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REVIEW

Employing extracellular vesicles for non-invasive renal monitoring: A captivating prospect

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Abstract

Extracellular vesicles (EVs) are fascinating nano-sized subjects extensively studied over the recent years across several disparate disciplines. EVs are endlessly secreted into the extracellular microenvironment by most cell types under physiological and pathological conditions. EVs encompass a variety of molecular constituents from their cell of origin, such as lipids, cell specific proteins and RNAs, thus constituting an informative resource for studying molecular events at the cellular level. There are three main classes of EVs classified based on their size, content, biogenesis and biological functions: exosomes, shedding microvesicles and apoptotic bodies. Besides cell culture supernatants, biological fluids have also been shown to contain different types of EVs. Amongst the various body fluids, the study of urinary extracellular vesicles (uEVs) as a source of candidate biomarkers gained much attention, since: (1) urine can be non-invasively collected in large amounts; and (2) the isolated uEVs are stable for a relatively long period of time. Here, we review the important aspects of urinary extracellular vesicles which are fast gaining attention as a promising future tool for the non-invasive monitoring of urinary tract. Recent advancements in the purification and analysis of uEVs and collection of their constituents in rapidly developing public databases, allow their better exploitation in molecular diagnostics. As a result, a growing number of studies have shown that changes in expression profile at the RNA and/or protein levels of uEVs reveal the molecular architectures of underlying key pathophysiological events of different clinically important diseases with kidney involvement.

Key words: Urine analysis; Extracellular vesicles; Biomarkers; Kidney disease; Quantitative proteomics; Exosomes

Core tip: Urinary extracellular vesicles research is a fast growing field of biomarker discovery providing new attracting prospective for monitoring tissue alteration in easily accessible clinical samples. Over the past ten years intense research has identified the various urinary vesicular cargo molecules (*i.e.*, RNAs, proteins and lipids) and detected their alterations upon a number of renal diseases. With the number of diseases relating to kidney increasing it is essential to effectively utilize this invaluable tool for the early diagnosis. Here we provide a comprehensive overview of uEVs nicely

setting the stage for their utility in future clinical diagnostics.

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EXTRACELLULAR VESICLES: WHY ARE THEY FASCINATING?

Extracellular vesicles (EVs) are membrane-bound secretory vesicles which exhibit an array of proteins, bioactive lipids, nucleic acids and metabolites[1-4]. EVs are considered to play an important role in intercellular communication[5-8], regulating immune response[9-12], antigen presentation[13-15], transport and propagation of infectious cargo such as prions and retroviruses[2,16-18]. Based on the size and origin, EVs are classified into three main types: (1) "exosomes" (40-100 nm) vesicles derived from the endosomal compartment and released *via* fusion of the multivesicular bodies with the plasma membrane; (2) "microvesicles" (50-1000 nm) vesicles that result from the direct budding from the plasma membrane; and (3) "apoptotic bodies" (800-5000 nm) vesicles released by cells undergoing programmed cell death[19,20]. Despite some distinct features, numerous similarities exist among the different classes of EVs with respect to their physical characteristics and biochemical composition, which make the separation of different subsets challenging[21].

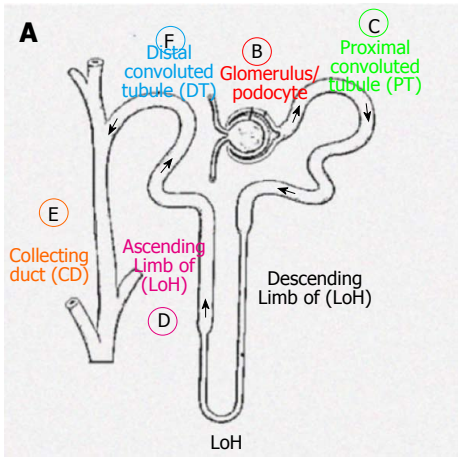
Nano- to micron-sized EVs have been isolated from many body fluids including urine[16,22-25], saliva[26,27], breast milk[9,27], cerebrospinal fluid[28], semen, pleural effusions and plasma[27,29]. Urinary extracellular vesicles (uEVs) are released from the renal epithelial cells, including glomerular podocytes, renal tubule cells, and the cells lining the drainage system[30]. They are promising starting material for biomarker discovery and also a great asset for non-invasive renal monitoring as they provide a full representation of the entire urinary system[3,16]. Recently developed quantitative workflows for uEVs transcriptomics, proteomics and lipidomics[31-34] including system biology approaches[35] enable researchers to study the expression profiles of the main bioactive constituents of uEVs in detail and with an increased rate and reproducibility than before. Research performed in the last 10 years highlighted that uEVs harbor 1%-3% of the total urinary proteins with a reduced dynamic range of protein concentration respect to the whole urine proteome. In this sense, the proteome of uEVs which today counts more than four thousand proteins can be considered as the urinary subproteome enclosed in double-layered vesicles. Despite the potential benefits of uEV analysis, there are barriers and limitations that must be dealt with. Foremost among these challenges is the reproducible isolation of pure vesicles suitable for downstream analysis[36]. In addition to proteins, uEVs encapsulate different RNA species amongst which messenger RNAs (mRNAs) and micro-RNAs (miRNAs) are the subjects of intense studies as possible biomarkers of renal diseases[3,37]. RNAs incorporated in uEVs are resistant to nuclease digestion and, similarly to proteins they originated from the different nephron regions[38]. For example, podocyte related mRNA expression of CD2-associated protein in uEVS was shown to correlate with renal function and level of proteinuria[39]. Moreover, miRNA-29c level in uEVs was shown to be a potential biomarker of renal fibrosis[40]. The relative abundance of different mRNAs do not necessarily correlate with cellular mRNA, suggesting a selective process of mRNA entry into vesicles[1]. As vesicle-enclosed mRNAs travel along the nephron, modification of nucleic acid cargo could permit targeted delivery of RNA to the kidney, a hypothesis which open a new area for the treatment of renal tubular disorders[41].

Here, we will focus on summarizing the current knowledge about uEVs, starting from the critical review of their isolation, biochemical characterization till their potential application in biomarker discovery and non-invasive renal monitoring.

URINARY SYSTEM ORIGIN OF uEVS

Exosomes are cell-derived secretory vesicles shed by proliferating cells through exocytosis[6,13,29]. During endosomal maturation, the formation of intraluminal vesicles (ILVs), ranging from 30-100 nm in diameter, inside the lumen of the endosome can be observed. The ILVs are generated by inward invagination of the endosomal membrane, and scission of vesicles from the limiting membrane into the endosomal lumen. The size of a fully matured late endosome, also called multivesicular body (MVB) is approximately 500 nm in diameter and, it contains several ILVs. The fate of MVBs may vary. They could fuse with lysosome and get degraded or fuse with the plasma membrane releasing the vesicles into the extracellular space. During the later process, the second inward budding of the endosome membrane results in a positive orientation of the ILVs lipid membrane. While the endosomal sorting complexes required for transport (ESCRT) machinery involved in sorting the cargo to lysosomes *via* MVBs within the endocytic system is well characterized (ESCRT-dependent endolysosomal pathway), protein sorting into exosomes until recently was less understood[6]. The involvement of ESCRT protein complexes and protein ubiquitination has been shown by different groups. Recently, Alvavez *et al*[3] revealed that syndecan-syntenin-*ALIX* might be a key regulator of the biogenesis of syntenin expressing exosomes. In particular, the interaction between syndecan and exosomes might thus support a novel role for proteoglycans in vesicular trafficking and cellular signaling. On the other hand, EVs displaying similar physicochemical characteristics like exosomes (density, size-distribution, presence of protein markers) have also been described to bud

A



B

UniProt Acc.Number	Protein name	Ref.
PODO_HUMAN	Podocin	48
PODXL_HUMAN	Podocalyxin	48
NPHN_HUMAN	Nephrin	48
CR1_HUMAN	Complement receptor type 1	48
KIRR2_HUMAN	Kin of IRRE-like protein 1	48
NEST_HUMAN	Nestin	98
VTDB_HUMAN	Vitamin D-binding protein	49
APT_HUMAN	Adenine phosphoribosyltransferase	62
LRRK2_HUMAN	Leucine-rich repeat kinase 2	50

C

UniProt Acc.Number	Protein name	Ref.
TR11B_HUMAN	Osteoprotegerin	49
LRP2_HUMAN	Megalin	44
CUBN_HUMAN	Cubilin	44
AMPN_HUMAN	Aminopeptidase N	44
AQP1_HUMAN	Aquaporin-1	44
CAH4_HUMAN	Type IV carbonic anhydrase	44
GGT1_HUMAN	γ-glutamyltransferase	44
SL9A3_HUMAN	Sodium/hydrogen exchanger 3	45
CD24_HUMAN	Signal transducer CD24	55
ANXA5_HUMAN	Annexin V	58
SCNNA_HUMAN	ENaC (epithelial sodium channel)	45
CHRDL1_HUMAN	Chordin-like protein 1	49
CYR61_HUMAN	Protein CYR61	49
ATF3_HUMAN	Activating transcription factor 3	22
LRG1_HUMAN	Leucine-rich alpha-2-glycoprotein-1	71
PKD1_HUMAN	Polycystin-1	45,46,54,62
MLL3_HUMAN	Isoform 1 of histone-lysine N-methyltransferase	16
GPC5B_HUMAN	G protein coupled receptor required for tubulogenesis	94
MMP9_HUMAN	Matrix metalloproteinase 9	77
CAH9_HUMAN	Carbonic Anhydrase 9	77
NGAL_HUMAN	Neutrophil gelatinase-associated lipocalin	91

D

UniProt Acc.Number	Protein name	Ref.
CD9_HUMAN	CD9 antigen	44
S12A1_HUMAN	Type 2 Na-K-2Cl cotransporter	44

E

UniProt Acc.Number	Protein name	Ref.
AQP2_HUMAN	Aquaporin-2	9
MUC1_HUMAN	Mucin-1	9
RHAG_HUMAN	Rh type C glycoprotein	9
TS101_HUMAN	Tumor susceptibility gene 101 protein	10-12
FLOT1_HUMAN	Flotillin	11,12

F

UniProt Acc.Number	Protein name	Ref.
S12A3_HUMAN	Thiazide-sensitive Na-Cl co-transporter	44

Figure 1 Schematics of Nephron showing the urinary extracellular vesicle proteins identified in the different segments. A: Core; B: Glomerulus/podocyte; C: Proximal convoluted tubule (PT); D: Ascending Limb of (LoH); E: Collecting duct (CD); F: Distal convoluted tubule (DT). UniProt Acc.Number: UniProt Database Accession Number. LoH: Loop of Henle.

from the plasma membrane. For example, podocyte-positive membrane vesicles in urine has been shown to originate from tip vesiculation of podocyte microvilli[42]. These vesicles however cannot be easily distinguished from endosome-derived exosomes and therefore their separate analysis remains a problem.

Exosomes are released from cells of different tissues or organs and while they share a common group of proteins related to their biogenesis they also harbor tissue specific proteins that reflect the origin and biological functions of their parental cells[43]. In fact, EVs isolated from biological fluids have organ and tissue specific protein and RNA profiles. The urinary system consists of the two kidneys, ureters, the bladder, and the urethra. Nephrons are the kidney functional and structural units with the main role to regulate water balance and inorganic ions by filtering the blood. More than a decade ago, the key finding of the first proteomic analyses performed on isolated uEVs was the presence of proteins known to be highly and differentially expressed in various kidney tissues from the glomerular podocyte to the epithelial cells lining the various nephron segments[44,45]. Later on proteins from the transitional epithelium of the urinary bladder were also identified, confirming uEVs may be shed from cells throughout the renal[46]. Targeted proteomics applied recently to perfused isolated rat kidney model to identified kidney originated proteins in human urine[47]. Out of the 990 kidney originated proteins with human analogues 74 were present only in plasma and 240 only in urine (including uEVs) but not in plasma. Screening the 240 kidney originated urinary proteins we have found that the major part (206) is listed in the human urinary dataset of EVpedia 2.0 database. This could be a core set of kidney originated vesicle proteins with high renal pathophysiology relevance for further studies.

Figure 1 shows a schematic diagram of a nephron

indicating sets of uEVs proteins highly expressed in the epithelial cells of glomerular podocyte[48] and to the different segments of renal tubule[44,45,49]. *Podocalyxin* (PODXL) specific for glomerular epithelial cells, aminopeptidase (AMPN) specific for proximal tubule cells, AQP2 specific for distal tubule, programmed cell death 6 interacting protein (PDCD6IP or ALIX) and *uroplakin-1* and *uroplakin-2* (UPK1 and UPK2) specific for bladder are frequently used as protein markers of human uEVs. Basal epithelial cells of collecting duct are hypothesized to be the major contributors toward the urinary pool of EVs including the expression of *leucine-rich repeat kinase-2* (LRRK2)[50]. More recently, Benito-Martin *et al*[49] showed that cultured human proximal tubular cell secrete exosomes containing osteoprotegerin (OPG) and this specific sub-group of exosomes can also be found in urine. Since, most of these have kidney relevant function, proteomic analysis of uEVs hold the promise to provide an insight into the physiological or pathophysiological processes in the various cell types facing the urinary space[44].

VESICLE ISOLATION: AN OBSTACLE TO OVERCOME

Isolation of EVs is still a major challenge in this rapidly growing field of research. The complex nature of body fluids and lack of standardized protocols makes isolation and characterization extremely difficult[34]. The research on EVs can be broadly divided into three categories depending on the end use of the vesicles: (1) discovery; (2) diagnostic; and (3) preparative, each of which demands a different level of purity, quality control and operating procedures. In most studies, ultracentrifugation is the commonly used technique for isolation of vesicles. Isolation of membrane vesicles by sequential differential centrifugations is complicated by the possibility of overlapping size distributions with microvesicles or macromolecular complexes. Recently, numerous alternate procedures were introduced, including immunoaffinity separation, filtration, microfluidic devices aided separation, and reagent based separation[20]. The choice of isolation procedure greatly depends on the source material and the goal of the EV research project.

Isolation of extracellular vesicles from urine has proven to be extremely difficult because of the presence of Tamm-Horsfall protein (THP), also known as uromodulin[36], and also due to very low vesicle load in this biofluid. Differential centrifugation is the most widely used technique for the isolation of uEVs. It includes several low-velocity centrifugation and ultracentrifugation steps with increasing centrifugal force; from 200 to 1500 g to remove cells and cellular debris, from 10000 to 20000 g to pellet microvesicles, and from 100000 to 200000 g to pellet nanometer-sized vesicles[50]. The efficiency to isolate EV depends not only on the size, shape and density of vesicles but on the volume, viscosity and temperature of the fluid in which the vesicles are present. Centrifugation time and the type of rotor used (fixed angle or swinging bucket) also influence the final yield and the purity of vesicles. Addition of dithiothreitol (DTT)[23,36,49,51] and 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS)[52] to the low-speed pellet has been shown to be efficient to disrupt the polymeric network formed by THP protein filaments, but do not solve the problem entirely[33,53]. Urinary EVs can be separated from non-membranous particles, such as protein aggregates by using their relatively low buoyant density, and differences in floatation velocity to separate differently sized classes of EV. Based on this, the crude uEVs containing pellet obtained by differential centrifugation can be further separated/purified using a sucrose density gradient[54,55] or sucrose cushion ultracentrifugation step[33,56,57]. It has been shown that both methods are efficient not only to separate exosomes from microvesicles but also to eliminate the interfering THP impurity[33,54-56]. The high sucrose concentration used in sucrose gradient centrifugation, however may negatively affect the biological function of EV. This can be avoided by layering the samples on top of the sucrose gradient or cushion in the tube subjected for centrifugation. Filtration through molecular filters (0.22-μm or 0.1-μm filters), which remove solvent and small molecule analytes while retaining and concentrating vesicles smaller than the pore size can also be used alone or in combination with centrifugation or ultrafiltration (MWCO 100 kDa) to isolate uEVs[25,58-60]. Among the primary advantages of the filtration method are the simplicity and easy scale-up. Though co-purification of abundant soluble urinary proteins and THP often compromises sample purity and the applied increasing forces with decreasing pore size has also been reported to result in artifacts[50]. The presence of characteristic surface proteins (CD9, CD63, CD81, Rab-5b, TSG101, Alix and A33) on certain EV classes is the basis for immunoaffinity isolation[57,61]. Immunoaffinity-based techniques employ antibodies thus have the potential for high specificity, an important consideration in the characterization of specific EV populations[57]. Because of the increasing interest in exosomes and other extracellular vesicles and their potential use in therapeutics or as biomarkers for disease, kits that allow for "easy isolation procedures" are being developed[16,62]. Commercial tests based on centrifugation (Total Exosome Isolation Reagent, Invitrogen™), filtration (Exomir ™ and Exo-Spin™), affinity capturing (Exotest™ and Exosome Dynabeads®) and proprietary exosome precipitation technologies (miRCURY™, ExoQuick-TC™ and DiagExo®) are already available in the market (Table 1). Such approaches for rapid purification are welcomed but they should be only considered as for bulk isolations because they often fail to pass quality test and fail to distinguish between differently sized EV and membrane-free macromolecular aggregates[50]. A widely accepted unique standardized protocol for the reproducible isolation and purification of uEVs suitable for the downstream analysis of the various RNA and protein constituents is still awaited.

Table 1 Methods used for the isolation of urinary extracellular vesicles

Technique	Type	Advantage(s)	Disadvantage (s)	Purity	Ref.
Centrifugation	Differential	Easy to perform	Co-purification of protein aggregates (THP) Inability to separate exosomes from microvesicles Lengthy Impractical for large-scale studies	++	[37,44,50,52,69,92]
	Sucrose gradient	No mechanical stress and hence allows the collection of morphologically intact particles Removes THP	High sucrose concentration may affect the biological functions of exosomes Inability to separate exosomes from particles with similar density and size Difficult and lengthy Impractical for large-scale studies	+++++	[23,50,54,55]
	Sucrose cushion	No mechanical stress and hence allows the collection of morphologically intact particles	Inability to separate exosomes from particles with similar density and size Difficult and lengthy Impractical for large-scale studies	+++++	[33,56,57,78,80]
Filtration	Nanofiltration	Easy to perform	Low purity grade Co-purification of protein aggregates (THP) Low exosome yield due to their lost on the surface of the nano-membrane	+++	[94]
	Microfiltration	Easy to perform Rapid Maintain vesicle structure	Co-purification of protein aggregates (THP) Inability to separate exosomes from microvesicles	+++	[59]
Immunoaffinity separation	Immunobeads	Allow rapid semi quantitative characterization of the surface phenotype can be tissue-specific	Not suited for large sample volumes Captured extracellular vesicles may not retain biological functionality even if successfully eluted from bead surface Co-purification of protein aggregates (THP) Low yield	++	[57,61,62]
Commercial kits	Total Exosome Isolation Reagent-Invitrogen™ Exomir™ Exo-Spin™ Exotest™ Exosome Dynabeads® miRCURY™ ExoQuick-TC™ and DiagExo®	Rapid and requires low sample volume Practical	Impractical for large-scale studies Reproducibility, yield and sample quality should be checked case by case Co-purification of protein aggregates (THP)	++	

THP: Tamm-Horsfall protein.

PHYSICOCHEMICAL AND ANALYTICAL CHARACTERIZATION OF URINARY EXTRACELLULAR VESICLES

Isolated uEVs are a heterogeneous vesicle population. Exosomes have a lipid bilayer membrane and a characteristic buoyant density ranging from 1.10 to 1.19 g/mL[55]. Determination of physicochemical properties, like integrity, morphology, size and concentration is generally the first step in the characterization of uEVs. Transmission electron microscopy (TEM)[16,23,33,52,58,59,63-65], dynamic light scattering (DLS)[66], nanoparticle tracking analysis (NTA)[67,68] and flow cytometry (FC)[69] are the methods most frequently used in this process. TEM imaging of numerous uEV samples has revealed intact vesicles with round morphology[16,23,33,52,58,59,63-65]. Depending on the isolation/purification method used, uEV samples typically show a heterogeneity in size ranging from approximately 30-100 nm in diameter (Figure 2A). Besides the determination of particle concentration and size distribution, the main advantage of using transmission electron microscopy (TEM) for uEV analysis is its ability to reveal the presence of known urinary contamination, like the long polymers of THP[36,52]. TEM[16,23,33,52,58,59,63-65] and cryo-EM[68,70] based morphological characterization, including observation of vesicle heterogeneity, therefore is a great support for the quality assessment of sample preparation[64]. Orthogonal techniques, like Dynamic Light Scattering (DLS) and nanoparticle Tracking Analysis (NTA) on the other hand, measures the Brownian motion of the particles in solution and calculate the hydrodynamic radii of the particles. Owning to its rapidity and simplicity, NTA is a quickly expanding in the field of exosome research. The mean diameter of human uEVs isolated by ultracentrifugation was measured by NTA

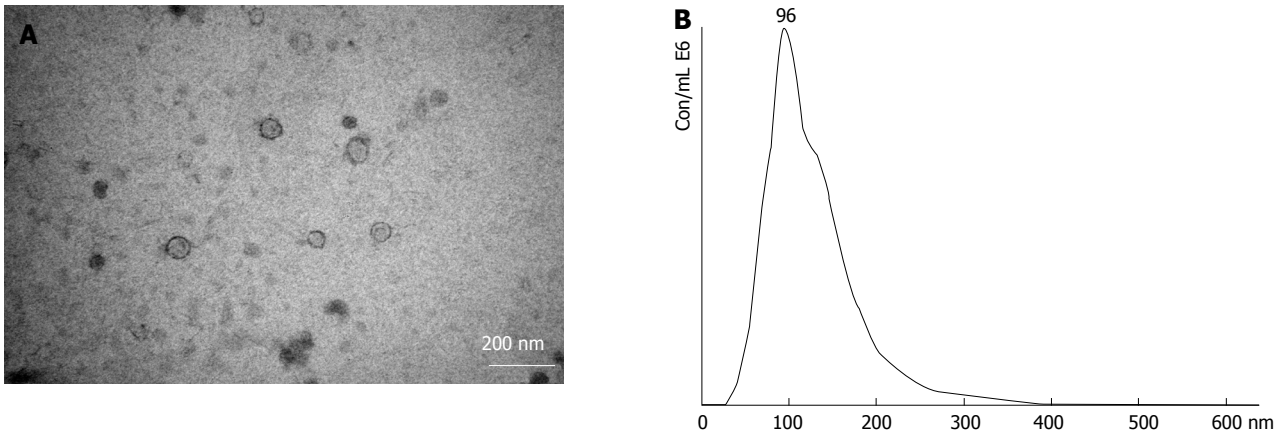


Figure 2 (A) Transmission electron microscopy and (B) nanoparticle tracking analysis images of urinary extracellular vesicles isolated by the sucrose cushion ultracentrifugation method.

around 150 nm ± 50 nm and concentration 10E9-10E11 particles/mL (Figure 2B)[68]. It should be noted that the hydrodynamic size of nanoparticles is expected to be higher to the core size measured with TEM, however the relationship between the two still need to be determined. Subpopulation of EVs can be studied by immunoelectron microscopy using antibodies to target membrane proteins, like aquaporin-2 (AQP2), aminopeptidase-N (AMPN), podocalixin (PODXL) and CD9[37,42,65,71]. Oosthuyzen *et al*[66] most recently demonstrated that NTA can also monitor specific subgroups of uEVs particles using fluorescent antibodies against specific surface proteins present on uEVs.

Analytical characterization of the components of urinary nano-vesicles (*i.e.,* proteins, mRNAs, mi-RNAs, lipids and small molecules) can be performed by transcriptomic[31,37,40,46,63,72,73], proteomic[16,19,23,25,31,33,35,44,47,48,50,52,59,60,62,63,65,66,71,74-79], metabolomic and lipidomic[2,31,46,68,75,80] tools[34]. One of the most exciting findings is that RNA transcripts exist in exosomes and maintain their function when transferred to other cells. Vesicle mediated route for cell-cell communication within the urinary tract opens new perspectives, yet its role in kidney development, function and pathogenesis needs to be elucidated[46]. Typically, commercially available extraction kits are used for total RNA isolation. Abundance of most RNAs is low in the in uEV which makes isolation and downstream RNA analysis challenging[37]. Most of the studies so far have used microarrays[72,81] or real time quantitative PCR (qPCR) assays[39,48,72] to examine exosomal RNAs, with a focus on miRNAs. Because of the inherent limitations of these methods, unknown miRNAs or other RNA species are often overlooked. To characterize RNA profiles systemically, Cheng *et al*[73] performed next-generation deep sequencing providing the base to identify miRNA biomarkers in urinary exosomes.

Since the first proteomic profiling reported by Pisitkun *et al*[44], protein content of urinary EVs has been extensively studied[16,19,23,25,31,33,35,41,45,47-50,52-54,59,60,62,63,65,66,70,71,74-80] both under healthy and numerous disease conditions. These studies let to comprehensive datasets of the identified proteins collected in the different exosome related public databases. It should be noted, that for the generation of many of the large datasets, uEV samples isolated by differential centrifugation (crude exosome preparation) was used[23,45]. As a consequence, public databases contain a relatively high percentage of urinary and other impurities related proteins. The protein content of urinary exosomes is about 1%-3% of that of total urine. The proteomic workflow generally starts with protein concentration assay (Bradford or BCA) followed by protein profile analysis which is usually performed by visualization of proteins separated by SDS-PAGE. The band at about 90 kDa corresponds to the highly glycosylated THP monomer[36] and its relative abundance is used to assess sample quality. Western blots are performed by using a set of urinary exosomal markers, such as Alix, CD9, TSG101, PODXL, AQP2, NES and Annexin V. Chemiluminescent western blotting gives valuable information for the quality of the preparation, but its use as a quantitative method for measuring the relative expression of the target proteins in the absence of appropriate normalization method is still debatable. To measure differences in protein expression between samples, quantitative proteomics is the method of choice. Both label-free quantitation[16,25,76] and stable isotope labeling by iTRAQ[33,78] and TMT reagents[50] in combination with SCX/C18 Multidimensional Protein Identification Technology (MudPIT) have been shown to be useful in the comparative analysis of uEVs. After appropriate enrichment steps, post translational modifications (PTMs), phosphorylation[45,50,79] and glycosylation[64,69] have also been studied by mass spectrometry-based proteomics in urinary exosomes. Since PTMs may affect important physiological process and its alterations may directly reflect early pathogenic events, further studies of PTMs alteration in uEV in various disease states are expected to come.

The bioactivity of exosomes is associated not only to their protein and RNA contents but also to their lipids. Compared to their cells, exosomes have been shown to be enriched in *Cholesterol* and *Sphingomyelin*. Total

exosomal lipids can be extracted by organic solvents according to Bligh and Dyer[82] or, alternatively using THF: H_2O (4:1)[2]. Different lipid classes (phospholipids and glycosphingolipids) can be purified and analyzed by thin layer chromatography (TLC), gas chromatography-mass spectrometry GC-MS and liquid chromatography-mass spectrometry (LC-MS). Recent progresses in electrospray ionization-tandem mass spectrometry-(ESI-MS/MS) based high-throughput lipidomics allowed the first comparative lipid analysis uEVs isolated from healthy individuals and renal cell carcinoma patient[2].

DATABASES FOR URINARY EXTRACELLULAR VESICLES RESEARCH

Recent studies have ignited significant interest on uEVs as possible players in kidney physiology and also as potential reservoirs of biomarkers. With such a huge interest, the amount of data accumulated has increased over time. High throughput proteomics study routinely identifies more than thousand proteins in human uEVs[23,45,50] and necessitates systematic classification of the data acquired[83]. Currently, there are two integrated manually curated web-based databases publicly available: Vesiclepedia[84] and EVpedia[85]. Both dedicate separate sections for the constituents of human urinary vesicles.

Vesiclepedia 2.1 (http://www.microvesicles.org) catalogs information from published non-mammalian eukaryotic and mammalian extracellular vesicles. Based on the 15 studies published on human uEVs, the current version contains 1162 unique proteins, 20 unique mRNAs and 690 unique miRNAs. To aid biomedical scientists in assessing the quality of the preparation Vesiclepedia also contains information on the methods used for the purification as well on the biophysical and molecular characterization of EVs.

EVpedia 2.0 (http://evpedia.info) provides a comprehensive lists of proteins, mRNAs, miRNAs, and lipids identified in EVs of both eukaryotic and prokaryotic (bacteria and archaea) origin, including non-mammalian eukaryotic extracellular vesicles[31,85]. Currently, out of the 263 studies included in this database 16 deal with urinary EVs. EVpedia 2.0 is cataloging a total of 12869 proteins isolated from human uEVs out of which 6275 are unique. 10 studies are related to the study of uEVs isolated from healthy donors and the number of unique proteins in this set is 4536. The most frequently identified 100 proteins are also listed in the database. Sets of proteins identified in different disease related samples (312 from early IgA nephropathy, 552 from autosomal recessive polycystic kidney disease, 621 from basement membrane nephropathy, 689 from bladder cancer, 480 from hernia, 184 from renal cell carcinoma) are particularly valuable components of this database. In the present 2.0 release of EVpedia there are no mRNAs, miRNAs and metabolites in the human urinary EVs subset reported. Regarding lipids, 28 different lipids were identified in human uEVs from healthy donors and 26 lipids were found in patients with renal cell carcinoma. Moreover, EVpedia offers a range of analytical tools: (1) search for and browse vesicular components; (2) Gene Ontology enrichment analysis of vesicular components; (3) network analysis of vesicular components; and (4) set analysis: a comparison of vesicular datasets by ortholog identification. Detailed methods for the isolation of extracellular vesicles and publications on extracellular vesicles are also listed in this database. An overall comparison of EVpedia with Vesiclepedia is beyond the scope of this paper and has recently been published[31,85]. Regarding urinary EVs, the current release of EVpedia contains more data on proteins and lipids while miRNAs and mRNAs are more represented in Vesiclepedia. There is a concern that a relatively high percentage of the entries in the single datasets included in the databases are not native constituents of uEVs but matrix impurities related to isolation/purification[83,86]. Complete workflow solution, starting from fast and efficient purification of EVs from urine without contamination by non-vesicular components (including abundant urinary proteins, protein aggregates), is a critical prerequisite for future high-throughput analyses. Important prerequisites for the efficient use of the databases are: (1) the definition of a reliable core set of uEVs' constituents; and (2) handling data redundancy of current databases, are still awaited.

EMPLOYING URINARY EXTRACELLULAR VESICLES IN BIOMARKER DISCOVERY

Biomarkers are defined as substances or characteristics that are objectively measured and evaluated as an indicator of normal or pathogenic processes, or pharmacologic responses to a therapeutic intervention. Amongst the different body fluid, urine is one of the most important sources of biomarkers for both urologic and non-urologic diseases. High throughput omics studies have resulted in a great number of potential urinary biomarkers which are publicly available[87]. While the characterization of the normal urinary proteome is steadily progressing, there are three major obstacles in the classical urine-based protein biomarker discovery: (1) sample instability; (2) high dynamic protein concentration range; and (3) the relatively high inter-individual and inter-gender variations of urinary proteome. Urinary EVs, on the other hand, possess some characteristics that make them particularly attractive for biomarker research, like increased stability, reduced complexity, lower dynamic range, and composition which is closely related to the cell of origin. Biomolecules enclosed in the extracellular vesicle are surrounded by a lipid bilayer which protects them against degradation by proteases and nucleases. Therefore, uEVs have been shown to be particularly stable over time[37,88]. In addition, extracellular vesicles exhibit a reduced complexity comparing that to the whole urine composition. The dynamic concentration range of proteins and RNAs was shown to be lower in uEVs when compared to whole urine[33,78]. Moreover, the content of EVs reflects

Table 2 Putative disease-associated protein and RNA biomarkers identified in urinary extracellular vesicles

Name of the putative marker	UniProt ID/RNA name	Molecular and biological function	Associated Kidney disease(s)	Disease/model	Ref.
Adenine phosphoribosyltransferase	APT_HUMAN	Enzyme involved in the purine nucleotide salvage pathway. Its deficiency can lead to urolithiasis and renal failure	APRT deficiency	Healthy	[62]
Low-density lipoprotein receptor-related protein 2 mRNA	LRP2_HUMAN (megalin), LRP2	Multi-ligand binding receptor; mediates endocytic uptake of complexes between the steroid 25(OH) vitamin D3 and vitamin D-binding protein in kidney proximal tubules	Donnai-Barrow syndrome; Renal aminoglycoside accumulation; Nephrotoxicity	Healthy	[38,78]
Polycystin-1, Polycystin-2	PKD1_HUMAN PKD2_HUMAN	Integral membrane glycoproteins, associated with structural and/or functional defects in the primary apical cilium of epithelia and polycystic kidney disease	ADPKD	Healthy	[45,46,54,62]
Solute carrier family 12 member 2 (Sodium potassium chloride cotransporter-2)	S12A2_HUMAN	Membrane transporter: aids in the active transport of sodium, potassium, and chloride into and out of cells; NKCC2 mutations lead to type I Bartter syndrome	Antenatal Bartter syndrome type 1	Healthy	[33,41,62]
Myosin-9	MYH9_HUMAN	MYH9 polymorphisms have been shown to associate with glomerulosclerosis and non-diabetic end stage renal disease	Fechtner syndrome and Epstein syndrome	Healthy	[62]
Aquaporin-2 mRNA	AQP2_HUMAN AQP2	Molecular water channel in the basolateral and apical plasma membranes of the collecting duct	Autosomal dominant and autosomal recessive nephrogenic diabetes insipidus	Healthy; Various kidney diseases	[24,33,38,39,44,54,58,62,95]
Neprilysin (CD10)	NEP_HUMAN	Surface metallo-endopeptidase highly expressed in the kidney brush-border membranes	Membranous glomerulonephritis	Healthy; Diabetic nephropathy, Renal cell carcinoma	[25,33,77,95]
Osteoprotegerin	TR11B_HUMAN	Decoy receptor of proximal tubular cells	chronic kidney disease-mineral and bone disorder	Healthy; ADPKD CKDD patients	[49]
Podocalyxin	PODXL_HUMAN	he major sialoprotein of kidney glomerulus; Involved in the regulation of cell adhesion, cell morphology and cancer progression	Autosomal recessive steroid–resistant nephrotic syndrome	Healthy; Glomerular disease; Renal cell carcinoma	[42,58,62,77,95]
Solute carrier family 12 member 3 (Thiazide-sensitive Na-Cl cotransporter; NCC)	S12A3_HUMAN	Membrane transporter highly expressed in the kidney: aids reabsorbing sodium and chloride ions from the tubular fluid into the cells of the distal convoluted tubule of the nephron	Gitelman syndrome; Aldosteronism	Healthy; High blood pressure; Aldosteronism	[41,62,79]
Protein AMBP (alpha-1-microglobulin; bikunin precursor)	AMBP_HUMAN	Membrane glycoprotein with serine protease inhibitor activity	Diabetic nephropathy	Healthy; DN patients in advanced disease stages	[16,45]
Leucine-rich alpha-2-glycoprotein-1	LRG1_HUMAN	Involved in protein-protein interaction, signal transduction, and cell adhesion and development; Expressed during granulocyte differentiation	NSCLC	Healthy; NSCLC	[71]
Matrix metalloproteinase 9	MMP9_HUMAN	Involved in extracellular matrix remodeling	Renal cell carcinoma	Healthy; Renal cell carcinoma	[77]
Basigin , Extracellular Matrix Metalloproteinase Inducer	BASI_HUMAN	Involved in extracellular matrix remodeling	Renal cell carcinoma	Healthy; Renal cell carcinoma	[77]
Ceruloplasmin	CERU_HUMAN	A ferroxidase enzyme in serum	Renal cell carcinoma	Healthy; Renal cell carcinoma	[77]
Dickkopf related protein 4	DKK4_HUMAN	Involved in extracellular matrix remodeling	Renal cell carcinoma	Healthy; Renal cell carcinoma	[77]
Carbonic Anhydrase 9	CAH9_HUMAN	A metalloenzyme that catalyzes the reversible hydration of carbon dioxide	Renal cell carcinoma	Healthy; Renal cell carcinoma	[77]
Dipeptidase 1	DPEP1_HUMAN	A kidney membrane enzyme that hydrolyzes a variety of dipeptides and is implicated in renal metabolism of glutathione and its conjugates	Renal cell carcinoma	Healthy; Renal cell carcinoma	[77]
Syntenin-1	SDCB1_HUMAN	Scaffold protein Pbp1 Syndecan-binding protein	Renal cell carcinoma	Healthy; Renal cell carcinoma	[77]

Leucine-rich repeatkinase2	LRRK2	A member of the leucine-rich repeat kinase family; Associated to risks to inflammation-linked diseases that include Crohn's disease and mycobacterium infection	Parkinson's disease	Healthy; Parkinson's disease	[50]
Activating transcription factor 3	ATF3_HUMAN miRNA494	Transcription factor with protective role in renal ischemia-reperfusion injury	AKI	AKI	[22]
Fetuin-A	FETUA_HUMAN	Plasma binding glycoprotein; inhibitor of calcification, regulator of cell-dependent process of osteogenesis	AKI	AKI	[46,96]
Wilm's tumor protein	WT1_HUMAN	Zinc finger protein with essential role in the normal development of the urogenital system	Early podocyte injury in diabetic nephropathy; steroid responsiveness or renal pathological conditions in patients with idiopathic nephrotic syndrome	FSGS patients; Diabetic Patients with or without Proteinuria; idiopathic nephrotic syndrome	[22,30,92]
Isoform 1 of histone-lysine N-methyltransferase	MLL3_HUMAN	Histonemethyltransferase which methylates "Lys-4" of histone H3, a specific tag for epigenetic transcriptional activation	Diabetic nephropathy	DN patients in advanced disease stages	[16]
Voltage-dependent anion-selective channel protein 1	VDAC1_HUMAN	Porin ion channel of the outer mitochondrial membrane	Diabetic nephropathy	DN patients in advanced disease stages	[16]
GPRC5B	GPC5B_HUMAN	G protein coupled receptor required for tubulogenesis	Acute Kidney Disease	AKD	[94]
Aquaporin-1 mRNA	AQP1_HUMAN AQP1	Molecular water channel in the basolateral and apical plasma membranes of the proximal tubules.	Renal ischaemia reperfusion injury	Renal transplantation; Renal cell carcinoma	[38,77,97]
Neutrophil gelatinase-associated lipocalin	NGAL_HUMAN	Iron-trafficking protein involved in multiple processes such as apoptosis, innate immunity and renal development. In the kidney it is produced by distal nephron	kidney transplantation	kidney allograft recipients	[91]
Immunoglobulin light chain	IGLL1_HUMAN IGKC_HUMAN	Immunoglobulin light chain is the small polypeptide subunit of an antibody	Light Chain Amyloidosis	Light chain amyloidosis; multiple myeloma; monoclonal gammopathy; non-paraproteinemia related kidney disease	[65]
Panel of 24 proteins _HUMAN	SERPINA1, A2M, APOA1, APOA2, CA1, CD5L, DPEP1, FGA, FGB, FGG, FN1, GSTT1, SLC2A1, HBA1, HBB, SERPIND1, HP, HPR, ITIH1, PON1, RAB27B, S100A8, S100A9, TACSTD2	-	Bladder cancer	Bladder cancer; Hernia	[77]
PSA	KLK3_HUMAN	Serine protease with diverse physiological functions; implicated in carcinogenesis	Prostate cancer	Prostate cancer	[56]
Glutamate carboxypeptidase 2 (Prostate-specific membrane antigen-PSMA)	FOLH1_HUMAN	Folate hydrolase and N-acetylated-alpha-linked-acidic dipeptidase activity; Involved in prostate tumor progression	Prostate cancer	Prostate cancer	[56]
microRNA	MicroRNA-29c	-	Renal fibrosis	Healthy; CKD	[40]
microRNA	miR-130A	-	Type 1 diabetes	type 1 diabetic patients with and without incipient diabetic nephropathy	[81]

microRNA	miR-145	-	Type 1 diabetes	type 1 diabetic patients with and without incipient diabetic nephropathy	[81]
microRNA	miR-155	-	Type 1 diabetes	type 1 diabetic patients with and without incipient diabetic nephropathy	[81]
microRNA	miR-424	-	Type 1 diabetes	type 1 diabetic patients with and without incipient diabetic nephropathy	[81]
mRNA (CD2-associated protein)	CD2AP	-	Podocyte injury	Healthy; diabetic nephropathy; focal segmental glomerulosclerosis; IgA nephropathy;membranous nephropathy	[39]
mRNA (Prostate cancer antigen 3)	PCA-3	-	Prostate cancer	Healthy; Prostate cancer	[32]
mRNA (TMPRSS2/ERGa fusion transcript)	TMPRSS2:ERG	-	Prostate cancer	Healthy; Prostate cancer	[32]

CKD: Chronic kidney disease; AKI: Acute kidney injury; PSA: Prostate specific antigen; NSCLC: Non-small cell lung cancer; APRT: Adenine phosphoribosyltransferase deficiency; CD2AP: CD2-associated protein; PCA-3: Prostate cancer antigen 3; ADPKD: Autosomal dominant polycystic kidney disease.

the cell of urinary tract they shed from. Therefore, it was expected that biomolecules of uEVs could provide clinically more specific information for both early diagnosis of disease and also for monitoring drug responsiveness than that of urine[89]. Since the first publication of uEVs[44], the majority of the works in this field is focused on exploring the potential use of uEVs in preclinical and clinical studies[2,6,24,25,33,35,37,39-41,71,74-78,81,89-92]. Since plasma derived EVs are too large to pass through the glomerular filtration barrier, urinary tract originated vesicles (Table 2)[41,44] carry cell specific markers from the specific regions of the kidney with relevant physiological and pathophysiological information[41].

Proteomic analysis has revealed proteins and RNAs that have been isolated from healthy individuals but are associated with different human diseases (Table 2): Adenine phosphoribosyltransferase in APRT deficiency[62]); Low-density lipoprotein receptor-related protein 2 in Donnai-Barrow syndrome, renal aminoglycoside accumulation and Nephrotoxicity; Polycystin-1 and Polycystin-2 in ADPKD[54]; Neprilysin in Membranous glomerulonephritis[41]; Non-muscle myosin heavy chain IIA in Fechtner syndrome and Epstein syndrome[62]; Sodium potassium chloride cotransporter-2 and Thiazide-sensitive Na-Cl cotransporter in Antenatal Bartter syndrome type 1 and Gitelman syndrome, respectively[41,62]. The presence of theses disease associated analytes in uEVs is promising but their expression levels in well-defined disease cohort and conditions still need to be determined.

In disease cohorts including bladder[57,74,93], prostate[32,56,75] and renal cell carcinoma[2,77] altered expressions of vesicle derived proteins, RNAs and lipids have been demonstrated (Table 2) and panels of putative protein biomarkers have been set up for validation. Vesicle expressed aquaporin water channel proteins (AQP1 and AQP2) have been found to be differentially expressed in a number of different renal diseases too. Other putative biomarkers related to urinary system diseases, like activating transcription factor 3 and fetuin-A in acute kidney injury[22,46]; podocalyxin in autosomal recessive steroid-resistant nephrotic syndrome[62]; and Wilm's tumor-1 in early podocyte injury[22,92] have also been described. There are only a few studies though which report putative biomarkers related to treatment, like the effect of low sodium diet and infusion of aldosterone on the phosphorylation of Thiazide-sensitive Na-Cl cotransporter and prostatin in uEVS[79], or renal transplantation[91].

The reservoir for biomarker discovery could however extend beyond diseases of the urinary tract[41]. Conde-Li *et al*[70] investigated a rat model of liver injury induced by galactosamine and reported a change in urinary exosomes that coincided with liver injury. Gildea *et al*[71] reported that leucine-rich a-2-glycoprotein (LRG-1) was increased in both human urinary exosomes from patients with lung cancer and the lung cancer tissue. High-level of LRRK2 was found in uEVs of patients affected by Parkinson's disease[50]. These observations open a novel

scenario towards the future application of uEVs also in non-renal diseases.

CONCLUSION

Chronic kidney disease (CKD) is increasingly recognized as worldwide public health problem. CKD increases the risk for many adverse health outcomes, including cardiovascular disease, end-stage renal disease, and mortality. Because CKD usually progresses asymptomatically until its advanced stages, detection of early-stage CKD requires laboratory testing. The two key markers used for the definition, classification, and monitoring of kidney function are urine albumin and estimated glomerular filtration rate (eGFR). Kidney dysfunction is indicated by eGFR of less than 60 mL/min per 1.73 m^2, while kidney damage most frequently is manifested as increased urinary albumin excretion. When less invasive blood and urine tests are insufficient fine-needle aspiration (FNA) or renal mass biopsy (RMB) is performed especially to substantiate the diagnosis in renal masses. While these traditional markers are certainly of great utility they also present several limitations. Protein (albumin) concentration in urine is not very specific. Levels may rise with use of certain non-steroidal anti-inflammatory drugs, rheumatoid arthritis, lupus and cancers etc. Creatinine level used to estimate GFR, on the other hand, provides little information about the underlying cause of kidney injuries, and lacks specificity in case of low muscle mass and unusual diets.

Because of the above limitations of currently used kidney function markers intensive research is going on to find more accurate ones. Many genes have been shown to be differentially expressed in the kidney upon glomerular injury, endothelial dysfunction, inflammation, fibrosis, cardiovascular dysfunction, metabolic disorders and cancer with the corresponding protein products appearing in plasma and urine. In urine, a unique biomarker source with great potential, a number of new candidate protein biomarkers have already been proposed: cystatin, N-acetyl-β-o-glucosaminidase (NAG) and liver-type fatty acid-binding protein (L-FABP), neutrophil gelatinase-associated lipocalin (NGAL) and kidney injury molecule 1 (KIM-1), just to mention a few, are potential biomarkers awaiting administration (FDA) approval. NGAL is suggested to be used as a urinary biomarker of delayed graft function (DGF), a frequent complication after kidney transplantation too. Recently Alvarez *et al*[90] have demonstrated that NGAL is mainly secreted into urinary vesicles and that the expression level of NGAL in uEVs is a sensitive measure of DGF, findings which might support the clinical management of patients undergoing kidney transplantation.

To become a clinically approved, a putative biomarker should be validated and implemented into clinical tests. Translating a novel discovery into clinical practice is however extremely challenging, consequently there are only a few urine-based protein biomarker assays which have been developed and approved so far (*i.e.*, BTA and NMP-22 for the diagnosis of bladder cancer and PCA3 for the diagnosis of prostate cancer). Rapidly expanding urinary extracellular vesicle research represents an interesting field relevant to the development of disease specific, non-invasive methods for clinical diagnostics as well as to the development of new therapeutic approaches. Especially, growing evidence suggest that EV-imprinted genetic and proteomic information may well reflect the state of their parental cells. In this sense, expression analysis of proteins and RNAs in circulating urinary vesicles could provide specific information about the change in the state of specific nephron segment(s) and/or of the epithelial cells of urogenital tract. Urinary EV-mediated cell-cell communication within the nephron is another interesting aspects which can have major impact in our current understanding in renal physiology. However, how to translate these captivating ideas to a non-invasive renal monitoring system, there is still a lot more to understand.

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