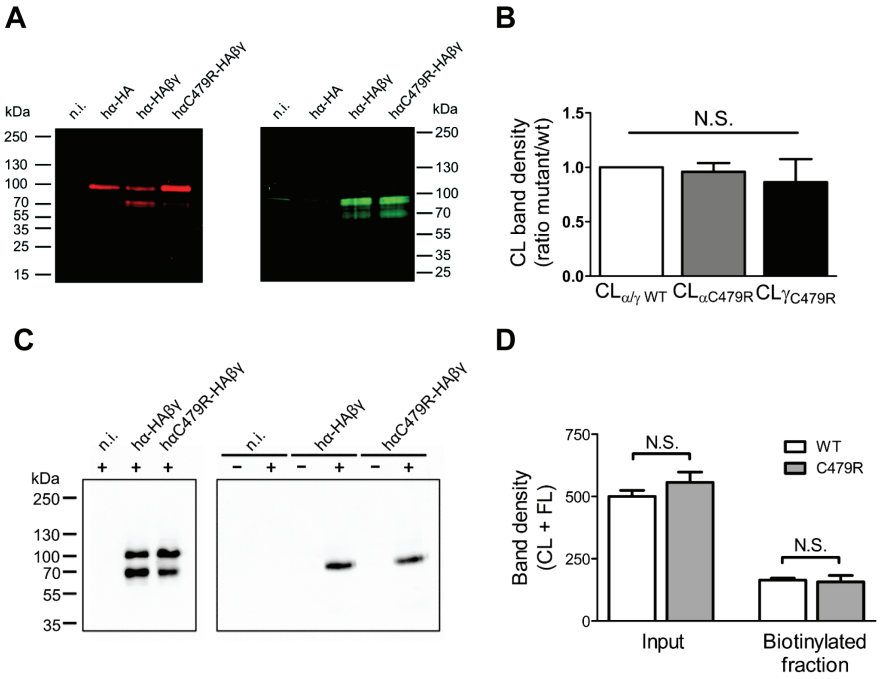


Figure 4. C479R does not increase channel surface density. *(**A**) Anti-HA-tag (red, left) and anti-γENaC (green, right) Western blot analysis of Triton-soluble fractions from Xenopus oocytes, non-injected (n.i.), or injected with cRNAs for either hα-HA alone or with β- and γENaC cRNAs, together with either wild-type or C479R mutant hα-HA. (**B**) The intensities of the bands corresponding to cleaved (CL) hα- and hγENaC were normalized to the amount of TCE-labeled, total protein obtained for each lane on the blot. The ratios between the thus calculated values for hα- and hγENaC in the $hα_{wt}βγ$ENaC and those for $hα_{C479R}βγ$ENaC are shown in the graph. Data correspond to mean ± SEM (10 blots from 7 independent experiments); differences are non-significant (N.S). (**C**) Right panel: Anti-HA immunoblot analysis of Neutravidin-bound fractions isolated from control (-) or cell-surface biotinylated (+) Xenopus oocytes, non-injected (n.i.), or injected with either $α_{wt}$-HA/β/γ or $α_{C479R}$-HA/β/γ cRNAs. Left panel: Inputs corresponding to 1% of the Triton-soluble preparations from biotinylated oocytes used in the pull-down experiments. (**D**) Values (mean ± SD) of CL+FL band intensities of inputs or of Neutravidin-bound fractions corresponding to experiments shown in C. Results from four blots with samples of four independent experiments. Differences are non-significant (N.S.).*

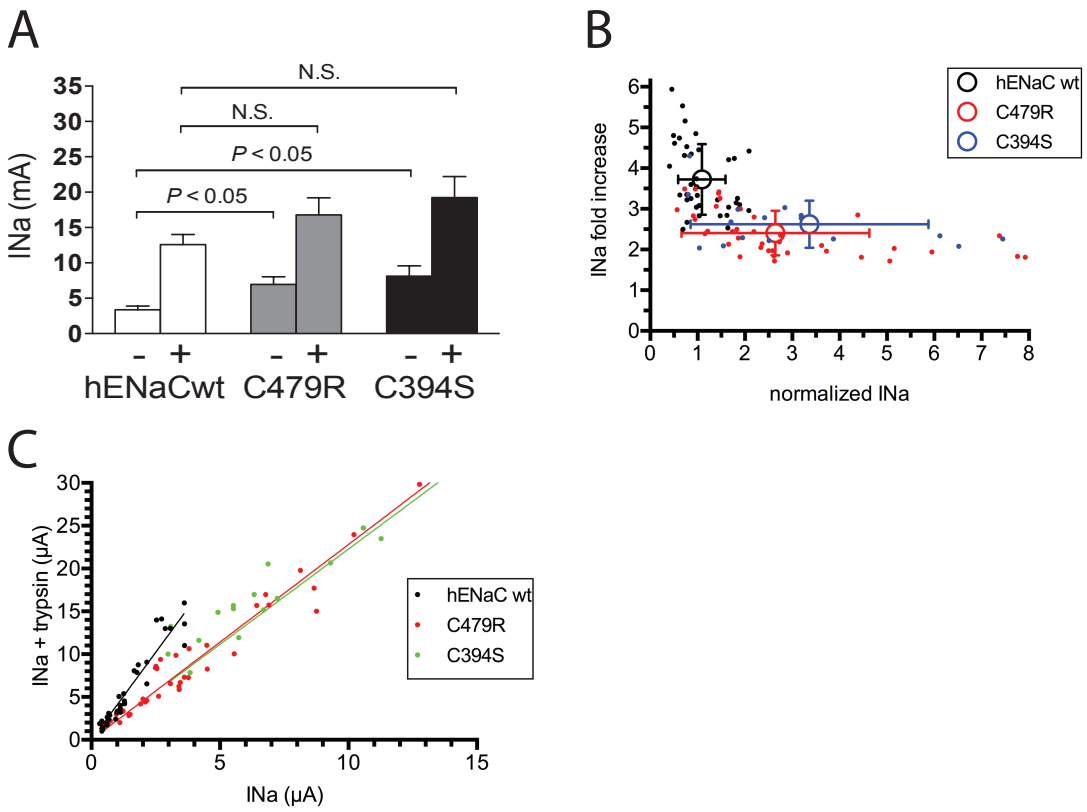


Figure 5. Sensitivity to trypsin of human ENaC C479R and C394S gain of function mutants. *(**A**) Amiloride-sensitive current (µA) of hENaC wild-type (wt, n = 17), hαENaC C479R (n = 17), hαENaC C394S (n = 18) in the absence (-) or presence (+) of trypsin. The magnitude of the currents in the absence of trypsin was significantly higher for C479R and C394S as compared to wt (* P < 0.01 by 1-way ANOVA), whereas the currents in oocytes expressing the mutant forms were no longer significantly higher after trypsin treatment. (**B**) Relationship between baseline INa in the absence of trypsin and fold increase in INa after the addition of trypsin in wild-type and mutant human ENaC (C479R and C394S). Current values for a single oocyte (filled symbols) and means ± SD (open symbols) are shown (P < 0.01 by ANOVA). (**C**) Correlation between current INa (µA) values in the absence and presence of trypsin. Linear regression analysis give the following best-fit values for the slopes $h\alpha_{wt:}$ 4.08 (95% CI 3.82-4.35); $h\alpha_{C479R}$ 2.28 (95% CI 2.14 – 2.42); $h\alpha_{C394S}$: 2.23 (95% CI 2.03 – 2.42). See* ***Supplemental Figure 3*** *for original traces.*

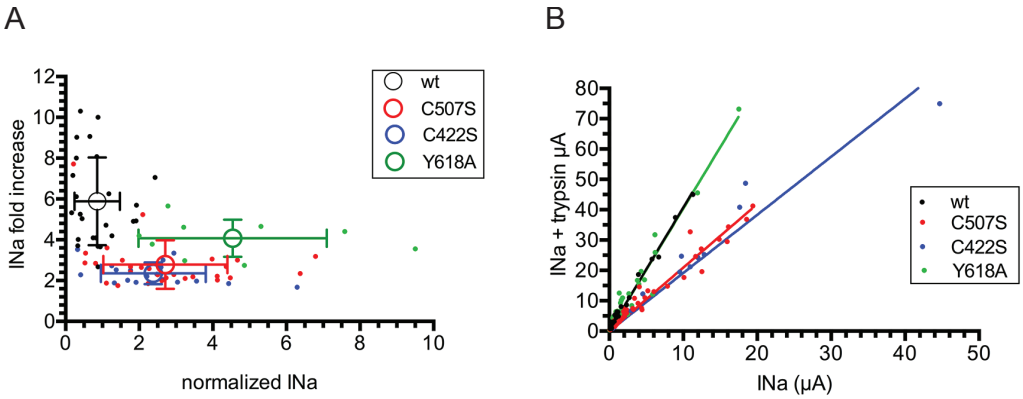


Figure 6. Sensitivities to trypsin of different types of rat ENaC gain of function mutants. *(A) Relationship between the baseline INa in the absence of trypsin and the fold increase in INa after the addition of trypsin for wild-type rat ENaC (rENaCwt), two αENaC mutants (C507S and C422S) and the βENaC Liddle mutant Y618A. Current values for a single oocyte (filled symbols) and means ± SD (open symbols) are shown (P < 0.01 by ANOVA).* ***(B)*** *Correlation between current INa values (µA) in the absence and presence of trypsin. Linear regression analysis give best-fit values for the slopes corresponding to* $r\alpha_{wt}/r\beta/r\gamma$, $r\alpha_{C507S}/r\beta/r\gamma$, $r\alpha_{C422S}/r\beta/r\gamma$, *and* $r\alpha_{wt}/r\beta/r\gamma_{Y618A}$ *and were, respectively, 4.07 (95% CI 3.92 – 4.23), 2.09 (95% CI 2.0 – 2.18), 1.92 (95% CI 1.74 – 2.09) , 4.04 (95% CI 3.79 – 4.30).*

DISCUSSION

Here, we report a mutation in αENaC associated with Liddle syndrome. Our functional investigations of the C479R mutation show that this Cys mutation in the second cysteine-rich domain of the extracellular domain of αENaC is a gain of function mutation. Our data are consistent with a higher intrinsic ENaC activity due to an increase in open probability (P_o), likely resulting from the disruption of the disulfide bonds between Cys479 and Cys394. The ENaC channel opener trypsin induces a lower response for the gain of function mutants $h\alpha_{C479R}$ and $r\alpha_{C507S}$, consistent with a higher channel P_o. This functional effect of the disruption of the Cys479–Cys394 disulfide bond is conserved in both rat and human ENaC channel isoforms. Our data are remarkably consistent with the mutational analysis of cysteine-rich domains of ENaC previously reported by Firsov *et al.*(14). They showed that the αC16S mutation

in rat αENaC (corresponding to the C479R and C507S mutations in our work) resulted in a 3-fold increase in amiloride-sensitive current without changing channel surface expression. Furthermore, mutation of the partner cysteine involved in the disulfide bridge (αC7S) had the same effects. Of interest, mutations of the cysteines in the extracellular cysteine-rich domains can result either in channel loss or gain of function. Indeed, mutation of the human Cys133 into a tyrosine causes the mirror image of Liddle syndrome, pseudohypoaldosteronism type 1, a severe salt losing syndrome in neonates (14, 21).

The majority of the previously reported Liddle mutations affect the intracellular PY-motif of β- or γENaC, impairing channel degradation by Nedd4-2 (9, 10). A mutation in αENaC causing Liddle syndrome has only been reported once previously, and was also located in the PY-motif (22). Therefore, this report on an αENaC mutation causing Liddle syndrome contains two novel aspects, including the location in the extracellular domain and the effect on open probability rather than surface expression. Of note, αENaC gain-of-function mutations have been identified previously in patients with cystic fibrosis-like symptoms, but it is not known whether these mutations also caused hypertension (23, 24). In addition, some of the mutations in α- or βENaC causing atypical cystic fibrosis were located outside the PY-motif and increased channel P_o (24, 25). A gain-of-function mutation (N530) in the putative extracellular domain of γENaC causing Liddle syndrome has also been reported previously (26). However, structural models of ENaC based on the crystal structure of the homolog ASIC1 predict that the N530 residue is located in the second transmembrane α helix of γENaC, and not in the extracellular domain of the channel. Finally, single nucleotide polymorphisms of the αENaC gene locus correlated with salt-sensitive hypertension in one Chinese study (27).

Of interest, the proband had the classical Liddle syndrome phenotype, whereas his sibling had a milder degree of salt-sensitive hypertension and no hypokalemia. Such differences between affected family members, including the

absence of hypokalemia, have been observed previously (28). The phenotypic differences between the proband and II-4 suggest variable expressivity, possibly related to gender, environmental factors, or genetic modifiers. Alternatively, the proband and his hypertensive siblings without the mutation (subjects II-2, II-3, and II-6, **Figure 1A**) may have an additional genetic variant predisposing to hypertension, although we did not identify this using whole-exome sequencing. Thus, in this family, the mutation segregated with suppressed renin and aldosterone but not with hypertension. In addition, our family appeared to have a milder phenotype compared to previous Liddle kindreds (9, 10, 28). This may also relate to the molecular mechanism of the αENaC mutation.

In vivo ENaC channel P_o determined by patch-clamp technique ranged from 0.3 to 0.7 (29). In Xenopus oocytes ENaC P_o under comparable conditions was estimated to be 0.3 (19). Assuming that trypsin stabilizes ENaC in the open conformation with a P_o of 1, then the slope current relations for the ENaC wild-type and mutants suggest that, under our experimental conditions, the P_o of wt ENaC is around 0.25, and that of the mutants around 0.5. Such an increase in P_o, estimated from the trypsin response, represents the main component of the higher ENaC current expressed by the C479R and the related mutants. Such a gain of function resulting from an increased P_o is certainly limited by the functional characteristics of the channels. Since ENaC likely never functions physiologically with a P_o of 1, a gain of function due to an increased channel gating would reasonably not exceed 3-4 fold. In contrast, Liddle gain of function mutation resulting from an increased ENaC retention at the cell surface may potentially enhance sodium transport to a much greater extent because the capacity of the membrane surface to accommodate a high density of ENaC channels is less restricted than the capacity of ENaC to increase in its P_o. Along this line we observed that the Y618A mutation, affecting channel interaction with Nedd4-2, is more efficient than C507S in increasing ENaC-mediated current (**Figure 6**). Although an increase in single channel conductance could theoretically explain the C479R channel gain of function, we know from previous structure-function studies on ENaC that such

mutations are restricted to the second transmembrane domain (TM2), a region that participates in the ion channel pore and selectivity filter (30).

In summary, we report a mutation in αENaC associated with Liddle syndrome. This represents the first ENaC gain of function mutation in the extracellular domain causing a higher intrinsic channel activity, a mechanism different from the previously reported mutations in β or γ associated with Liddle syndrome (9, 10). This study raises the question about the necessity of genotyping unexplained cases of hypertension associated with suppressed plasma renin and aldosterone and with a poor response to standard antihypertensive therapy. Furthermore, this mutation provides novel insight in ENaC activation and potentially in distal nephron sodium reabsorption and salt-sensitive hypertension.

CONCISE METHODS

Studies in patients

The patient studies were performed in accordance with the declaration of Helsinki. All patients provided written informed consent, and the ethics committee approved the study. Plasma renin concentration and plasma aldosterone were measured by enzyme-kinetic assay and LC/MS, respectively. These measurements were performed in the absence of interfering drugs (renin-angiotensin system inhibitors or diuretics). The triamterene test was based on the thiazide test (31). Results were compared to a historic cohort of healthy subjects receiving 100 mg triamterene in a similar setting (13).

Exome sequencing

The analysis of the exome data was divided into two steps: the renal gene panel analysis and the exome analysis. In the renal gene panel analysis, an in silico enrichment of genes associated with genetic renal disorders was performed

(version: DGD141114). After the patient consented for the second step, exome analysis, likely pathogenic variants in all coding genes were analyzed. Exome sequencing was performed using a Illumina HiSeq2000TM sequencer at BGI-Europe (Copenhagen, Denmark). Read alignment to the human reference genome (GrCH37/hg19) and variant calling was performed at BGI using BWA and GATK software, respectively. Variant annotation was performed using a custom designed in-house annotation and variant prioritization pipeline.

Site-directed mutagenesis and expression in Xenopus laevis oocytes

Mutant forms of the wild-type forms of human α-, β-, and γENaC subunits had been cloned in the pBSK(+)_Xglob vector (32). In these vectors, the ENaC cDNAs are flanked by sequences corresponding to the 5' and 3' non-coding stretches of Xenopus β-globin, which boosts protein expression when injected into Xenopus oocytes (33). Plasmids suitable for *in vitro* transcription of wild-type and mutant forms of the human αENaC subunit (hαENaC) were generated as described in the Complete Methods (**Supplemental Material online**). All constructs were verified by sequencing. Subsequently, healthy stage V and VI Xenopus laevis oocytes were pressure-injected with mixes containing equal amounts of cRNAs encoding α-, β-, and γ-subunits for a total of (unless stated otherwise) 1 or 3 ng cRNA for human and rat ENaC, respectively. Mutant and control cRNAs were prepared in parallel and ≥ 3 independent batches of cRNA were used.

Electrophysiology

Electrophysiological measurements were made 24-32 h after injection with the standard two-electrode voltage clamp technique, using a TEV-200A voltage clamp amplifier (Dagan, Minneapolis, MN), an ITC-16 digitizer interface (Instrutech Corp. Elmont, NY), and the PatchMaster data acquisition and analysis package (HEKA Elektronik Dr. Schulze GmbH, Ludwigshafen/Rhein, Germany). The amiloride-sensitive currents were measured in the presence of 10 μM of this blocker adjusted in a separate solution. Inward Na^+ currents were generated by switching from the amiloride-containing perfusion solution to that

without amiloride. In the experiment with proteases, the oocytes were perfused for 2 min with the amiloride-free solution supplemented with 2 µg/ml trypsin (Sigma-Aldrich Chemie GmbH, Buchs, Switzerland). All INa values were normalized in all experiments to the mean of the amiloride-sensitive currents measured for wildtype ENaC with the same oocyte batch.

Isolation of ENaC-enriched fractions

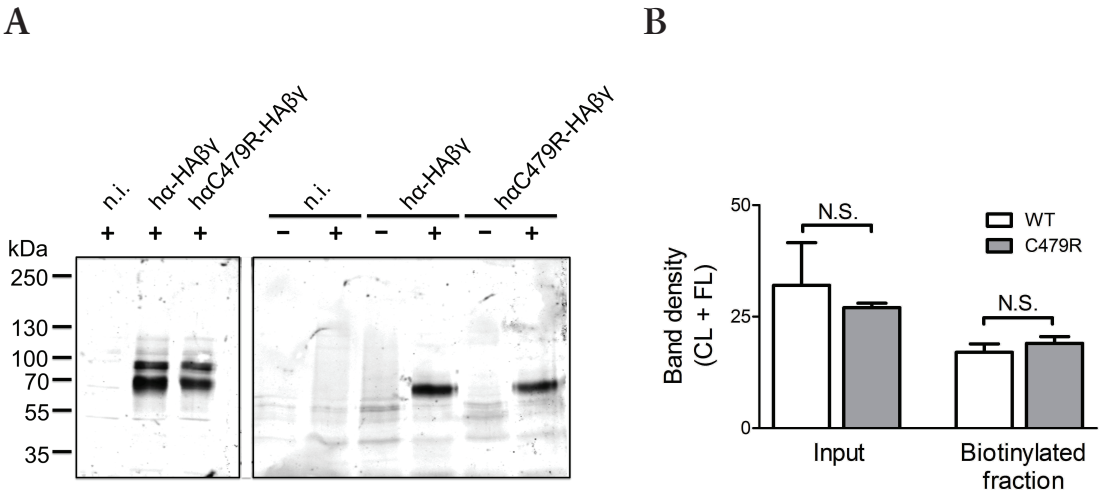
To isolate membrane fractions, 15-30 oocytes were disrupted by pipetting in 1.5 ml of membrane isolation buffer followed by centrifugation through cell shredders. The membrane pellets obtained after 30 min centrifugation at 20,000 g (4°C) were resuspended in membrane solubilization solution. Proteins in samples were resolved, along with pre-stained molecular weight markers by SDS-PAGE on 5–15% acrylamide gradient minigels supplemented with 0.5% (v/v) of 2,2,2-Trichloroethanol (TCE) for subsequent, in-gel, fluorescently labelling of proteins (34). Total protein per lane was assessed densitometrically from these images using ImageJ. Band intensities were assessed with the Odyssey v2.1 software and normalized with the amount of total, TCE-labelled, protein in the corresponding lanes.

Analysis of cell-surface biotinylated fractions

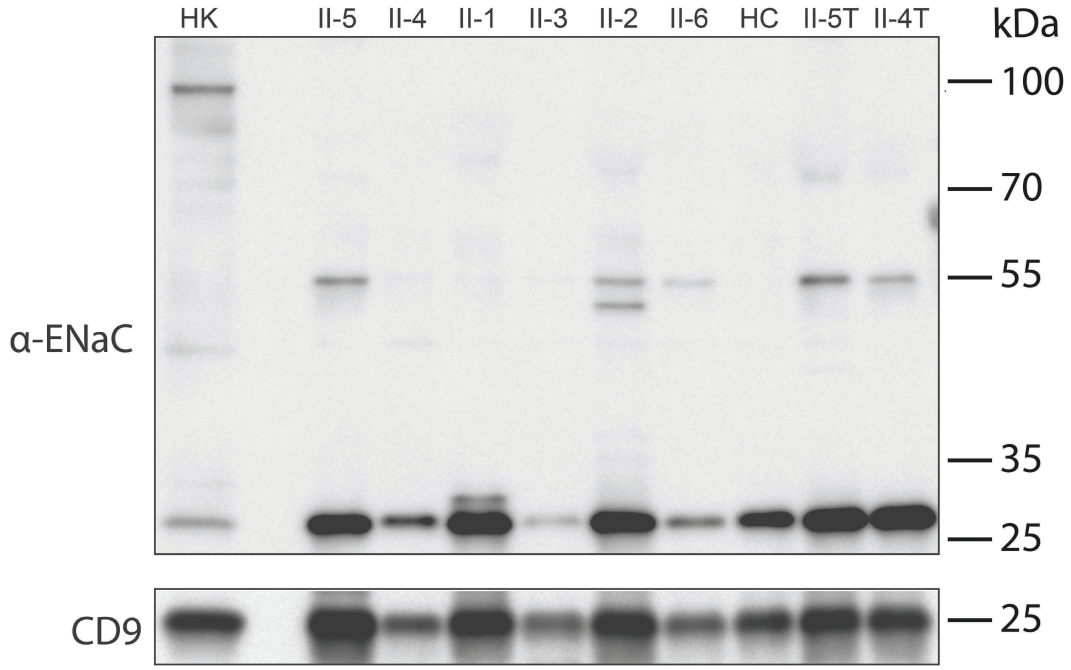
Control or injected oocytes (~25 per condition) were incubated for 15 min on ice in 1 ml Biotinylation buffer. Biotinylated fractions were isolated from ENaC-enriched fractions which had been purified as described before (35). Neutravidin-bound fractions and Triton-soluble fractions (1% of total) were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and blocked as described before. To account for non-specific binding to Neutravidin beads, band intensity values from control, non-biotinylated samples were subtracted from the values of the corresponding, biotinylated samples.

Statistics

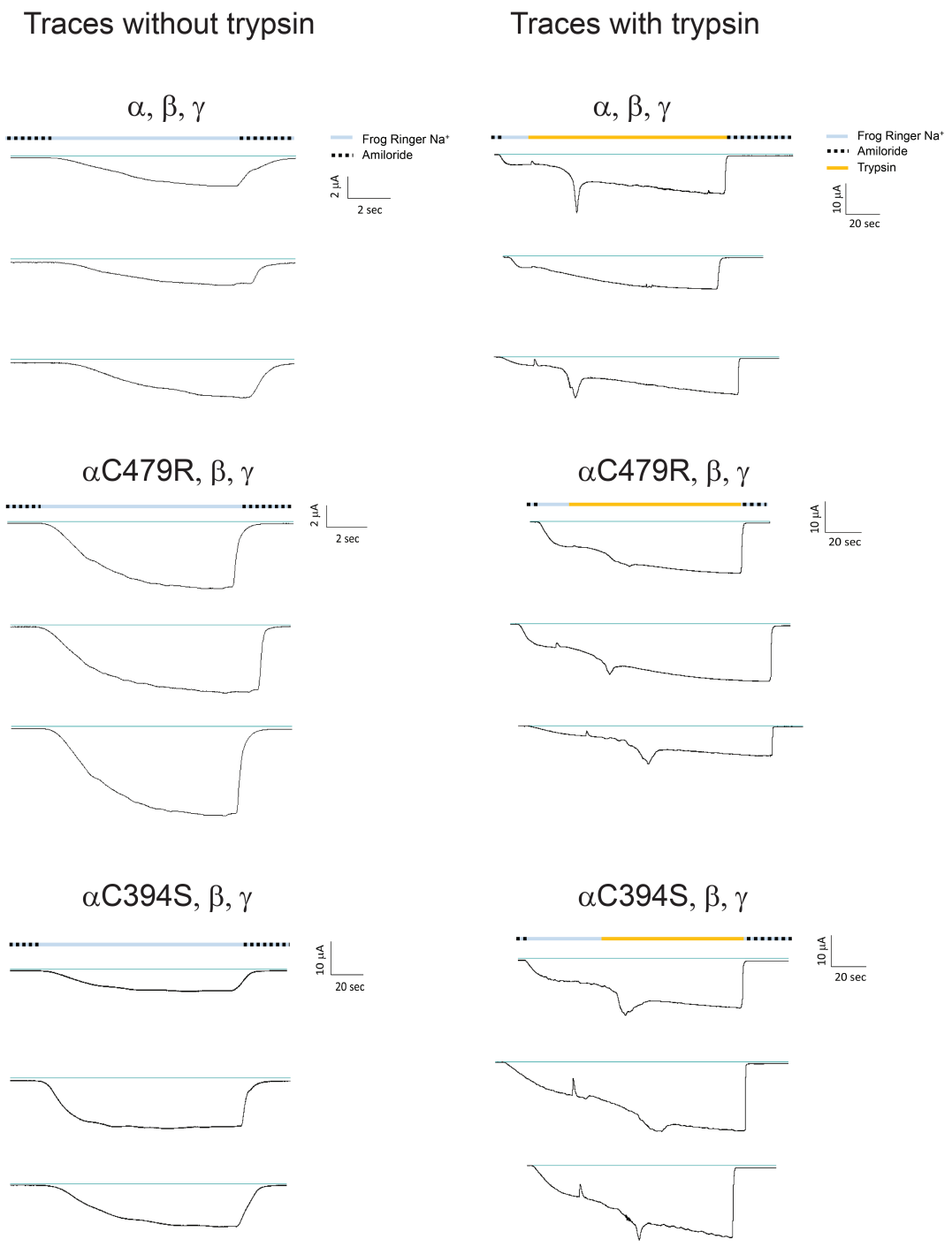
Statistical analyses were performed using GraphPad Prism software (GraphPad Software). Differences between groups were assessed by 1-way ANOVA and the Tukey post-hoc test. *P* values of less than 0.05 were considered statistically significant



Supplemental Figure 1: αENaC C479R mutation does not result in increased γENaC levels at the cell surface of injected oocytes. *After probing with anti-HA antibody (see* ***Figure 4****), blots were re-blocked and analyzed with anti-γENaC antibody as described for lysates in the Materials and Methods section. (**A**) Right panel, immunoblot analysis of Neutravidin-bound fractions isolated from control (-) or cell-surface biotinylated (+) Xenopus oocytes, non-injected (n.i.), or injected with either α_{wt}-HA/β/γ or α_{C479R}-HA/β/γ cRNAs. Left panel. Inputs corresponding to 1% of the Triton-soluble preparations from biotinylated oocytes used in the pull-down experiments. (**B**) Values (mean ± SEM) of CL+FL band intensities of inputs or of Neutravidin-bound fractions corresponding to experiments shown in A. Results from three blots with samples of three independent experiments. Differences are non-significant (N.S.).*



Supplemental Figure 2: αENaC in human urinary extracellular vesicles. *αENaC in urinary extracellular vesicles isolated from the reported family. Urinary extracellular vesicles were collected in the absence of interfering medication (no renin-angiotensin inhibitors or diuretics). A human kidney (HK) sample was included as positive control and showed the expected bands at 100 kDa (full-length αENaC) and ~30 kDa (cleaved αENaC). It is unclear what the low intensity band at ~45 kDa represents. Of these bands, only the 30 kDa band was detected in human urinary extracellular vesicles. Although no firm conclusions can be drawn based on the small number of individuals, no clear difference in the abundance of this 30 kDa band was visible between the two subjects carrying the C479R mutation (II-5 and II-4) compared to those without the mutation (II-1, II-2, II-3, and II-6) and a healthy control (HC). II-5T and II-4T are urinary extracellular vesicles isolated after the standardized diuretic test with triamterene (T). Of note, an additional band at ~55 kDa was visible in some of the subjects. Although this band could represent partially or atypically cleaved αENaC, it could also be non-specific, as it was also vaguely visible in kidneys of αENaC -/- mice (Sorensen et al., Kidney Int, 2013).*



Supplemental Figure 3: *Three original traces from hENaCwt, C479R, and C394S in the absence (left) or presence (right) of trypsin. Results from independent experiments; please note the current and time scales.*

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SECTION

03

URINARY EXTRACELLULAR VESICLES AND OTHER MARKERS FOR POLYCYSTIC KIDNEY DISEASE

CHAPTER

08

PROTEOMICS OF URINARY VESICLES LINKS PLAKINS AND COMPLEMENT TO POLYCYSTIC KIDNEY DISEASE

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ABSTRACT

Novel therapies in autosomal dominant polycystic kidney disease (ADPKD) signal the need for markers of disease progression or response to therapy. This study aimed to identify disease-associated proteins in urinary extracellular vesicles (uEVs), which include exosomes, in patients with ADPKD. We performed quantitative proteomics on uEVs using a labeled approach (healthy vs. ADPKD) and then using a label-free approach in different subjects (healthy vs. ADPKD vs. non-ADPKD chronic kidney disease [CKD]). In both experiments, thirty proteins were consistently more abundant (≥ 2-fold) in ADPKD-uEVs compared with healthy and CKD uEVs. Of these proteins, periplakin, envoplakin, villin-1, complement C3 and C9 were selected for confirmation, because (1) they were also significantly overrepresented in pathway analysis, and (2) they have been previously implicated in the pathogenesis of ADPKD. Immunoblotting was used to validate the proteomics results, confirming higher abundances of the selected proteins in uEVs from three independent groups of ADPKD-patients. While villin-1, periplakin, and envoplakin were increased in advanced stages of the disease, complement was already higher in uEVs of young ADPKD patients with preserved kidney function. Furthermore, all five proteins correlated positively with total kidney volume. The proteins of interest were also analyzed in kidney tissue from kidney-specific-tamoxifen-inducible *Pkd1*-deletion mice, demonstrating higher expression in more severe stages of the disease and correlation with kidney weight. In summary, proteomic analysis of uEVs identified plakins and complement as disease-associated proteins in ADPKD. These proteins are new candidates for evaluation as biomarkers or targets for therapy in ADPKD.

INTRODUCTION

Autosomal dominant polycystic kidney disease (ADPKD) is the most common inherited kidney disease, affecting approximately 4 in 10,000 individuals (1). It is caused by mutations in the *PKD1* or *PKD2* gene, encoding for polycystin-1 and polycystin-2 proteins (2). Both proteins associate with primary cilia and are thought to play a role in stretch-activated signaling. Loss of function of polycystins results in the development of fluid-filled cysts, ultimately leading to disruption of the normal kidney parenchyma. In the last decade, urinary extracellular vesicles (uEVs, which also include the so-called "exosomes" (3)) have emerged as promising markers for kidney disease (4-6). These nanosized vesicles are released by direct shedding or by fusion of multivesicular bodies with the plasma membrane (7). Their content comprises proteins and nucleic acids, both of which have been explored as biomarkers (5). More specific, uEVs appear to mirror the cellular make-up of renal epithelial cells. For example, we previously showed that aldosterone increased the sodium chloride cotransporter both in the kidney and in uEVs (8). Twenty to sixty percent of renal cysts in ADPKD remain connected with the parent nephron (9, 10), so that a significant portion of uEVs in ADPKD may be derived from cyst epithelial cells. Studying uEVs in ADPKD may address the pathophysiology of the disease, as uEVs contain polycystins and have been shown to interact with primary cilia (11). We therefore hypothesize that studying uEVs in ADPKD is more advantageous than studying whole urine. Accordingly, the aims of this study were to (1) compare the proteome of whole urine with the proteome of uEVs (2), identify disease-associated proteins in uEVs from ADPKD patients.

RESULTS

Characteristics of participants

uEVs were isolated in four groups of ADPKD patients due to a *PKD1* mutation to identify and confirm disease-associated proteins (**Table 1, Figure 1, Supplementary Table 1 online**). In the *identification cohort* we used labeled proteomics to identify proteins with higher or lower abundance in uEVs of patients with ADPKD. We also analyzed the proteome of whole urine to compare it with the uEV proteome. In confirmation cohort 1 we used label-free proteomics, and included different patients with ADPKD and also included patients with non-ADPKD chronic kidney disease (CKD). CKD patients were matched by age, sex, and estimated glomerular filtration rate (eGFR) and this group was used to exclude proteins that may be related to impaired kidney function in general. The reason to use both labeled and label-free proteomics techniques is that the two approaches complement each other in terms of quantitation (labeled) and sensitivity (label-free). In *confirmation cohort 2*, uEVs were isolated and compared with the uEVs of the CKD patients from the validation cohort. Finally, *confirmation cohort 3* cohort consisted of healthy control subjects, young ADPKD patients with preserved renal function (CKD stage 1) and those with more progressive disease (CKD stages 2-4).

Qualitative comparison of the urinary proteome: whole urine versus uEVs

Figure 2A shows that 1048 proteins were identified in whole urine and 1245 proteins in uEVs of which 527 overlapped (see www.proteomexchange.org, identifier PXD003298 for a list of all proteins). The total number of identified proteins in urine was therefore 1766. Of interest, despite the fact that whole urine still contained uEVs, 718 proteins were *only* identified in uEVs. This suggests that isolation of uEVs results in a different set of proteins not found in whole urine. **Figure 2B** characterizes how the unique proteins distribute to the different cellular components. Whole urine showed more cell surface, plasma membrane, and extracellular proteins, whereas uEVs showed more cytoplasmic, cytoskeletal, endosomal, and mitochondrial proteins. Of the 517 unique

proteins in whole urine, the majority (389 proteins, 75%) had a molecular weight below the cut-off for glomerular filtration (< 70 kDa) (12). We also performed a qualitative comparison to identify over-represented pathways in whole urine and uEVs of patients with ADPKD (**Table 2**) (13). Using the DAVID annotation tool (14), we identified that significantly over-represented pathways in ADPKD-uEVs consisted of actin-related processes and immune system processes, including complement activation. The latter finding was confirmed by an analysis using the Kyoto Encyclopedia of Genes and Genomes (KEGG), which also indicated that 'complement and coagulation cascades' (hsa04610) were over-represented in ADPKD (37 proteins, P = 0.012).

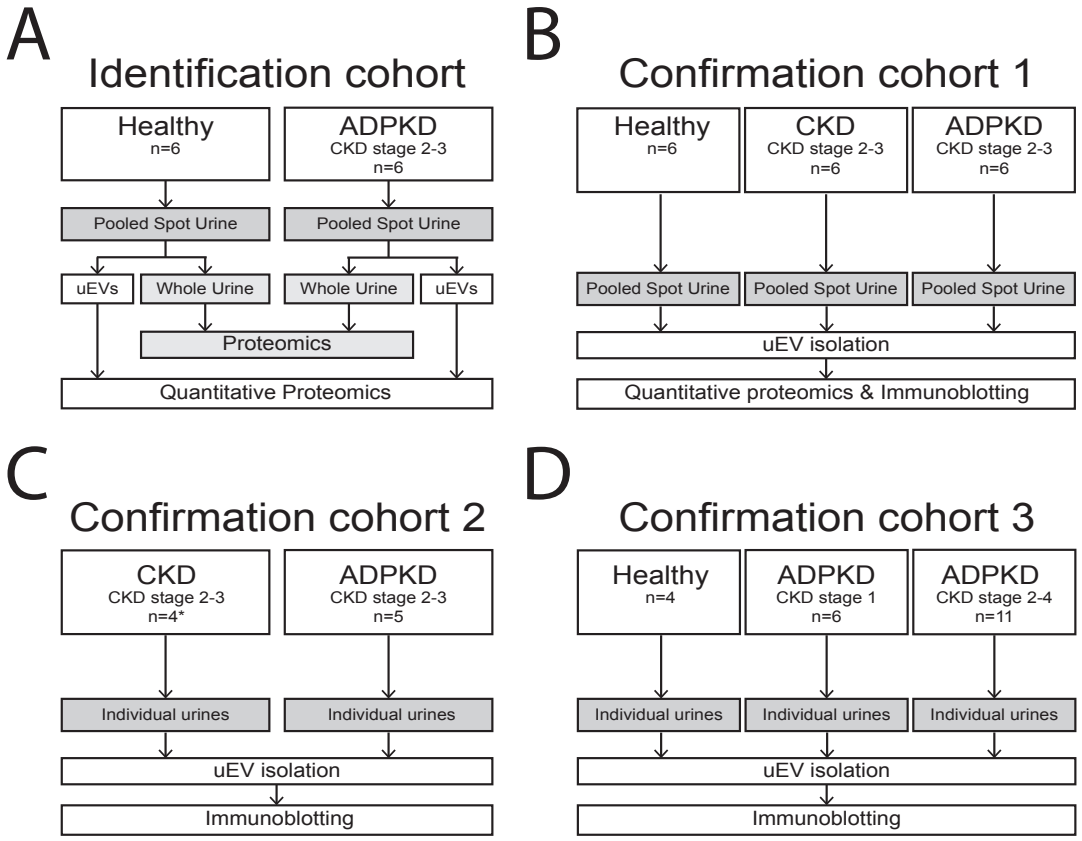


Figure 1: Sample collection, processing, and analysis in the four study groups. ***A and B:*** *Identification and confirmation cohorts used for proteomic analysis. Quantitative proteomics was performed using dimethyl-labeling in the identification cohort and label-free methods in confirmation cohort 1 (see Concise Methods).*

C and D: *Confirmation cohorts 2 and 3 were used for the immunoblotting analysis.*

** Patients from validation cohort.*

Quantitative proteomics of ADPKD-uEVs

We performed quantitative proteomics in the identification cohort and confirmation cohort 1 in order to select proteins that were consistently higher or lower in uEVs of patients with ADPKD and not related to CKD (**Figure 1**). **Table 3** shows the 30 proteins with consistently higher abundance in ADPKD-uEVs and the 4 proteins with lower abundance, including the ratios for the mean ion intensities (ratios were not calculated if proteins in one of the control groups were absent or very low abundant). Among the identified proteins were the actin modulating protein villin-1, as well as plakins such as envoplakin, and periplakin. Complement-related proteins were also more abundant in ADPKD-uEVs, including complement C3 and C9.

Cohort	Subjects	Age	% Male	CKD stage	eGFR	HtTKV	U_{Alb}
Identification cohort	Healthy (n = 6)	49.2 ± 5.1	50	-	N.M.	-	2.2 ± 1.2
	ADPKD (n = 6)	48.7 ± 5.5	50	2 - 3	47.5 ± 4.2	1101 ± 346	3.0 ± 1.4
Confirmation cohort 1	Healthy (n = 6)	49.7 ± 3.5	50	-	N.M.	-	0.5 ± 0.1
	CKD* (n = 6)	48.8 ± 3.2	50	3	45.7 ± 3.5	-	8.2 ± 4.8
	ADPKD (n = 6)	50.3 ± 3.1	50	2 - 3	49.8 ± 4.5	1481 ± 223	2.3 ± 0.4
Confirmation cohort 2	CKD (n = 4)	44.3 ± 2.1	75	3	48.5 ± 4.6	-	6.5 ± 6.0
	ADPKD (n = 5)	47.6 ± 3.3	60	2 - 3	44.2 ± 10.9	1258 ± 355	6.0 ± 2.8
Confirmation cohort 3	Healthy (n = 4)	30.0 ± 0.7	50	-	N.M.	-	6.3 ± 1.8
	ADPKD (n = 6)	27.8 ± 2.0	50	1	109.8 ± 4.3	622.3 ± 48.2	7.5 ± 2.0
	ADPKD (n =11)	43.6 ± 1.8	45	2 - 4	45.1 ± 3.8	1475 ± 297.4	2.7 ± 1.3

Table 1: Characteristics of patients and healthy subjects. ** CKD due to hypertensive nephropathy in all patients.*
ADPKD, autosomal dominant polycystic kidney disease; CKD, chronic kidney disease; eGFR, estimated glomerular filtration rate (calculated by the 4 variable Modification of Diet in Renal Disease formula); HtTKV, height adjusted total kidney volume; N.M., not measured; U_{Alb}, urinary albumin.

Confirmation of plakins and complement in ADPKD-uEVs

Five proteins that were more abundant in uEVs of ADPKD-patients were selected for confirmation and further characterization based on the pathway analysis and their possible involvement in the pathophysiology of ADPKD (15-19). These proteins included villin-1, envoplakin, periplakin, C3, and C9 (**Table 3**). To confirm the quantitative proteomics results, we immunoblotted the five proteins using the same pooled uEV-samples as were used for the proteomics studies (**Figure 1B**, **Figure 3**). Indeed, the abundance of all five proteins was higher for the ADPKD-group compared to the two other groups. Similar abundances of CD9 suggested comparable number of vesicles in the three groups (20). Subsequently, the five proteins of interest were analyzed in uEVs from a third group of ADPKD patients (**Figure 1C**) and compared with the CKD group (**Figure 4**). Again, this analysis confirmed the higher abundance of the five selected proteins in ADPKD-uEVs, but now also on an individual basis. Because our identification and confirmation cohorts 1 and 2 consisted of older ADPKD patients with CKD stage 2-3, we also analyzed our proteins of interest in a third confirmation cohort (**Figure 1D**) consisting of younger ADPKD patients with preserved renal function and additional APDKD patients with CKD stages 2-4 (**Figure 5**). As CD9 declined with progressive CKD, we analyzed the ratio between uEV protein abundance and CD9. Villin-1, periplakin, and envoplakin were increased only in progressive CKD, whereas complement was already increased in uEVs from ADPKD patients with preserved renal function. In addition, all five proteins correlated with height adjusted total kidney volume (**Figure 5B**).

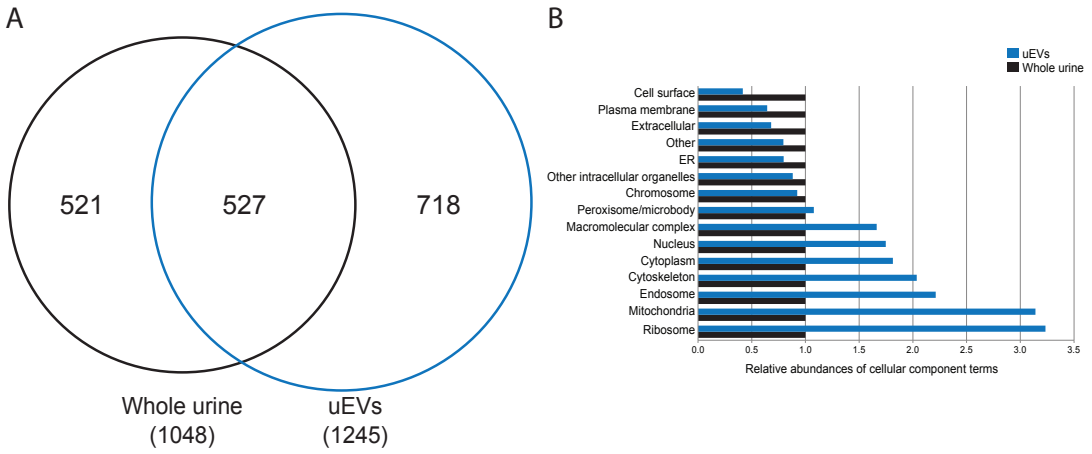


Figure 2: A: *Venn diagram showing that urinary extracellular vesicles (uEVs) contained a different set of unique proteins compared to whole urine.* ***B:*** *Comparison of the cellular components to which the unique proteins identified whole urine (n=521) and uEVs (n=718) belong. For comparison whole urine is set at a relative abundance of 1.*

Biological process	**GO numbers***	**Number of proteins**
Urinary extracellular vesicles		
Actin filament-based process	0030029, 0030832, 0030036, 0008064, 0030833	60
Lymphocyte mediated immunity	0002449	25
Activation of plasma proteins involved in acute inflammatory response	0002541	24
Adaptive immune response	0002250, 0002460	24
Complement activation	0006956	23
Oxidation reduction	0055114	81
Positive regulation of hydrolase activity	0051345	22
Regulation of protein polymerization	0032271	22
Whole urine		
Innate immune response	0045087, 0006955, 0050778, 0002252	107
Programmed cell death	0012501, 0016265, 0008219	48
Response to extracellular stimulus	0009991, 0031667	29
Carbohydrate catabolic process	0016052	28
Proteolysis	0006508	93

Table 2: Gene Ontology (GO) biological process terms over-represented in uEVs and whole urine of patients with ADPKD. ** Several GO-terms were combined if processes were similar and directly linked. P-value ≤ 0.05 for all GO-terms as calculated by DAVID annotation tool. P ≤ 0.05 is considered strongly enriched.*

Characterization of plakins and complement in uEVs

We further characterized the five proteins by density-based fractionation using sucrose (**Figure 6**). This was done to analyze with which type of vesicles the proteins associate, using CD9 and CD63 as markers for exosomes (20), and NHE3 and AQP2 as markers for vesicles derived from the proximal tubule or collecting duct, respectively. An additional advantage of density-based fractionation is that protein complexes and large protein aggregates may be co-isolated during ultracentrifugation, but do not float on a sucrose gradient (21). This analysis suggested the presence of two populations of vesicles. More specific, periplakin, envoplakin, villin-1, and C9 were detected in CD9 and CD63 positive fractions, suggesting their presence in exosomes (20). All six proteins were also detected in the fractions representing denser vesicles (fractions 2 – 4). The presence of AQP2 in these denser vesicles (mainly in fraction 3) suggests that some of these vesicles are derived from the collecting duct (22).

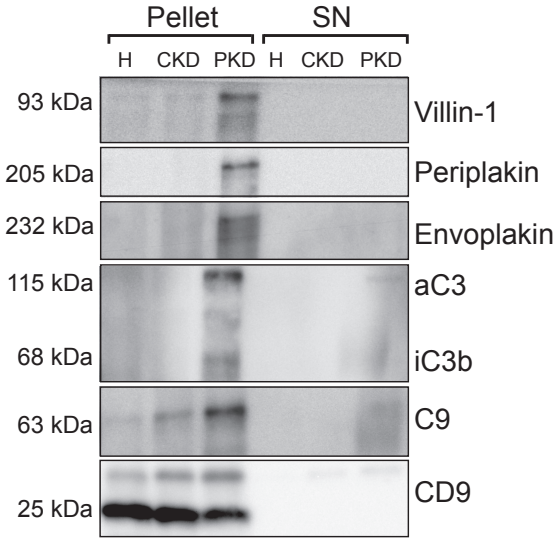


Figure 3: Immunoblot analysis of the five selected proteins using the same pooled urine as was used for quantitative proteomics in the validation group. *The first three rows show results for isolated uEVs (Pellet), while the last three rows show results for the supernatant (SN), which was used as negative control. Anti-complement C3 antibody recognizes the C3 alpha chain (aC3) and its split product iC3b. H, healthy subjects; CKD, chronic kidney disease; PKD, polycystic kidney disease.*

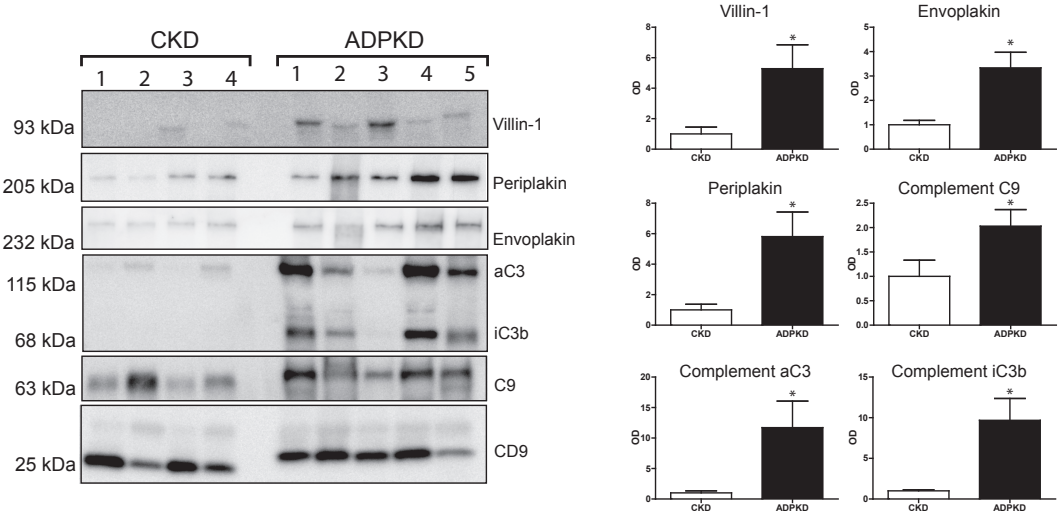


Figure 4: *Immunoblot analysis comparing the proteins of interest between individual CKD and ADPKD patients. uEVs were isolated from individual spot urines of CKD and ADPKD patients (confirmation cohort 2). Anti-complement C3 antibody recognizes the C3 alpha chain (aC3) and its split product iC3b. * $P < 0.05$.*

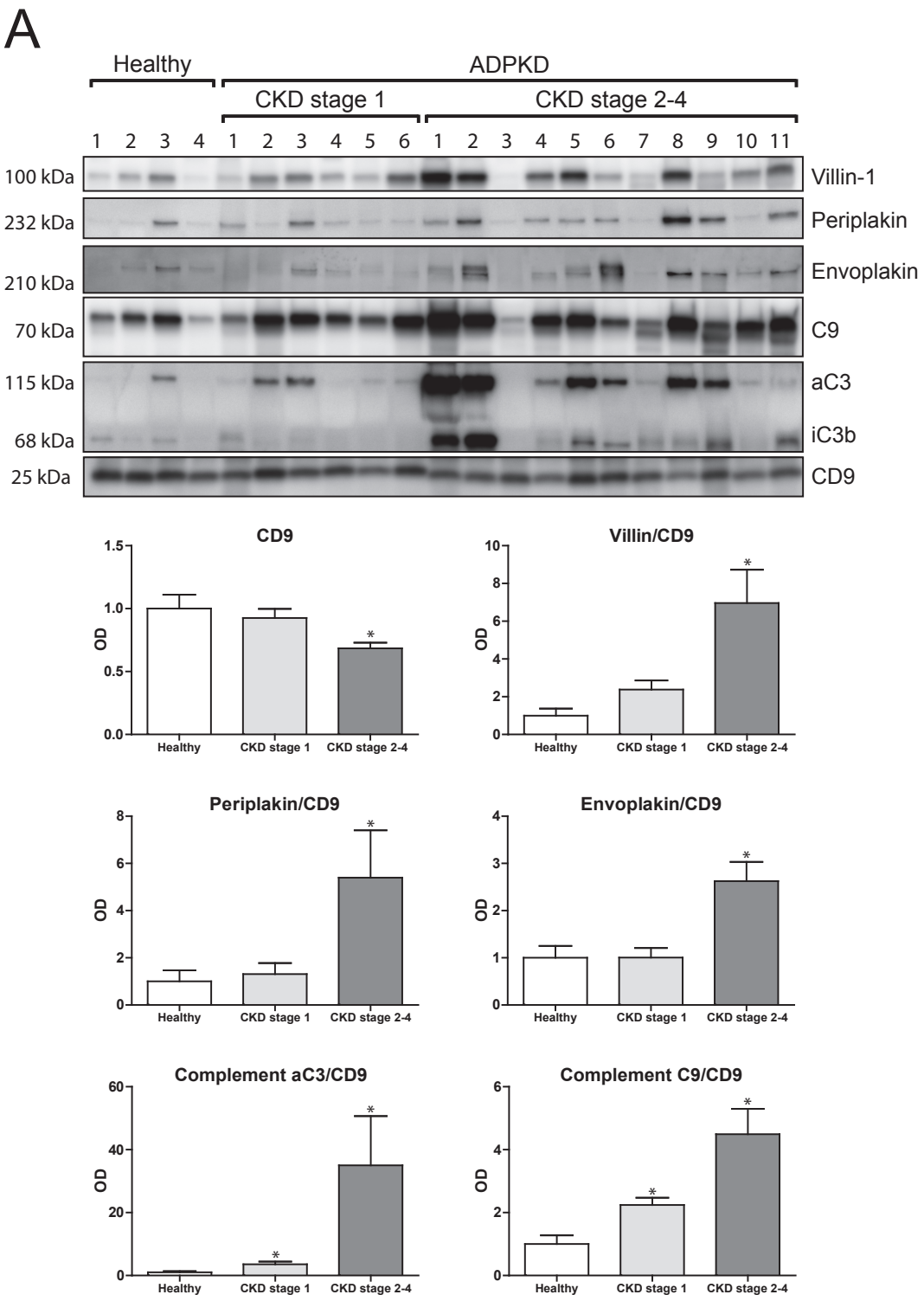


Figure 5: A: *Immunoblot analysis comparing the proteins of interest in uEVs from individual healthy subjects, young ADPKD patients with preserved renal function and ADPKD patients with CKD stage 2-4. * P < 0.05.*
ADPKD, autosomal dominant polycystic kidney disease; CKD, chronic kidney disease

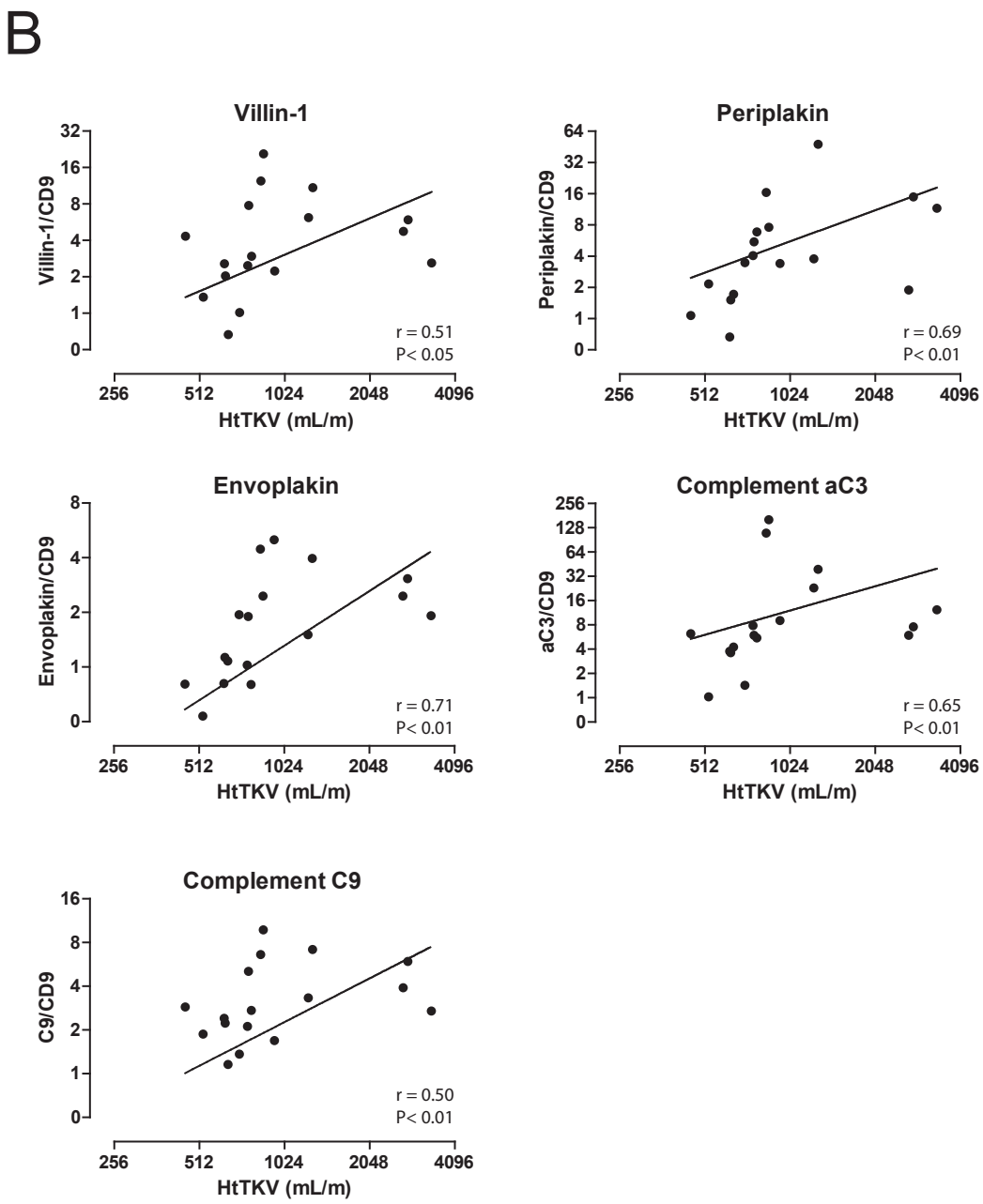


Figure 5: B: *Correlations of abundances of the uEV proteins of interest compared to height adjusted total kidney volume. Spearman ρ and P-values are shown.*
HtTKV, height adjusted total kidney volume.

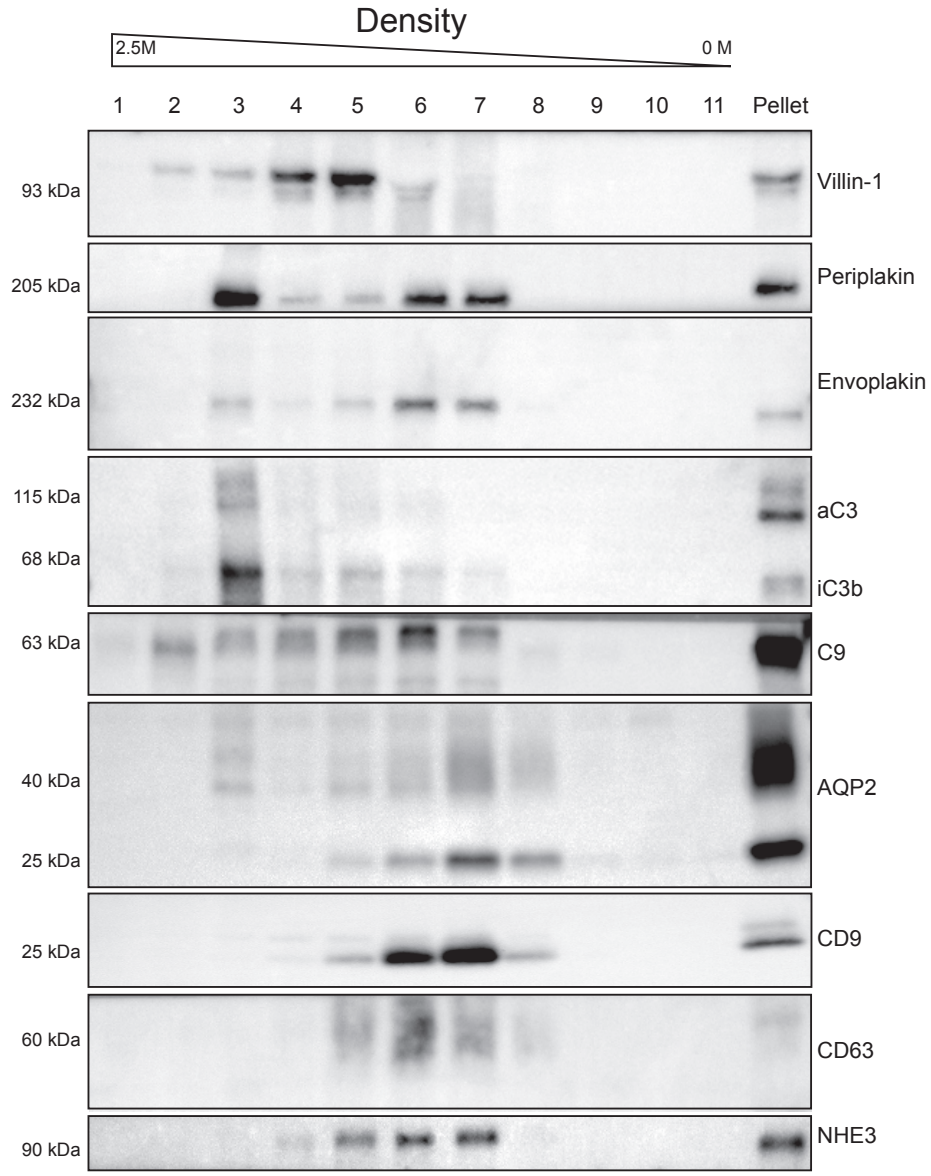


Figure 6: Sucrose gradient fractionation was performed to analyze with which type of vesicles the identified proteins associate. *Fraction 1 represents the most dense fraction. In addition to the six proteins of interest, we also analyzed CD9 and CD63 (markers for urinary exosomes) and NHE3 and AQP2 (markers for proximal tubule and collecting duct). 'Pellet' refers to a part of the pooled ultracentrifugation pellet used for direct immunoblotting (positive control). Anti-complement C3 antibody recognizes the C3 alpha chain (aC3) and its split product iC3b.*

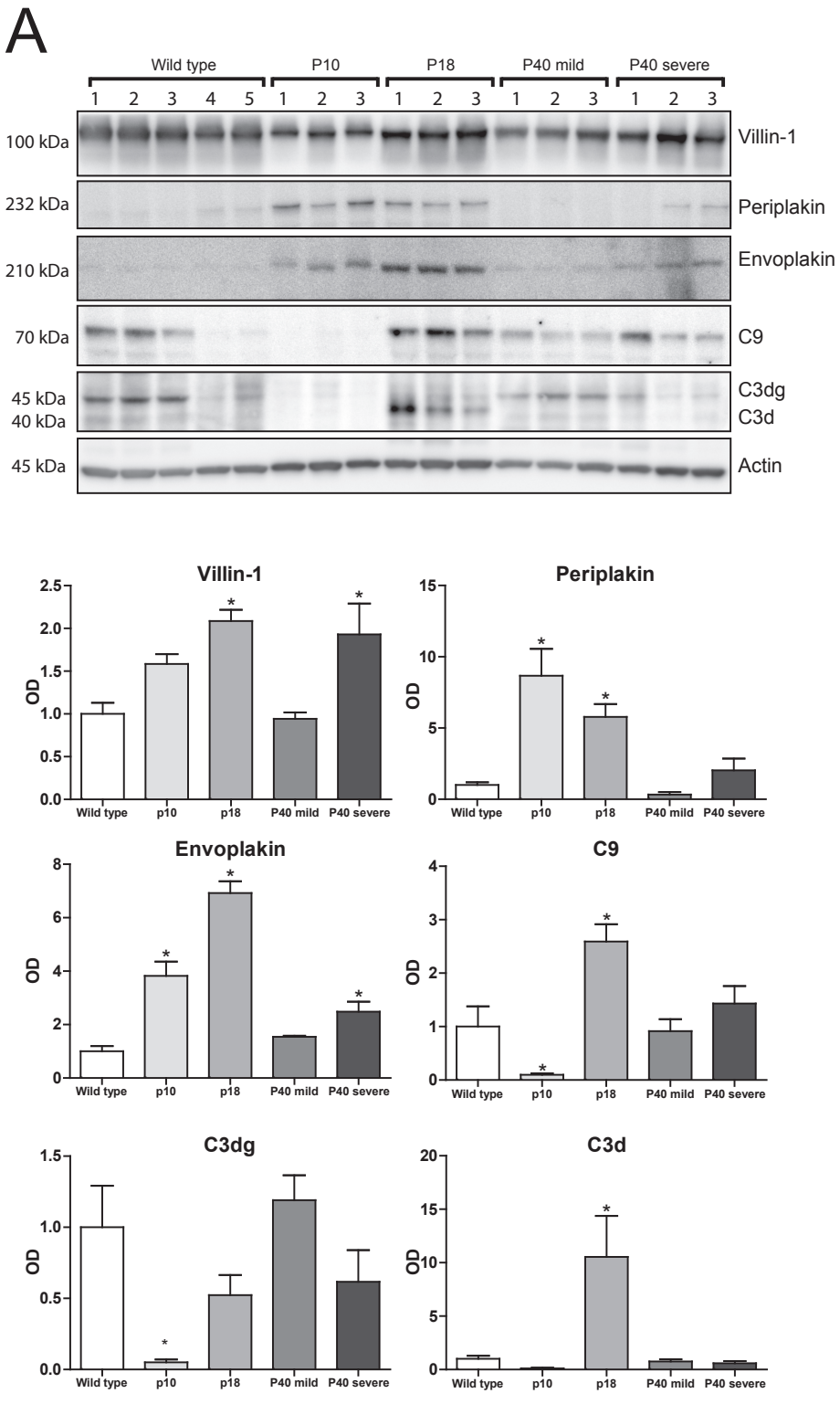


Figure 7: A: *Immunoblot analysis comparing the proteins of interest in kidney homogenates of three inducible ADPKD mouse models. 'P' indicates the post natal day at which the Pkd1-gene was inactivated with tamoxifen. The P40 mice were sacrificed at two different time points, producing a mild (normal blood urea) or severe (elevated blood urea) phenotype. Each ADPKD group was compared to wild-type. * P < 0.05.*

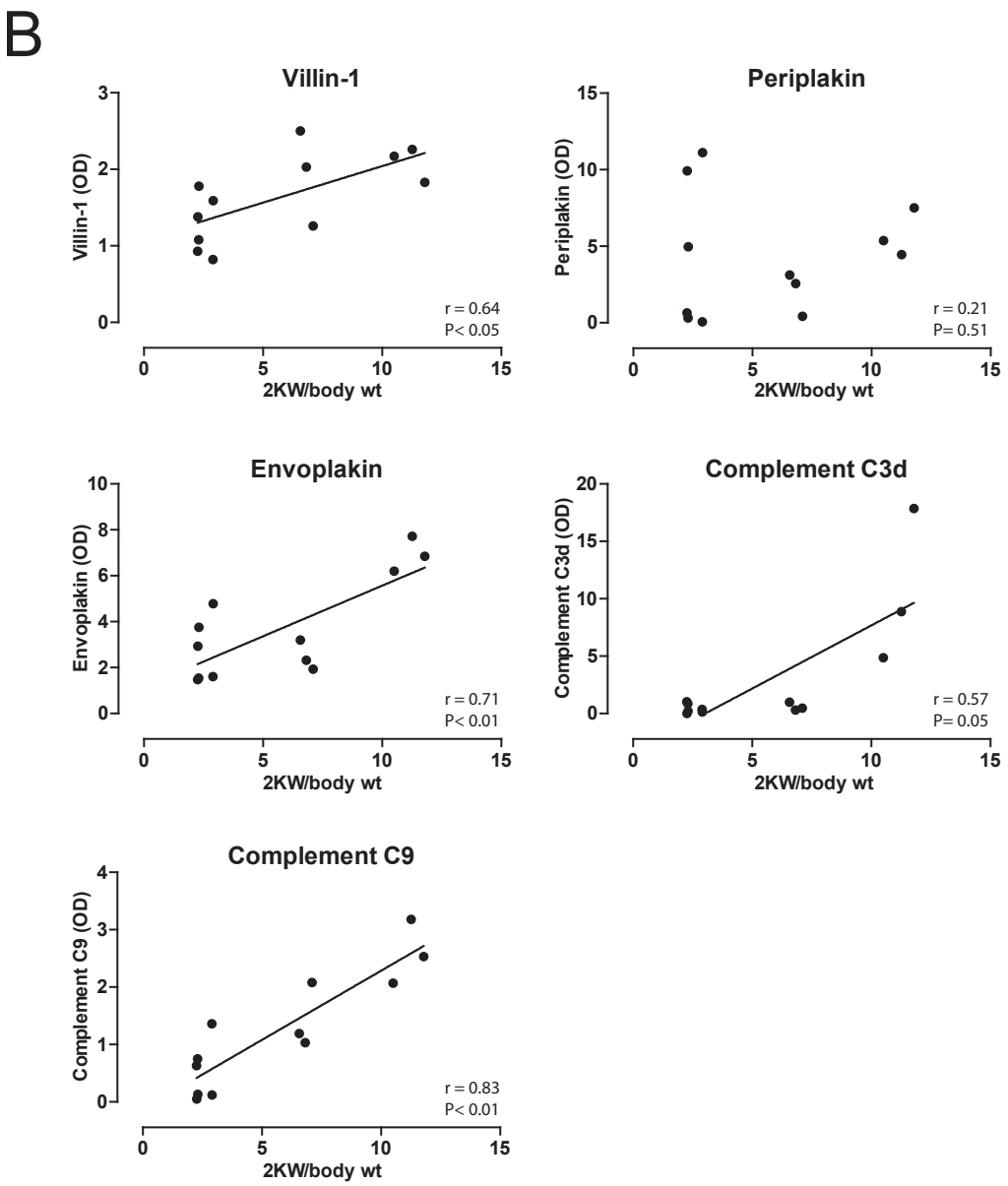


***Figure** 7: **B:** Correlations between kidney weights and kidney abundances of villin-1, envoplakin, periplakin, complement C3d, C9. Spearman ρ and P-values are shown. 2KW/BW: 2 kidney weight to body weight ratio.*

Increased plakins and complement in ADPKD mouse models

To analyze whether the proteins of interest were also more abundant in polycystic kidneys, we used three variants of kidney-specific-tamoxifen-inducible *Pkd1*-deletion (iKsp-*Pkd1*^{del}) mice (See **Supplementary Table 2 online**) (23). *Pkd1* inactivation in these mice was induced at either *post natal* day (P) 10, 18 or 40, which results in distinct PKD phenotypes. The P10 model rapidly develops cysts primarily from distal tubules and collecting ducts (24), whereas the P40 model has a much slower progression with cysts derived primarily from the proximal part of the nephron and to a lesser extent from distal tubules and collecting ducts (25). The P40 mice were sacrificed after 117 or 140 days resulting in a mild (normal blood urea) or severe (elevated blood urea) phenotype. In addition, the P18- iKsp-*Pkd1*^{del} mice is an adult onset model with cysts from all different tubular segments (26). The abundances of both periplakin and envoplakin were significantly higher in the P10, P18, and to a lesser extent the P40 severe models (**Figure 7A**). Villin-1 was increased in P18 and P40. Complement C3d (the final breakdown product of activated C3) and C9 were increased in the P18 model, but decreased in P10. Of note, the lower abundances of C3d and C9 may be related to age rather than to ADPKD (wild type mice 4 and 5 were younger than the other wild type mice, but had the same age as the P10 mice, **Supplementary Table 2 online**). No changes in C3 and C9 were found in the P40 models. Furthermore, we found that protein abundance correlated positively with total kidney weight for villin-1, envoplakin, complement C3d and C9 (**Figure 7B**).

Group	Protein	Group 1 (vs. hlty)*	Group 2 (vs. hlty)*	Group 2 (vs. CKD)*
Plakins	Desmoplakin	4.4	N.C.	N.C.
	Envoplakin† ¶	12.7	2.2	N.C.
	Periplakin†	10.8	2.0	N.C.
Complement	Complement C3†	2.5	13.8	7.4
	Complement C5	3.9	N.C.	74.8
	Complement C4-B	3.1	5.9	4.3
	Complement C9†	7.5	10.2	5.8
	Complement factor B	13.7	20.3	8.5
	Complement C1q subcomponent subunit A	2.0	N.C.	N.C.
Glycoproteins	Protein tweety homolog 3 ¶	2.2	2.1	2.5
	Isoform 2 of Solute carrier family 22 member 13 ¶	5.4	2.2	N.C.
	V-set domain-containing T-cell activation inhibitor 1	3.1	2.45	3.09
	Retinoic acid-induced protein 3 ¶	6.6	2.15	3.45
	Pigment epithelium-derived factor	6.0	5.23	8.27
	Heparin cofactor 2	2.1	30.32	N.C.
	Inter-alpha-trypsin inhibitor heavy chain H1	3.2	N.C.	21.69
Miscellaneous	Villin-1† ¶	3.0	2.4	11.8
	Tyrosine-protein phosphatase non-receptor type 13 ¶	2.6	2.36	N.C.
	Soluble of Catechol O-methyltransferase ¶	2.1	4.10	2.98
	Protein crumbs homolog 3 ¶	2.6	N.C.	N.C.
	Aconitate hydratase, mitochondrial ¶	3.0	3.05	N.C.
	Apolipoprotein A-IV ¶	2.6	4.30	2.82
	Cysteine-rich C-terminal protein 1 ¶	15.8	13.05	N.C.
	Glycogen phosphorylase, brain form	4.1	2.03	N.C.
	Angiotensinogen	10.5	11.70	9.95
	Calpain-5 ¶	2.3	2.1	9.0
	EH domain-containing protein 4	2.6	2.2	15.1
	Gamma-synuclein	2.2	3.4	N.C.
	Prothrombin	6.0	3.96	2.82
	Plasminogen	23.6	4.84	2.77

Table 3A: Proteins more abundant in uEVs of patients with ADPKD. *No ratio was calculated (N.C.) if the protein was identified in patients with ADPKD but was absent in the healthy subjects or patients with non-ADPKD CKD (or identified at very low levels). † These proteins were selected for confirmation and further characterization.*

Protein	Group 1 (vs. hlty)*	Group 2 (vs. hlty)*	Group 2 (vs. CKD)*
Annexin A2	0.12	0.41	0.49
Contactin-1	0.43	0.26	0.14
Syndecan-4 ¶	0.40	0.08	0.19
Granulins OS	0.34	0.50	0.23

Table 3B: Proteins less abundant in uEVs of patients with ADPKD. ** Mean ion intensities were compared and are reported as ratios. ¶ These proteins were not identified in whole urine. CKD, chronic kidney disease group; Hlty, healthy subjects.*

DISCUSSION

Urinary extracellular vesicles (uEVs) are increasingly used to identify non-invasive markers of disease, including ADPKD. Here, we show higher abundances of complement-related proteins (C3 and C9) and cytoskeletal proteins (villin-1 and plakins) in ADPKD-uEVs, and suggest these proteins may be used as disease markers. Complement C3 and C9 increase already early in the disease, whereas the cytoskeletal proteins increased with more progressive disease. The interpretation of proteomic analyses of uEVs deserves to be critical, because many aspects of uEVs remain unclear (5). However, we believe the strength of the approach in this study was that (1) two complementary proteomics approaches were used to identify proteins of interest, (2) the proteins of interest were higher in four independent groups of ADPKD patients, (3) results were compared with non-ADPKD CKD-uEVs to exclude proteins related to kidney function decline in general, (4) the proteins of interest correlate to total kidney volume, and (5) the proteins of interest were also increased in mouse models of ADPKD.

The identification of villin-1, plakins, and complement in uEVs of patients with ADPKD may be biologically plausible. Villin-1 is an actin-modifying protein that is involved in cell morphology, actin reorganization and cell motility; in the

kidney, it is mainly expressed in the brush border of the proximal tubules (27). Polycystin-1 is implicated in the regulation of actin cytoskeleton organization, migration and cell adhesion (28). Defects in polycystin-1 results in cell-polarity defects (29) and aberrant cell growth (30), which may explain the increase of villin-1. The desmosomal plaque consists of several transmembrane proteins belonging to the cadherin family (also called plakins (31)). Desmosomes form an adhesive junction at the basolateral membrane and are vital for stabilizing the epithelial sheet. Polycystin-1 is associated with desmosomal proteins and is required for the establishment of cell polarity (19, 32). In ADPKD, polycystin-1 no longer co-localizes with desmosomes leading to mispolarization of desmosomal proteins from the basolateral side to the apical domain (17). This may explain why we found higher abundances of plakins in uEVs of patients with ADPKD. Complement activation has previously been implicated in both ARPKD and ADPKD (15, 16, 18). Gene expression analysis in cpk mice, a model for PKD, identified the innate immune response to be highly activated, specifically complement factors such as C3 (16). In humans, cyst epithelial cells produce complement components, including C3 and C9, as shown in immunohistochemical staining (15, 18). Many of the complement components are abundantly present in human ADPKD kidney cyst fluid (33, 34). Furthermore, inhibition of the complement system by rosmarinic acid in $Pkd1^{-/-}$ mice and Han:SPRD Cy/+ rats reduced cyst growth (18), suggesting that the complement pathway is involved in the pathogenesis of ADPKD. We identified nearly all complement proteins in uEVs, both from the classical (C1q, C2, and C4) and alternative pathways (complement factors B and D), along with inhibitors of this system (plasma protease C1 inhibitor, C4 binding protein and complement factor B). Complement C9 co-localized with the exosomal markers CD9 and CD63, suggesting its presence in exosomes. However, complement C3 was mainly identified in a denser fraction, as was previously demonstrated (35). Other studies also reported the presence of complement in uEVs (35-37) and circulating EVs (38, 39). It is unclear why complement is isolated in ultracentrifuged urine and whether complement is physically associated with uEVs. In theory, complement may be filtered from plasma and end up non-

specifically attached to uEVs. However, most complement proteins are large in size (the majority exceeding 70 kDa), and therefore unlikely to be filtered. A more plausible explanation is the local production and excretion of complement by renal epithelial cells (40), possibly to opsonize pathogens as a defense mechanism for urinary tract infections (41, 42). The higher abundance of components of the complement system in uEVs of patients with ADPKD may reflect increased production by renal cyst epithelial cells.

Recently, Hogan *et al.* also studied uEVs as a source of biomarkers for ADPKD (43). In patients with a *PKD1* mutation, they found polycystin-1 and polycystin-2 to be decreased, and transmembrane protein 2 (TMEM2) to be increased. The ratios between the two polycystins and TMEM2 allowed differentiation between patients with ADPKD and healthy subjects. In our study, polycystins were not identified in our first quantitative proteomics experiment (identification cohort), and therefore not selected for further confirmation. In the second analysis (confirmation cohort 1), polycystin-2 was identified and 83% lower in ADPKD compared to healthy subjects, but was not identified in the CKD-group. Polycystin-1 was only identified in the control group, and TMEM2 was not identified at all. We propose the following explanations for these differences. First, in order to analyze the complete spectrum of vesicles, we did not fractionate uEVs, whereas Hogan *et al.* isolated polycystin-positive uEVs (43). This difference in isolation methods may explain why polycystin-1 and polycystin-2 were 5- to 7-fold more abundant in the study by Hogan *et al.* (43). Second, our patients with ADPKD had a lower eGFR. In addition to the mutation, the decreasing eGFR may explain why we observed a larger decrease in polycystin-2 and failed to identify polycystin-1 in patients with ADPKD.

The reason to focus on uEVs is illustrated by the comparison of the urinary proteome of whole urine with that of uEVs (**Figure 2**). The proteins identified in whole urine by mass spectrometry primarily consisted of extracellular proteins with low molecular weight and were therefore most likely plasma-derived (12). Despite the fact that whole urine still contained uEVs, the isolation of uEVs

yielded a set of 718 unique proteins that were primarily of intracellular origin. These differences underscore that the isolation of uEVs increases the identification rate of low-abundant proteins that are likely derived from renal epithelial cells. These proteins were not identified in whole urine, probably because they were masked by more abundant plasma-derived proteins. To our knowledge only one study has compared these two proteomes, using less sensitive mass-spectrometers (< 100 identified proteins in each fraction) (44). Gradient fractionation showed that all proteins were present in CD9+ and CD63+ vesicles, compatible with exosomes, but also revealed their presence in denser vesicles. These might be ectosomes from the glomerulus (35), but electron microscopy would be necessary to confirm this. The distinction in vesicle subtype is relevant, because polycystin 1 and 2 are excreted in exosomes. This implies that selectively isolating CD9, CD63 or polycystin-positive vesicles may be a useful strategy to more specifically analyze disease-associated proteins.[43]

Our study has a number of limitations. Pooled urine was used for proteomics analysis to increase homogeneity, but this did not allow statistical analysis of the proteomics results. This was addressed by analyzing the candidate proteins in individual patients from a separate ADPDK group (**Figures 4 and 5**). Although the inclusion of two proteomics studies reduced the list of candidate proteins, we believe this approach excluded proteins related to inter-subject variation and non-ADPKD CKD. It is unlikely that uEV-markers will be used as diagnostic tool in ADPKD, as the diagnosis can usually be established by family history, ultrasound, or CT-scan (2). Therefore, to be clinically useful, uEV-markers should correlate with disease progression or response to therapy The proteins identified in this study should therefore be considered candidate markers and require further evaluation in larger prospective studies using serial urine samples (45). Unlike the complement proteins, villin-1 and the plakins appear unsuitable for early monitoring of ADPKD. They may be evaluated in more advanced stages of ADPKD, for example for monitoring therapeutic response. Importantly, kidney injury unrelated to ADPKD may also increase

complement in uEVs, although several studies have indicated that complement may play a more specific role in the disease. These aspects should be taken into consideration in future evaluation of the candidate proteins identified in this study.

In conclusion, we have demonstrated the advantage of uEVs to enrich the urinary proteome. We explored uEVs as potential biomarkers for ADPKD and identified several classes of proteins to be specifically increased, including plakins and components of the complement system. These findings warrant further investigation of these proteins as potential biomarkers in a larger prospective cohort.

CONCISE METHODS

Participants and isolation of uEVs

The Medical Ethics Committee of the Erasmus Medical Center approved this study (MEC-2012-313 and MEC-2013-370). Patients with ADPKD were recruited from the ongoing DIPAK-1 and DIPAK-observational studies, which include ADPKD patients with preserved renal function as well as those with CKD stage 2-4 (46). Genetic analysis was performed for all patients. To increase homogeneity, we only included patients with a confirmed *PKD1* mutation (Supplementary Table 1 online). Two patients with a negative *PKD2* mutation and an ADPKD phenotype were also considered *PKD1* mutation (47). Each individual patient was age- and gender-matched to a healthy control (identification cohort) or an age-, gender- and eGFR-matched patient with CKD (confirmation cohorts 1 and 2). Additional inclusion criteria for the patients with CKD were the absence of ADPKD and minimal proteinuria (< 1 gram/10 mmol creatinine). Previously, we successfully used spot urines for uEV analysis (normalized by urinary creatinine) and prefer this over 24-hour urine to limit protein degradation and the risk of incomplete collections (8). Therefore,

second morning spot urines were collected, a protease inhibitor (cOmplete, Roche diagnostics) was added and samples were immediately stored at -80°C until further processing. uEVs were isolated using high-speed centrifugation and ultracentrifugation (see **Supplemental File online** for complete protocol) (8). Our protocol differed from a recent uEV-study in ADPKD, (48) which used density gradient fraction to enrich for PKD-positive uEVs. We did not use this approach because we were interested in all uEVs. A recent study compared different uEV isolation protocols and concluded they were comparable (49). Dithiothreitol was used in our protocol because this disrupts uromodulin which may entrap uEVs (20), although it may also cause loss of certain uEV proteins. Obtained pellets were processed for mass-spectrometry or solubilized in Laemmli buffer for immunoblot analysis. **Supplementary Figure 2** shows a representative SDS-Page gel for three uEV samples stained with Coomassie Blue.

Mass spectrometry

Samples were prepared for mass spectrometry as described previously. Briefly, uEVs and acetonone-precipiated whole urine were lysed and trypsinized. Tryptic peptides were fractionated by HILIC and each fraction was then analyzed by LC-MS. For quantitative dimethyl labeling of uEVs (identification cohort), desalting and reductive dimethylation was performed on the SPE cartridge (as described in (50)) before peptide fractionation by HILIC. All LC–MS/MS analyses were performed on a Q Exactive mass spectrometer (Thermo Scientific, San Jose, CA). Each Data collection cycle in the Q Exactive consisted of 1 full MS scan (300-1750 m/z) followed by 15 data dependent MS/MS scans. The proteomics data have been submitted to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD003298 (51).

Bioinformatics

Raw data files were processed and analyzed using Proteome Discoverer 1.4 (Thermo Fisher Scientific) or MaxQuant (52). MS/MS spectra were searched against the Uniprot database (taxonomy: *Homo sapiens*) with the following

search parameters: 15 ppm precursor ion tolerance and 0.02 Da fragment ion tolerance, fully tryptic digestion, up to two missed cleavages were allowed, posttranslational static modifications of 57.02146 Da on cysteine (carbamidomethyl), dynamic modifications of 15.99491 Da on methionine (oxidation). For dimethylation labeled uEV samples 28.031 Da on lysine and the peptide N-terminus (light) and 32.056 Da on lysine and the peptide N-terminus (heavy) were added to the search parameters. The resulting data were analyzed using the DAVID bioinformatics tool (Database for Annotation, Visualization and Integrated Discovery, NIAID, Bethesda, MD) to determine which Gene Ontology terms are over-represented relative to the complete set of identified proteins. For this analysis, we excluded those pathways that were similarly enriched in healthy subjects or the CKD group. Gene Ontology terms were retrieved by the software tool for rapid annotation of proteins (STRAP version 1.5.0.0) (53).

Immunoblotting and sucrose gradient fractionation

The solubilized uEV pellet was preheated at 60°C for 15 min. SDS/PAGE was carried out on a 4-20% gradient gel and proteins were transferred to Trans-Blot® Turbo™ (Biorad, United States). The membranes were blocked in 5% milk and were probed overnight at 4°C with the antibody of choice. The following antibodies were used: envoplakin (Santa Cruz, 1:200), periplakin (Abcam, 1:2000), complement C3 (Abcam, 1:1000), complement C9 (Abcam, 1:1000), Villin-1 (anti-human: abcam, 1:10000, anti-mouse: Cell signaling, 1:1000), CD9 (Santa Cruz, 1:200) and CD63 (BD biosciences, 1:500), NHE3 (Stressmarq, 1:10,000), AQP2 (Stressmarq, 1:1000). The antibodies against mouse C3dg, C3d, and C9 were generated by one of the investigators (CvK) (54). After three washes (3 x 10 minutes in TBS-Tween 20), membranes were incubated with the secondary antibody in 5% milk (Thermo Scientific, Rockford, IL USA, 1:3000) for 1 hour and washed again. For visualization, blots were exposed to Pierce® enchanced chemiluminescent substrate and measured by Uvitec Alliance 2.7 (Cambridge, UK). Chemiluminescence was quantified using Imagequant TL (Life Sciences version 8.1), background was

substracted and ratios were measured in Excel. For the sucrose gradient fractionation, we used spot urine samples from 4 ADPKD patients. After pooling the samples and obtaining the uEV pellets, we ultracentrifuged uEVs overnight on top of a 2.5M to 0.25M sucrose gradient, corresponding to a density of 1.32 g/m^3 to 1.03 g/m^3. Each fraction was carefully removed, diluted in PBS and ultracentrifuged again to obtain the pellet. Fractions were solubilized in Laemmli for immunoblot analysis.

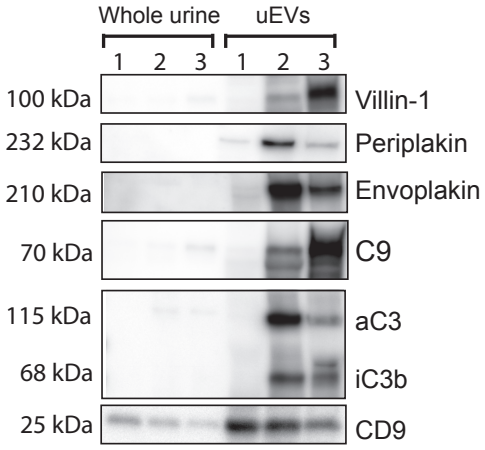
Mouse models of ADPKD

The local animal experimental committee of the Leiden University Medical Center and the Commission Biotechnology in Animals of the Dutch Ministry of Agriculture approved the animal experiments. The generation of the iKsp-*Pkd1*^{del} mice and tamoxifen administration to these mice were described previously (23). On three consecutive days the mice received a tamoxifen dosage of either 6 mg/kg at P10-12, 150mg/kg at P18-20, or 200 mg/kg tamoxifen at P40-P42 (**Supplementary Table 2 online**). The P10 mice were euthanized at 33 days of age at which the mice have relatively severe PKD. The P18 and the P40 mice were euthanized at the onset of renal failure (defined as a blood urea level > 20 mmol/L, as assessed by Reflotron technology; Kerkhof Medical Service). An additional time-point with mild PKD of the P40 mice (11 weeks after Tamoxifen) was included. Protein extraction from these kidneys was performed as described previously (25).

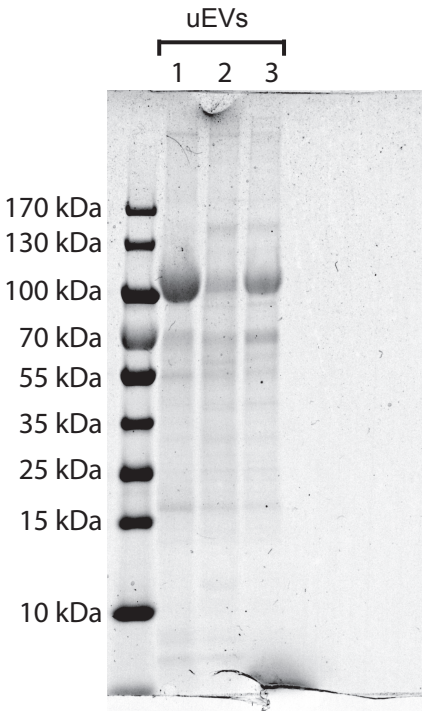
Statistical analysis

Immunoblotting results were analyzed by Student's T-test or Mann-Whitney U-test, as appropriate. A P-value ≤ 0.05 was considered statistically significant. Gene ontology enrichment was calculated by the DAVID bioinformatic tool, which applies the Fisher Exact test ($P \leq 0.05$ is considered strongly enriched).

Supplementary Figures:



Supplementary figure 1: *immunoblot of whole urine or urinary extracellular vesicles loaded with similar protein concentrations*



Supplementary figure 2: *SDS-PAGE of three uEV samples stained with Coomassie blue*

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CHAPTER

09

URINARY RENIN-ANGIOTENSIN MARKERS IN POLYCYSTIC KIDNEY DISEASE

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ABSTRACT

In autosomal dominant polycystic kidney disease (ADPKD) activation of the renin-angiotensin aldosterone system (RAAS) may contribute to hypertension and disease progression. Although previous studies focused on circulating RAAS-components, preliminary evidence suggests APDKD may increase urinary RAAS-components. Therefore, our aim was to analyze circulating and urinary RAAS-components in ADPKD. We cross-sectionally compared 60 patients with ADPKD to 57 patients with non-ADPKD chronic kidney disease (CKD). The two groups were matched by gender, estimated glomerular filtration rate (eGFR), blood pressure, and RAAS-inhibitor use. Despite similar plasma levels of angiotensinogen and renin, urinary angiotensinogen and renin excretion were 5- to 6-fold higher in ADPKD (P<0.001). These differences persisted when adjusting for group differences, and were present regardless of RAAS-inhibitor use. In multivariable analyses, ADPKD, albuminuria, and the respective plasma concentrations were independent predictors for urinary angiotensinogen and renin excretion. In ADPKD, both plasma and urinary renin correlated negatively with eGFR. Total kidney volume correlated with plasma renin and albuminuria, but not with urinary renin or angiotensinogen excretions. Albuminuria correlated positively with urinary angiotensinogen and renin excretions in ADPKD and CKD. In three ADPKD patients who underwent nephrectomy, the concentrations of albumin and angiotensinogen were highest in plasma followed by cyst fluid and urine; urinary renin concentrations were higher than cyst fluid. In conclusion, this study shows that, despite similar circulating RAAS-component levels, higher urinary excretions of angiotensinogen and renin are a unique feature of ADPKD. Future studies should address the underlying mechanism and whether this may contribute to hypertension or disease progression in ADPKD.

INTRODUCTION

Hypertension develops early in autosomal dominant polycystic kidney disease (ADPKD), usually occurring before a reduction in glomerular filtration rate (GFR) with an average age of onset of 30 years (5, 9). Increased activity of the renin-angiotensin-aldosterone system (RAAS) has been implicated in the pathogenesis of hypertension in ADPKD. One hypothesis is that cyst expansion results in areas of local renal ischemia, which increases renin release (3, 5). In addition, renin has also been suggested to be produced by the epithelial cells lining the cysts and active renin can be found within the cyst fluid (12, 36). However, measurement of plasma renin and aldosterone in patients with ADPKD yielded equivocal results (**Table 1**). Several studies found that plasma renin and aldosterone concentrations were not higher in hypertensive ADPKD patients when compared to controls, even during specific interventions (low or high sodium diet, ACE-inhibition, angiotensin II infusion) (1, 7, 21, 29, 37, 40). Different control groups were used for these studies, including normotensive ADPKD patients, normotensive siblings without ADPKD, patients with essential hypertension, or healthy volunteers (**Table 1**). The observation that plasma renin activity was not consistently higher in hypertensive ADPKD patients is notable, because this is contrary to what would be expected if cysts caused local renal ischemia (3, 5). Therefore, to further address the role of the RAAS in ADPKD, it may be informative to analyze RAAS-components in urine, as urinary angiotensinogen and renin have previously been used as markers of the intra-renal renin-angiotensin system (32). Emerging data suggest that filtered or locally produced RAAS-components may activate this intrarenal renin-angiotensin system and thereby contribute to hypertension (11). Two recent studies reported higher urinary angiotensinogen concentrations in hypertensive ADPKD patients (15, 25). However, despite its postulated central role in the pathogenesis of hypertension, urinary renin has never been measured in patients with ADPKD. We recently showed that it is important to measure urinary renin with standardized assays, because commercial assays may produce ≥ 10-fold higher results (31). Therefore, here, we measured urinary

renin in patients with ADPKD using a validated renin immunoradiometric assay and an in-house enzyme kinetic assay. In addition, we also measured multiple other RAAS-components in plasma and urine, including plasma renin and aldosterone, and urine angiotensinogen, prorenin, and aldosterone. As a comparator, and for the first time, we used matched patients with non-ADPKD chronic kidney disease (CKD) to address whether the type of kidney injury affects the RAAS differently.

Study	Cases	CKD stage	Controls	Numbers	Measurement(s)	Difference
Valvo (37)	ADPKD + HT	1-3	ADPKD + NT	20 vs. 12	PRA	↔
Bell (3)	ADPKD + HT	1-2	ADPKD + NT	9 vs. 7	PRA during low/ high Na^+ diet + ACEi	↔ but ↑ during ACEi + high Na^+ diet
Chapman (5)	ADPKD	1	Essential HT + healthy controls	14 + 11 vs. 9 + 13	PRA and aldo during ACEi	PRA and aldo ↑ in ADPKD + HT
Harrap (13)	ADPKD	1	Siblings	19 vs. 20	PRA and aldo	PRA and aldo ↑
Watson (40)	ADPKD	1-2	Siblings	13 vs. 10	PRA	↔
Barrett (1)	ADPKD	1-2	Siblings	21 vs. 12	PRA and aldo during low/high Na^+ diet, ACEi, Ang II infusion	↔
Martinez-Vea (21)	ADPKD + HT	2-3	Essential HT	20 vs. 20	PRA, aldosterone, ANP, Ang II	↔
Ramunni (29)	ADPKD + HT	1-2	ADPKD + NT	17 vs. 17	PRA	↔
Doulton (7)	ADPKD + HT	1-2	Essential HT	11 vs. 8	PRA during low/high Na^+ diet and ACEi	↔
Kurultak (17)	ADPKD	1	Healthy controls	20 vs. 20	Urinary AGT	↔
Kocyigit (15)	ADPKD + HT	1-2	ADPKD + NT, healthy controls	43 vs. 41 + 40	Plasma and urinary AGT	Urinary AGT ↑
Park (25)	ADPKD	1-5	None	186	Plasma renin+ aldo, urinary AGT	Correlation urinary AGT with eGFR, TKV, BP
Present study	ADPKD	3	CKD	69 vs. 58	Plasma + urinary AGT, renin, aldo	Urinary AGT + renin ↑

Table 1: Comparison of studies measuring RAAS-components in patients with ADPKD. *ADPKD, autosomal dominant polycystic kidney disease; ACEi, angiotensin converting enzyme inhibitor; AGT, angiotensinogen; aldo, aldosterone; Ang II, angiotensin II; CKD, chronic kidney disease; eGFR, estimated glomerular filtration rate; HT, hypertension; NT, normotension; PRA, plasma renin activity; BP, blood pressure.*

MATERIALS AND METHODS

Patients

Patients with ADPKD were recruited from one of the centers (Erasmus Medical Center, Rotterdam, The Netherlands) participating in a national ADPKD consortium (DIPAK study, with inclusion criteria CKD stage 3 at entry into the study and age ≤ 60 years) (23). Patients were matched to non-ADPKD CKD patients (referred to hereafter as 'CKD') from the PREVEND cohort (University Medical Center Groningen) (18). The Medical Ethics Committees of the Erasmus Medical Center and University Medical Center Groningen approved the studies (MEC-2012-313 and METC-90/01/022). Patients with ADPKD and CKD were individually matched for gender, eGFR (using the Modification of Diet in Renal Disease equation (19)), use of RAAS-inhibitors (defined as the use of angiotensin-converting enzyme inhibitors or angiotensin receptor blockers) and blood pressure (difference in systolic blood pressure ≤ 5 mmHg). Patients with a history of diabetes mellitus, or those using insulin or oral glucose lowering drugs were excluded, because diabetes mellitus may activate the intrarenal renin-angiotensin system (38). From three ADPKD patients who underwent elective nephrectomy (not part of the DIPAK cohort), we collected plasma, cyst fluid, and urine samples. For this part of the study, a separate approval from the Medical Ethics Committee of the University Medical Center Groningen was obtained (METC 2014.396).

Data Collection

Detailed description of data collection for both the DIPAK and PREVEND studies has been described elsewhere (18, 23). Briefly, participants of both studies collected 24-hour urine and visited the outpatient clinic for blood sampling and blood pressure measurements using an automatic oscillometric device. Hypertension was defined as a blood pressure > 140/90 mmHg or the use of anti-hypertensive medication. Participants were instructed to store urine at 4°C during collection. Upon arrival in the university medical center, blood and urine samples were immediately stored at -80°C until further use. We have

previously shown that in 24-hour urine, prorenin is not converted into renin prior to freezing (38). To determine adequate 24-hour urine collection, we calculated the expected 99% quantile of creatinine excretion based on previously defined criteria (10). Patients who exceeded the expected range were excluded (8 patients with ADPKD and 1 patient with CKD). ADPKD patients underwent magnetic resonance imaging to determine total kidney volume (23). The World Health Organization defined daily dose (DDD) was used to calculate daily RAAS-inhibitor use.

Measurements

Plasma and urine samples from the DIPAK and PREVEND cohorts were measured simultaneously. Renin in plasma was measured with a commercially available immunoradiometric kit (Renin III; Cisbio, Gif-sur-Yvette, France), making use of an active site-directed radiolabeled antibody (4). Total plasma renin was determined simultaneously using the same kit after the induction of a conformational change in the prorenin molecule with aliskiren (10 μmol/l for 48 hours at 4°C), which enabled its recognition by the active site-directed radiolabeled antibodies applied in the Cisbio kit (2). The detection limit of this assay is 1 pg/ml with intra- and inter-assay coefficients of variation (CVs) of 2.4% and 7.2%. Urinary renin and urinary total renin (after prorenin activation with trypsin) were measured with an in-house enzyme kinetic assay (EKA) (16). This measurement involves the incubation of the urine sample with excess sheep angiotensinogen and angiotensinase inhibitors and the subsequent detection of the generated Ang I by radioimmunoassay. The detection limit of the EKA is 0.05 ng Ang I/ml per hour with intra- and inter-assay CVs of 2.9 and 12.6%. Ang I–generating activities were converted to renin concentrations based on the fact that 1 ng Ang I/ml per hour corresponds with 2.6 pg human renin/ml (16). Prorenin was determined by subtraction of renin from total renin. Angiotensinogen in plasma and urine was measured as the maximum quantity of Ang I that was generated during incubation with excess recombinant renin (6). The detection limit of this assay is 0.50 pmol/ml with intra- and inter-assay CVs of 4 and 10%. Aldosterone was measured by solid-

phase radioimmunoassay (Diagnostic Products Corporation, Los Angeles, California, USA), with a detection limit of 25 pg/ml with intra- and inter-assay CVs of 3.3 and 8.4% (14).

Statistical Analyses

Results are expressed as mean and standard deviation or median and range, as appropriate. Data were logarithmically transformed before analysis in case of non-normal distribution. Levels that were below the detection limit were considered to be half the detection limit to allow for statistical analysis (8). Analysis of variance (ANOVA) was used for group comparison (using log-transformed data as appropriate). Further analysis was performed using analysis of covariance (ANCOVA) to adjust for covariates. To analyze which parameters independently predicted urinary angiotensinogen or renin excretion, we performed multivariable linear regression. Finally, the Pearson correlation coefficient was analyzed for selected variables. A P-value < 0.05 was considered statistically significant. Statistical analyses were performed with SPSS (version 21, IBM).

RESULTS

ADPKD Increases Urinary Angiotensinogen and Renin Excretion

Table 2 shows the baseline characteristics and RAAS-component measurements for the ADPKD and CKD groups. Patients with ADPKD were younger (47 vs. 68 years), taller (175 vs. 169 cm), and used more RAAS-inhibitors. While plasma levels of angiotensinogen, renin, and aldosterone were similar between the two groups, 24-hour urine volume, and urinary albumin, angiotensinogen, renin, and aldosterone excretions were significantly higher in patients with ADPKD (P < 0.05 for all). Similarly, when expressed as ratio with creatinine, urinary angiotensinogen and renin were also significantly higher in ADPKD (urinary angiotensinogen 14.6 vs. 3.3 pmol/mol creatinine, urinary renin 204

vs. 44 pg/mol creatinine, p < 0.01 for both). Because of the group differences in age, height, DDD, and albuminuria, we also performed a second analysis adjusting for these factors (**Table 2**). This analysis showed that urinary angiotensinogen and renin excretion were still significantly higher in ADPKD than CKD ($P < 0.001$ for both). In addition, a subanalysis was performed in patients (n = 17 vs. 11) with similar age, gender, height and a similar degree albuminuria , which also showed that urinary angiotensinogen (286.2 vs. 38.7 pmol/day) and renin (1874 vs. 398.6 pg/day) excretions were significantly higher in ADPKD compared to CKD ($P < 0.05$ for both).

Effects of RAAS-Inhibitors

Because the use of RAAS-inhibitors increases plasma renin, this may also increase urinary renin. Therefore, we also report the plasma and urinary RAAS-components in patients with and without RAAS-inhibitor use (**Figure 1**). Plasma renin was indeed significantly higher in both ADPKD and CKD patients using RAAS-inhibitors. In patients *without* RAAS-inhibitors, plasma renin was significantly *lower* in ADPKD than in CKD. Despite these differences in plasma renin, urinary renin excretion was consistently higher in the patients with ADPKD than in the patients with CKD regardless of RAAS-inhibitor use (**Figure 1**). Urinary angiotensinogen excretion was significantly higher only in patients with ADPKD and RAAS-inhibitor use, but this may be a power issue, as few patients were without RAAS-inhibitors.

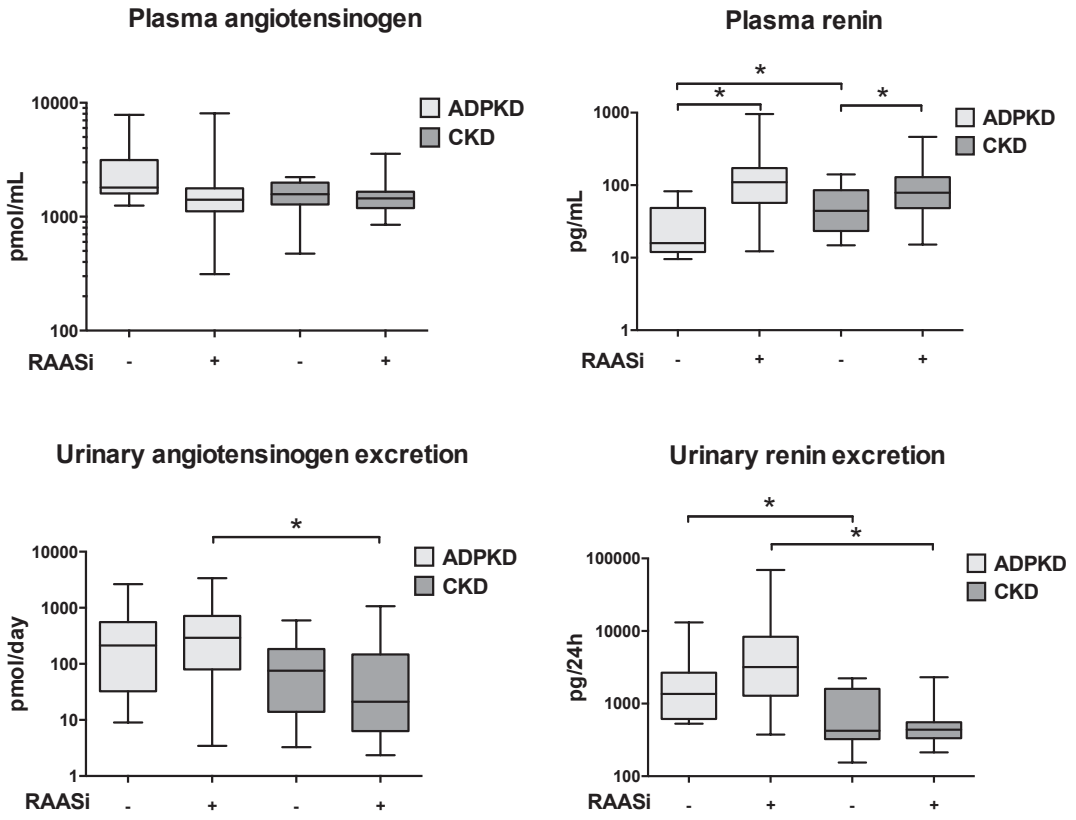

Figure 1: Plasma concentrations and urinary excretions of angiotensinogen and renin in patients with ADPKD or CKD and with or without RAAS-inhibitors. *Box-and-whisker plots of plasma concentrations and urinary excretions of renin and angiotensinogen for ADPKD (light grey) and CKD (dark grey). Groups were subdivided into those with (+) and without (-) use of RAAS-inhibitors (RAASi). Of the 60 patients with ADPKD, 10 patients did not use RAASi; of the 57 CKD patients, 13 patients did not use RAASi. Boxes show the median, interquartile range and range. ANOVA was used for comparison with * $P < 0.05$.*

Category	Parameter	ADPKD (n = 60)	CKD (n = 57)	P-value*	P-value**
Clinical data	Age, years	47 ± 8	68 ± 8	< 0.001	
	Male gender, n (%)	25 (42)	25 (44)	N.T.¶	
	Height, cm	175 ± 10	169 ± 9	0.001	
	Weight, kg	81.6 ± 17.0	81.2 ± 11.9	0.9	
	Hypertension, n (%)†	56 (93)	49 (86)	N.T.	
	SBP, mmHg	131 ± 14	134 ± 14	N.T.	
	DBP, mmHg	79 ± 9	76 ± 7	N.T.	
	RAAS-inhibitors, n (%)	50 (83)	44 (77)	N.T.	
	DDD RAAS-inhibitors, n	1.9 ± 1.5	1.1 ± 0.9	< 0.001	
Plasma	Creatinine, mg/dL	1.5 ± 0.4	1.4 ± 0.4	0.6	
	eGFR, ml/min per 1.73 m^2	48 ± 11	46 ± 9	N.T.	
	Angiotensinogen, pmol/mL	1599 (313–8067)	1455 (474–3567)	0.3	
	Renin, pg/mL	81.3 (9.6–950.0)	68.3 (14.8–464.5)	0.3	
	Aldosterone, pg/mL	121.7 (16.5–470.1)	105.9 (16.6–546.4)	0.2	
Urine	Volume, mL/day	2233 (800–6500)	1652 (530–3140)	< 0.001	0.2
	Creatinine, mmol/day	13.3 (5.2–21.2)	10.9 (6.0–18.2)	< 0.001	0.8
	Albumin, mg/day	40.0 (3.1–266.4)	26.7 (3.4–293.2)	0.05	-
	Sodium, mmol/day	150 (40 – 354)	142 (60 – 371)	0.5	
	Angiotensinogen, pmol/day	194.4 (3.5–3384.0)	36.0 (2.3–1070)	< 0.001	< 0.001
	Renin, pg/day	2717 (375.7–69248.0)	485.5 (154.7–2293.0)	< 0.001	< 0.001
	Aldosterone, µg/day	4.6 (0.9–32.8)	3.5 (1.0–18.0)	0.02	0.2

Table 2: Patient characteristics and renin-angiotensin-aldosterone system measurements. ** Using analysis of variance (ANOVA) with log-transformed data as appropriate. ** Using analysis of covariance (ANCOVA) with log-transformed data as appropriate and adjustments for age, height, defined daily dose, and albuminuria. ¶ N.T., not tested (matching criteria).*
† Defined by use of antihypertensive drugs. ADPKD, autosomal dominant polycystic kidney disease; CKD, chronic kidney disease; DBP, diastolic blood pressure; DDD, defined daily dose; eGFR, estimated glomerular filtration rate; SBP, systolic blood pressure.

Predictors of Urinary Angiotensinogen and Renin Excretion

Two multivariable linear regression analyses were performed to analyze which factors independently predict urinary angiotensinogen or urinary renin excretion (**Table 3**). In the model we included the presence of ADPKD, eGFR, age, DDD, plasma concentrations of angiotensinogen and renin, and urinary sodium and albumin excretion. For urinary angiotensinogen excretion, ADPKD, eGFR, and albuminuria were identified as independent predictors. For urinary renin excretion, ADPKD, plasma renin, and albuminuria were identified

as independent predictors. When the analyses were restricted to patients with ADPKD, only albuminuria predicted urinary angiotensinogen excretion, and only plasma renin predicted urinary renin excretion (data not shown).

Variable	Urinary angiotensinogen excretion		Urinary renin excretion	
	β	***P*-value**	**β**	***P*-value**
Presence of ADPKD	9.6 (3.7 – 24.5)	< 0.001	4.9 (2.6 – 9.0)	< 0.001
eGFR, ml/min/1.73 m^2	0.96 (0.94 – 0.99)	0.002	0.99 (0.98 – 1.01)	0.3
Age, years	1.0 (0.9 – 1.1)	0.2	0.99 (0.97 – 1.02)	0.6
DDD RAAS-inhibitors, n	0.8 (0.7 – 1.0)	0.1	0.9 (0.8 – 1.1)	0.2
Plasma renin, pg/mL	0.5 (0.3 – 1.0)	0.05	1.9 (1.2 – 2.9)	0.007
Plasma AGT, pg/mL	3.0 (0.9 – 10.7)	0.08	1.1 (0.5 – 2.4)	0.9
Urinary sodium, mmol/day	1.6 (0.4 – 7.1)	0.5	1.0 (0.4 – 2.8)	0.9
Albuminuria, mg/day	6.2 (3.6 – 10.7)	< 0.001	1.6 (1.1 – 2.3)	0.01

Table 3: Multivariable analysis of factors predicting urinary angiotensinogen and renin excretion. *ADPKD, autosomal dominant polycystic kidney disease; AGT, angiotensinogen; DDD, defined daily dose; eGFR, estimated glomerular filtration rate; RAAS, renin-angiotensin-aldosterone system*

Correlations with Total Kidney Volume and Kidney Function

Within the ADPKD group, we analyzed whether circulating or urinary RAAS-components correlated with total kidney volume and kidney function (eGFR). A higher total kidney volume correlated with higher plasma renin, and more albuminuria, but not with urinary angiotensinogen or renin excretion (**Figure 2A**). Both higher plasma renin and higher urinary renin excretion correlated with lower eGFR (**Figure 2B**). To analyze the possible mechanism of urinary angiotensinogen and renin excretion, we analyzed in the ADPKD and CKD groups whether these two urinary RAAS-components correlated with albuminuria. Indeed, both in patients with ADPKD and CKD, a higher degree of albuminuria correlated with higher urinary angiotensinogen or renin excretion, although the strength of this correlation was modest (**Figure 2C**). For urinary renin excretion, this correlation was of borderline significance in patients with ADPKD ($P = 0.06$).

Comparison of Concentrations in Plasma, Cyst Fluid, and Urine

In three patients with ADPKD who underwent nephrectomy, we measured albumin, angiotensinogen, prorenin, and renin in plasma, cyst fluid (average concentration of five cysts) and urine. For all four parameters, the concentrations were highest in plasma followed by cyst fluid and urine (**Figure 3**). Of interest, urinary concentrations were lower than cyst concentrations except for renin. Urinary prorenin concentrations were close to or below the detection limit.

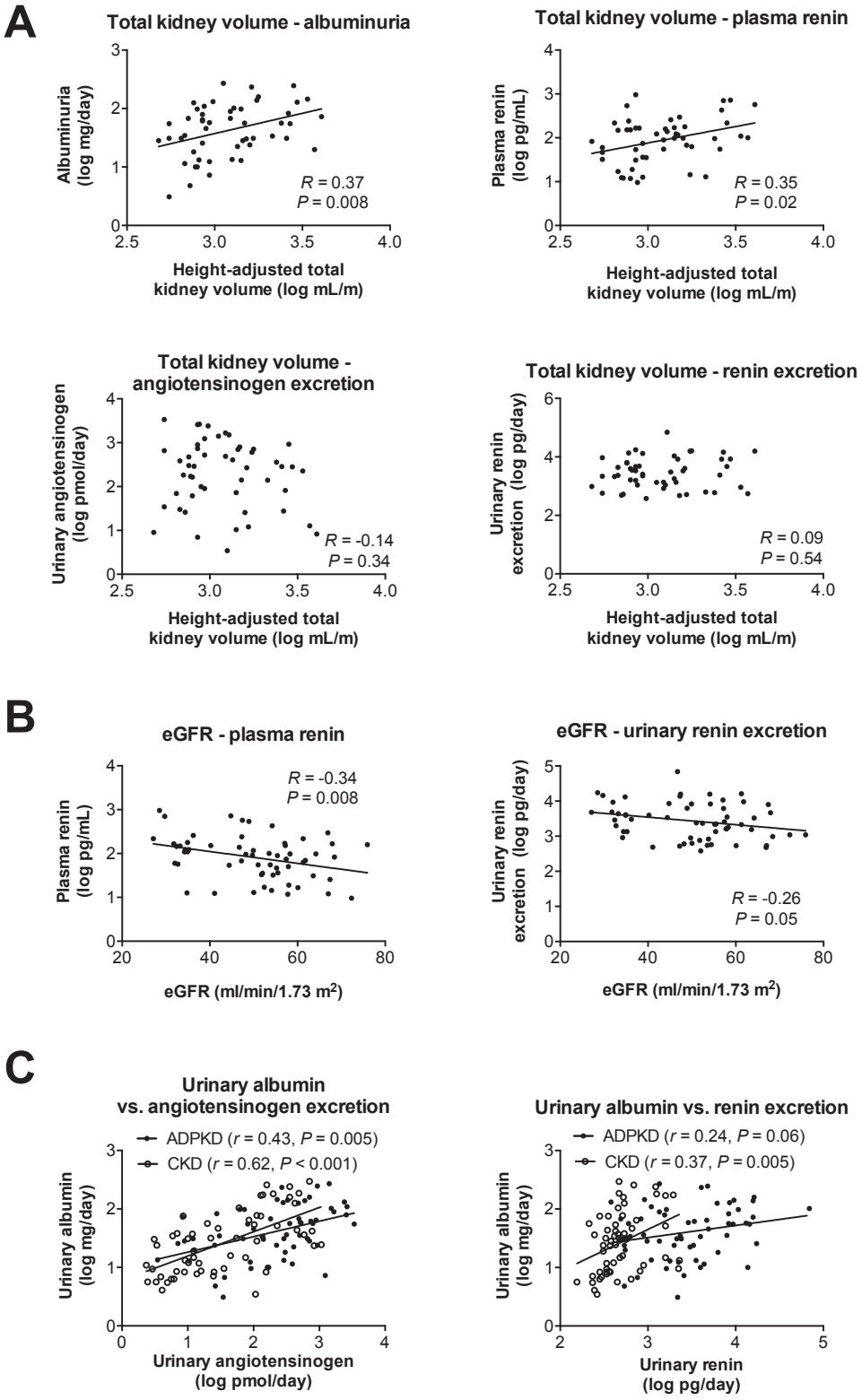

Figure 2: Correlations of RAAS-components with total kidney volume and kidney function. Pearson correlation coefficients were calculated using log-transformed data. Height-adjusted total kidney volume was available in 51 patients with ADPKD. Correlations between urinary albumin, angiotensinogen, and renin excretion are shown both for patients with ADPKD and CKD.

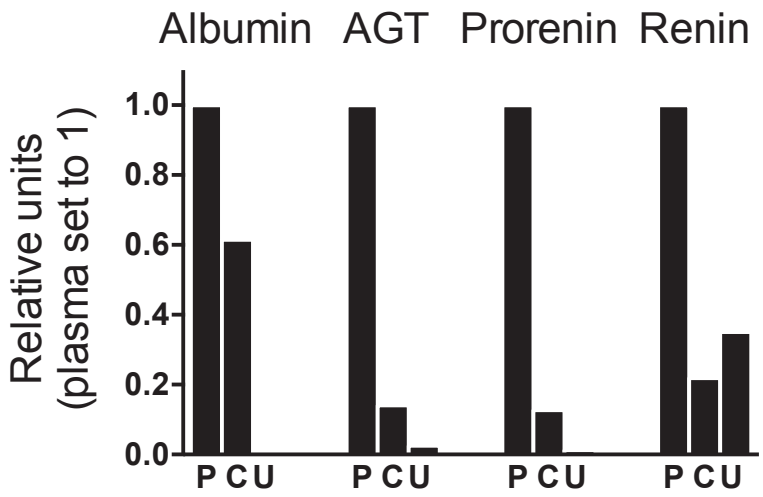

Figure 3: Concentrations of Albumin and RAAS-components in plasma, cyst fluid, and urine. *With plasma values set to 1, this figure shows the relative mean concentrations of albumin, angiotensinogen (AGT), prorenin, and renin in plasma (P), cyst fluid (C) and urine (U). The actual mean plasma concentrations were 42 g/L, 1814 pmol/L, 573.8 pg/mL, and 81.3 pg/mL, respectively. Measurements were performed in three ADPKD patients, who underwent elective nephrectomy to create space for kidney transplantation (45 and 52 year-old males with eGFRs of 12 and 9 mL/min/1.73 m^2, respectively) or because of mechanical discomfort (71-year-old female, eGFR 18 mL/min/1.73 m^2).*

DISCUSSION

This study reveals a unique feature of patients with ADPKD, namely a consistently higher urinary excretion of angiotensinogen and renin compared to patients with CKD. Urinary angiotensinogen and renin excretions were 5- to 6-fold higher in patients with ADPKD than in patients with CKD, who were matched by eGFR, blood pressure, and RAAS-inhibitor use (**Table 2**). ADPKD remained a significant predictor for urinary angiotensinogen and renin excretion in adjusted and in multivariable analyses (**Tables 2** and **3**), and regardless of RAAS-inhibitor use (**Figure 1**). Recent studies found higher urinary angiotensinogen to creatinine ratios in normotensive ADPKD patients compared to healthy controls (17), or higher levels within ADPKD patients in the presence of hypertension (15) or reduced kidney function (25). The

magnitude of the urinary angiotensinogen levels reported in these previous studies are comparable to our data. Our study is the first to analyze urinary renin and to use patients with CKD as control group (**Table 1**). Although our cross-sectional study cannot give definitive answers, our data give directions on the possible mechanisms and potential clinical implications of the increased urinary excretions.

In principle, urinary angiotensinogen and renin excretion can increase in ADPKD because of (1) damage to the glomerular filtration barrier, (2) reduced proximal tubular reabsorption, (3) enhanced tubular secretion by intact nephrons, (4) differences in degradation, or (5) ectopic production by cyst-lining epithelial cells.

When evaluating the first two possibilities, it is important to correct for differences in the plasma levels. Although the correlation between total kidney volume and plasma renin in ADPKD patients indeed suggests that renal ischemia by cysts can increase plasma renin (**Figure 2A**), patients with ADPKD in general do not have higher plasma renin concentrations than patients with CKD (**Table 2**). In fact, patients with ADPKD *without* RAAS-inhibitors had significantly *lower* plasma renin concentrations than patients with CKD (**Figure 1**). Thus, the higher urinary excretions of angiotensinogen and renin in ADPKD are not simply the consequence of elevated plasma RAAS concentrations exposed to the same degree of filtration and reabsorption as in CKD patients. In addition, increase of cysts (i.e. total kidney volume) did not correlate with increased excretion of urinary angiotensinogen or renin. Next, it is important to emphasize that ADPKD is a primarily a tubular disorder that is less likely to damage the glomerular filtration barrier (24). Indeed, previous studies have attributed albuminuria in animal models of ADPKD to disturbed endocytosis of albumin in the proximal tubule (24, 41). In these studies, immuno-histochemistry showed less expression of the chloride channel ClC-5 and megalin, which are both involved in the reabsorption of low-molecular weight proteins. Because albumin, angiotensinogen, and renin are all reabsorbed by a

megalin-dependent pathway, a proximal tubular disorder should by definition result in higher urinary angiotensinogen and renin excretion, even in the face of identical or lower plasma RAAS-component levels (28, 30). In agreement with this concept, we recently showed that patients with Dent's disease (who lack ClC-5) displayed a 20-40-fold rise in urinary angiotensinogen and renin levels, although their plasma RAAS levels were in the normal range (30). Similarly, other urinary markers of proximal tubule damage, such as fetuin-A and β2-microglobulin, are increased in ADPKD (22, 27). The ADPKD component that independently predicted urinary angiotensinogen and renin excretion in our multivariable regression analysis therefore possibly reflects a difference in tubular reabsorption. We showed that albuminuria correlated with total kidney volume (**Figure 2A**), as was shown previously (34). In addition to ADPKD, albuminuria also independently predicted urinary renin and angiotensinogen excretion. This also suggests that the urinary excretions of albumin, angiotensinogen, and renin was at least in part due to similar mechanisms, and argues against selective tubular secretion of RAAS-components. This leaves the issue of altered degradation. Reduced reabsorption would also be expected to result in elevated levels of RAAS-component degrading enzymes, leading to enhanced degradation. Yet, higher levels of urinary RAAS-components were found in ADPKD. Combined with data from previous studies showing no evidence for urinary degradation of renin or prorenin (38), and identifying all urinary angiotensinogen as intact (and not cleaved) (39), it appears that reduced degradation does not underlie the increased urinary excretion of angiotensinogen and renin in ADPKD.

The possibility of ectopic RAAS-component production by cyst-lining epithelial cells has been suggested by several investigators (20, 35). Although we cannot entirely exclude this possibility, such local production should have resulted in angiotensinogen and renin concentrations in cyst fluid that would have been at least comparable to, if not far above, their plasma concentrations. Remarkably, this was not the case (**Figure 3**). In fact, relative to albumin, the concentrations of angiotensinogen, renin and prorenin were lower in cyst fluid (**Figure 3**). In

other words, when using cyst albumin concentrations as a measure of blood-derived proteins, cyst RAAS-component concentrations can be entirely explained on the basis of leakage from blood plasma. Of interest, the urinary concentrations of albumin, angiotensinogen, and prorenin were lower than their concentrations in cyst fluid. This most likely reflects further dilution and/or tubular reabsorption. Surprisingly, this was not the case for renin: its concentrations in cyst fluid and urine were similar (**Figure 3**). Moreover, the correlation between albumin and renin excretion, although modest, showed a different pattern in ADPKD than in CKD (**Figure 2C**). Taken together, these data suggest that ADPKD affects the urinary excretion of renin differently than of albumin, angiotensinogen and prorenin. This difference between renin and prorenin excretion (resulting in urinary prorenin levels that are virtually undetectable), has been observed before (30, 38). Of note, we did not measure the sodium concentration in cyst fluid; previously, more active renin was found in so-called "gradient cysts" in which the sodium concentration is low (36).

Many aspects of the intra-renal renin-angiotensin system remain unclear, although some groups have suggested that angiotensinogen and renin in tubular fluid may lead to high tubular angiotensin II levels (11, 20). Such high local angiotensin II levels could promote renal sodium retention, hypertension, kidney damage, or even cystogenesis (20). Of interest in this regard is a recent report where targeting the intra-renal renin-angiotensin system reduced cyst growth in an animal model of polycystic kidney disease (33). Based on our cross-sectional data, we cannot conclude whether the higher urinary angiotensinogen and renin excretions are a cause or a consequence of the kidney damage in ADPKD. In other words, it is unclear if higher urinary angiotensinogen and renin excretion should be considered as a damage marker or as a potential contributor to kidney damage. Given the experimental link between the intra-renal renin-angiotensin system and cystogenesis, this deserves further study.

A number of limitations of this study should be mentioned. First, RAAS-inhibitor use could have influenced our results. Ideally, urinary RAAS-components should be measured before and after starting a RAAS-inhibitor. Previous studies have shown a decrease of urinary angiotensinogen and no effects on urinary renin after initiation of an angiotensin receptor blocker in patients with chronic kidney disease (26, 42). Therefore, if anything, our ADPKD group would be expected to have lower urinary angiotensinogen excretion, which was not the case. Second, despite matching of ADPKD with CKD patients by the most relevant parameters (eGFR, blood pressure, RAAS-inhibitors), several differences remained. The difference in age was inevitable, as eGFR decline occurs much earlier in ADPKD than in other forms of CKD. We addressed these differences by using an adjusted analysis, multivariable analyses, and a subanalysis (**Table 2, Table 3**). Finally, although significant, the strength of the correlations observed in this study were modest, suggesting high inter-individual variability or a multifactorial origin.

In conclusion, ADPKD uniquely increases urinary angiotensinogen and renin excretion despite their circulating levels being comparable to those in CKD. We believe these findings warrant further analysis in mechanistic or intervention studies.

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CHAPTER

10

SUMMARY, DISCUSSION AND FUTURE DIRECTIONS

SUMMARY

uEVs are increasingly used for biomarker purposes in renal disorders. The studies presented in this thesis were aimed to develop a novel method to isolate and characterize uEVs, as well as to explore their use as biomarkers for salt-sensitive hypertension and ADPKD.

Chapter 2 provides an overview of uEVs, with two main focuses: 1) it compares different techniques currently available to isolate and characterize uEVs and 2) it provides an overview of the use of uEVs for biomarker discovery and therapeutic purposes in nephrology. Different methods are currently being used to isolate uEVs, including ultracentrifugation, filtration, precipitation, and immune-isolation. The choice of method depends on the starting material and the specific aim of the study. For example, ultracentrifugation requires a larger starting volume of urine (minimum 5 mL, usually > 20 mL) and is time-consuming, but has the advantage of a high yield and an unselected population of uEVs. In contrast, immuno-isolation requires a low starting volume (0.05 mL), is less time-consuming, but has the disadvantage of selecting a subpopulation of uEVs, and is unsuitable for RNA isolation. Another issue dealt with in this chapter is the normalization of samples, which remains the 'holy grail' in the field of uEVs. The commonly used indirect normalization methods include urinary creatinine, timed urine collection, and the use of uEV markers (e.g., CD9 or CD63). Because these methods do not directly count the number of uEVs, each method has its own limitations, and should therefore be used with caution.

In ***chapter 3*** we present a novel method to quantify, normalize, and characterize uEVs. Because the different techniques mentioned in the previous chapter are time-consuming and require large starting volumes, they are unsuitable for high-throughput clinical application. Therefore, we developed an immunoassay that captures uEVs using an anti-CD9 antibody. Subsequently, uEV proteins of interest can be quantified. As proof-of-principle, we analyzed the sodium

chloride cotransporter (NCC) and the water channel aquaporin-2 (AQP2). Our immuno-assay allows for the simultaneous quantification of CD9 count by using a different label (called time-resolved fluorescence). We show that CD9 correlates to urine creatinine, and can therefore be used to normalize for differences in urine concentration within the same assay, thus omitting the need to measure urine creatinine. We further demonstrate its applicability in the physiological regulation of AQP2 and NCC, and show it correlated with the conventional method (ultracentrifugation and followed by immunoblotting). Similarly, we demonstrate that our immuno-assay was capable of detecting dysregulation of AQP2 and NCC in human disease. We therefore conclude that this platform is a promising technique that can be used for high-throughput clinical application for potential uEV biomarkers.

Chapter 4 introduces the second section of this thesis. Here, we provide an overview of studies in which uEV sodium transporters were analyzed in hypertensive disorders. We conclude that the analysis of sodium transporters in uEVs allows for a non-invasive read-out of renal epithelial transport, and may therefore be used as 'liquid biopsy'.

In ***chapter 5*** we analyze uEV sodium transporters in CS. Glucocorticoid excess in CS causes hypertension. Due to the pleiotropic effects of glucocorticoids, the pathogenesis of hypertension in CS is multifactorial and may be related to an increase in vasoconstriction or renal sodium reabsorption. We show that CS patients with hypertension could be divided on the basis of a suppressed or non-suppressed renin-angiotensin-aldosterone system (RAAS). The sodium hydrogen exchanger type 3 (NHE3) in uEVs was higher in all patients with CS compared to controls. Of note, only patients with suppressed RAAS had higher abundances of the sodium potassium chloride cotransporter (NKCC2) and NCC in uEVs. We reveal that lower serum potassium concentrations were correlated with higher abundances of NKCC2 and NCC. This suggests a role of potassium deficiency in the salt-sensitivity and extracellular fluid volume expansion in hypertensive CS patients (1).

In ***chapter 6*** we test the hypothesis that primary aldosteronism increases the phosphorylation of NCC (pNCC) and the abundance of prostasin (a serin protease involved in the activation of the epithelial sodium channel) in uEVs. Using two animal models of aldosteronism (infusion of aldosterone and low sodium diet), we demonstrate that pNCC, and to a lesser extent prostasin, increased both in kidney and in uEVs. Similarly, pNCC was significantly higher in uEVs of patients with primary aldosteronism than in patients with essential hypertension. We conclude that pNCC in uEVs may be used as a urinary biomarker for aldosteronism.

Chapter 7 reports a family with a Liddle phenotype (hypokalemic hypertension with suppressed renin and aldosterone), but without mutations in the genes encoding the β- or γ-subunit of ENaC (*SCNN1B* or *SCNN1G*). Whole exome sequencing identified a novel heterozygous missense mutation in αENaC that results in the substitution of cysteine 479 to arginine (C479R). We further characterize this mutation using electrophysiology and showed a 2-fold increase in amiloride-sensitive sodium current in oocytes. Subsequently we show that the mutation did not increase αENaC abundance at the plasma membrane, but did decrease trypsin sensitivity. This suggests that increased αENaC activity is due to higher open probability rather than increased channel number. This finding was also confirmed in analysis of αENaC in the uEVs isolated from the patients, as the subjects carrying the mutation had similar αENaC abundance as their non-affected siblings.

The third section focuses on urinary markers of ADPKD, including uEV-associated markers of ADPKD. In ***chapter 8*** we use a quantitative proteomics approach to identify disease-associated markers of ADPKD in uEVs. With four different cohorts and two proteomic methods (labeled and label-free), we compare uEVs isolated from patients with ADPKD, non-ADPKD chronic kidney disease (CKD), and healthy controls. We identify several upregulated proteins, including complement and plakins, which were consistently higher in ADPKD in subsequent validations. More importantly, the identify proteins

correlated with total kidney volume, suggesting they may be used as a marker of disease progression. These findings were also confirmed in whole kidney homogenates from ADPKD mouse models. The subpopulation of uEVs that contained complement was of higher density than CD9+ vesicles, suggesting they are not of exosomal origin.

Finally, in ***chapter 9***, we tested the hypothesis that increases in the urinary excretion of renin-angiotensin components are a unique feature of ADPKD. We therefore analyze markers of both the circulating and urinary renin-angiotensin-aldosterone system (RAAS) in 60 patients with ADPKD, and compare those to 57 matched patients with non-ADPKD CKD. In three patients with ADPKD, we also compare angiotensinogen and renin concentrations in plasma, cyst fluid, and urine. Our main conclusions were that, despite similar circulating levels, urinary angiotensinogen and renin were consistently higher in patients with ADPKD. Importantly, albuminuria correlated with urinary angiotensinogen and renin suggesting they may be plasma derived. Also, cyst concentrations of angiotensinogen and renin were consistently lower than plasma, and urinary renin was higher than cyst concentrations. These results challenge the hypothesis of production of renin-angiotensin components by cyst epithelial cells.

The ***appendix*** reports the design of the DIPAK1 study, which is a randomized controlled trial in 300 subjects with ADPKD (eGFR between 30-60 mL/min/1.73 m^2). The patients included in ***chapters 8 and 9*** participated in this study.

In conclusion, this thesis illustrates the use of uEVs in biomarker discovery, including the development of a novel isolation technique, and the application of uEV analysis in clinical disorders of salt-sensitive hypertension and polycystic kidney disease.

DISCUSSION AND FUTURE DIRECTIONS

Urinary extracellular vesicles: potential for nephrology & novel isolation method

A common and continuing issue in EV research is the method by which uEVs are isolated and quantified, which was discussed in *chapters 2 and 3*. In brief, it may be stated that the use of the different available platforms depends on availability of equipment (such as an ultracentrifuge), volume of stored urine and the ultimate purpose (i.e., protein or RNA quantification or *in vitro* use). It also depends on the research question at hand, as a proteomic-based untargeted approach may be more suitable for biomarker discovery, whereas a targeted approach, such as our immunoassay described in *chapter 3*, is more appropriate for diagnostic or monitoring purposes. The ideal platform should be able to quantify multiple uEV proteins of interest without the need for isolation. This poses technical challenges with the low abundance of uEVs. Here, the advances in proteomics may provide the solution. Qi *et al*. used multiple reaction monitoring, which is a targeted form of proteomics, to quantify several proteins of interest (2). They, however, still relied on isolating uEVs by ultracentrifugation. With increases in sensitivity and novel labeling methods in proteomics, co-labeling of specific uEV markers and markers of interest, may bypass the need for their isolation in the future (3).

Another challenge is the normalization of uEVs, as commonly used methods do not actually count uEV number. These include timed urine collection, urine creatinine or protein markers (4). With timed collection or urine creatinine, it is assumed that uEVs are excreted in a constant manner, and are independent of glomerular filtration rate (GFR), which is not necessarily true. This is illustrated in *chapter 8*, where despite normalizing for urinary creatinine, the tetraspanin CD9 decreased with deteriorating GFR, suggesting a decrease in uEV number. Two methods have been developed to count uEV number, including the nanoparticle-tracking analysis (Nanosight, UK), which measures Brownian motion in fluids, and qnano (Izon, New Zealand), which uses nanopores that

measures change in ionic current flow (5, 6). A limitation of these methods is their lower detection limit of 50 nm, excluding smaller particles. A standardized, easy and reliable technique to sort, normalize and quantify uEVs is therefore still needed.

Finally, although a standardized nomenclature for uEV subpopulations was proposed, there are no universally accepted morphological or structural markers (7). Studying subpopulations of uEVs may therefore help distinguish and ultimately better understand their origin and role in the kidney (8-10).

Urinary extracellular vesicles: markers for salt-sensitive hypertension

The majority of secondary forms of hypertension are due to excessive renal sodium reabsorption by activated sodium transporters (11). With the use of uEVs, it is now possible to analyze renal sodium transporter abundances (12, 13). In this thesis, we demonstrated that abundances of renal sodium transporters are reflected in uEVs for renal tubular disorders (chapter 3), CS (chapter 5), and primary aldosteronism (chapter 6). The abundance of a sodium transporter, however, does not always equal activity. This may be overcome by analyzing post-transcriptional modifications that do reflect transporter activity, such as phosphorylation (demonstrated in chapters 5 and 6). Still, true transporter activity cannot be measured in uEVs. The activating mutation of αENaC in chapter 7, which increased its activity, but not its abundance, would therefore be missed. Despite these limitations, the analysis of renal sodium transporters in uEV may help to differentiate the cause of hypertension, but also to personalize therapy. This was recently demonstrated, as NCC abundance in uEVs predicted the blood pressure response to thiazides (14). More studies are needed to analyze renal sodium transporters in uEVs in hypertensive disorders, and response to therapy. High throughput platforms, such as demonstrated in chapter 3, may be used for this purpose.

Urinary extracellular vesicles and other markers for polycystic kidney disease

Studying urinary markers, including uEVs, in ADPKD could serve to predict and monitor disease progression and response to therapy, but also provide

insights in the pathophysiology of the disease. For example, uEVs are enriched for polycystins and fibrocystin, both implicated in the pathogenesis of ADPKD, and recently shown to inversely correlate to total kidney volume (8, 10). We observed an increase in plakins and complement in uEVs of patients with ADPKD (chapter 8). In particular, complement C3 and C9 was increased at early stages of disease and further increased with progression of CKD. Interestingly, complement has previously been linked to ADPKD, where its gene expression is highly activated, and its inhibition results in reduced cyst growth in animal models (15, 16). Further studies are needed to understand the pathophysiological role of complement in ADPKD and explore if complement could be a therapeutic target.

Finally, in chapter 9, we demonstrated increased urinary RAAS components in ADPKD, in particular angiotensinogen and renin. This is a unique feature of ADPKD and not related to CKD. How and why these urinary RAAS components are increased is unknown, but circumstantial evidence indicates it is due to reduced proximal tubular reabsorption rather that cyst-derived production. Whether or not this accounts for increased blood pressure is yet unknown. Future studies should address whether higher urinary excretion of angiotensinogen and renin contribute to hypertension and cyst progression in ADPKD.

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CHAPTER

11

NEDERLANDSE SAMENVATTING
CURRICULUM VITAE
LIST OF PUBLICATIONS
PHD PORTFOLIO
DANKWOORD

SAMENVATTING

Extracellulaire vesikels (EVs) zijn minuscule blaasjes die door alle lichaamscellen worden uitgescheiden. Hoewel men voorheen aannam dat het cellulair afval betrof, wijst recent onderzoek uit dat EVs veel functies hebben binnen het menselijk lichaam. EVs komen ook voor in urine. Deze zogenaamde urine EVs (uEVs) worden door de niercellen uitgescheiden en geven een indruk van de cellen waarvan zij uitgescheiden worden. Daardoor bieden uEVs perspectief als potentiële biomarkers voor nieraandoeningen. In dit proefschrift werden verschillende aspecten van uEVs onderzocht. Zo werd een nieuwe methode ten behoeve van het isoleren en karakteriseren van uEVs ontwikkeld. Ook werden potentiële biomarkers in uEVs onderzocht voor zout-gevoelige hypertensie en autosomaal dominante polycysteuze nierziekte (ADPKD).

Hoofdstuk 2 geeft een overzicht van uEVs met aandacht voor twee aspecten: 1) het vergelijkt de beschikbare technieken om uEVs te isoleren en karakteriseren en 2) het geeft een overzicht van het gebruik van uEVs als potentiële biomarkers binnen de nefrologie. Verschillende methoden zijn in gebruik om uEVs te isoleren, inclusief ultracentrifugatie, filtratie, precipitatie en immuno-isolatie. Welke methode je dient te gebruiken hangt af van de hoeveelheid beschikbare urine, alsook het doel van de studie. Voor bijvoorbeeld ultracentrifugatie heb je relatief veel urine nodig (minimaal 5 mL, maar meestal > 20 mL) en neemt de isolatie ervan veel tijd in beslag. Het heeft echter als voordeel een hoge opbrengst van niet-geselecteerde uEVs. Voor immuno-isolatie daarentegen heb je weinig urine nodig (0,05 mL), neemt de isolatie minder tijd in beslag, maar heeft als nadelen dat je selecteert op een uEV subpopulaties en dat het ongeschikt is voor RNA onderzoek. Een ander onderwerp waar dit hoofdstuk aandacht aan besteedt is het normaliseren van uEVs. De meest gebruikte en indirecte methoden hiervoor zijn het meten van urine creatinine, het normaliseren op basis van tijdgsgebonden verzameling van urine en het gebruiken van uEV eiwit markers (zoals CD9 of CD63). Aan al deze methoden

zijn nadelen verbonden aangezien zij niet het daadwerkelijk aantal uEVs tellen, waar rekening mee gehouden dient te worden.

Hoofdstuk 3 beschrijft een nieuwe methode om uEVs te kwantificeren, normaliseren en ook te karakteriseren. De beschreven immunoassay isoleert uEVs door een anti-CD9 antilichaam. Vervolgens worden de eiwitten van interesse, de natrium chloride cotransporter (NCC) en het waterkanaal aquaporine-2 (AQP2), gekwantificeerd. Tegelijkertijd kan het CD9 eiwit gekwantificeerd worden door gebruik te maken van een andere label (de zogenaamde "*time-resolved fluorescence*"). Omdat CD9 correleerde met urine creatinine, was het mogelijk om binnen dezelfde assay CD9 te gebruiken als maat voor normalisatie tussen de urines. De toepasbaarheid van deze immunoassay wordt aangetoond door de bevindingen van fysiologische regulatie van het waterkanaal AQP2 en zout transporter NCC te correleren met de conventionele methode van uEV isolatie en karakterisatie: de ultracentrifuge en immunoblot methode. Ook aandoeningen die AQP2 en NCC verstoren worden gedetecteerd met deze immunoassay. We concluderen dat deze nieuwe methode een veelbelovende techniek is die mogelijk klinisch toegepast zou kunnen worden voor potentiële uEV biomarkers.

Hoofdstuk 4 introduceert het tweede thema van dit proefschrift. Hier wordt een overzicht gegeven van de studies waarin zout-transporters in uEVs werden bestudeerd bij hypertensieve aandoeningen. Het meten van zout-transporters in uEVs biedt een non-invasieve uitleesmaat van renale epitheliale (zout) transport en zou daarom gebruikt kunnen worden als potentiële biomarker.

In ***hoofdstuk 5*** worden zout-transporters in uEVs van patiënten met het syndroom van Cushing bestudeerd. Het syndroom van Cushing veroorzaakt hypertensie als gevolg van een overschot aan glucocorticoïden. Doordat glucocorticoïden een pleiotroop effect hebben, kan het mechanisme van het ontstaan van hypertensie gerelateerd zijn aan toename van vasoconstrictie, en ook toegenomen heropname van zout reabsorptie door de nieren. Patiënten met

het syndroom van Cushing en hypertensie konden onderverdeeld worden in een groep met onderdrukt en een groep met niet-onderdrukt renine-angiotensine-aldosteron systeem (RAAS). De zout-transporter die natrium en waterstof uitwisselt (NHE3) blijkt in uEVs hoger te zijn in alle patiënten met het syndroom van Cushing vergeleken met gezonden. Daarentegen hebben alleen patiënten met een onderdrukt RAAS meer natrium kalium chloride cotransporter (NKCC2) en NCC in uEVs. Ook correleert een lagere serum kalium-concentratie omgekeerd aan NKCC2 en NCC in uEVs. Dit suggereert een rol voor kalium-deficiëntie in de zout-gevoeligheid en extracellulaire volume expansie bij patiënten met het syndroom van Cushing met hypertensie.

Hoofdstuk 6 onderzoekt de hypothese dat primair hyperaldosteronisme de fosforylering (activatie) van NCC en de hoeveelheid prostasin (een serine protease betrokken bij de activatie van het epitheliale natrium kanaal) doet toenemen in uEVs. Daarvoor wordt gebruik gemaakt van twee diermodellen van toegenoemen aldosteron: directe infusie van aldosteron en een laag zout dieet. We laten zien dat pNCC, en in mindere mate prostasin, beide toenemen in zowel de nieren, maar ook in uEVs. Deze toename wordt voor pNCC ook aangetoond bij patiënten met primair hyperaldosteronisme wanneer zij worden vergeleken met patiënten met essentiële hypertensie. Concluderend lijkt pNCC in uEVs een potentiële biomarker voor hyperaldosteronisme.

Hoofstuk 7 beschrijft een familie met het fenotype van het syndroom van Liddle (hypokalïemische hypertensie met onderdrukking van renine en aldosteron), maar zonder mutaties in de verwachte genen coderend voor het β- of γ-subunit van ENaC (*SCNN1B* or *SCNN1G*). Een nieuwe missense mutatie in αENaC wordt geidentificeerd door middel van whole exome sequencing. Deze mutatie veroorzaakt een vervanging van cysteïne 479 naar arginine (C479R), met als gevolg een tweevoudig toename van amiloride-gevoelige natrium opname in oöcyten. De mutatie zorgt niet voor toename in de hoeveelheid αENaC kanalen op de plasma membraan, maar wel een afname van trypsine gevoeligheid. Dit suggereert dat de toename van αENaC activiteit wordt veroorzaakt door een

toegenomen *open probability* van het kanaal en niet een toegenomen aantal kanalen op de cel membraan. Deze bevinding wordt bevestigd doordat het aantal αENaC kanalen in uEVs vergelijkbaar is tussen de patiënten met en zonder mutatie in αENaC.

Het derde thema richt zich op de urine markers voor ADPKD. In ***hoofdstuk 8*** wordt gebruik gemaakt van een kwantitatieve proteomics methode om ziekte-gerelateerde biomarkers te vinden in uEVs van patiënten met ADPKD. Bij vier verschillende cohorten en gebruik makend van twee labeling methoden, worden uEVs van patiënten met ADPKD, chronische nierinsufficiëntie (CKD) en gezonden vergeleken. Enkele uEV eiwitten, waaronder complement en plakines, waren consistent hoger bij patiënten met ADPKD. Ook correleren de gevonden eiwitten met totaal niervolume, suggererend dat zij mogelijk toegepast kunnen worden als markers voor ziekteprogressie. Deze bevindingen worden ook bevestigd in nierhomogenaten van ADPKD muis modellen. Concluderend zouden complement eiwitten en plakines in uEVs kunnen dienen als biomarkers voor patiënten met ADPKD.

In ***hoofdstuk 9*** wordt onderzocht of de excretie van urine renine-angiotensine componenten een unieke eigenschap is van patiënten met ADPKD. Daarvoor wordt plasma en urine markers van het renine-angiotensine-aldosteron systeem (RAAS) gemeten bij 60 patiënten met ADPKD en vergeleken met 57 vergelijkbare patiënten met CKD. Bij drie ADPKD patiënten wordt ook angiotensinogeen en renine concentraties gemeten in plasma, cyste-vloeistof en urine. Ondanks vergelijkbare plasma-waarden, waren urine angiotensinogeen en renine excreties significant hoger waren bij patiënten met ADPKD. Ook blijkt albuminurie te correleren met zowel urine angiotensinogeen als renine, suggererend dat het mechanisme berust op glomerulaire lek. Ook waren de cyste-concentraties van angiotensinogeen en renine lager dan plasma concentraties, maar urine renine concentratie hoger dan de concentratie in cysten. Deze bevinding is in strijd met de gedachte dat cysten angiotensinogeen en renine zouden produceren.

Het ***appendix*** beschrijft de DIPAK1 studie: een gerandomiseerde studie in 300 patiënten met ADPKD (eGFR tussen 30-60 mL/min/1.73 m^2). De geïncludeerde patiënten uit ***hoofdstuk 8 en 9*** deden mee aan de DIPAK1 studie.

Concluderend illustreert dit proefschrift het gebruik van uEVs als materiaal voor biomarkers, het ontwikkelen van een nieuwe isolatie- en kwantificatie-methode en de klinische toepassing van uEVs bij patiënten met zoutgevoelige hypertensie en polycysteuze nierziekte.

CURRICULUM VITAE

The author of this thesis was born on the 5th of June 1985. He graduated from secondary school at the Stedelijk Dalton Lyceum in Dordrecht in 2002, after which he started his medical training at the Erasmus Medical Center in Rotterdam, The Netherlands. Supported by prof.dr. A.J. van der Heijden, he co-founded 'Student-Tolk' during his studies, in which medical students were trained as hospital interpreters. This project was nominated for the national 'medical communication award' in 2012.

After graduating in 2010 (*cum laude*), he commenced clinical work at the departments of intensive care unit and internal medicine of the Albert Schweitzer Hospital in Dordrecht. His PhD began in 2012 at the division of Nephrology and Transplantation of the Erasmus Medical Center, supervised by prof.dr. E.J. Hoorn and prof.dr. R. Zietse and supported by the Dutch Kidney Foundation. His work consisted of both laboratory and clinical studies, ultimately resulting in this thesis.

In 2016 he started his training in Internal Medicine at the Maasstad Hospital, Rotterdam (supervisor: dr. M.A. van den Dorpel) and the Erasmus Medical Center Rotterdam (supervisor: dr. S.C.E. Klein-Nagelvoort Schuit).

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1. **Salih M**, van Kinschot CMJ, Peeters RP, de Herder WW, Duschek EJJ, van der Linden J, van Noord C. Thyrotoxic periodic paralysis: an unusual presentation of hyperthyroidism. *Neth J Med,* Epub Oct 2017.

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PHD PORTFOLIO

PhD candidate:	Mahdi Salih
PhD period:	2012-2015
Erasmus MC department:	Internal Medicine Division of Nephrology
Research school:	Cardiovascular Research School Erasmus University Rotterdam (COEUR)
Promotors:	Prof.dr. R. Zietse Prof.dr. E.J. Hoorn

PHD TRAINING

	Year	Workload (ECTS)
Clinical research courses		
- Basic course on rules and organization of clinical trials (BROK)	2012	1
- Rotterdam Course in Electrolyte and Acid-Base Disorders	2013	0.3
- Pre-ASN education: Intensive Care Nephrology	2014	0.9
- Nephrology course (Harvard, Boston, USA)	2015	1.8
Scientific courses		
- Winter school, Dutch Kidney Foundation	2012	1.2
- Cardiovascular Pharmacology (COEUR)	2013	1.5
- Biostatistical Methods I: Basic Principles (NIHES)	2014	5.7
- ERA-EDTA (WGIKD) course: renal fluids and electrolytes: from genes to bedside	2014	0.9
- Research seminar: SALT (COEUR)	2014	0.4
- Molecular biology in cardiovascular research	2014	1.5

	Year	Workload (ECTS)
- **National and international conferences**		
- Annual Dutch Nephrology Days	2012	0.6
- Kidney week (American Society of Nephrology)	2012	1.5
- International Society of Extracellular Vesicles meeting*	2014	1.5
- Kidney week (American Society of Nephrology)**	2014	1.8
- Annual Dutch Nephrology Days**	2014	0.9
- Kidney Benelux meeting**	2015	0.6
- International society of extracellular vesicles meeting**	2015	1.5
- Frontiers in Regenerative Medicine*	2015	1.2
- Kidney week (American Society of Nephrology)*	2015	1.5
- 'The New Kids on the Block' scientific symposium in Nephrology	2015	0.3
Teaching activities		
- Supervising 2 laboratory students	2013-2015	1.2
- Minor Immunology	2015	0.3
Other attendances		
- Annual DIPAK research meetings*	2012-2015	1.2
- PLAN (platform researchers in Nephrology)*	2013-2015	1.0
- Erasmus MC Internal Medicine science days*	2014-2015	1.2
- COEUR lectures	2013-2015	0.3
- Department of pharmacology weekly research meetings	2012-2015	2.7
Total		34.5

**oral presentation, ** poster presentation*

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APPENDIX

RATIONALE AND DESIGN OF THE DIPAK1 STUDY: A RANDOMISED, CONTROLLED CLINICAL TRIAL ASSESSING THE EFFICACY OF LANREOTIDE TO HALT DISEASE PROGRESSION IN ADPKD

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Am J Kidney Dis. 2014; 63(3): 446-55.

ABSTRACT

Background: There are limited therapeutic options to slow the progression of autosomal dominant polycystic kidney disease (ADPKD). Recent clinical studies indicate that somatostatin analogues are promising for treating polycystic liver disease and potentially also for the kidney phenotype. We report on the design of the DIPAK 1 (Developing Interventions to Halt Progression of ADPKD 1) Study, which will examine the efficacy of the somatostatin analogue lanreotide on preservation of kidney function in ADPKD.

Study design: The DIPAK1 study is an investigator driven, randomized, multicenter, controlled clinical trial.

Setting & participants: We plan to enroll 300 individuals with ADPKD and estimated glomerular filtration rate (eGFR) of 30-60 mL/min/1.73 m2 who are aged 18-60 years.

Intervention: Patients will be randomized (1:1) to standard care or Lanreotide 120 mg sc every 28 days for 120 weeks in addition to standard care.

Outcomes: Main study outcome is the slope through serial eGFR measurements starting at week 12 until end of treatment for lanreotide versus standard care. Secondary outcome parameters include change in eGFR from pretreatment versus 12 weeks after treatment cessation, change in kidney volume, change in liver volume, and change in quality of life.

Measurements: Blood and urine will be collected and questionnaires will be filled in following a fixed scheme. MRI's will be made for assessment of kidney and liver volume.

Results: Assuming an average change in eGFR of 5.2 ± 4.3 (SD) mL/min/1.73 m2 per year in untreated patients, 150 patients are needed in each group to detect a 30% reduction in the rate of kidney function loss between treatment groups with 80% power, 2-sided $\alpha = 0.05$, and 20% protocol violators and/or dropouts

Limitations: The design is an open randomized controlled trial and measurement of our primary end point does not begin at randomization.

Conclusion: The DIPAK 1 Study will show whether subcutaneous administration of lanreotide every 4 weeks attenuates disease progression in patients with ADPKD.

BACKGROUND

Autosomal dominant polycystic kidney disease (ADPKD) is the most common hereditary kidney disease (1, 2). It is characterized by progressive cyst formation in both kidneys, often leading to end-stage kidney disease between the fourth and seventh decades of life (1-3). Approximately 10% of patients receiving renal replacement therapy have ADPKD as the underlying disease (1). Cyst formation also is found in the liver, with an overall prevalence of 83% in a cohort of patients with early ADPKD (4). Symptoms of polycystic liver disease include abdominal distension, early satiety, dyspnea, and pain (5).

The development of renoprotective treatments for ADPKD is of major importance for patients with ADPKD. Increasing knowledge of the pathophysiology of ADPKD has allowed the identification of several potential therapeutic targets, and animal experiments have confirmed that drugs directed at these targets are renoprotective. Three drug classes have been tested in clinical trials: mammalian target of rapamycin (mTOR) inhibitors, vasopressin V2 receptor (V2R) antagonists, and somatostatin analogues (6-9).

Despite encouraging animal data with mTOR inhibitors (10-12), 2 controlled trials recently failed to show a beneficial effect on decline in kidney function in patients with ADPKD (13,14). A post hoc analysis of 2 open-label studies involving V2R antagonists, with matched untreated controls from historical ADPKD cohorts, suggested that these agents had renoprotective effects (15). Recently, a large randomized clinical trial with V2R antagonist treatment showed a reduction in kidney growth and preservation of kidney function in

1,445 patients with ADPKD with a mean estimated creatinine clearance of 81 mL/min (16). These results are promising because for the first time, a drug was shown to slow the decline in kidney function in patients with ADPKD.

However, there are a number of limitations to the use of V2R antagonists. First, the effect of these drugs probably is limited to renal tubular cells in the distal nephron and collecting duct (7). Although these are the predominant cysts in adult patients with ADPKD, kidney cysts also may originate from other parts of the nephron (17). Whether V2R antagonists will be effective in patients with chronic kidney disease (CKD) stages 3-4 is not known. Furthermore, V2R antagonists have adverse effects that may limit widespread clinical use, such as thirst, polydipsia, polyuria, and nycturia, which can cause sleep disturbance. A final consideration is that polycystic liver disease is a common manifestation of ADPKD and curtailing the growth of the liver is a desirable therapeutic target. Because the V2R is not expressed in liver tissue, no liver-specific therapeutic action of V2R antagonists may be expected. Recent randomized clinical trials suggest that somatostatin analogues ameliorate polycystic liver disease (18-22). These trials included only a limited number of patients with ADPKD, making it difficult to reach a definitive conclusion on the possible renoprotective efficacy of these drugs. Therefore, these trials do not allow one to conclude that somatostatin analogues should be standard care for patients with ADPKD at high risk of disease progression.

The DIPAK (Developing Interventions to Halt Progression of Autosomal Dominant Polycystic Kidney Disease) 1 Study is designed to validate the efficacy of the somatostatin analogue lanreotide to reduce disease progression in patients with ADPKD with CKD stage 3.

METHODS

Study setting and population

The DIPAK 1 Study is designed as a multicenter, open-label, randomized, controlled, parallel-arm trial in 300 participants with ADPKD and a high likelihood of disease progression. It will include individuals with an ADPKD diagnosis based on Ravine criteria, aged 18-60 years, with an estimated glomerular filtration rate (eGFR) of 30-60 mL/min/1.73 m2 (23, 24). Detailed patient inclusion and exclusion criteria are listed in **Box 1**. The eGFR cutoff values in combination with the age criteria ensure that only individuals with a high likelihood of disease progression will be included. Also, the inclusion criteria are easy to translate into clinical practice (in contrast to inclusion criteria based on total kidney volume), which increases the external validity of the data. Furthermore, interventions initiated in individuals with eGFR < 30 mL/min/1.73 m2 are less likely effective.

Study Design

Individuals meeting the entry criteria and completing baseline assessments will be enrolled in 1 of the 4 participating University Medical Centers in the Netherlands (Groningen, Leiden, Nijmegen, and Rotterdam). The planned recruitment period is 21 months. After informed consent is obtained and eligibility is assessed (**Box 1**), patients will be randomly assigned to standard care (control) or standard care plus 4-weekly lanreotide injections. Randomization will be performed using an interactive voice response system, with stratification for eGFR at time of screening (≤45 and >45 mL/min/1.73 m2), sex (male/ female), and age (≤45 and >45 years). There are no specific demands set to the number of patients to be included per stratum.

Figure 1 presents a schematic of the trial design. One week after the first injection, the patient will receive a telephone call to assess adverse events. Participants will be evaluated in person at weeks 4 (T4), 8 (T8), and 12 (T12) and every 12 weeks thereafter until the end of the trial (end-of-treatment visit

scheduled to be at week 120). The last dose of lanreotide will be given at week 116. Participants will be seen 12 weeks after the end of the trial for a follow-up visit. Total duration of the study therefore will be 132 weeks. In case a participant does not tolerate medication and treatment ends, an early end-of-treatment visit will be performed within 1 week after the next injection should have been administered and the participant will continue regular study visits.

Trial treatments

Treatment will consist of 120 mg of lanreotide administered subcutaneously every 4 weeks. The dosage will be eGFR (body surface area unadjusted) dependent. Participants who reach for the second time an eGFR < 30 mL/min during the study will receive lanreotide, 90 mg, subcutaneously every 28 days. Participants experiencing intolerable adverse effects will also have their medication dose adjusted (stepwise, from 120 to 90 to 60 to 0 mg). Lanreotide will be administered by trained nurses.

The dosage and frequency of treatment with lanreotide is based on a pilot study (18) in which a dosage of 120 mg subcutaneously once every 28 days was effective in decreasing the rate of liver and kidney volume growth in individuals with polycystic liver disease. The dosing scheme of 120 mg once every 28 days furthermore is approved by the European Medicines Agency and US Food and Drug Administration for other indications. There is only limited information on the use of lanreotide in individuals with decreased kidney function (25). Although the therapeutic index of lanreotide is broad, we decided to adjust the dose of lanreotide to kidney function given the limited pharmacokinetic data.

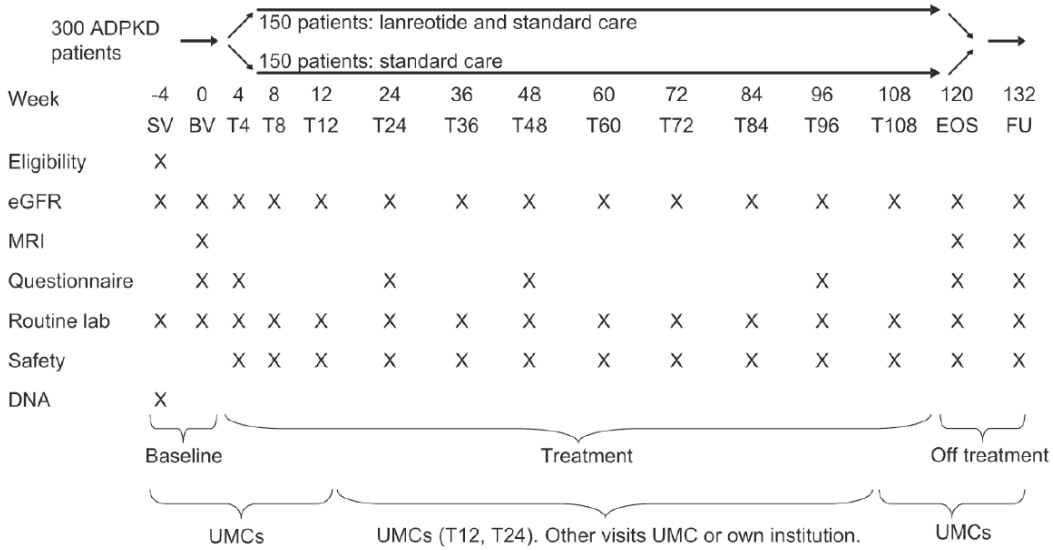

Figure 1: Trial design of the DIPAK-1 study. *ADPKD, autosomal dominant polycystic kidney disease; BV, baseline visit before start of treatment; eGFR, estimated glomerular filtration rate; EOS, end-of-study; FU, follow-up; lab, laboratory; MRI, magnetic resonance imaging; SV, screening visit; UMC, University Medical Center.*

Box 1. Eligibility criteria of the DIPAK1 study

Inclusion criteria

- Diagnosis of ADPKD, based upon the modified Ravine criteria23
- Age between 18 and 60 years.
- eGFR (MDRD) between 30 and 60 ml/min/1.73 m2.
- Providing informed consent.

Exclusion criteria

- Patients who, in the opinion of the study investigator may present a safety risk.
- Patients who are unlikely to adequately comply with the trial's procedures [due for instance to medical conditions likely to require an extended interruption or discontinuation, history of substance abuse or noncompliance).
- Patients taking medications likely to confound endpoint assessments (e.g. chronic NSAID, cyclosporine, lithium, immunosuppressant use)
- Patients having other systemic diseases that have the potential to influence kidney function (e.g. SLE, diabetes mellitus requiring treatment and patients with proteinuria > 1 g /24hr).
- Patients who underwent surgical or drainage interventions for cystic kidney disease the year before study-entry or are likely candidates for these procedures within 2 years of start of the study. (e.g. a patient that had previous successful cyst reduction surgery and now pain attributed to one dominant cyst.)
- Patients taking other experimental (i.e., not approved by FDA or EMA for the indication of ADPKD) therapies aimed at attenuating disease progression in ADPKD.
- Patients having used Lanreotide (or another somatostatin analogue) in the 3 months before study start.
- Patients with known intolerance for Lanreotide (or another somatostatin analogue).
- Unwillingness to comply with reproductive precautions. Women who are capable of becoming pregnant must be willing to comply with approved birth control from two-weeks prior to, and for 60 days after taking investigational product.
- Women, who are pregnant or breastfeeding.
- Patients, who suffer from cardiac arrhythmia's that are thought to be dangerous in combination with Lanreotide administration
- Patients, who ever suffered from symptomatic gallstones and did not undergo cholecystectomy.
- Patients, who have a medical history of pancreatitis.

Patients having contraindications to, or interference with MRI assessments can enter the study, but will not be assessed for change in kidney and/or liver volume.

Standard care

Participants will not be allowed to participate in other (experimental) trials investigating pharmaceutical agents or strategies aimed at intervening with the natural disease course of ADPKD. Participants with hypertension (defined as systolic blood pressure ≥ 140 mm Hg and/or diastolic blood pressure ≥ 90 mm Hg) will be treated with angiotensin-converting enzyme inhibitors, or in case of intolerance for angiotensin-converting enzyme inhibitors, with angiotensin receptor blockers. Although definitive evidence is lacking, these 2 classes of antihypertensive drugs are regarded as first-line agents for the treatment of hypertension in individuals with CKD, including ADPKD (1, 26, 27). If hypertension remains despite the use of these agents, the choice of additional antihypertensive medication will be at the discretion of the treating physician. Use of estrogens and oral contraceptives is discouraged per protocol in women with significant liver cysts because these drugs may increase liver cyst growth in women with ADPKD (28, 29). However, the decision to prescribe these drugs will be at the discretion of the treating physician. Similarly, dietary advice (reduction in sodium, caffeine, and protein intake and increase in water intake) will be at the discretion of the treating physician because dietary interventions have not yet been proven to decrease the rate of disease progression in ADPKD.

Primary endpoint

The primary outcome variable is rate of change in kidney function for lanreotide-treated versus control patients. This is defined as the slope through serial eGFR values over time during the treatment phase of the study. The value obtained at week 12 will be used as the first eGFR for slope analysis. If participants reach end-stage kidney disease or die, only eGFR values before these events will be taken into account.

Kidney function has been chosen as the primary end point instead of total kidney volume because the clinical relevance of this latter parameter is still uncertain. eGFR values obtained at weeks 4 and 8 during the treatment phase of the study will be used for safety analyses, but not for efficacy analysis.

Kidney function will be estimated using the creatinine-based 4-variable MDRD (Modification of Diet in Renal Disease) Study equation (24). This equation is validated in individuals with eGFR < 60 mL/min/1.73 m2 and generally is used in Dutch clinical practice. Furthermore, to rule out an effect of change in muscle mass or tubular creatinine secretion due to treatment, as a sensitivity analysis, cystatin C will be measured to estimate GFR (using the CKD-EPI [CKD Epidemiology Collaboration] equation).

Secondary endpoints

Secondary end points are separated into end points for the kidney, liver, and quality of life and are listed in **Box 2**.

We thought that it was useful to assess change in liver volume only in participants who have a polycystic liver and therefore decided not to analyze this secondary end point in those who have no or only a limited number of liver cysts because this will only dilute the effect size of the drug under investigation.

Box 2. Primary and secondary end points in the DIPAK1 study

Primary end point

Rate of change in kidney function for lanreotide-treated vs control patients (ie, the slope through serial eGFR values over time during treatment phase of study, where value obstained at week 12 is used as first eGFR for slope analysis and only eGFR values prior to end-stage kidney disease or death are taken into account).

Secondary end points

Kidney function

1. Change in kidney function as assessed as pretreatment eGFR (the average of the screening visit (SV) and at the baseline visit (BV)) versus eGFR 12 weeks after cessation of treatment (obtained at the follow-up visit, FU).
2. Incidence of a confirmed 30% decrease in eGFR and/or need for kidney replacement therapy computed from pretreatment.

Kidney volume

- Change in total kidney volume (MRI) as assessed at the baseline visit before start of treatment (BV) versus the value obtained 12 weeks after cessation of treatment (obtained at the follow-up visit, FU).

Liver

- Change in total liver volume (MRI) in the subset of subjects with moderate to severe polycystic liver disease (defined as a liver volume ≥2000 ml), as assessed at the baseline visit before start of treatment (BV) versus the value obtained 12 weeks after cessation of treatment (at the follow-up visit, FU).

Quality of life

- Change in quality of life as assessed at the baseline visit (BV) versus the value obtained 12 weeks after cessation of treatment (obtained at the follow-up visit)

Table 1. Specified flow chart of the DIPAK1 study

Visit	SV	BV	T1	T4	T8	T12	T24	T36	T48	T60	T72	T84	T96	T108	EOS	FU	EET
Weeks	-4	0	1	4	8	12	24	36	48	60	72	84	96	108	120	132	NA
Time window (from baseline or date of approved MRI (whichever comes last), for FU from EOS)	NA	Within 4 weeks after SV	± 1 day**	± 4 days	± 4 days	± 4 days	± 1 wk	± 1 wk	± 1 wk	± 2 wks	± 2 wks	± 2 wks	± 2 wks	± 2 wks	± 2 wks	± 1 wk	NA
Activity																	
Demographics	X																
Informed Consent	X																
In/Exclusion criteria	X	X*															
Medical History (ADPKD related, Risk factors, Other current & Past)	X																
Questionnaire		X				X			X				X		X	X	X
Physical Examination	X					X			X				X		X	X	X
Vital Signs	X	X		X	X	X	X	X	X	X	X	X	X	X	X	X	X
MRI (BV: -8 days till + 4 days after BV, EOS/EET/FU: +/- 8 days from visit)		X													X	X	X
Randomisation		X															
Study Completion																X	
Study treatment			X														
Concomitant Medication	X	X		X	X	X	X	X	X	X	X	X	X	X	X	X	X
Study Medication Summary				X	X	X	X	X	X	X	X	X	X	X	X		X
(Serious) Adverse Events		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X

X* Only pregnancy test in women with childbearing potential

Abbreviations are: SV: screenings visit; BV: baseline visit; EOS: end of study visit; FU follow-up visit; EET: early end of treatment visit

Data collection

Figure 1, **Table 1**, and **Table S1** (provided as online supplementary material) show the data to be obtained during study visits. Health-related quality of life will be assessed using an ADPKD-specific questionnaire, including questions regarding polycystic liver disease (30). Blood pressure will be assessed with an automatic device for 10 minutes during study visits. Blood and urine chemistry will be analyzed locally. In addition, a blood sample will be shipped to the core laboratory for storage (–80°C), and assessment of key efficacy variables (creatinine and cystatin C) will be performed after completion of the study in one run per participant to minimize interlaboratory and interassay variation. These centrally assessed laboratory variables will be used for efficacy analyses. Of note, storing blood samples at room temperature for up to 4 days does not influence creatinine (31) and cystatin C concentrations, nor does frozen storage at –80°C for prolonged periods (32).

At the baseline visit, at the end of the treatment phase of the study (week 120 or at early termination visit), and at follow-up, magnetic resonance (MR) imaging (MRI) will be performed, using a standardized protocol without use of intravenous contrast. The MRI acquisition protocol includes T2-weighted single-shot fast gradient spin-echo images with fat-saturation. MR images will be sent to the central reading facility, using a secure server. MRI end point data will be analyzed and read centrally using Analyze 11 software (AnalyzeDirect Inc) to assess total kidney volume with a stereology method. To ensure that valid MR images are obtained, quality control will be performed within 48 hours by trained personnel. In case a scan is rejected, it will be repeated before the injection of lanreotide.

Lanreotide serum levels will be measured after completion of the trial using blood samples for post-hoc assessment of the association between drug blood levels and efficacy. A web-based electronic case report form has been designed to enter study data to ensure correct and timely data collection in a central database.

Estimation of power and sample size

In a cohort of patients with ADPKD participating in the MDRD Study (baseline measured GFR, 25-55 mL/min), the mean slope of GFR decline on treatment was 5.2 mL/min per year with a calculated standard deviation of 4.3 (33). In the recent Everolimus ADPKD Study (baseline eGFR, 30-90 mL/min/1.73 m2), mean change in eGFR was 4.2 mL/min/1.73 m2 with a similar standard deviation of 4.3 (13). The annual slope of GFR in this study is expected to be similar to the MDRD Study and steeper than in the Everolimus ADPKD Study because in the present study, only individuals with decreased kidney function will be included (CKD stage 3). Assuming an average change in eGFR of 5.2 mL/min/1.73 m2 per year in untreated patients and a standard deviation of 4.3 in both treatment groups, 120 individuals per study group are needed to detect a 30% reduction in the rate of kidney function loss between treatment groups, with 80% power to detect this difference and 2-sided $\alpha = 0.05$. Taking into account the possibility of 20% protocol violators and/or dropouts, our aim is to include 150 participants per group.

Statistical analysis

Analyses will be done after completion of the study. To assess differences between treatment groups in baseline characteristics for continuous data, t test will be used for normally distributed data, and Mann-Whitney U test, for non-normally distributed data. The χ^2 test will be used to compare dichotomized variables between groups. (Generalized) mixed models will be used to analyze the primary end point (difference in change in kidney function in lanreotide-treated patients vs controls). All available eGFR values will be taken into account until a participant reaches end-stage kidney disease. We will explore whether missing eGFR values are random, and if necessary, we will use other statistical models that handle informative dropout.

In addition, we will perform linear regression analysis (calculating a slope through the available eGFR values per individual) as sensitivity analysis for the primary end point and secondary end points involving change in a variable. Incidences of worsening kidney function, end-stage kidney disease, and death

will be investigated using Cox proportional hazard models. Kaplan-Meier graphs will be prepared. All P values will be 2 tailed, and the level of significance will be set at $P < 0.05$. All analyses will be performed as intention-totreat analyses. Perprotocol analyses will be done as secondary analyses. The main analyses also will be performed in a priori–defined subgroups: baseline age younger than or equal to/ older than median, baseline eGFR less than or equal to/greater than median, baseline total kidney volume less than or equal to/greater than median, and men versus women. Of note, we will perform analyses for change in liver volume as a secondary analysis, with a sensitivity analysis with adjustment for use of estrogens or oral contraceptives. To control for type I errors, $P < 0.01$ will indicate statistical significance for the subgroup analyses. Furthermore, we will investigate correlations investigating changes in kidney volume versus changes in liver volume over time.

Ethical considerations

The Medical Ethics Committee of the University Medical Center Groningen approved the protocol and informed consent form. The trial is to be conducted in accordance with the International Conference of Harmonization Good Clinical Practice Guidelines and will adhere to the ethical principles that have their origin in the Declaration of Helsinki.

All participants have the right to withdraw at any time during the study. Further stopping rules for patients and the trial are given in **Table S2 online**.

Study organization

A steering committee oversaw the design and will overview the conduct of the study; a central study coordinator will coordinate the study. An independent data safety monitoring board has been established to monitor the safety and efficacy of the trial and can advise to stop the study based on serious adverse events and/or interim analysis of adverse effects. An academic contract research organization will monitor study progress and quality and completeness of study data.

DISCUSSION

The DIPAK 1 Study seeks to determine whether lanreotide attenuates kidney function deterioration in patients with ADPKD.

In ADPKD, well-described genetic defects initiate the formation of cysts (34-37). Cysts further expand due to disturbances in cell proliferation, apoptosis, cell-matrix interactions, and fluid secretion. One of the factors that potentially can affect these processes is 3′,5′-cyclic adenosine monophosphate (cAMP). Elevated cAMP levels might hasten cyst growth and overall kidney enlargement in patients with PKD (38). cAMP production can be inhibited by blocking the V2R, but also by activation of the somatostatin receptor (6, 39, 40). There are 5 receptors for somatostatin. Octreotide and lanreotide bind with high affinity to somatostatin receptor 2 (39, 41, 42). Detection of the somatostatin receptor 2 in kidney tubules and its inhibitory effect on cAMP production suggest a potential effect of somatostatin on cyst fluid secretion and enlargement in patients with ADPKD (43, 44). In experimental models of PKD, somatostatin analogues have been shown to inhibit hepatorenal cystogenesis (45, 46).

In humans, to date, only 3 small-scale studies have been performed with somatostatin analogues in ADPKD. In these studies, kidney function was not a primary outcome measure. Ruggenenti et al. (8) performed a randomized crossover study comparing the effect of a 6- month treatment regimen of octreotide, a long-lasting somatostatin analogue, with no treatment in 14 patients with ADPKD (mean baseline measured GFR, 57.1 [range, 24.4-95.3] mL/min). GFR, measured using iohexol clearance, did not change significantly during both treatment periods. Although total kidney volume increased significantly in both groups, the increase in kidney volume was reduced, with 60% reduction by administration of octreotide ($P < 0.05$). van Keimpema et al. (18) performed a randomized clinical study with a 6-month regimen of lanreotide, administered in the normal clinical dose of 120 mg once every 28 days subcutaneously in 54 patients, 32 of whom had ADPKD. In participants

with ADPKD, total liver volume decreased significantly with lanreotide compared to placebo ($P < 0.01$), and total kidney volume decreased by 17 mL (1.5%) in the lanreotide group and increased by 50 mL (3.4%) in the placebo group (absolute difference, $P < 0.02$). This beneficial effect was maintained in the following 6 months (47). Lanreotide treatment decreased serum creatinine levels ($P = 0.079$). In addition, at 6 months, lanreotide improved general healthy perception.

Hogan et al. (19) randomly assigned 42 patients with polycystic liver disease (of whom 34 had ADPKD) to 12 months' treatment with octreotide or placebo. Mean baseline GFR was 71 (range, 20-124) mL/min/1.73 m2 . Total liver volume decreased 4.95% in the octreotide group compared with an increase of 0.92% in the placebo group ($P = 0.048$). Among patients with ADPKD, the kidney growth rate was significantly reduced in the octreotide group compared with nontreated patients (0.25% vs 8.61%, respectively; $P = 0.045$). GFR decreased by 5.1% with octreotide and 7.2% with placebo (difference not statistically significant) (19). After 2 years of octreotide treatment, the reduction in total liver volume was maintained (–5.96% compared to baseline), but the inhibition of kidney growth during the first year was not sustained during the second year (48).

Caroli et al. (22) recently reported results of a single-blind randomized controlled trial involving 79 patients with ADPKD with eGFR $\geq$ 40 mL/min/1.73 m2 . Total kidney volume increased significantly less with octreotide compared to placebo after 1 year of treatment. After 3 years of treatment, the mean increase in total kidney volume again was smaller in the treated group, but results were not statistically significant. During the entire follow-up period, the rate of eGFR decline (measured by iohexol clearance) tended to be slower in the octreotide group than in the placebo group, but the difference was not statistically significant. After 1 year of treatment, there was no difference in GFRs. The long-term GFR decline from year 1 to year 3 was almost 50% slower in the octreotide group than in the placebo group (2.28 vs

4.32 mL/min/1.73 m2 per year, respectively; P = 0.03). It should be noted that at baseline in the placebo group, GFR was lower and total kidney volume was higher, which may have led to a worse prognosis in the placebo group independent of treatment. These data led the authors to conclude that their findings present the background for large randomized controlled trials to assess the protective effect of somatostatin analogues against loss of kidney function and progression to end-stage kidney disease (22).

To our knowledge, 3 trials are ongoing with somatostatin analogues in patients with ADPKD (n = 48 with pasireotide and n = 43 with lanreotide, both directed at liver volume (49, 50) and n = 80 with octreotide directed at kidney volume and rate of GFR decline (51)). Although these trials are important, the lower number of included patients may preclude definitive conclusions on the efficacy of somatostatin analogues for renoprotection in this patient group.

The most common adverse effects of lanreotide are injection-site discomfort and erythema, diarrhea, abdominal cramping, (asymptomatic) biliary sludge or gallstones, and abnormal glucose metabolism (52). Less common adverse effects are allergic skin reactions and acute pancreatitis. Diarrhea and abdominal cramping are expected to occur in the first days after the first injections when lanreotide reaches peak blood concentrations. These symptoms resolve spontaneously in most cases during continued use when more stable blood concentrations are reached (steady-state phase) (53). In case these symptoms do not resolve, pancreatic enzymes may be prescribed, which generally improve these symptoms (18). Of the 118 patients with ADPKD who were included in the 3 aforementioned studies with somatostatin analogues, only 2 patients stopped study medication permanently, and in only 4 patients did dosages have to be lowered (8, 18, 19).

The present costs associated with lanreotide are a disadvantage. In the Netherlands, a 120- mg lanreotide injection costs $2,310. This is approximately $30,000 per year for an injection schedule of once every 4 weeks. If proved

effective, new price agreements may be necessary to improve the cost-effectiveness ratio of lanreotide administration for ADPKD.

Recently, the Tolvaptan Efficacy and Safety in Management of ADPKD and Its Outcomes (TEMPO) 3-4 Study, a randomized controlled trial in 1,445 patients with ADPKD, showed renoprotection of the V2R antagonist tolvaptan (16). Inclusion criteria were different from those in this study. Only patients with ADPKD with estimated creatinine clearance > 60 mL/min were included. The efficacy and safety of tolvaptan in patients with ADPKD with CKD stages 3-4 are unproved to date. Patients with ADPKD with lower eGFRs have higher vasopressin levels (54, 55). Consequently, such individuals might require higher dosages of a V2R antagonist to effectively block the receptor. We found in an experimental model for ADPKD that a fixed dose of a vasopressin receptor antagonist showed less efficacy when administered at a later stage of disease (56). Because V2R antagonists might be less effective in a later stage of disease, we chose to compare lanreotide with standard care and not in addition to vasopressin receptor antagonists. The positive findings in the TEMPO 3-4 Study nevertheless are encouraging for the present study because both V2R antagonists and somatostatin analogues lower intracelluar cAMP levels (6).

Study limitations include the design as an open randomized controlled trial. Administration of lanreotide, which is a gel, will result in temporary injection infiltrates in the majority of actively treated individuals. Manufacturing a placebo that has a similar effect is not possible from a technical point of view. This precludes execution of this trial as a double-blinded randomized controlled trial. To minimize bias, efficacy end points will be assessed in a blinded fashion (eGFR and MRI kidney and liver volume measurements will be done centrally by personnel blinded for treatment allocation). Furthermore, the primary outcome is change in kidney function from 12 weeks after the start of treatment, instead of from randomization. This is done because in the first 3 months during treatment, dose adjustments of lanreotide and/or antihypertensive drugs may be needed, which may induce acute renal

hemodynamic effects that may compromise an accurate assessment of eGFR slope (57). However, a necessary assumption for this end point to be valid is that changes in eGFR during the first 12 weeks after randomization are fully reversible during the 12 weeks after discontinuation of the drug, after completion of the intervention. We cannot prove this assumption until the trial has finished. Therefore, after completion of the trial, this will be studied extensively, and in case the change in eGFR is not fully reversible, our primary end point requires support by one or more secondary kidney end points.

Another limitation is that kidney function will be estimated and not measured, potentially inducing more variability. However, serial measurement of kidney function in 300 patients with ADPKD in 4 different centers is not feasible, and it recently has been shown that measured GFR and eGFR in ADPKD are highly correlated.58 Finally, in this study, total kidney volume will be measured using MRI, a method that is well validated (4, 14).

In conclusion, to our knowledge, the DIPAK 1 Study is the first larger scale clinical study that will investigate the efficacy of somatostatin analogue on attenuation of kidney function decline in ADPKD.

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