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Urinary extracellular vesicles: Origin, role as intercellular messengers and biomarkers; efficient sorting and potential treatment options

Per Svenningsen¹, Rugivan Sabaratnam¹² and Boye L. Jensen¹

¹Department of Cardiovascular and Renal Research, Institute of Molecular Medicine, University of Southern Denmark, Odense, Denmark, ²Steno Diabetes Center Odense, Odense University Hospital, Section of Molecular Diabetes & Metabolism, Institute of Clinical Research, 5000 Odense C, Denmark.

Short title: Extracellular vesicles as messengers

Correspondence:

Per Svenningsen, PhD,
Department of Molecular Medicine, University of Southern Denmark
J.B. Winsloews vej 21.3, DK-5000 Odense C, Denmark

e-mail: psvenningsen@health.sdu.dk, Phone: +45 6550 7905
Abstract
Urinary extracellular vesicles (uEVs) are a heterogeneous group of vesicles consisting mainly of microvesicles and exosomes that originate predominantly (99.96%) from kidney, the urinary tract epithelium, and the male reproductive tract. Secreted EVs contain molecular cargo from parental cells and provide an attractive source for biomarkers, a potential readout of physiological and pathophysiological mechanisms, and events associated with the urinary system. uEVs are readily enriched and isolated from urine samples and we review six standard methods that allow for downstream analysis of the uEV cargo. Although the use of uEVs as a surrogate readout for physiological changes in tissue protein levels is widespread, the protein abundance in uEVs is affected significantly by mechanisms that regulate protein sorting and secretion in uEVs. Data suggest that baseline kidney tissue and uEV levels of apical membrane-associated electrolyte transport proteins are not directly related in human patients. Recent evidence indicates that EVs may contribute to physiological and pathophysiological intercellular signaling and EVs confer protection against renal ischemia-reperfusion injury. The therapeutic use of EVs as information carriers has mainly been explored in vitro and a major hurdle lies in the translation of the in vitro findings into an in vivo setting. Thus, the EV research field is moving from a technical focus to a more physiological focus, allowing for a deeper understanding of human physiology, development of diagnostic tools and potential treatment strategies for precision medicine.

Key words: exosomes, microvesicles, intercellular communication.
**Introduction**

Urinalysis provides an important screening and diagnostic tool as the urine composition reflects the overall need to maintain solute and water balance. The homeostatic control is accomplished by tight autoregulation of filtration of plasma/extracellular fluid and selective renal tubular secretion and reabsorption by specific transport mechanisms. In the early 1990s, it was found that human and rat urine contained renal epithelial water channel proteins, aquaporin (AQP) 1 and AQP2. The urinary excretion of AQP2 displayed the expected physiological regulation of AQP2 protein abundance by arginine vasopressin (AVP) in patients with central diabetes insipidus, but not nephrogenic DI. Subsequent studies showed that membrane fraction from rat urine samples contained Na/H exchanger (NHE)3, Na/K/2Cl (NKCC2) and NaCl cotransporter (NCC) proteins, demonstrating that also electrolyte transporters from the major nephron segments are present in urine. Interestingly, urinary AQP1 and AQP2 proteins were associated with small vesicles, larger membrane structures and intracellular organelles resembling multivesicular bodies (MVB) that were pelleted by ultracentrifugation (i.e. 200,000g) of urine samples. In the landmark study by Pisitkun et al. the membrane fraction from healthy individuals was identified as “exosomes”, a type of extracellular vesicle (EVs) originating from MVB secretion, indicating that urine samples could provide a non-invasive snapshot of the protein complement of urogenital epithelial cells. Since then many reports have described, not just how epithelial proteins, but also how lipids and RNAs are excreted in urinary EVs (uEVs). Thus, evidence suggest that EVs might contribute to intercellular signaling and also provide a novel therapeutic strategy for targeted delivery of bio-active molecules.

In this review, we will provide a comprehensive overview of advantages and limitations of the current methods available for isolation of uEVs. Additionally, we present novel *in silico* analysis of their potential cell of origin and their molecular cargo based on available public databases. Lastly,
we will review the current literature that describes uEV application for understanding mechanisms in renal physiology and pathophysiology. The role as potential biomarkers for malignant diseases in the urogenital system have been covered by multiple recent reviews\textsuperscript{10-12} and will not be discussed here. In perspective, a short paragraph covers potential use as novel treatment strategies.

\textit{Classification and nomenclature of extracellular vesicles}

EVs are a heterogeneous group of membrane-enclosed vesicles that can mainly be divided into microvesicles and exosomes based on their biogenesis, morphological and physical features. Microvesicles bud off directly from the plasma membrane, and the membrane of the microvesicles has been suggested to reflect the plasma membrane of the parent cell\textsuperscript{13}. Exosomes, on the other hand, are of endosomal origin and are generated when the endosomal membrane fuses with the membrane of the MVB, generating intralumenal vesicles (ILV).\textsuperscript{14} Microvesicles and exosomes differ in size and molecular constituents, and a combination of these features has been used to discriminate between the two classes of EVs. Microvesicles have a typical diameter of 200 -1000 nm, whereas exosomes are smaller with a diameter of 30-150 nm. However, there is no evidence that vesicles with a diameter < 100 nm cannot directly bud off from the plasma membrane\textsuperscript{15}, and analysis of the size distribution of the EVs cannot alone determine the vesicular entities. In addition to size, antibodies against known markers have been used to discriminate between the two EVs\textsuperscript{13}; thus, exosomes express endosomal markers e.g. tetraspanin CD63,\textsuperscript{13} while e.g. \(\alpha\)-actinins-1 and -4 are enriched in microvesicles\textsuperscript{16}. The EV field is relatively young and the nomenclature has changed over the years, so in this review we will use the term extracellular vesicles (EVs) when the experimental details of the paper does not allow for discrimination between the two vesicle types.
The molecular cargo and secretion of extracellular vesicles

EVs are actively released from cells into their surroundings and carry biomolecules, e.g. lipids, nucleic acids and proteins, from their parental cell. The exact molecular mechanisms by which EVs are generated has not been resolved. However, in vitro, the mechanism of exosome generation involves components of the endosomal-sorting-complex-required-for-transport (ESCRT). ESCRT complex is comprised of roughly 30 proteins, which assembles into five functional subcomplexes. The ESCRT-0 sequesters ubiquitinated cargo, ESCRT-I/II/III directs ILV budding and Vps4 complex ensures final membrane scission and/or ESCRT recycling. In addition to the ESCRT proteins, lipids, e.g. ceramides, and tetraspanins, e.g. CD9 and CD63 are also involved in the formation of intraluminal vesicles.

The lipid composition of EVs varies considerably from the parental cells. EV are enriched in cholesterol, sphingomyelin, glycosphingolipids and phosphatidylycerine (PS) and the lipid profile of uEVs has been used in biomarker discovery of prostate and breast cancer, and renal cell carcinoma. Pharmacological manipulation of cellular lipid homeostasis affects exosomes biosynthesis rate, however, the effects depend on the cell type. Thus, endosomal cholesterol appears to regulate exosome production, and the cationic amphiphile compound U18666A that accumulates cholesterol into endosomes increases exosome secretion in oligodendroglial cells, while U18666A reduces exosome secretion in B-cell lymphoma cells. Similarly, inhibition of ceramide production through blocking of neutral sphingomyelinase (nSMase) or knockdown of nSMase expression reduces exosome section in some cells types, while exosome secretion from other cell types was unaffected by similar treatments. Not only the cell type is important, but the lipid profile of EVs derived from biological fluids and conditioned cell culture medium varies considerably and cell culture conditions are unlikely to mimic the in vivo situation and EV lipid content is sensitive e.g. to oxygen tension. Thus, these findings highlight the importance of
cell culture conditions for the EV lipid content implying that kidney cells that reside in areas with very different oxygen tensions (cortex vs outer medulla) and house cells with exquisite oxygen sensitivity might produce EV with very different lipid composition.

RNA is the dominant nucleic acid in EVs. RNA associated with EVs are mainly non-coding RNA species, but protein encoding mRNAs are also present. EV-associated RNAs in human and rat urine contain segment-specific mRNA transcripts from glomerulus, proximal, thin descending, medullary thick ascending, distal convoluted tubules and collecting ducts. The RNA content of parental cells and their EVs is poorly correlated indicating that sorting mechanisms exist for the loading of EVs. In agreement, a short RNA motif, EXOmotif, that determined the sorting of miRNA into exosomes has been identified. Quantitative analysis of miRNA contents of EVs from the plasma of metastatic prostate cancer patients, however, indicates that the stoichiometry of a given miRNA inside individual EVs is below 1, proposing that either few miRNAs are present within each EV or that a restricted subtype of EVs contain significant amount of miRNA. Thus, similar to the lipid content, the EV content of nucleic acid is enriched in specific RNAs and correlates poorly with the parental cells.

EVs are enriched in specific proteins and ESCRT-I components Tsg101, ESCRT-accessory protein Alix, tetraspanins CD9 and CD63 are among the most frequently used exosomal markers. Post-translational modification of proteins, e.g. ubiquitination and oligomerization of membrane proteins promote their loading into EVs; however, to date no specific protein motif that directs proteins into EVs has been identified.

Emerging evidence from in vitro studies using cultured cells and cell conditioned medium showed that EVs are stimulated and released by various stimuli including hypoxia, oxidative stress or an acute increase in intracellular Ca²⁺. Thus, only small changes in environmental factors are predicted to change the uEV abundance. Exosome release has been studied in more detail and
occurs upon fusion between MVB membrane and cell membrane and is accomplished by the activity of specific Rab GTPases including Rab11, Rab27a, Rab27b and Rab35. However, the polarity of epithelial cells adds an extra layer of complexity and proteomic analysis of polarized retinal pigmented epithelium has shown that the cargo of basolaterally and apically secreted EVs is different. In agreement, apical AQP2, but not basolaterally expressed AQP3, is excreted in uEVs. Other proteins, such as Wnt3a, are present in both apical and basolateral EVs, but disruption of a site for posttranslational lipidation of Wnt3 eliminates its basolateral, but not apical, secretion. It is still unresolved how the polarity in EV secretion is established, emphasising that EV secretion is likely to be cell-type specific and that the mechanism identified in un-polarized cells cannot be directly translated into polarized epithelial cells.

Methods for uEV isolation

Proteomic characterization of uEV proteins is by far the most studied cargo species of EVs and our overview of uEV isolation methods will mainly focus on their advantages and limitations with respect to isolation and analyses of EV proteins. Sample collection and subsequent analysis of EV proteins can be separated in that EVs remain intact during long-term storage at -80 °C and protein integrity is preserved by addition of protease inhibitors. Although protease inhibitors are important for storage, EV proteins are necessarily exposed to luminal soluble proteases and/or cell-attached, e.g. GPI-anchored or transmembrane, proteases along the urinary tract and/or during storage in the bladder. EVs isolated from the renal pelvis by intrarenal catheters and voided urine samples differ with respect to proteolysis of specific membrane proteins. The EV protein concentration in biological fluids is relatively low and the EV content in 100 ml human blood plasma corresponds to ~60 µg; thus, methods to enrich for EVs are required to
analyze their cargo. The six most common methods to enrich/isolate EV from human urine samples are shown in Figure 1. The methods can be broadly divided into isolation methods based on physicochemical properties (size and density), affinity enrichment and volume-exclusion polymers (Figure 1a-f) and each has advantages and limitations (Table 1) as to the subsequent downstream application of uEVs, e.g. is high purity or high yield needed?

The classical, and still mostly used method (Figure 1g), is differential ultracentrifugation (dUC) in which centrifugation steps at increasing speed is used to pellet EVs based on size density; thus, cell and debris are first pelleted (<1,500g), next large EV (10,000-20,000g) and small EVs are pelleted at speeds above ~100,000g \(^{50}\) (Figure 1a). Protein aggregates are pelleted at these high speeds as well, and if discovery-based approaches like mass spectrometry is to be performed on the pellet, means to reduce contaminating aggregates need to be performed. Moreover, the recovery rate of EVs is variable and rates of 2% to 80% has been reported for isolation of EV from human plasma \(^{51}\). dUC is low-throughput and requires specialized equipment (ultracentrifuge).

Density gradient centrifugation (DGC) can isolate EVs based on size and mass density with sucrose and iodixanol being the most commonly used density media (Figure 1b). In contrast to iodixanol, sucrose-gradients are not iso-osmotic, and the osmotic differences changes EV volume/density during centrifugation. DGC reduces protein contamination of the samples but is time-consuming and low throughput. Moreover, for many applications the density medium has to be removed by e.g. dialysis and an ultracentrifugation step is needed to pellet EVs.

Size-exclusion chromatography (SEC) separates according to the size allowing for separation of protein aggregates and EVs (Figure 1c). The exclusion matrix determined the size cut-off and Sepharose 2B is commonly used matrix with a cut-off of ~60 nm. SEC effectively depletes soluble protein content of the sample, including the most abundant protein in healthy urine, Tamm-Horsfall
protein (THP)\textsuperscript{52}. SEC is relatively fast and only requires 10-20 min per sample and gentle to the EV morphology; however, the isolation is at the expense of dilution\textsuperscript{53}.

Ultrafiltration using nanomembranes is effective at concentrating EVs by passing soluble components e.g. proteins, through a filter with suitable size cut-off e.g. 100 kDa\textsuperscript{54} (Figure 1d). The EV recovery is good and the processing time is short, and ultrafiltration is an attractive means for uEVs in that large sample volumes can be effectively concentrated.

EV can be isolated by addition of a precipitant (Figure 1e). EVs are in the nano-meter size, similar to viruses, and standard protocol for isolation of viruses using water exclusion polymers such as polyethylene glycol (PEG) can be used to effectively enrich EVs\textsuperscript{55}. PEG is a non-toxic, non-denaturing, water-soluble and volume-excluding polymer that is added to samples, incubated at 4°C and EVs are precipitated by a short centrifugation step at relatively low speed. PEG precipitation is effective for sample EV concentration, but not suitable for high-throughput identification of biomarkers in that co-precipitation of contaminating proteins is high. However, it is an attractive option to use after e.g. SEC. The EV recovery is high\textsuperscript{56}, sample preparation is inexpensive and requires no special equipment.

Immuno-affinity using antibodies or other affinity reagents against marker proteins has been utilised for the enrichment of EVs from urine (Figure 1f) e.g. heat-shock protein affinity venceremim (Vn) peptides\textsuperscript{57}, lectins\textsuperscript{58} anti-podocyte CR1\textsuperscript{59}, anti-CD133\textsuperscript{60}. Moreover, lipids in the plasma membrane are asymmetrically distributed and there is an enrichment of negatively charged lipids on the inner leaflet in living cells\textsuperscript{61}. In EVs, this asymmetric lipid distribution is lost and negatively charged PS is exposed on the surface\textsuperscript{61}. Using an endogenous PS receptor, Tim4, as affinity reagent\textsuperscript{62}, EVs were isolated in high purity from cell conditioned media and biological fluids\textsuperscript{63}. The affinity is in the nanomolar range, and since the interaction is Ca\textsuperscript{2+} dependent, elution can be accomplished by chelation of Ca\textsuperscript{2+}\textsuperscript{63}. However, it should be noted that the presence of PS on
the surface of EVs is controversial and several studies indicate that only a subset of EVs contain PS
64,65. Nonetheless, the high selectivity of the affinity isolation yields high sample purity, but the
isolated EV may only represent a subtype of the EVs.

Using the EV knowledgebase EV-TRACK66, we retrieved data on the isolation methods used for
enrichment of human uEVs (Figure 1g). dUC is the most common isolation method and has also
been proposed as the “gold” standard against which other techniques must be judged 67. Strikingly,
a large fraction of the studies used a combination of isolation methods, suggesting that for many
downstream applications, such as biomarker discovery, EV purity is valued higher than yield.

EV isolation from plasma and urine poses different challenges. EV preparation from urine samples
is challenged by THP. THP is a highly abundant protein in urine samples from normal healthy
persons and polymerizes to form 3D matrices entrapping the EVs 68. The polymeric THP is prone to
precipitation, and at 4 °C and “high” salt concentration, similar to e.g. phosphate buffered saline,
significant amounts of THP precipitate25,26. EVs are released from the polymeric THP by addition
to the urine of the reducing agent dithiothreitol (DTT), however, monomeric THP is still in the
high-speed EV pellet 68. Although DTT treatment releases EV, the treatment is harsh and can
destroy protein activity and structure of proteins stabilized by intramolecular disulphide bridges. As
an alternative to DTT, the detergent CHAPS (3-[(3-Cholamidopropyl)dimethylammonio]-1-
propanesulfonate hydrate) has been shown to release EVs from the THP trap, while preserving
vesicle morphology and protein activity 69.

EVs isolated from plasma are often co-isolated with lipoprotein particles if the isolation is only
carried out using 1-step isolation like for example ultracentrifugation or density centrifugation 70.
Since lipoprotein particles in plasma outnumber plasma EVs by a factor 106, their removal and
means to assess the contamination is therefore important 70. Apolipoprotein B is a major protein
constituent of chylomicrons (apo B-48), LDL (apo B-100) and VLDL (apo B-100) and has been
detected in mass-spectrometric analyses of human urine samples using differential ultracentrifugation \(^{71,72}\) and ultrafiltration\(^{73}\). Means to assess the purity of EVs/contamination by lipid particles are therefore important \(^{74}\) in that significant amount of the particle could bias e.g. the analysis of lipids in uEVs. For protein abundance studies by e.g. western blotting, single step purification may be sufficient, while large-scale proteomic studies require separation of highly-abundant soluble proteins in that they may mask genuine EV-associated proteins. Therefore, a combination of purification methods can be employed to ensure purity of the sample.

**uEVs - where are the parental cells located and what do they contain?**

Cultured proximal tubular cells release basolateral EVs \(^{75}\). The basolaterally secreted EVs are expected to be present in blood plasma samples, but no plasma analyses of *in vivo* secreted EVs from the urinary system have been reported. In the following, we will focus on urinary EVs, which contain predominantly apical epithelial markers, while basolateral markers are less dominant \(^6\). It is assumed that the uEVs contain proteins derived from the epithelial cells lining the urinary system; however, intravascular injection of isolated and labelled EV in anaesthetized rats resulted in urinary excretion of the particles.\(^{76}\) This finding indicates that there might be a route allowing for urinary excretion of EVs derived from cells outside the urinary system, but whether endogenous non-urinary system-derived EVs reach the urine under physiological conditions is unanswered.

To explore the potential parental cells both within and outside the urinary tract, we cross-referenced publicly available databases *Vesiclepedia* (http://microvesicles.org) \(^{77,78}\) and the Human Protein Atlas \(^{79}\). In Vesiclepedia, a total of 5113 identified uEV proteins from 90 studies are deposited and we analysed for each of the identified protein their tissue RNA expression by cross-referencing to the Human Proteins Atlas. Of the 5113 identified uEV proteins, 5090, or 99.96\%, showed RNA expression within the urinary tract epithelium, i.e. kidney, urinary bladder, and from male: testis,
prostate, epididymis and seminal vesicle. Proteins with highly enriched RNA expression in testis (71 genes), kidney (23 genes), prostate (7 genes), seminal vesicles (2 genes), urinary bladder (2 genes) and epididymis (2 genes) were present and indicated that each of these tissues were represented in uEVs. This confirms that the urinary tract and male seminal and reproductive gland epithelia are the major source of uEVs (Figure 2a,b). The remaining 0.04% of the proteins (23 proteins of the 5113 identified proteins) showed no RNA expression in the urinary tract using the RNA sequencing data from the Human Protein Atlas. Further inspection of the tissue-specific RNA expression in the Bgee gene expression data base showed that 21 of them were either expressed in one of more of the tissues of the urinary tract or expressed in skin and therefore potential contaminant from sampling detected by mass spectrometry. RNA expression was, however, not detected for the G-coupled receptor GRP101 and the Olfactory receptor 11L1 (ORL11L1), which have been detected in uEVs by mass spectrometry. According to the Human Protein Atlas, GRP101 and OR11L1 expression is highly enriched in cerebral cortex and thyroid gland, respectively. Both proteins are multi-pass membrane protein and their identification in uEV is therefore not likely to be the result of a glomerular filtration as circulating proteins; however, the route of obtained by the two proteins is not known. Nonetheless, since only 2 of the 5113 identified uEVs proteins were not detected at the RNA level in the urinary tract, this indicated that the tissues outside the urinary system do not contribute quantitatively to the urinary content of EVs.

**Nephron segment-specific enrichment of uEVs**

Initially, antibody-based methods demonstrated that AQP1 and -2, as well as NHE3, NKCC2 and NCC were excreted in urine from human and rats. Since then several large-scale mass-spectrometry-based studies have been conducted on uEVs from healthy persons and patients with e.g. hypertension; Cushing’s syndrome; diabetic nephropathy and adrenal adenoma.
Application of antibody-based techniques, such as ELISA and western blotting resulted in detection of NCC \(^{8,44,45}\), NKCC2 \(^{8,44}\), TMEM16a \(^{87}\), AQP2, \(^{88,89}\) ENaC, \(^{48,89,90}\) prostasin, \(^{89}\) and ROMK \(^{48}\) indicating that uEVs from all tubular segments were present. Even podocytes appear to release EVs into urine \(^{91}\). In addition, the uEV-associated proteins showed post-translational modifications. The phosphorylation status of uEV proteins has been studied with mass-spectrometry \(^{81}\) and western blotting, \(^{86}\) and ubiquitinated proteins \(^{37}\) were also present in uEVs. Proteolytic cleavage of ENaC subunits is a potent mechanism for activation of ENaC \(^{92}\). We and other have shown that uEVs isolated from human and rats have almost fully cleaved \(\gamma\)ENaC subunit \(^{48,89,90,93}\). EVs isolated from intrarenal pelvic catheter urine samples showed the presence of more full-length forms indicating that at least some of the proteolytic cleavage occurs along the ureters and/or in the bladder \(^{48}\). Thus, physiological/pathophysiological extracellular proteolytic processing of channels can be also be studied in uEVs. Glycosylated EV proteins are also present at the EV surface \(^{58,94,95}\) and their interaction with specific carbohydrate-binding lectins has been used for their isolation and detection \(^{58,94,95}\). The lectins *Lotus tetragonolobus lectin* (LTL), *Dolichos Biflorus Agglutinin* (DBA) and *Peanut agglutinin* (PNA) label proximal tubular cells, principal collecting duct cells and intercalated collecting duct cells, respectively \(^{96-98}\) and have been used to enrich for specific cell types in dissociated kidneys. \(^{34,35}\) We tested whether these lectins could also be used for affinity isolation of segment-specific EVs. Thus, we isolated uEVs from urine samples from three healthy volunteers using PEG-precipitation \(^{55}\), and incubated the isolated uEVs with PBS, biotinylated LTL, DBA or PNA and used magnetic Dynabeads to isolate the lectin-bound uEVs. As shown in Figure 3, LTL lectin isolated SGLT2 expressed in proximal tubule; DBA isolated AQP2 expressed in principal cells and PNA isolated intercalated cell-expressed Carbonic anhydrase 2 (CAII). These observations indicate that cell type-specific uEVs can be enriched by their lectin affinity and in future human studies the lectin-enrichment of segment-specific EVs
could be used to provide detailed information on e.g. RNA expressional regulation in low abundant cells types.

*Physiological regulation of proteins associated with uEV and challenge of normalization across individual samples*

The protein expression level in renal epithelial cell is dynamically regulated to meet the physiological requirements, thus a key question in the uEV research field is to elucidate whether kidney tissue protein abundance is reflected proportionally in uEVs, i.e. do kidney tissue /cell membrane protein levels directly correlate with uEV level of specific transporters?

The epithelial protein level is the difference between its production rate and its rates of degradation and secretion. The secretion of protein in EVs is a multistep process that involves the packaging and subsequent release. Thus, for a direct correlation between cell and EV level to occur would require that a constant fraction of the cellular pool of protein to be packed into EV and secreted with no regulation of the steps involved. *In vitro* experiments using cultured cells and cell-conditioned medium indicate that EVs are released by constitutive mechanism but also stimulated by e.g. hypoxia \(^3\) and acute increases in intracellular \(\mathrm{Ca}^{2+}\) \(^4\). Thus, only small changes in environmental factors would be predicted to change the correlation between epithelial and uEV protein abundance. Nonetheless, cultures of murine cortical collecting duct cells showed a direct correlation between the cellular and culture medium EV-associated AQP2 level after desmopressin-stimulation \(^9\). In rats, uEV levels of NKCC2 and NCC excretion correlated with their kidney tissue abundance \(^1\) and aldosterone infusion in adrenalectomized rats showed that NCC and phosphorylated NCC were increased in uEVs \(^8\), while kidney tissue but not uEV levels of prostasin increased \(^8\). The phosphorylated form of NCC protein was significantly higher in uEVs from patients with primary aldosteronism than in patients with essential hypertension \(^8\). Moreover, patients with loss of
function mutation in AVP2R which predicts lower membrane association and lower protein abundance of AQP2 in the kidney collecting duct principal cells, generally displayed a significantly lower uEV AQP2 abundance by semiquantitative immunoblotting. A high-aldosterone condition, normal pregnancy, was associated with increased abundance of αENaC, the aldosterone-sensitive subunit in two studies of uEVs and by mass spectrometry, uEV-associated ENaC was increased by a low sodium intake and aldosterone administration. Although these studies indicate that aldosterone signaling results in the predicted response in uEV protein abundance, other studies have found that uEV levels of NKCC2 and NCC did not increase in response to a low sodium diet. The low sodium differs in the above studies, i.e. 10 mmol vs <100 mmol sodium per day, and may explain the difference in response. We have recently used paired analysis of uEVs and kidney tissue from a random sample of nephrectomy patients to establish the correlation between kidney and uEV abundance of Na/Phosphate cotransporter NaPi-2a, THP and ROMK, NCC, intercalated cell expressed proton-pump subunit ATP6V1G3 and AQP2. The uEV abundance of the tested proteins showed higher inter-patients variability than the kidney abundance and we did not detect a correlation between the kidney and uEV protein abundance of the tested proteins.

Thus, although data from cell cultures and animal studies support a correlation between kidney and uEV level of some proteins in paired samples with well controlled stimuli, in humans the responses to lower stimuli might be masked by individual and environmental differences indicating that uEV secretion is not a simple “overflow” of excess or waste proteins but is a tightly and dynamically regulated process. Further human studies are warranted to establish whether uEV-associated proteins can be used as a read-out of physiological regulation of electrolyte transport proteins.

The quantitative comparison of uEV levels between individuals require normalization at least with regard to the degree of urine concentration. One approach is to measure the EV concentration in
urine using tunable resistive pulse sensing \(^{104}\) or nanoparticle tracking analysis \(^{105}\). In patients with albuminuria, nanoparticle tracking analysis overestimates the concentration of EV particles and depletion of albumin and immunoglobulin from the samples reduces the particle count\(^{106,107}\), indicating that an accurate EV count is difficult to obtain in urine from patients with kidney disease. A second approach is to normalize to EV protein amount; however, if techniques, such as PEG precipitation is used to isolate EV, the contribution of contaminating proteins is significant and might yield incorrect normalization factors. Tamm-Horsfall protein, nonetheless, has been suggested as a normalization factor for uEV \(^{68}\) and since it is easily measured by a simple Coomassie stained SDS-PAGE, this approach provides an easy normalization factor. Normalizing uEV input to urinary creatinine concentration, instead of e.g. uEV protein concentration, is widely used and good correlations between uEV levels and urinary creatinine concentration exist. Most data have been obtained using differential ultracentrifugation\(^{48,82,88,100,108}\) and immune-capture methods. \(^{109}\) Thus, no gold standard for normalization exists and many questions remain unanswered: Are uEV release rates from nephron segments with high degree of hormonal regulation of membrane protein trafficking from minute to minute (DCT and CDs) versus more “constitutive” segments that, on the other hand, display high endocytosis rate (Proximal tubule, urinary tract) differentially regulated? How is EV stability affected by urine osmolality that can vary by a factor of 20 with EVs having high degree of aquaporins and thus predicted osmotic sensitivity? Is the release rate and specific protein density altered as uEVs are synthesized and released? Does the composition of the glomerular filtrate that contains many small peptides and signaling molecules affect the release rate through apical signaling?

**Biological functions associated with uEVs**
While most of the studies on uEVs have focused on technical aspect of isolation, characterization and biomarker discovery, the putative function of EVs and their therapeutic use are beginning to be explored. Cell-to-cell communication of renal tubular epithelial cell-derived EVs has been demonstrated in vitro in cultures occurring from human proximal tubular cells to distal and collecting duct cells \cite{110}, between cultured mCCDc11 collecting duct cells \cite{99} and from human bone marrow mesenchymal stem cells to cultured tubular epithelial cells \cite{111}. In the two latter studies, EV concentrations of 50 µg protein/ml was used \cite{99,111}, but is this a physiological relevant EV concentration? It has been estimated that 1 µg exosome protein corresponds to \(2 \times 10^9\) exosomes \cite{112}, indicating that a concentration of \(\sim 10^{11}\) exosomes/ml was necessary to demonstrate intercellular EV communication. The EV concentration in human blood plasma \cite{113} and urine \cite{104} has been estimated to \(\sim 10^9\) EV/ml, thus, the concentration used to demonstrate the intercellular EV communication by cell-specific EV is at least \(\sim 100\)-fold higher than the EV level in urine. Although the concentration used was supra-physiological, the EV uptake was blocked by incubation with soluble hyaluronic acid. Anti-CD44 and -CD29 blocking antibodies inhibited EV incorporation in tubular epithelial cells, whereas anti- 4-integrin and 5-integrin did not, suggesting that expression of CD44 and CD29 is critical for EV internalization \cite{111}. A situation more closely resembling the in vivo situation for EV communication has been demonstrated elegantly using a Boyden chamber assay: Proximal tubular cell grown on filters in the upper chamber secreted EVs that were transferred to fibroblasts grown in the lower chamber and induced proliferation via TGF-b1 expression, \(\alpha\)-smooth muscle and F-actin expression, and type I collagen production \cite{39}. The EV transfer occurred with only minimal intervention and imply that the epithelial-derived EVs reach the fibroblasts on the basolateral side and thereby mediate cell-cell communication \cite{39}. In vivo support of this mode of communication is still lacking.
The block of EV uptake by anti-CD44 and anti-CD29 antibodies\textsuperscript{111} support a role for receptor-mediated targeting and uptake of EV. It has been demonstrated that modification of EV surface proteins allow for redirection of EVs to the brain\textsuperscript{114,115} and skeletal muscle\textsuperscript{115} which provides a future treatment strategy for delivery of custom-made designer EVs that are specifically directed to reach specific tissue targets.

Interestingly, administration of renal tubular cell-derived EVs after renal ischemia in rats limited the severity of the reperfusion injury\textsuperscript{116} and EVs isolated from endothelial progenitor cells, but not fibroblast-derived EVs, improved acute kidney damage after ischemia-reperfusion\textsuperscript{117}, cisplatin-induced injury\textsuperscript{118} and Thy1.1 glomerular nephritis\textsuperscript{119}. Although the mode of action is not clear, the experimental bioassay data point to a protective effect of EVs derived from cultured cells, particularly endothelial cells, which could potentially be used in future treatment strategies. Along this avenue, the elusive serum factor that confers “preconditioning” cardiac protection against ischemic insults could include exosomal miRNAs in pigs.\textsuperscript{120} The beneficial action of ischemic preconditioning appears not as significant in kidney as compared with heart\textsuperscript{121}.

In addition to intercellular communication, uEVs have been suggested as urinary immune effectors in that uEVs from healthy persons were enriched in proteins involved in host defense and immunity with bacteriostatic/bactericidal functions and protein that bind to bacterial surface molecules\textsuperscript{122}. Moreover, addition to cultures of pathogenic and commensal E. coli of uEVs potently inhibited bacterial growth and induced cell lysis\textsuperscript{122} indicating they have a protective effect.

To gain further insight into the physiological and therapeutic relevance of EVs, the mechanisms underlying EV-specific release and uptake needs to be identified, since this will allow the testing of hypotheses that can be falsified by experimental data. Most studies have been performed \textit{in vitro} using cultured cells and have revealed that a significant cellular heterogeneity exists with respect to
EV secretion and that current culture conditions are not likely to mimic the *in vivo* situation. Thus, new animal models for the study of EV biology are important to establish and will allow the field to move from correlation analysis to intervention studies and thereby expand our knowledge on the potential future use of EVs based treatments for disease.

**Summary and conclusion**

uEVs originate from the geno-urinary tract epithelium and the contribution of cells outside of the urinary system is negligible. The surface of uEVs is glycosylated similarly to their renal epithelial parental cells, which allow for segment-specific mRNAs and proteins isolated using lectin affinity-precipitation. Although uEVs contain proteins from their cells of origin, there is not a general, quantitative, direct correlation between kidney tissue and uEV protein abundances. The urinary EV field is transforming from a technical focus into a focus on the physiological role of EVs. EVs emerge as conveyers of information, in some cases even tissue protection, between cells from different tubular segments, but the research is still in its infancy and largely based on *in vitro* studies in cultured cells. Many unknowns need elucidation: Control of release rate of EVs; The physiological concentration of cell-specific EVs; the correlation with tissue level, receptors and ligands that mediate direct transfer; however, consensus is established that urine extracellular vesicles are a biological entity that may have physiological, pathophysiological and diagnostic/therapeutic relevance.

**Conflicts of interest**

None
References

19. Kim, H., Min, H. K., Kong, G. & Moon, M. H. Quantitative analysis of phosphatidylcholines and phosphatidylethanolamines in urine of patients with breast cancer


85. van der Lubbe, N. *et al.* The phosphorylated sodium chloride cotransporter in urinary exosomes is superior to prostasin as a marker for aldosteronism. *Hypertension* **60**, 741–748 (2012).


### Table 1: Overview of methods for enrichment of uEVs

<table>
<thead>
<tr>
<th>Method</th>
<th>Principle</th>
<th>Special equipment</th>
<th>Throughput</th>
<th>Advantage</th>
<th>Limitation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Differential ultracentrifugation</td>
<td>Size density</td>
<td>Ultracentrifuge</td>
<td>Low</td>
<td>Simple and relatively high yield</td>
<td>Co-isolation of non-EV proteins</td>
</tr>
<tr>
<td>Density gradient centrifugation</td>
<td>Size/mass density</td>
<td>Ultracentrifuge</td>
<td>Low</td>
<td>High purity of EVs</td>
<td>Low yield, Density medium has to be removed</td>
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<tr>
<td>Size-exclusion chromatography</td>
<td>Size</td>
<td>None</td>
<td>High</td>
<td>High purity</td>
<td>Sample dilution</td>
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<tr>
<td>Ultrafiltration</td>
<td>Cut-off size</td>
<td>None</td>
<td>High</td>
<td>High yield</td>
<td>Enrichment of non-EV proteins</td>
</tr>
<tr>
<td>Precipitants</td>
<td>Solubility</td>
<td>None</td>
<td>High</td>
<td>High yield</td>
<td>Low purity, co-precipitation of non-EV proteins</td>
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<tr>
<td>Immuno-affinity</td>
<td>Affinity</td>
<td>None</td>
<td>High</td>
<td>High purity</td>
<td>Sub-types of EVs are isolated</td>
</tr>
</tbody>
</table>
Figure legends

**Figure 1: Common methods for isolation of uEVs**

The enrichment of EVs (green) from urine samples is based on size, density and surface markers/post-translation modifications. (a) Differential ultracentrifugation (dUC) separate particles based on size and large particles (red) are pelleted at lower centrifugation speed than uEVs. (b) Density gradient centrifugation (DGC) separate particle based on density and uEV will migrate to bands with similar density. (c) Size-exclusion chromatography (SEC) separate particles based on size by running samples through porous matrix (light gray, dotted circles). Small particles will enter the porous matrix and their path through the matrix is longer than particles with a diameter greater than the porous matrix and they are therefore eluted faster. (d) Ultrafiltration (UF) uses a filter with a size cut-off below the size of EV. EVs are therefore retained in the filter, while soluble proteins and smaller particles passes the filter. (e) Precipitation of uEVs is done by adding a precipitant, e.g. polyethylene glycol (PEG), which induces aggregation and precipitation of EVs, other particles and proteins. (f) In affinity isolation, beads conjugated to a binding agent, e.g. an antibody or peptide, are incubated with uEV samples and uEV bound beads are then isolated. (g) Using EV-TRACK 66, we retrieved data on the methods used for isolation of EVs from human urine from 100 publication published between 2010-2018. A total of 136 experiments were included and showed that dUC was the most common method for uEV isolation (50%) and that many of the experiments used a combination of 2 or 3 isolation methods.

**Figure 2: Tissue-specificity of urinary EVs**

(a) Proteomic data retrieved from Vesiclepedia was cross-referenced with transcriptional RNA data retrieved from the Human Protein Atlas. A total of 5113 uEV proteins previously identified in uEVs were included in the analysis. While genes for only 0.04% (2) uEV proteins were not detected at the
RNA expressional level, 99.96% (5111) uEV detected proteins were expressed at the RNA level in the urinary system. (b) Of the 5113 uEV detected proteins, 105 had specific expression cell from the urinary system, i.e., 71, 23, 7, 2, 1 and 1 of the identified proteins had highly enriched RNA expression in testis, kidney and prostate, seminal vesicle, urinary bladder and epididymis, respectively.

Figure 3: Renal tubular segment-specific enrichment of urinary EV by lectin affinity precipitation.

(a) Urine from 3 healthy male individuals were pooled and ExtraPEG 55 was used to precipitate uEVs. The uEV enriched pellet was resuspended in phosphate buffered saline (PBS) and divided in 4 fractions, which were added PBS (control), biotinylated DBA (Vector biolabs), biotinylated LTL (Vector biolabs) and biotinylated PNA (Vector biolabs). The biotinylated lectins were isolated using streptavidin-conjugated Dynabeads and lectin bound molecules were eluted twice (indicated with 1 and 2 in using Galactose, L-fucose, N-Acetylgalactosamine, Galactose, respectively. (b) LTL precipitated EVs contained SGLT2 (rabbit anti-SGLT2, Cat no. 14210, Cell Signaling), DBA precipitated EVs contained AQP2 (goat anti-AQP2, Cat no. sc-9882, Santa Cruz Biotechnology), and PNA precipitated EVs contain CAII (rabbit anti-CA2, Cat no. HPA001550, Sigma-Aldrich). Fractions with PBS added did not contain any of the markers.
99.96% of the uEV proteins are expressed in the urinary system.

0.04% of the uEV proteins are not expressed in the urinary system.

5113 uEV proteins analysed.

Number of tissue enriched proteins in uEVs.