Urinary extracellular vesicles and the kidney: biomarkers and beyond

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Submitted 27 February 2014; accepted in final form 28 March 2014

Salih M, Zietse R, Hoorn EJ. Urinary extracellular vesicles and the kidney: biomarkers and beyond. Am J Physiol Renal Physiol 306: F1251–F1259, 2014.—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 pathophysiological state of cells lining the nephron. Because urine is a noninvasive 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 of using vesicles as a therapy for kidney disorders.

exosomes; mesenchymal stem cells; microvesicles; ultracentrifugation

URINE CONTAINS VESICLES derived from the kidney and urinary tract. Vesicles may be released into the preureine by direct shedding or budding from the plasma membrane or through the fusion of intracellular multivesicular bodies with the plasma membrane (29). The latter resembles a process of exocytosis, and the vesicles formed in this way are often called “exosomes” (53). Exosomes are vesicles with a lipid bilayer membrane that are 30–120 nm in size and float at a density of 1.15–1.19 g/ml in continuous sucrose gradient (69). Although these characteristics are ascribed to exosomes, other vesicles are usually copurified. This was illustrated by one study (24) 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. 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 (20, 22, 77). Figure 1 shows uEVs as visualized by electron microscopy. Proteomic analysis has shown that uEVs contain proteins from glomerular, tubular, prostate, and bladder cells (21, 53). 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 tetraspansins, apoptosis-linked gene-2-interacting protein X (ALIX), and tumor susceptibility gene (TSG)-101, which are now commonly used as uEV markers (17, 84). In addition to proteins, uEVs also contain nucleic acids (41). Although mRNA profiles from whole cells in urine are being studied as disease markers (67), 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 (12, 41). Of interest, uEVs also contain microRNA (miRNA), which are small noncoding RNAs that regulate mRNA processing (12). It appears that small RNAs, including miRNAs, make up the majority of RNA species contained in uEVs (12). In addition to analyzing proteins, mRNA, or miRNA in uEVs, analysis of posttranslational modification of plasma membrane proteins in uEVs may be another method for biomarker discovery, as was recently illustrated for polycystic kidney disease (PKD) (20). 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 uEVs

Methods used to isolate EVs from biofluids and cell culture media include ultracentrifugation, filtration, precipitation, and immunoisolation (Table 1) (42, 77). 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 the degradation of uEVs (50, 84). In addition, storage at −80°C, compared with +4 or −20°C, seemed better to pre-
vent degradation, although freshly processed urine is most favorable (50). Importantly, it seems that degradation of uEVs could occur within 2 h of urine collection (50). It is therefore recommended that urine samples are stored immediately after the addition of a protease inhibitor. uEVs can be recovered from urine up to 7 mo after freezing (84). 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 observations).

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 (62). This precipitate cosediments proteins such as Tamm-Horsfall protein (THP; also called uromodulin) at low-speed centrifugation (62). THP is a high-molecular-weight polymeric glycoprotein secreted by epithelial cells lining the thick ascending limb of the loop of Henle. Under physiological conditions, THP is the most abundant protein in urine and is excreted at a rate of ~20–100 mg/day (56). THP is capable of entrapping uEVs by forming polymers that precipitate at low-speed centrifugation (relative centrifugal force: 17,000 g), leading to a reduced yield of isolated uEVs in the final ultracentrifugation pellet (17). 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-containing precipitates (62), thereby reducing EV entrapment in THP (84). A further increase of the uEV yield can be achieved by adding DTT, a strong reducing agent capable of disrupting the zona pellucida disulphide bonds of THP (17). DTT will release entrapped uEVs from the low-speed pellet (17,000 g) into the high-speed pellet (200,000 g). Of note, CD9-positive uEVs seem to escape the low-speed entrapment (17). CHAPS 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 that of dipeptidyl peptidase IV (45), a potential biomarker for diabetes mellitus (66).

Ultracentrifugation. Ultracentrifugation is still the most commonly used method to isolate EVs from biofluids. Before actual ultracentrifugation, most protocols use one or more centrifugation steps to eliminate whole cells, large vesicles, and debris. Ultracentrifugation is then performed, usually at 100,000–200,000 g for at least 1 h (42, 69). Of note, ultracentrifugation does not isolate all uEVs, because 40% of uEVs could still be isolated from the supernatant using ammonium sulphate precipitation (46). This is in line with earlier reports from cell lines, in which ultracentrifugation recovered only 5–25% of total EVs (36). 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 (31). Because the buoyancy of vesicles depends on the density of urine, adjusting this density may increase the uEV yield with ultracentrifugation.

Table 1. Methods for isolation of uEVs

<table>
<thead>
<tr>
<th>Technique</th>
<th>Minimum Starting Volume, ml</th>
<th>Isolation Time, h</th>
<th>Suitable for RNA</th>
<th>Advantage</th>
<th>Disadvantage</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>UC</td>
<td>5</td>
<td>1–2</td>
<td>Yes</td>
<td>High yield</td>
<td>Tamm-Horsfall protein entrapment</td>
<td>35, 53, 69, 84</td>
</tr>
<tr>
<td>UC with reducing agent or detergent</td>
<td>5</td>
<td>1.5–2.5</td>
<td>Yes</td>
<td>Very high yield</td>
<td>Low of functionality</td>
<td>1, 17, 45</td>
</tr>
<tr>
<td>Sucrose gradient UC</td>
<td>25</td>
<td>2.5–24</td>
<td>Yes</td>
<td>High purity</td>
<td>Low yield, time consuming</td>
<td>1, 11, 27, 28, 55, 69</td>
</tr>
<tr>
<td>UC with size-exclusion chromatography</td>
<td>45</td>
<td>Unknown</td>
<td>Unknown</td>
<td>Suitable for proteinuria</td>
<td>Need for HPLC</td>
<td>61</td>
</tr>
<tr>
<td>Filtration</td>
<td>Microfiltration</td>
<td>12.5</td>
<td>Unknown</td>
<td>Unknown</td>
<td>No need for UC</td>
<td>Low throughput</td>
</tr>
<tr>
<td></td>
<td>Nanofiltration</td>
<td>0.5</td>
<td>5</td>
<td>No</td>
<td>No need for UC</td>
<td>Unsuitable for proteinuria</td>
</tr>
<tr>
<td></td>
<td>Precipitation ExoQuick TC</td>
<td>5</td>
<td>12*</td>
<td>No</td>
<td>No need for UC</td>
<td>Not suitable for urine</td>
</tr>
<tr>
<td></td>
<td>Modified ExoQuick TC</td>
<td>5</td>
<td>13*</td>
<td>Yes</td>
<td>No need for UC</td>
<td>Unknown reagent</td>
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<tr>
<td></td>
<td>Immunoisolation ELISA</td>
<td>0.05</td>
<td>5.5</td>
<td>No</td>
<td>High throughput</td>
<td>Selection of subpopulation</td>
</tr>
</tbody>
</table>

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 (69), double (55), and gradient (1, 11, 27, 28) sucrose in heavy water giving rise to different populations of uEVs. One study (11) 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, especially proximal tubules), and aquaporin-2 (collecting duct origin). While these methods enrich the final pellet of uEVs, they are time consuming (up to 24 h) and require larger starting volumes of ~25 ml urine compared with 5 ml urine for other ultracentrifugation protocols (Table 1). This may result in a lower yield of uEVs, even <1% compared with crude pellets (28).

Ultracentrifugation with size-exclusion chromatography. Special handling is required for the 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-abundance interfering proteins, respectively (61). This method resulted in higher EV purity compared with nanomembrane ultrafiltration or standard ultracentrifugation methods, with and without DTT (61). Although ultracentrifugation with size-exclusion chromatography is considered the technique of choice for proteinuric urine, it has been 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 a 13-nm pore size, which requires only 0.5 ml urine as a 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 (13). Compared with other isolation methods, the purity of recovered protein in uEVs remains low (1), and this method therefore seems less suitable for nephrotic urine (61) and to isolate RNA from uEVs (1). Hydrophilized polyvinylidene difluoride microfilters, which have a 100-nm pore size, have been successfully used to isolate uEVs (40). 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.

Precipitation. ExoQuick is a commercially available precipitation kit to isolate microvesicles (System Biosciences, Mountain View, CA). For urine, its current protocol results in a very low yield of both protein and RNA (1). Modification of the sample workup resulted in the highest quantities of mRNA and miRNA and an acceptable protein yield compared with ultracentrifugation methods (1). 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 (AKI).

Immunoisolation. For clinical applications, it is desirable to be able to directly isolate vesicles from urine without the need for ultracentrifugation. To this end, immunoisolation methods may be especially attractive, as has been successfully performed for the water channel aquaporin-2 (34). 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 have used ELISA with different capture antibodies, including AD-1 (an antibody raised against human membrane bound liver alkaline phosphate) (66) and the NaCl  cotransporter (NCC) (30). In the first study (66), 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. In the second study (30), however, urine was first ultracentrifuged, and the pellet was then lysed and finally added to a plate coated with an antibody against NCC (30). This lysis step was necessary because the antibody targets the intracellular domain of NCC, which is located inside 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 (30). 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 (31). Normalization methods that have been used include urinary creatinine, timed urine collection, THP, use of “household” uEV markers, timed urine collection, THP, use of “household” uEV markers, (e.g., CD9), and estimation of the actual number of uEVs (17, 29, 65). Urinary creatinine has been used most often and has the advantage that it allows for the use of spot urines (30, 73, 84). 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 intraday variability, it is notoriously incomplete (29). It is also unknown if nephrosis results in chronic kidney disease results in the formation of fewer 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) (1, 65). 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 (43, 74, 77). Currently, resistive pulse sensing and nanoparticle track-
NHE3 in uEVs was more specific than the fractional excretion of Na⁺, 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 2 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 (83). The same group (81) also studied activated transcription factor 3 in experimental and clinical AKI. Although transcription factors are undetectable in whole urine and uEVs from healthy subjects, even when using proteomics (21), they are readily detectable in uEVs during AKI, thereby representing a new class of biomarkers in kidney disease. The water channel aquaporin-2 in mice after treatment of desmopressin was readily detectable. This method also reduced the intra-assay variability due to the interference of larger particles, one of the limiting factors for nanoparticle tracking analysis in unprocessed samples (50). 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 (52).

Use of uEVs in Nephrology

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

AKI. AKI is characterized by a sudden deterioration in kidney function. It is common in hospitalized patients and associated with high morbidity and mortality (5). Because the serum creatinine concentration starts to increase only when glomerular filtration rate has decreased by >50%, earlier biomarkers of reduced kidney function are being sought (5). In one study (16), increased abundance of Na⁺/H⁺ exchanger type 3 (NHE3) in uEVs was able to differentiate between prerenal azotemia, acute tubular necrosis, and other causes of renal failure in 68 patients admitted to the intensive care unit. NHE3 in uEVs was more specific than the fractional excretion of Na⁺, 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 2 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 (83). The same group (81) also studied activated transcription factor 3 in experimental and clinical AKI. Although transcription factors are undetectable in whole urine and uEVs from healthy subjects, even when using proteomics (21), they are readily detectable in uEVs during AKI, thereby representing a new class of biomarkers in kidney disease. The water channel aquaporin-2 in mice after treatment of desmopressin was readily detectable. This method also reduced the intra-assay variability due to the interference of larger particles, one of the limiting factors for nanoparticle tracking analysis in unprocessed samples (50). 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 (52).

Table 3. Potential biomarkers in uEVs for different kidney disorders

<table>
<thead>
<tr>
<th>Disorder</th>
<th>Potential biomarkers in uEVs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acute kidney injury</td>
<td>Na⁺/H⁺ exchanger type 3, activated transcription factor-3, fetuin-A, aquaporin-1</td>
</tr>
<tr>
<td>Focal segmental glomerulosclerosis</td>
<td>αK-Antitrypsin, aminopeptidase N, actorsin precursor, ceruloplasmin, podocalyxin</td>
</tr>
<tr>
<td>IgA nephropathy</td>
<td>Podocalyxin, dipetidyl peptidase IV, miR-145, Wilm’s tumor-1</td>
</tr>
<tr>
<td>Diabetic nephropathy</td>
<td>Aquaporin-2</td>
</tr>
<tr>
<td>Nephrogenic diabetes insipidus</td>
<td>Na⁺/K⁺/Cl⁻ cotransporter type 2</td>
</tr>
<tr>
<td>Bartter syndrome</td>
<td>Na⁺/Cl⁻ cotransporter</td>
</tr>
<tr>
<td>Gitelman syndrome</td>
<td>Na⁺/Cl⁻ cotransporter</td>
</tr>
<tr>
<td>Familial hyperkalemic hypertension</td>
<td>Na⁺/Cl⁻ cotransporter, prostasin</td>
</tr>
<tr>
<td>Primary aldosteronism</td>
<td>Polyoxin-1, polyoxin-2, fibrocytin/polyductin, surface glycosylation profiles</td>
</tr>
<tr>
<td>Polycystic kidney disease</td>
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</table>
undetectable after 48 h. 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 (2, 57, 70, 76). 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 (6, 7). There is evidence to suggest that EVs play a pivotal role in this paracrine effect (8–10, 19, 58, 85). Bruno et al. isolated EVs derived from human bone marrow MSCs and used these EVs to test their effect in tubular epithelial cells (TECs) and immunodeficient mice with glycerol-induced AKI (9). After EVs were labeled 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 were 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 control mice. Interestingly, RNase treatment abrogated the protective effects of MSC EVs in all studies, suggesting an RNA-dependent mechanism (8–10, 19, 58, 85). Indeed, mRNA and miRNA seem to be specifically incorporated into EVs, suggesting a horizontal transfer of MSC EVs to target cells (65).

Fig. 2. Illustration of the many functions of uEVs in kidney disorders. Shown is a schematic drawing of the nephron with the glomerulus and kidney tubule. Five magnifications illustrate local processes in which uEVs are involved along the nephron. A: uEVs containing podocalyxin and Wilm’s tumor-1 protein, which are excreted after podocyte injury (3, 23–25, 33, 81, 82). B: uEVs containing the Na\(^+\)/Cl\(^-\) cotransporter (NCC). These uEVs may be used in primary or secondary aldosteronism (Fig. 3), Gitelman syndrome, or familial hyperkalemic hypertension (Table 2) (30, 32, 39, 73). C: 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 (8–10, 19, 58, 85)]. D: how uEVs may interact with primary cilia and possibly exert a downstream effect, for example, in polycystic kidney disease (28, 38, 78). E: uEVs may possibly transfer functional aquaporin-2 (AQP2) between kidney collecting duct cells [based on in vitro findings (65)].
by MSCs. When fibroblast cell lines derived from mice lacking the IGF-1 receptor were incubated with MSC-derived EVs, functional IGF-1 receptors were transcribed. Furthermore, incubation of these cells with EVs led to an increase in proliferation in cisplatin-treated proximal tubular epithelial cells. This effect was enhanced when IgF-1 was coincubated. This also suggests that MSC-derived EVs contribute to the repair of damaged cells in an RNA-dependent fashion.

**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 (18, 51, 80, 82). A number of specific biomarkers for glomerular disease in uEVs have been identified, including Wilm’s tumor (WT)-1, podocalyxin, α1-antitrypsin, aminopeptidase N, vasorin precursor, ceruloplasmin, and miR-145 (Table 3). In animal models of focal segmental glomerulosclerosis and collapsing glomerulopathy, WT-1 protein in uEVs, a transcription factor required for kidney development, increased 1 wk earlier than urinary albumin (81) and predicted the effect of angiotensin receptor blocker treatment (82). One study found that WT-1 in uEVs was increased in patients with focal segmental glomerulosclerosis but not in patients with AKI or in healthy volunteers (81, 82); this finding, however, was not confirmed by others (37). The increase in uEV WT-1 was also associated with a decline in kidney function in diabetic nephropathy, suggesting early podocyte damage (33). Another podocyte injury marker is podocalyxin, which, unlike WT-1, was found in shedding vesicles (no expression of CD24 and CD63) (24, 25). In addition to diabetic nephropathy, podocalyxin was also increased in uEVs isolated from patients with IgA nephropathy (3, 23). 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 α1-antitrypsin, aminopeptidase N, vasorin precursor, and ceruloplasmin (44). In type 1 diabetics with diabetic nephropathy, miRNA expression profiling revealed differential expression of 22 of 226 miRNAs isolated from uEVs (4). The 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 (21, 29, 53). Less clear is if the abundance of transporters in uEVs correlates with their transport activity in the kidney, although a number of studies have suggested this may be so (26, 65). In 1995, Kanno et al. (34) were the first to identify aquaporin-2 in urine. Aquaporin-2 is a vasopressin-sensitive water channel localized in 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 were 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 (65), the abundance of aquaporin-2 in uEVs highly correlated with the abundance in murine kidney collecting duct cells after desmopressin stimulation. Interestingly, the transfer of uEVs from desmopressin-treated cells to untreated cells resulted in an increase of functional aquaporin-2 expression in untreated cells (Fig. 1). Similar effects were seen in rats after desmopressin treatment, because the excretion of aquaporin-2 in uEVs increased (65). The good correlation between desmopressin stimulation and aquaporin-2 excretion in uEVs was confirmed by others (26). In addition to plasma vasopressin, aquaporin-2 in uEVs was also influenced by alkalization of the urine (26). 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 has also been illustrated by the absence of the Na\(^+\)-K\(^+\)-Cl\(^-\) cotransporter (NKCC2) in urinary exosomes from patients with Bartter syndrome type 1, a rare disease caused by mutations in the gene encoding for NKCC2 (21). Similarly, NCC was absent in urinary exosomes of patients with Gitelman syndrome, who have an inactivating mutation in the gene encoding for NCC (32). The opposite was also true, because patients with familial hyperkalemic hypertension, which is characterized by NCC overactivity, had increased NCC in urinary exosomes (30, 39). 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 (73). Indeed, rats fed a low-Na\(^+\) diet or infused with aldosterone showed increases in total and phosphorylated NCC in kidney tissue and uEVs (Fig. 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 prostanin, a serine protease known to regulate the epithelial Na\(^+\) channel, which is also sensitive to aldosterone. Another disorder in which kidney transporters in uEVs were analyzed is American cutaneous leishmaniasis (49). 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, whereas 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.

**PKD.** PKD is the most common inherited kidney disease and is caused by dysfunction of cilia (72). EVs may be important in cilia biology and as biomarkers for PKD (Fig. 3). Primary cilia are flow-sensing organelles and are expressed in nearly all renal epithelial cells except for intercalated cells of the collecting duct (54, 60, 75). Mutations in genes encoding for proteins involved in the function of primary cilia lead to PKD, including polycystin-1, polycystin-2, and fibrocystin/polyductin (79). The proteins polycystin-1 and polycystin-2 form a complex that is involved in mechanosensing by cilia. Changes in luminal flow induce a Ca\(^{2+}\) influx through the polycystin-2 channel, which is a nonselective cation channel (47). 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 (68). 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 Ca\(^{2+}\) elevation in the cells of the left ventral node. Mutations in polycystin-2 hamper this increase in intracellular Ca\(^{2+}\), disturbing the left-right breaking process. This, in turn, could lead to situs inversus, a congenital condition in which the major organs are reversed (48). Furthermore, so-called PKD-positive vesicles seem to specifically interact with primary cilia of kidney and biliary epithelial cells, as observed in transmission electron microscopy images (28, 78). 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 (38). Interestingly, the proteins involved in the pathogenesis of PDK, polycystin-1, polycystin-2, and fibrocystin/polyductin, have also been found in uEVs (21, 53). Characterization of uEVs in which polycystin-1, polycystin-2, and fibrocystin/polyductin were present identified two additional proteins previously known to be involved in PKD, namely, cystin and ADP-ribosylation factor-like 6 (28). 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 of 43 lectins studied, illustrating potential biomarker use. Our group is currently searching for potential biomarkers in uEVs for PKD using quantitative proteomics (63).

**ACKNOWLEDGMENTS**

The authors thank Prof. Dr. R. Willemsen for help with the electron microscopic image.

**GRANTS**

M. Salih is supported by the Dutch Kidney Foundation (Developing Intervention Strategies to Halt Progression of Autosomal Dominant Polycystic Kidney Disease Consortium). E. J. Hoon is supported by a grant from the Netherlands Organisation of Scientific Research (VenI).

**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).

**AUTHOR CONTRIBUTIONS**

Author contributions: M.S. prepared figures; M.S. and E.J.H. drafted manuscript; M.S., R.Z., and E.J.H. edited and revised manuscript; M.S., R.Z., and E.J.H. approved final version of manuscript.

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