Journal of Food and Drug Analysis

Volume 30 | Issue 2 Article 3

2022

Exosomal proteomics in kidney disease: From technical approaches to clinical applications

REVIEW ARTICLE

Exosomal proteomics in kidney disease: From technical approaches to clinical applications

Ageng Brahmadhi [a,b,c], Yung-Kun Chuang [d,e,f,1], San-Yuan Wang [g,1], Chih-Chin Kao [h,i,j,*], I-Lin Tsai [a,b,g,k,l,**]

[a] Department of Biochemistry and Molecular Cell Biology, School of Medicine, College of Medicine, Taipei Medical University, Taipei, Taiwan
[b] International Ph.D. Program for Cell Therapy and Regeneration Medicine, College of Medicine, Taipei Medical University, Taipei, Taiwan
[c] Faculty of Medicine, Universitas Muhammadiyah Purwokerto, Purwokerto, Central Java, Indonesia
[d] School of Food Safety, College of Nutrition, Taipei Medical University, Taipei, Taiwan
[e] Nutrition Research Center, Taipei Medical University Hospital, Taipei, Taiwan
[f] Master Program in Food Safety, College of Nutrition, Taipei Medical University, Taipei, Taiwan
[g] Master Program in Clinical Genomics and Proteomics, College of Pharmacy, Taipei Medical University, Taipei, Taiwan
[h] Division of Nephrology, Department of Internal Medicine, School of Medicine, College of Medicine, Taipei Medical University, Taipei, Taiwan
[i] Division of Nephrology, Department of Internal Medicine, Taipei Medical University Hospital, Taipei, Taiwan
[j] Taipei Medical University-Research Center of Urology and Kidney (TMU-RCUK), Taipei Medical University, Taipei, Taiwan
[k] Pulmonary Research Center, Wan Fang Hospital, Taipei Medical University, Taipei, Taiwan
[l] Graduate Institute of Medical Sciences, College of Medicine, Taipei Medical University, Taipei, Taiwan

Abstract

Exosomes are small extracellular vesicles (sEVs) secreted from cells and have a general diameter ranging from 30−150 nm. It was reported that exosomes have essential roles in intercellular communication and can be targeted as biomarkers of disease or as therapeutic agents. Among the different techniques used for exosome investigation, the mass spectrometry-based proteomics approach has accelerated the unraveling of the molecular composition of exosomes and has contributed to improved knowledge of molecular processes in various diseases. In this review, we focused on proteomics-based studies of exosomes and clinical applications in kidney diseases. A general introduction of exosomes, isolation and characterization techniques, and proteomics-based study workflows are included in this article. We also categorized applications in acute kidney injury, chronic kidney disease, renal transplantation, congenital kidney disease, and malignant kidney disorder to show the important findings from proteomics-based exosomal investigations.

Keywords: Exosome, Extracellular vesicle, Kidney-related diseases, Mass spectrometry, Proteomics

1. Introduction

Living cells can secrete particles called extracellular vesicles (EVs). EVs are categorized into two major categories based on their origin: exosomes and ectosomes. Exosomes are 30−150 nm in diameter and originate from the endosome, while ectosomes are larger vesicles (50 nm−1 μm in diameter) that originate from plasma membrane budding [1].

Exosomes were discovered 30 years ago. The term "exosome" was first introduced in 1981 to describe plasma membrane-derived vesicles [2]. In 1983, two study groups, Harding's group and Johnstone's group, isolated transferrin receptor-associated vesicles from reticulocytes [3,4].

Received 16 October 2021; revised 16 January 2022; accepted 21 March 2022.
Available online 15 June 2022.

* Corresponding author at: Department of Internal Medicine, Taipei Medical University Hospital, 252 Wuxing Street, Taipei 11031. Taiwan.
** Corresponding author at: Department of Biochemistry and Molecular Cell Biology, School of Medicine, College of Medicine, Taipei Medical University, 250 Wuxing Street, Taipei 11031. Taiwan.
E-mail addresses: 121008@h.tmu.edu.tw (C-C. Kao), isabel10@tmu.edu.tw (I-L. Tsai).
[1] Both authors contributed equally to this manuscript.

https://doi.org/10.38212/2224-6614.3409

However, the term "exosome" was not used until 1984, when Salem and colleagues described the exfoliation of membrane vesicles in cultured rat glioma cells. This group referred to exfoliated membrane vesicles as exosomes [5]. Later, in an article published by Johnstone and colleagues in 1987 [6], the term exosome was highlighted and became widely used to describe EVs that originate from multivesicular bodies (MVBs).

In recent years, scientists began studies of exosomes to investigate their biological functions, and techniques used for exosome isolation and characterization also were being developed. Although the heterogeneous properties of exosomes make them difficult to isolate, important findings have been continually reported that point out their roles in cellular communication, immune modulation, and pathological/therapeutic effects [1].

Several review articles have summarized and discussed the application of omics analyses to kidney disease-related exosomes to understand pathological mechanisms and the potential of discovering exosomal biomarkers for various disorders. In this review article, we focused on proteomics-based exosomal studies and their applications to clinical kidney diseases. General introduction to exosomes and isolation methods are included, followed by characterization techniques, proteomics study workflows, and key findings from kidney disease-related exosomal studies.

1.1. Exosome biogenesis

Exosome formation occurs within the endosomal complex. The endosomal complex can be distinguished into three groups: early endosomes or sorting endosomes, late endosomes, and recycling endosomes [7]. The early endosome undergoes several transformations in progressing to the late endosome. During the process, early endosomes might fuse with endocytic vesicles and sort their contents for recycling, degradation, or exocytosis. Contents designated for exportation are further sorted into 30–150-nm vesicles. These multiple vesicles are referred to as intraluminal vesicles. Together with the late endosome structure, these structures are referred to as multivesicular bodies (MVBs) [8]. Late endosomes fuse with lysosomes or plasma membranes. Fusion with lysosomes results in the degradation of contents in late endosomes. Fusion with the plasma membrane leads to the secretion of intraluminal vesicles or exosomes (Fig. 1).

1.2. Literature survey for proteomics-based exosome studies and kidney diseases

Generally, exosomes used for clinical studies are mostly obtained from blood or urine. To further understand the trends and applications of exosome and proteomics research in kidney-related diseases, a literature survey was performed in the PubMed database through July 2021. The keywords "exosome", "kidney disease", and "proteomic" were used as advanced search options, and 115 articles were identified, among which 85 articles involved human subjects (Fig. 2). To expand the search results, additional keywords addressing kidney-related diseases were used, including "acute kidney injury", "diabetic nephropathy", "glomerular disease", "polycystic kidney disease", "renal cell carcinoma", and "renal fibrosis". Some inclusion criteria were adopted during manual screening: the article had to be an exosome-related original article, published in 2000–2021, with humans as the main subject of the research. In addition, articles were excluded if they were not a mass spectrometric (MS)-based proteomics study. After expansion, 105 human-related articles were collected. The collected articles consisted of 33 original articles, 35 review articles, and 37 nonrelated articles. Even though we used exosome as keyword for the literature survey, some screened-out articles used EV instead of exosome to represent their purified vesicles. It needs to be emphasized here that the consensus of nomenclature in using the term "EV, exosome, or microvesicle" has just been discussed in recent years [9]. It is suggested to use the name "EV" if there is no clear evidence of biogenetic origin or characterization for the purified vesicles. Although we aimed to collect articles focusing on small EVs or exosomes, the studies we found used different terms to represent their purified vesicles; therefore, we will use the term "exosome/sEV" in the clinical application part to prevent confusion.

In the collected literature, the general workflow covered exosome/sEV isolation, characterization, and bottom-up proteomics. Urine samples were the most common source of exosomes/sEVs in these studies. Other biofluids, such as plasma, amniotic fluid, and peritoneal dialysis effluent (PDE), also were utilized in some cases. Since plasma and urine are still commonly used in many clinical studies, general sample pretreatment methods, isolation and characterization techniques for exosomes/sEVs from both sample types are introduced in the following section (Section 2). The

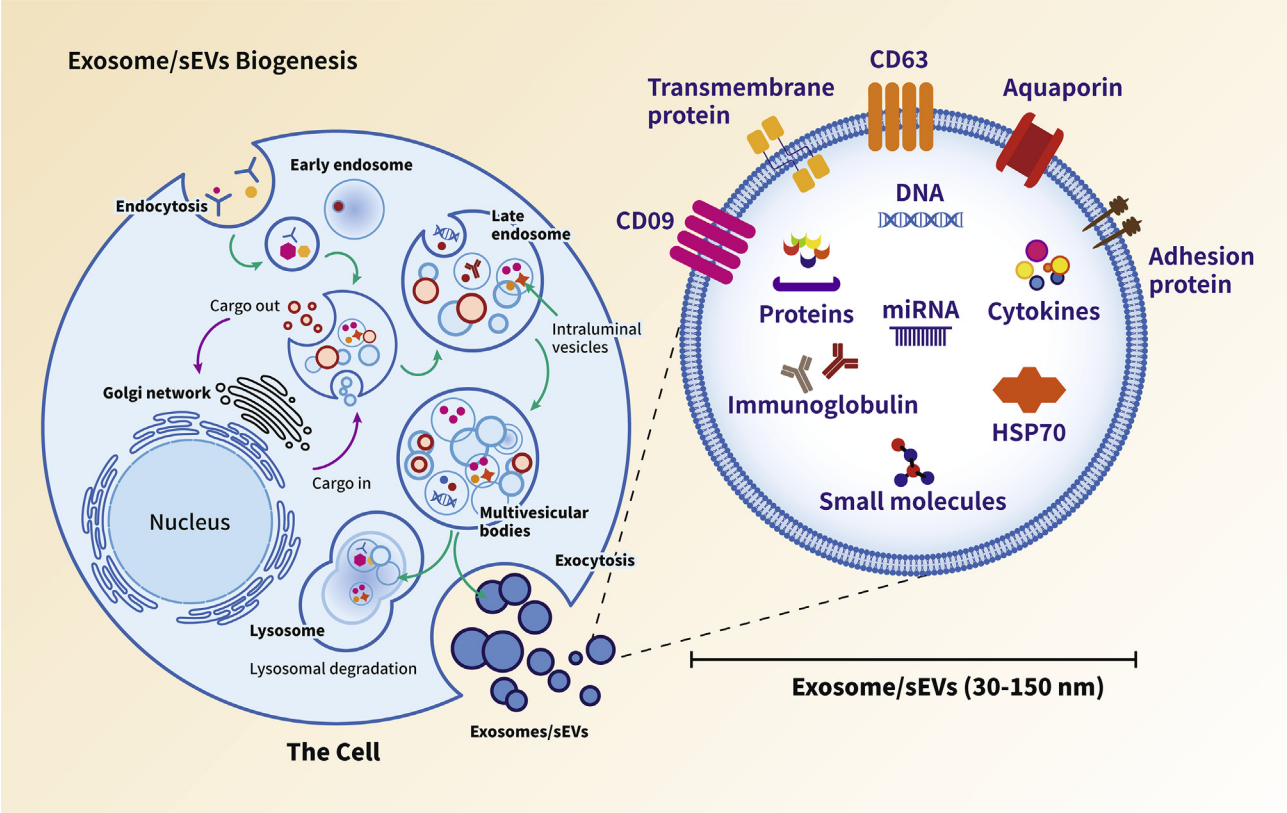

Fig. 1. Schematic representation of exosome/sEVs biogenesis. Exosome/sEVs biogenesis involves the formation of endocytic vesicles, endosome maturation, and multivesicular body (MVB) formation. Subsequently, MVBs fuse with plasma membranes and release vesicular contents, which also are called exosomes.

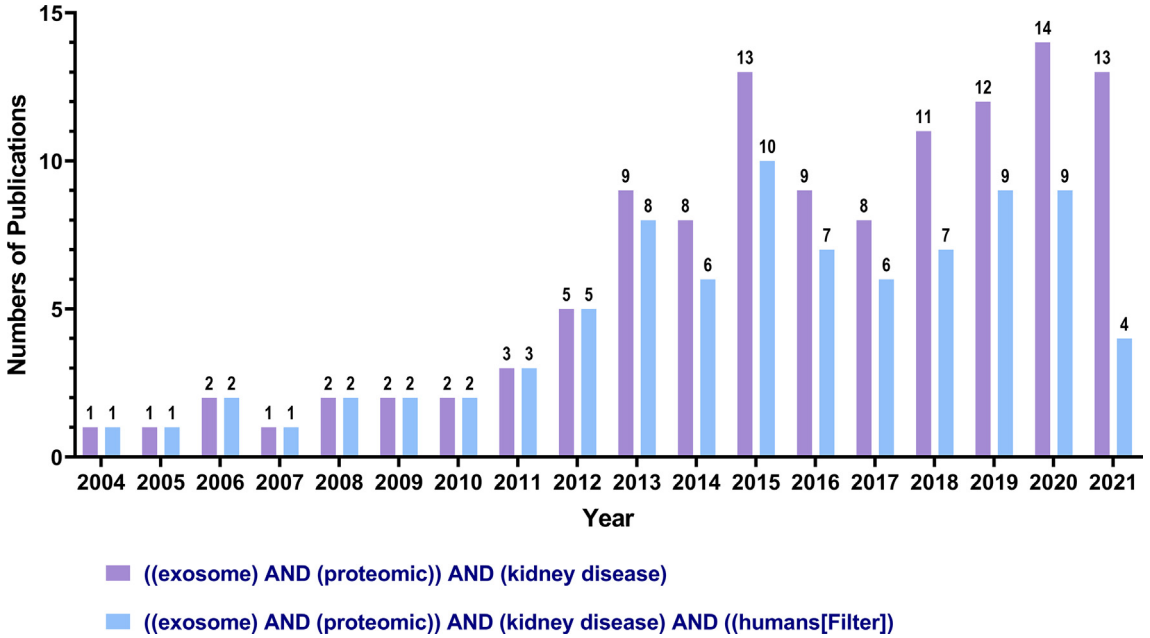

Fig. 2. Bibliographic visualization of publication trends in proteomics-based exosomal studies related to kidney diseases. Publication numbers for each year are indicated at the top of the purple bars, and publication numbers for studies related to human species are indicated at the top of the blue bars.

proteomics workflow and useful tools are introduced in Section 3. Selected studies that applied these methods to clinical investigations are summarized and discussed in Section 4.

2. Sample pretreatment and exosome isolation

Exosomes can be purified from biological samples based on their nanoscale size, density, and specific surface markers. Ultracentrifugation (UC), precipitation reagents, size-exclusion chromatography (SEC), and immunoaffinity capture methods have been frequently reported. Other techniques, such as microfluidic binding affinity isolation [10], field flow fractionation, and fluorescence-activated cell sorting (FACS), also have been developed [11]. Before applying isolation techniques to target samples, a pretreatment step is important in achieving higher-quality exosome purification.

2.1. Sample pretreatment for urine and plasma samples

The pretreatment stage is intended to preserve exosomes and reduce cell debris. The general process usually includes the addition of protease inhibitors followed by sequential low-speed centrifugation to obtain a clearer sample source [12].

2.1.1. Urine sample pretreatment

Second-morning urine is preferred to be used for isolating urinary exosomes. Protease inhibitors are added immediately after urine collection to preserve them [13]. The collected urine can be stored at −80 °C until extraction. Vigorous vortexing is suggested after samples are thawed, followed by centrifugation at 17,000×*g* for 10–15 min to remove large particles and cell debris [14,15]. A lower speed of initial centrifugation also was purposed at 4,000×*g* for 15 min at 4 °C. This approach was proven to clear cell debris without decreasing the purification yield [13].

The major obstacle of urine exosome isolation is the presence of the Tamm–Horsfall protein (THP) or uromodulin. This protein has polymeric networks that might bind the exosome. Vesicle entrapment can be visualized during TEM observations [16]. This entrapment not only disturbs exosome visualization but also affects total protein yields and identified proteins in proteomic studies. To minimize the disturbance due to THP, dithiothreitol (DTT) is added as a reducing agent to the sample mixture, which helps dissolve the THP polymeric network. This approach was successfully used for samples before applying UC and precipitation methods. Since DTT is a strong reducing agent, it can affect the identified proteins in proteomic studies and might lead to false identification or detection. Therefore, the use of DTT requires further consideration. We also found one study that showed that it is also possible to perform a proteomic study while allowing uromodulin to remain in samples. Dr. Lilley et al. developed an approach to overcome uromodulin in proteomic experiments by applying a list of excluded uromodulin-related peptide ions. By including a uromodulin-related exclusion list during MS analyses, the number of identified proteins was increased by 29.7%, for a total of 288 identified proteins, compared to the conventional method with 222 proteins [17].

2.1.2. Plasma sample pretreatment

It was suggested to utilize plasma, instead of serum, as the main source when isolating exosomes from the blood of patients and healthy individuals. The majority of vesicles in serum might be released from platelets during clot formation due to the blood-collection process [18]. Plasma from blood samples can be collected by using tubes with anticoagulants and a low speed of centrifugation (1,200–1,800×*g* for 10 min). Before introducing plasma to exosome isolation, additional low-speed centrifugations, such as 1,500×*g* for 10 min followed by 10,000×*g* for 20 min, can be used to remove platelets or large vesicles [19]. Centrifugation at 2,500×*g* for 10 min also was conducted in some studies [20].

2.2. Exosome isolation techniques

Exosome can be isolated from multiple types of biofluids, including plasma/serum, saliva, urine, pleural effusions, and amniotic fluids, and they also can be isolated from conditioned medium of cell cultures. Until recently, there was no single best practice for retrieving exosomes from original sample sources, and all methods required fine-tuning of the experimental conditions.

2.2.1. Ultracentrifugation (UC)

UC is the most commonly used technique for isolating exosomes. Based on a survey reported in 2016, UC applications accounted for 81% of all methods worldwide [21]. Centrifugation utilizes centrifugal force to separate a heterogeneous mixture based on the particle density, size, and shape. The term UC refers to remarkably high-speed centrifugation up to 100,000–120,000×*g*. The pellets of dead cells and debris were discarded with

a series of low-speed centrifugations as described in the sample pretreatment section, and the supernatant was used for subsequent high-speed UC to obtain exosomes. It usually takes 1–2 h to get exosome fraction. A wash step with phosphate-buffered saline (PBS) is recommended to eliminate contaminating proteins, and the sample is again centrifuged at high speed ($10^5 \times g$) to recover the exosome fraction [15]. This method results in relatively pure exosomes and is considered the gold standard of isolation techniques. However, possible contaminants were reported to be lipoproteins and aggregate particles with similar densities when using UC for blood exosome isolation [22].

Sucrose density gradient UC was developed to achieve better purities. With this method, 30% sucrose cushioning is added to the bottom of the UC tube. The exosome sample purified by traditional UC is added on top of the sucrose cushion, and the tube is subjected to UC. Exosomes are trapped in the sucrose cushion and can be collected carefully. However, co-isolation of high-density lipoprotein (HDL) still occurs because exosomes and HDL have similar densities (1.06–1.21 g/mL). Therefore, exosomes and HDL can be separated only based on size differences, since a single vesicle has a larger diameter (30–1,000 nm) than an HDL particle (7–13 nm) [23]. Although UC is considered the gold standard of exosome isolation, it is relatively time-consuming and requires high volumes of starting materials, and vesicles can rupture due to the high velocities in some cases [24].

2.2.2. Precipitation method

Polyethylene glycol (PEG) is one of the most commonly used reagents in precipitation methods. The PEG polymer has the capability to interact with water molecules to create a polymer network for trapping exosomes, and the trapped exosome aggregates can be precipitated using low-speed centrifugation at $1{,}500 \times g$ [22].

Commercially available precipitation reagents include ExoQuick (System Biosciences), Total Exosome Isolation (Invitrogen, Thermo Fisher Scientific), RIBO™ Exosome Isolation Reagent (REI), Trident High-Efficiency Exosome Precipitation Reagent (GeneTex), and Minute™ High-Efficiency Exosome Precipitation Reagent (Invent Biotechnologies). The technique is quick, simple, and requires relatively small amounts of starting materials. However, the isolated exosome sample is less pure due to the co-precipitation of other EVs, proteins, and aggregates. In addition, residual polymers might be retained in the final isolated exosome sample [24].

2.2.3. Size-exclusion chromatography (SEC)

Exosome-specific sizes of 30–150 nm are advantageous for size-based particle separation. SEC is a liquid chromatographic (LC) technique that separates molecules based on their size, which depends on the molecular weight and shape. It utilizes a porous polymer as the stationary phase to separate particles in sample mixtures. The advantages of SEC are that it is simple and faster, no specialized equipment is required, and it is less destructive for isolated exosomes than UC [25]. However, since limited sample volumes can be loaded into an SEC column, SEC is more suitable for isolating exosomes from biological fluids, such as serum/plasma and saliva, rather than conditioned medium. Biological fluids usually contain relatively abundant EVs; therefore, small volumes of 0.5–2 mL are sufficient for subsequent analyses. In contrast, exosomes are relatively diluted in conditioned medium, and the elution process results in more diluted isolated exosomes [26]. For example, single-step exosome isolation using SEC was demonstrated by the team of Dr. Nieuwland. Sepharose CL-2B pores were used as the porous polymer inside a 10-mL syringe as the SEC column of 6.2 cm in height and 1.6 cm in diameter. Sepharose CL-2B has a pore size diameter of approximately 75 nm, which limits particles with a diameter larger than 75 nm from entering the pores and traveling along with the elution buffer. With this approach, vesicles with diameters of >75 nm can be successfully isolated [27]. The qEV SEC column from Izon Science is one of the examples of commercial SEC-based exosome isolation kits. Other products include ExoPure™ from BioVision and SmartSEC™ from System Biosciences.

2.2.4. Immunoaffinity-based exosome isolation

Immunoaffinity exosome isolation relies on specific markers expressed by the exosome membrane. Specific surface markers include cluster of differentiation 63 (CD63), CD09, and CD81, which are targeted by immunoaffinity isolation using magnetic beads [28]. The generic workflow for exosome isolation using magnetic capture is divided into three steps. The first step is the pre-enrichment of the initial sample, which is performed by several options, including UC, precipitation, filtration, and SEC. The second step is the magnetic capture of exosome-specific surface markers, which requires the overnight incubation of samples and magnetic beads (Dynabeads) coated with CD9 or CD81 antibodies. The following day, the isolation tube is placed on a magnet, and the magnetic beads are washed by removing the supernatant and adding 0.1% bovine serum albumin (BSA) in PBS. The final

steps are labeling the captured exosomes and downstream analysis, such as the Western blotting of bead-bound exosomes [28].

Many studies also have developed novel affinity purification methods for higher exosome isolation purity. For instance, Dr. Hanayama's team utilized an affinity protein named T cell immunoglobulin domain and mucin domain-containing protein 4 (Tim4), which strongly binds phosphatidylserine to purify EVs. The binding is Ca^{2+} dependent, and the captured EVs can be easily recovered by adding Ca^{2+} chelators [29]. Dr. Kuroda et al. also found that lysine peptides that bind phospholipids could be used to isolate exosomes. In their study, immobilized peptides containing 8 and 16 lysine residues on magnetic beads were able to capture small EVs with a size of <0.2 μm [30].

2.2.5. Sequential isolation method and novel techniques

In addition to single isolation methods, combined methods also should be considered. To separate exosomes into different size groups, additional fractionation is required. Asymmetric flow field-flow fractionation is able to separate exosomes into subpopulations: large exosome vesicles (90–120 nm), small exosome vesicles (60–80 nm), and non-membranous nanoparticles termed exomeres (<35 nm) [31]. Dr. Klein et al. compared nanomembrane ultrafiltration, UC, and the combined methods of UC-SECs for exosome isolation from nephrotic urine. The combined UC-SEC method enhances the enrichment and purification of microparticles. With the proteomics analysis performed using tandem matrix-assisted laser desorption ionization-tandem time of flight mass spectrometry (MALDI-TOF/TOF MS), neprilysin, aquaporin-2, and podocalyxin were highly enriched by the UC-SEC method compared to the other two isolation methods. The improvement in vesicular isolation supported the identification of nephrotic syndrome biomarkers [32].

2.3. Exosome characterization

Exosome characterization in terms of identification, biomolecules carried, total particle numbers, and size distributions requires multiple complementary techniques. In the following paragraphs, we focus only on techniques used for exosome characterization. Other information such as purity, copurified components, and contaminants also should be clarified. Although these are not discussed here, more information can be found in the *Minimal Information for Studies of Extracellular Vesicles (MISEV) guidelines* (2018) [9].

Exosomes have simple spherical characteristics with a lipid bilayer membrane as they originate from the endosome. Even though secreted from the same cell type, the sizes of exosomes are significantly heterogeneous. The total particle number and size distribution of exosomes can be determined using nanoparticle tracking analysis (NTA), standard and high-resolution flow cytometry, and tunable resistive pulse sensing (TRPS). At minimum, it is important to report the obtained concentration (particle numbers/mL) along with the maximum and minimum diameters of the exosomes. Electron microscopy (EM) is considered a standard tool for examining exosome morphology [33]. EM techniques include scanning electron microscopy (SEM), transmission electron microscopy (TEM), and cryo-EM. Under SEM, exosomes appear round-spheroid-shaped and relatively uniform. Meanwhile, TEM imaging displays a central depression [34]. Enhanced visualization by TEM might be achieved using cryo-TEM. Under cryo-TEM, the majority of extracellular exosomes appear as round-shaped vesicles with double-layered electrodense plasma membranes [35]. In several cases, immunolabeling was added to improve the EM-specific output or function-dependent morphology confirmation [36].

Exosomes contain diverse molecules, including DNA, messenger (m)RNA, micro (mi)RNA, lipids, metabolites, and proteins [37]. Specific proteins enriched in exosomes can be used for vesicle characterization. Examples of membrane proteins include tetraspanins, such as CD63, CD81, and CD9. There also are cell-specific membrane proteins, such as epithelial cell-associated epithelial cell adhesion molecule (EpCAM) and mesenchymal stem/stromal cell (MSC)-associated CD90. Examples of soluble or cytosolic proteins include endosomal complexes required for transport (ESCRT-I/II/III), flotillins, apoptosis-linked gene 2 interacting protein X (ALIX), etc. Immunoblotting or Western blotting is widely used to analyze exosome markers [9], and multiple exosome markers also can be detected using enzyme-linked immunosorbent assays (ELISAs) [38].

Total protein amounts of isolated exosomes are important to be determined for purification evaluation and are useful in the following proteomics analysis. It can be measured by colorimetric methods such as the Bradford assay and bicinchoninic acid (BCA) protein assay [39]. By calculating the ratio of exosome particle numbers and protein concentrations, the purity of exosomes can be determined [39]. One must consider that protein quantification can be interfered with by protein contaminants from plasma/serum or the culture

medium. In addition to total biomolecule quantification, detailed profiling of exosome components can be performed through transcriptomics, lipidomics, metabolomics, and proteomics analyses.

3. Proteomics analysis of exosomes

The protein contents of exosomes can be studied using MS-based analyses. With the advantages of the sensitivity and selectivity of MS, a wide range of proteins can be identified and quantified [40]. After isolating exosomes from biofluids, a fraction of the sample is used for exosome characterization. A sufficient amount of exosomal proteins can be used for proteomics analysis. Although protein analysis by Western blotting or ELISA is also categorized as conventional proteomics analysis, comprehensive and quantitative bottom-up (shotgun) proteomics studies using MS have more frequently been applied recently. A successful proteomic experiment requires suitable protein sample preparation and accurate instrumental analysis, including peptide separation and MS detection, protein identification, pathway mapping, and pathway interaction analysis [41]. In Fig. 3, we summarize the workflow of proteomics-based exosomal studies, and detailed information on sample preparation, instrumentation, and data analysis are introduced in the following subsections.

3.1. Sample preparation for exosomal proteomics analysis

Since the sensitivity and discovery depth of MS in peptide analysis are critically affected by the sample quality, fractionation of proteins and sample cleanup are usually necessary steps during preparation. Sample pretreatment steps prior to the MS analysis include (1) protein extraction, (2) protein preparation, (3) protein digestion, (4) peptide cleaning, detergent removal, and desalting, and (5) peptide separation [42].

After exosome isolation, protein isolation is the first step of sample preparation. Exosomal proteins are usually extracted using lysis buffer such as sodium dodecyl sulfate (SDS) or 8 M urea [43]. Another option is to use physical lysis equipment such as a homogenizer or sonicator. The combination of chemical and physical lysis also can be applied. During lysis, enzymes such as proteases and phosphatase are released and may affect protein profiles. Placing the sample on ice and adding a cocktail of inhibitors are useful to halt enzyme activity and prevent protein loss [41].

Crude extracted proteins are contaminated with salts, detergents, and other contaminants, which make samples incompatible with MS. Removal of those components is necessary, and this can be achieved using gel electrophoresis, chromatography, membrane filtration, and protein precipitation [41]. Gel electrophoresis can remove low-molecular-weight contaminants. In addition, this protein preseparation is beneficial by increasing the number of identified proteins, which eventually increases the depth of the analysis. A more advanced approach can be achieved using filter-aided sample preparation (FASP). FASP utilizes an ultrafiltration device to separate proteins from contaminants. In FASP, the sample is solubilized in 4% SDS, contaminants pass through an ultrafilter during centrifugation, and proteins are retained on the filter unit [44]. The filter unit provides the function of detergent removal, buffer exchange, and solid support during protein digestion. Unlike in-gel digestion, FASP enables consecutive digestion using different enzyme combinations [45]. Although FASP is versatile and efficient, sample loss occurs when the sample load contains less than 10 μg protein. This is suspected due to nonspecific protein binding to the inner membrane of the ultrafiltration device. High protein concentrations also lead to irreversible protein aggregation, which results in additional sample loss [45]. The addition of polyvinylpyrrolidone-40 (PVP-40) to the protein sample before FASP digestion improved peptide recovery and identification. This approach also produces cleaner samples than traditional FASP [46]. Dr. Lasch's team developed a detergent-free sample preparation method called sample preparation by easy extraction and digestion (SPEED). It uses pure trifluoroacetic acid (TFA) for protein extraction. Compared to detergent-based sample preparations, the number of identified peptides and proteins was increased by >40%, and it was claimed to have simplified sample preparation and enhanced the reproducibility of the results [47].

In the protocols and methods mentioned above, protein reduction using DTT and protein alkylation with iodoacetamide are commonly conducted. The prepared proteins are then digested with different enzymes. Trypsin is the most widely used digestion enzyme due to its high specificity, availability, and practicality. However, not all sequences are detectable by protein tryptic digestion. Missing sequence coverage might occur due to the uneven distribution and number of arginine and lysine residues [48], with the limitation restricting the total elucidation of protein segments, proteome information, and post-translational modification sites. Therefore, alternative enzymes in addition to trypsin, such as chymotrypsin, Lys-C, Lys-N, Asp-N, Glu-C, and

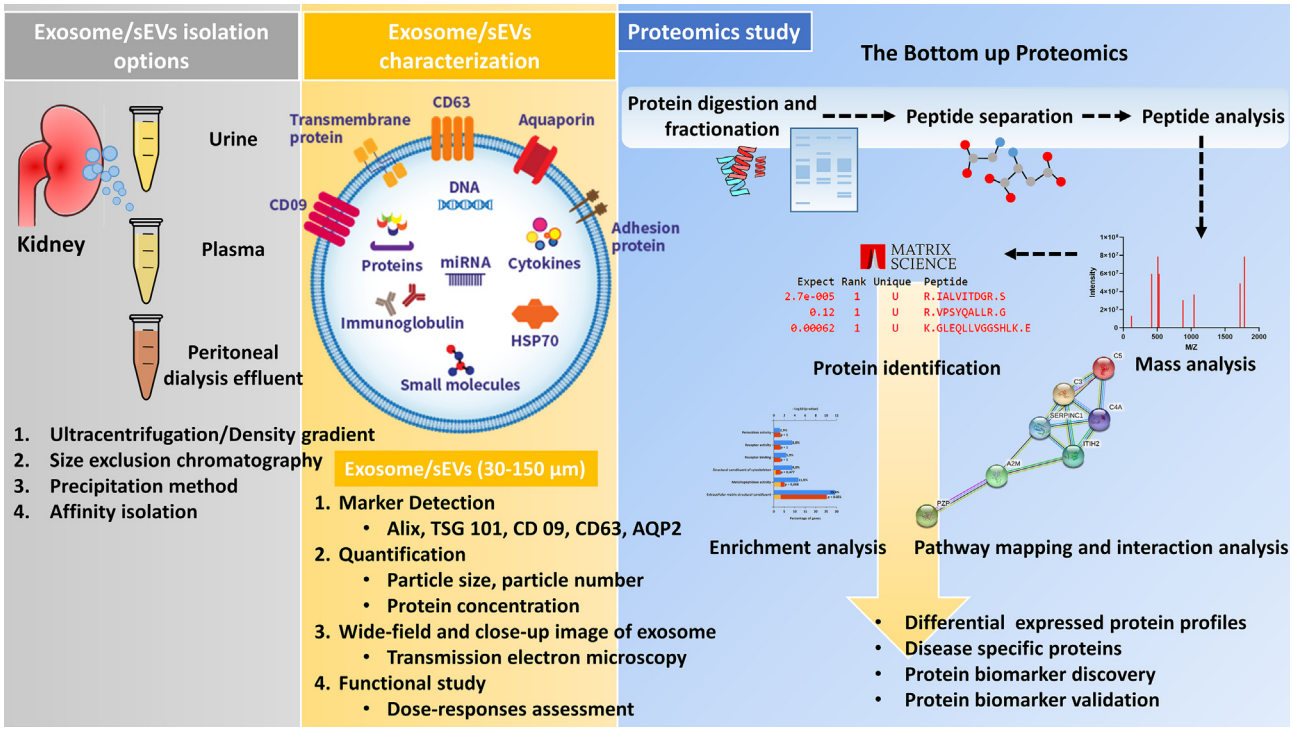

Fig. 3. General workflow of proteomics-based exosomal studies of kidney diseases. Exosomal proteomic studies were initiated by exosome isolation from body fluids. Exosomes/sEVs can be isolated from urine, and alternative sources are plasma and peritoneal fluids. Ultracentrifugation, size-exclusion chromatography, affinity purification, and polymer precipitation are commonly used purification methods. Exosomes/sEVs should be characterized as a method of confirmation. After an LC-MS/MS analysis, proteins can be identified, thus enabling further data analyses and pathway interpretation.

Arg-C, began to garner interest in proteomic studies [49]. Multiple protease utilization might be beneficial in large-scale mass spectrometry-based protein sequencing, as there is an approximately 20% increase compared to the single protease approach, and a twofold increase in proteome sequence coverage [50]. The peptides are further collected and desalted using solid-phase extraction, followed by liquid chromatographic tandem MS (LC-MS/MS) analysis.

3.2. LC-MS/MS analysis

LC-MS is a high-throughput, sensitive analytical technique. The technique utilizes the physical separation by LC, coupled with the measurement of the mass-to-charge ratio of ionized molecules by MS. The aims of MS-based proteomics studies are to determine proteins and posttranslational modifications of those proteins [51]. Reversed-phase LC columns, such as C18 packing material, are usually used as the stationary phase for peptide separation [52]. Compared to conventional high-performance LC (HPLC) columns, nanoscale LC coupled with tandem MS (nano LC-MS/MS) has become the method of choice because of its better sensitivity. The inner diameter of the separation column can be $\leq$ 0.1 mm, a typical flow rate is $<$ 1 μL/min, and it can be utilized with limited sample volumes [53]. Typical commercial nano-LC columns are 150 mm long with a 50–75 μm inner diameter.

Electrospray ionization (ESI) is the most commonly used ion source for peptide analyses. Multiple positive charges of peptides are generated when the nano-LC eluate passes through the ESI ion source. Charged peptides are then analyzed by a mass analyzer, such as a TOF mass analyzer, Orbitrap, and Fourier transform-ion cyclotron resonance (FT-ICR) mass spectrometers. Some of the high-resolution MS techniques can provide resolving power of 10^5 resolution with a mass accuracy of 2 ppm and 5 $\times$ 10^5 resolution with a mass accuracy of <2 ppm [54].

There are two types of data acquisition modes for bottom-up proteomics: data-dependent acquisition (DDA) and data-independent acquisition (DIA). The DDA strategy selects precursor ions for fragmentation based on the topmost abundant precursor ions in MS1, the absolute signal intensity, and the charges of the precursor ions. Peptides are identified by matching the fragment spectra with a database of protein sequences [55]. DDA can identify

thousands of proteins; however, minor contents might be missing in some cases. This issue is caused by excessive coelution of peptide species and appears in a single MS1 scan. Since DDA stochastically selects the most abundant peptides, peptides with lower abundance might be neglected [56]. In contrast to DDA, isolation windows are predetermined for the MS2 scan in the DIA approach. All precursor ions within the isolation windows are scanned regardless of their intensity [57]. This strategy ensures the identification of all peptides within the selected *m*/*z* range, generally within 300–1200 *m*/*z* isolation windows [52]. However, DIA MS2 scans contain peptide mixtures, which makes them difficult to analyze. This condition also affects the measurement precision of very low abundance peptides [56]. Among proteomics-based exosomal investigations related to kidney diseases, most scientists use DDA proteomics approaches.

3.3. Data interpretation and normalization

After obtaining MS/MS spectra, protein sequences can be identified through *de novo* sequencing or a database search. With *de novo* sequencing, peptide sequences are obtained directly from the spectra. Database searching is a common strategy for protein identification where a spectrum is matched against a protein database. A score is given for each match between theoretical and acquired spectra, and peptides with high matching scores are used for protein identification [58].

Many protein identification software programs have been developed, such as Mascot, X!Tandem, SEQUEST, and OMSSA. From the open-source lineup, Skyline and MaxQuant are the most popular platforms for MS-based proteomics analysis. Skyline was designed for targeted proteomics studies. Meanwhile, MaxQuant is a quantitative proteomics software for mass spectrometry-based shotgun proteomics. Database matching is robust for already identified proteins but not for unknown proteins. This makes the number of identified proteins reliant on the availability of known proteins in corresponding databases, such as the UniProt and NCBI-nr protein sequence databases. The choice of proteolytic enzymes used in digestion also alters the identified peptide results in the database search.

With proteins identified from experiments, it is important to perform data interpretation and pathway analysis. General and kidney-specific protein expression databases are both useful in related research [59]. General protein expression or sequence databases include PRIDE (PRoteomics IDEntifications) [60], the Human Protein Atlas [61], and UniProt [62]. For kidney-specific proteome databases, HKUPP (Human Kidney and Urine Proteome Project) (http://www.hkupp.org/) and the Urinary Protein Biomarker Database are available [63]. ExoCarta is a compendium of exosomal cargoes and is devoted to covering exosomal proteins, RNAs, and lipids. The current ExoCarta database hosts 286 studies and records approximately 41,860 protein entries. This database also comes with the FunRich (Functional Enrichment Analysis Tool), which is useful for analyses of functional enrichment and interaction networks [64]. Other databases used for functional pathway analyses include the Database for Annotation, Visualization and Integrated Discovery (DAVID) [65] and Reactome [66].

To discover biomarkers for clinical diseases, a comparison of biomolecules among different groups or samples is usually conducted. A key issue for biomarkers from urine exosomes/sEVs is that the components and concentrations of urine are always variable due to many factors, such as fluid intake, time of urine collection, biological status of the individual, diet or medication, and even age. The isolation approach also results in variation, which requires normalization. Normalization approaches include evaluating the *relative* excretion rate or *absolute* excretion rate. The relative excretion rate can be calculated by dividing the abundance of biomarkers by the number of exosomes/sEVs or total proteins from exosomes/sEVs. Meanwhile, the absolute excretion rate refers to biomarkers in exosomes/sEVs excreted per unit of time, and the evaluation can be achieved through a timed collection [67]. A recent report showed that urine creatinine is highly correlated with urine EV concentrations, suggesting the reliability of using urine creatinine as a normalization factor for spot samples [68]. Normalization should be taken into account carefully during the study to achieve a more accurate investigation.

4. Proteomics-based studies of exosomes/sEVs for kidney diseases

In this section, we summarized isolation techniques used in the collected clinical studies, followed by categorizing these studies into acute kidney injury, chronic kidney disease, renal transplantation, congenital kidney disease, and malignant kidney disorder. We also summarized the related information of these studies in Table 1.

Urine is one of the common sample types for kidney disease studies, and among the screened-out articles, ultracentrifugation (UC) is the most

Table 1. Proteomics-based exosomal studies in kidney related diseases.

Disease	Sample type	Isolation technique	Characterization of exosome[a]	Analytical instrument	Potential finding/biomarkers	Ref.
Acute kidney injury (AKI)						
Acute kidney injury	Urine	Ultracentrifugation	WB	MALDI-TOF/TOF and LC-MS/MS	Increase of exosomal fetuin-A.	[15]
Vancomycin associated AKI	Urine	Polyethylene glycol (PEG)-induced precipitation	–	LC-MS/MS	• Increase of complement C3, complement C4, galectin-3-binding protein, fibrinogen, alpha-2 macroglobulin, immunoglobulin heavy constant mu, serotransferrin. • Inflammatory and coagulation pathways.	[75]
Acute kidney injury	Conditioned medium of primary human renal tubular cells	Sequential centrifugation	NTA, EM, WB	LC-MS/MS	Injecting exosomes from human renal tubular cells prevented most protein alterations during severe kidney ischemia in animal model.	[76]
Chronic kidney disease (CKD)						
IgA nephropathy (IgAN) vs Thin basement membrane nephropathy (TBMN)	Urine	Ultracentrifugation	WB, EM	nano LC-MS/MS	Comparing the two groups: • Increase of α-1-antitrypsin and ceruloplasmin for IgAN group. • Increase of aminopeptidase N and vasorin precursor for TBMN group.	[83]
Glomerular membrane disease	Urine	Density centrifugation	WB, EM, size distribution characterization	LC-MS/MS	Potential biomarkers from glomerular membranous vesicles: Nephrin, TRPC6, INF2, PLA2R, etc.	[13]

(*continued on next page*)

Table 1. (continued)

Disease	Sample type	Isolation technique	Characterization of exosome[a]	Analytical instrument	Potential finding/biomarkers	Ref.
Hypertensive nephropathy (albuminuria)	Urine	Ultracentrifugation	EM, Proteomic exosomal marker detection	LC-MS/MS	Pathway and proteins in response to hypertension and albuminuria: • Glycosaminoglycan degradation: Beta-galactosidase (GLB1), beta-glucuronidase (GUSB), N-acetyl-glucosamine-6-sulfatase (GNS), N-acetyl-galactosamine-6-sulfatase (GALNS), etc. • Coagulation/Complement system: Antithrombin III, protein C inhibitor, C4a, C4b, C3, etc. • Oxidative stress: Ceruloplasmin, transferrin myeloperoxidase (MPO), etc.	[85]
Diabetic nephropathy	Urine and kidney biopsy	Ultracentrifugation	EM, WB	MALDI-TOF MS	Downregulation of regucalcin.	[88]
Diabetic nephropathy	Urine	Ultracentrifugation	EM, WB	nano LC-MS/MS	• Increase of α-microglobulin/bikunin precursor (AMBP), histone-lysine N-methyltransferase (MLL3). • Downregulation of voltage-dependent anion-selective channel protein 1 (VDAC1).	[14]
Immunoglobulin light chain amyloidosis (AL)	Urine	Density gradient centrifugation	WB, Immuno-gold EM	nano LC-MS/MS	High molecular weight light chain oligomers were found for AL patients (active disease).	[90]
Immunoglobulin light chain amyloidosis (AL)	Urine	Density gradient centrifugation	WB	nano LC-MS/MS & MALDI-TOF MS	Two monoclonal lambda light chains were identified.	[91]
End-stage renal disease	Peritoneal dialysis effluent (PDE)	Size-exclusion chromatography (SEC)	Flow cytometry analyses (anti-CD9, anti-CD63)	nano LC-MS/MS	Enriched proteins were found in the PD patients with stable peritoneal membrane (PM) functions: Endoglin (ENG), Thy-1 membrane glycoprotein (THY-1 or CD90), biglycan (BGN), kininogen-1 (KNG1).	[94]
Renal transplantation						
Renal transplantation	Conditioned medium of human proximal tubular epithelial cells, serum (murine)	Ultracentrifugation	EM, Immunoblotting, Flow cytometry	nano-LC-MS/MS	Vascular injury enhanced the proteasome activity in apoptotic exosome-like vesicles which increased the production of renal damage-related autoantibodies (anti-LG3).	[96]

Acute T cell-mediated rejection in kidney transplant (TCMR)	Urine	Ultracentrifugation	EM, WB	nano-UPLC MS/MS	Apolipoprotein A1, Hemopexin, Polymeric immunoglobulin receptor, Lectin galactoside-binding soluble 3 binding protein, Tetraspanin-1 were potential diagnostic proteins.	[97]
Renal transplantation and medication	Urine	Size-exclusion chromatography (SEC)	Flow cytometry (CD9 and CD63)	LC-MS/MS	Proteins of uroplakin (UPK) and plakin families were significantly upregulated in the patients with calcineurin inhibitors toxicity.	[99]
Kidney related congenital diseases						
Autosomal dominant polycystic kidney disease (ADPKD)	Urine	Sucrose density gradient ultracentrifugation	EM, WB	Mass spectrometry	Polycystin−1 (PC-1), PC2, fibrocystin/polyductin (FCP), and their interacting proteins were identified. Cystin and ADP-ribosylation factor-like 6 were detected which were involved in cystic disease.	[102]
Autosomal dominant polycystic kidney disease (ADPKD)	Urine	Sucrose density gradient ultracentrifugation	EM, WB	nano-LC-MS/MS	Post-translational proteolytic processes were discovered for polycystin−1 (PC1), PC2, and fibrocystin in human urine exosome like vesicles.	[103]
Autosomal dominant polycystic kidney disease (ADPKD)	Urine	Sucrose density gradient ultracentrifugation	EM, WB	nano-LC-MS/MS	Ratios of exosomal polycystin-1/transmembrane protein 2 (PC1/TMEM2) or polycystin-2/transmembrane protein 2 (PC2/TMEM2) have potential to be indicators for polycystic kidney disease.	[104]
Autosomal dominant polycystic kidney disease (ADPKD)	Urine	Ultracentrifugation	WB	LC-MS/MS	Periplakin, envoplakin, villin-1, and complement C3 and C9 were highly expressed in exosomes from ADPKD patients.	[105]
Autosomal dominant polycystic kidney disease (ADPKD) & Medullary sponge kidney (MSK)	Urine	Ultracentrifugation	Dynamic light scattering, WB, Flow cytometry, ELISA	nano LC-MS/MS	• Higher level in ADPKD: Prominin 1 (CD133), cellular repressor of E1A stimulated genes 1 (CREG1), Inter-alpha-trypsin inhibitor heavy chain 5 (ITIH5), Guanylate cyclase activator 2B (GUCA2B), Myelin and lymphocyte protein (MAL). • Higher level in MSK: Osteopontin (SPP1).	[38]

(*continued on next page*)

Table 1. (continued)

Disease	Sample type	Isolation technique	Characterization of exosome[a]	Analytical instrument	Potential finding/biomarkers	Ref.
Medullary sponge kidney (MSK) disease	Urine	Ultracentrifugation	Dynamic light scattering, WB, ELISA	nano LC-MS/MS	Ficolin 1 (FCN1) and complement component 4-binding protein beta (C4BPB) were more abundant in the exosomes from MSK patients. Lectin complement pathway was pointed out to be associated with MSK.	[106]
Ureteropelvic junction obstruction (UPJO)	Amniotic fluid	Exosome extraction kit	EM, WB	LC-MS/MS	Amniotic fluid exosomes from patient with congenital UPJO had less angiotensin-converting enzyme (ACE) and aminopeptidase N (AP-N).	[108]
Galactosemia related kidney insufficiency	Urine	Differential centrifugation	WB	LC fractionation, MALDI-MS/MS	Premature protein dysglycosylation disrupts the renal basement membrane.	[109]
Cystinuria	Urine	Ultracentrifugation	EM, WB	nano LC-MS/MS,	Protein rBAT which was coded by the SLC3A1 gene, was detected only in healthy controls. The other 38 proteins which were overexpressed in patients with cystinuria were associated with kidney injury, circulating proteins, and neutrophil signature.	[111]
Kidney malignancy						
Renal cell carcinoma (RCC)	Urine	Differential centrifugation	EM, WB, exosome size characterization	LC-MS/MS	Exosomal proteins from RCC patients were different from healthy controls: Matrix metalloproteinase 9 (MMP-9), Ceruloplasmin (CP), Podocalyxin (PODXL), Dickkopf related protein 4 (DKK4), and Carbonic Anhydrase IX (CAIX).	[113]
Kidney cancer and Chronic kidney disease (CKD)	Plasma	Chemical affinity purification (amphiphilic beads)	EM, NTA, Tunable resistive pulse sensing, WB	nano LC-MS/MS,	The cardiomyopathy-associated protein 5 (CMYA5), phosphorylated Crk-like protein (CRKL), and LYRIC (MTDH) were higher in the kidney cancer group. The increase of apolipoprotein A-IV (APOA4) was found for CKD.	[20]

[a] Some studies provided concise methods or the characterizations were referred to their previously published work. WB: Western blot; EM: Electron microscopy; NTA: nanoparticle tracking analysis.

commonly used method to purify uEVs. However, the recovery yield needs to be evaluated carefully for UC to prevent the loss of important proteins [69]. Other isolation methods include sucrose density gradient ultracentrifugation, differential centrifugation, size-exclusion chromatography, polyethylene glycol (PEG)-induced precipitation, and exosome extraction kits were used in clinical studies as well (Table 1).

4.1. Acute kidney injury (AKI)

AKI is characterized by swift escalation of serum creatinine, an accelerated reduction in urine output, or a combination of these two conditions. AKI was mainly caused by the mismatch of oxygen/nutrient delivery and nephron energy demand [70]. Common causes include cardiorenal syndrome, shock, transplantation, sepsis, drug-related necrosis, and urinary tract obstruction, which cause impaired perfusion [71]. Novel AKI biomarkers, namely, tissue inhibitor of metalloproteinase-2, neutrophil gelatinase-associated lipocalin, kidney injury marker-1, and cystatin C, may provide earlier diagnostic and prognostic value. However, more investigations are needed to define the clinical roles of these biomarkers [72].

Many drugs have been reported to cause AKI, including antimicrobial agents, NSAIDs, herbal medicines, chemotherapeutic agents, and checkpoint inhibitors [73]. Proteomics analyses of urinary exosomes/sEVs have been applied to discover medication-induced AKI biomarkers such as cisplatin and vancomycin. Renal tubular cells are vulnerable to the toxic effects of medications. Dr. Star et al. found that fetuin-A was increased in urinary exosomes/sEVs in both an animal model and patients with cisplatin-induced AKI. The exact functions of fetuin-A in AKI remain elusive; however, it was hypothesized that exosomes/sEVs containing fetuin-A may be secreted from damaged proximal tubule cells [15]. Increased urinary ATF3 (activating transcription factor 3) protein was reported in acute kidney injury by cisplatin or ischemia-reperfusion injury [74]. The increase in ATF3 in urinary exosomes/sEVs precedes serum creatinine elevation and may serve as an early biomarker. Therefore, urinary exosomal fetuin-A and ATF3 might serve as indicators of kidney tubular injury. In the case of vancomycin-related (V)-AKI, complement C3 and C4 are significantly increased in urinary exosomes/sEVs. According to the results, inflammation and the complement system might play important roles in vancomycin-related kidney injury [75].

In addition to research on biomarkers, the application of exosomes/sEVs in AKI therapy also was evaluated. In a hypoxic AKI model of athymic nude rats, injections of exosomes/sEVs originating from harvested human kidneys prevented apoptosis of kidney cells and preserved the microvascular and renal tubular structures. These results suggested that exosomes/sEVs might act as therapeutic agents in kidney diseases [76].

4.2. Chronic kidney disease (CKD)

CKD is a pathological condition characterized by the gradual loss of normal kidney functions and kidney structural changes. While this medical condition slowly progresses, the damage is irreversible. The disease prevalence is approximately 10%–13% of the population and is associated with increased cardiovascular risks [77]. Currently, CKD is defined by a glomerular filtration rate (GFR) of <60 mL/min/1.73 m^2, microalbuminuria, or an indication of kidney failure [78]. Glomerulonephritis, hypertension, and diabetes are common causes of CKD.

4.2.1. Glomerulonephritis

Glomerulonephritis (GN) refers to inflammation of glomeruli in response to infections, malignancies, drugs, toxins, or autoimmune diseases [79]. Primary GN is kidney oriented without accompanying conditions, such as idiopathic nephrotic syndrome, thin basement membrane nephropathy (TBMN), IgA nephropathy (IgAN), Alport syndrome, and membranous nephropathy. Secondary GN is more related to systemic diseases such as lupus nephritis, diabetic nephropathy, and postinfectious GN [80]. Glomerular injuries can disturb the glomerular filtration barrier and result in a decreased glomerular filtration rate (GFR), hematuria, and proteinuria [81]. Currently, the gold standard for diagnosing glomerulonephritis is kidney biopsy, which is invasive and inconvenient. Therefore, novel biomarkers would substantially improve the diagnosis and therapy of this disease [82]. Isolated microscopic hematuria is mostly due to Alport syndrome, immunoglobulin A nephropathy (IgAN), and thin basement membrane nephropathy (TBMN). Although IgAN and TBMN have identical clinical presentations, patients with IgAN develop worse clinical outcomes than those with TBMN. Currently, there are no diagnostic biomarkers, and a renal biopsy is the only diagnostic tool for differentiating IgAN from TBMN. From the proteomics profiling of urinary exosomes/sEVs, aminopeptidase N, vasorin precursor, α-1-antitrypsin, and ceruloplasmin were shown to be potential biomarkers to differentiate

early IgAN from TBMN. IgAN was found to have higher α-1-antitrypsin, which could induce fibrinolysis in response to hematuria, and the increase in ceruloplasmin is an indication of mesangial inflammation. The TBMN group with higher aminopeptidase N is capable of modulating MAPK pathways, galectin (Lgals) molecules, Fc receptors, and immune regulators. The higher vasorin precursor was reported to be associated with the vessel repair process [83].

Dr. Hogan et al. isolated glomerular membrane vesicles (GMVs) from human urine samples and applied proteomics analysis for biomolecule investigation. In the GMV-enriched samples from 3 patients with glomerular membrane diseases, 5657 unique proteins were identified. Some of them were associated with nephrotic syndrome, including nephrin, short transient receptor potential channel 6 (TRPC6), inverted formin 2 (INF2), and secretory phospholipase A2 receptor (PLA2R). Although these identified proteins require further investigation as clinical biomarkers, the results of this study showed that GMVs have the potential to be used for biomarker discovery [13].

4.2.2. Hypertensive nephropathy and diabetic nephropathy

The relationship between hypertension and chronic kidney disease (CKD) is a reciprocal causation. Hypertension can result in renal impairment, and CKD can contribute to the development of secondary hypertension [84]. The renin-angiotensin system (RAS) plays an important role in blood pressure regulation. RAS suppression is usually found in hypertensive patients. However, even with the presence of RAS suppression, albuminuria occurs in some cases in which the mechanism is unclear. Dr. Gonzalez-Calero and Ruilope et al. investigated urinary exosomes/sEVs from patients with normoalbuminuria, *de novo* albuminuria, and maintained albuminuria. In their study, three functional clusters, including glycosaminoglycan degradation, the coagulation/complement system, and oxidative stress, were identified [85]. The hit proteins and mapped pathways were mostly related to glomerular basement membrane (GBM) disruption and proinflammatory signs.

Diabetic nephropathy (DN), also known as diabetic kidney disease, is characterized by perpetual albuminuria and a gradual diminution of normal renal functions. DN occurs in 20%–50% of patients with diabetes mellitus (DM), and disease progression arises in both type 1 and 2 DM. Since DN silently progresses and shows no obvious clinical symptoms in the early stages, a potential biomarker of this disease needs to be discovered. The current noninvasive marker is microalbuminuria; however, this marker is not very sensitive and cannot predict the prognosis [86,87]. Dr. Alvarez-Llamas et al. found that the expression of regucalcin was reduced in human kidney tissues and in urinary exosomes/sEVs from DN patients. Regucalcin plays essential roles in regulating intracellular calcium transport, cell signaling, DNA synthesis, gene expression, proliferation, and apoptosis in kidney cells. Since urine is an easily accessible and noninvasive biofluid, regucalcin of urinary exosomes/sEVs might be a novel target for the early diagnosis of DN [88]. In addition to regucalcin, α-microglobulin/bikunin precursor (AMBP) and histone-lysine N-methyltransferase (MLL3) were found at higher levels in DN patients. AMBP is a membrane glycoprotein with serine protease inhibitor activity, and MLL3 is a histone methyltransferase. In contrast, voltage-dependent anion-selective channel protein 1 (VDAC1) was decreased in DN patient urinary exosomes/sEVs. It is a transmembrane channel that plays a crucial role in regulating cell survival, growth, and death [14].

4.2.3. Amyloidosis nephropathy

Amyloidosis is a rare disease characterized by misfolded amyloid deposition in particular tissues, and it can lead to organ failure. Kidney tissues are one of the main sites of amyloid deposition [89]. Dr. Ramirez-Alvarado et al. investigated the proteomic profiling of urinary exosomes/sEVs from AL patients and revealed enormous numbers of free light-chain immunoglobulins. In addition, high-molecular-weight oligomers (light chain decamers) were specifically found in patients with light-chain amyloidosis [90]. They further analyzed proteins of urinary exosomes/sEVs by MS and identified two monoclonal lambda light chains that could not be detected by current clinical methods. Immunoglobulin light-chain oligomers in urinary exosomes/sEVs were correlated with the formation of amyloid and might predict the prognosis of immunoglobulin light-chain (AL) amyloidosis [91].

4.2.4. End-stage renal disease (ESRD)

ESRD is referred to as the final stage of chronic kidney disease. To define ESRD, uremia must be present, and the patient needs renal replacement therapy. Alternatively, ESRD might be defined as a condition with an estimated glomerular filtration rate (eGFR) < 15 mL/min/1.73 m^2 [92]. Hemodialysis and peritoneal dialysis are two treatment options for ESRD patients. Peritoneal dialysis effluent (PDE) contains a diverse variety of cells and

secretomes that reflect the pathophysiological state of patients, and exosomes/sEVs can be isolated from PDE [93]. A longitudinal study of peritoneal dialysis patients was conducted by Dr. Troya-Saborido and Dr. Borràs et al. to compare peritoneal dialysis effluent containing extracellular vesicle (PDE-EV) proteomes related to the stability of peritoneal membrane (PM) functions. The results revealed that PDE-EVs of patients with stable PM functions were enriched in several proteins, including endoglin (ENG), Thy-1 membrane glycoprotein (THY-1 or CD90), biglycan (BGN), and kininogen-1 (KNG1). These proteins were found to be associated with the ultrafiltration capacity and the functional status of PM [94].

4.3. Renal transplantation

Large-scale analysis of urinary exosomes/sEVs from renal allografts has been used to discover biomarkers of graft rejection or tubular injury [95]. Vascular injury enhanced proteasome activity in apoptotic exosome-like vesicles, which further increased the production of renal damage-related autoantibodies, such as anti-LG3 IgG. The increase in anti-LG3 is known to be correlated with an increased risk of rejection post-transplantation [96]. In another report, tetraspanin-1 (TSPAN1), hemopexin (HPX), and the other 3 proteins were significantly increased in urine exosomes/sEVs from patients with acute T cell-mediated rejection (TCMR) compared to those with stable graft function. The results revealed that exosome proteins could be potential markers for TCMR [97].

A proteomics study of urine exosomes/sEVs also was conducted to discover biomarkers of immunosuppressant toxicity for renal transplant recipients. It was found that the use of calcineurin inhibitors (CNIs) might lead to nephrotoxicity (CNI toxicity, CNIT) [98]. There were differences in proteomic profiles among renal transplant recipients with normal kidney function, the CNIT group, and the interstitial fibrosis/tubular atrophy (IFTA) group. Proteins of the uroplakin (UPK) and plakin families, including UPK1A, UPK1B, UPK2, UPK3A, envoplakin (EVPL), and periplakin (PPL), were significantly upregulated in the CNIT group, which might have an important role in CNIT processes [99].

4.4. Congenital kidney diseases

Autosomal dominant polycystic kidney disease (ADPKD) is an inherited genetic disorder characterized by numerous cysts and enormous bilateral kidney enlargement. Exosomes/sEVs from ADPKD urine samples can potentially serve as a noninvasive disease biomarker [100,101]. Dr. Ward et al. successfully identified polycystin-1 (PC1), PC2, fibrocystin/polyductin (FCP), and their interacting proteins from urinary exosome-like vesicles of ADPKD patients [102]. Posttranslational proteolytic processes also were found for PC1, PC2, and FCP proteins by studying urinary exosome-like vesicles that affect the progression of cystic disease [103]. In addition, PC1 and PC2 were found to be lower in urinary vesicles from patients with PKD1 mutations, and transmembrane protein 2 (TMEM2) was found to have higher levels. The PC1/TMEM2 or PC2/TEME2 ratios were indicated as potential indicators for ADPKD [104]. Comparing the exosomal proteins between patients with ADPKD and medullary sponge kidney (MSK), Dr. Zaza et al. found higher expression of prominin-1 (CD133), cellular repressor of E1A-stimulated genes 1 (CREG1), interalpha-trypsin inhibitor heavy chain 5 (ITIH5), and guanylate cyclase activator 2B (GUCA2B) in ADPKD patients [38]. In addition, complement C3, C9, plakins, and villin-1 were highly expressed in urinary exosomes/sEVs from ADPKD patients compared to healthy controls [105].

Medullary sponge kidney (MSK) disease is a rare kidney disease characterized by cystic anomalies in the precalyceal ducts and nephrocalcinosis. By analyzing the proteomics profiles of urine exosomes/sEVs, Dr. Zaza and the team found that the lectin complement pathway was associated with the pathogenesis of the disease. Ficolin 1 (FCN1), mannan-binding lectin-associated serine protease-2 (MASP2), and complement component 4-binding protein beta (C4BPB) are three proteins that can be used to discriminate the MSK group and the group of patients with idiopathic calcium nephrolithiasis (ICN) [106].

Ureteropelvic junction obstruction (UPJO) is an obstruction of the urinary tract that results in reduced urine flow from the renal pelvis into the ureter. Antenatal ultrasound can be used to detect congenital UPJO during the second trimester [107]. Dr. Wei et al. found that exosomes/sEVs from the amniotic fluid of patients with congenital UPJO have significant alterations in the protein profiles in terms of decreases in angiotensin-converting enzyme (ACE) and aminopeptidase N (AP-N). The biological effects of ACE and AP-N reduction were studied *in vitro* and were discovered to be associated with the curb of renal tubular epithelial cell proliferation, overproduction of reactive oxygen species, and enhanced inflammation [108].

Galactosemia is caused by a deficiency of galactose-1-phosphate uridyltransferase due to a mutation of the GALT gene, which causes severe defects in galactose metabolism. The proteomic profile from urinary exosomes/sEVs depicts alterations related to renal failure, indicating drastic increases in abundant serum glycoproteins, including leucine-rich α-2-glycoprotein, albumin, fetuin, prostaglandin H2 D-isomerase, α-1-microglobulin protein, and immunoglobulins. It was hypothesized that renal failure might occur due to premature protein dysglycosylation, which interferes with the formation of the renal basement membrane. However, markers from urinary exosomes/sEVs of galactosemia patients overlap with urinary protein markers for IgA nephropathy, thin basement membrane nephropathy, and acute kidney injury, making it difficult to differentiate proteomic profiles across these diseases [109].

Cystinuria is an autosomal recessive disorder with defects in the *SLC3A1* and *SLC7A9* genes [110]. The results of the exosomal proteomics study showed that the rBAT protein, encoded by the *SLC3A1* gene, was detected only in healthy individuals. Meanwhile, 38 proteins were overexpressed in the cystinuria groups. The results suggested that one-quarter of overexpressed proteins in cystinuria patients were neutrophil-derived, which might indicate intrarenal inflammatory reactions due to microscopic crystal formation [111].

4.5. Kidney malignancies

The roles of exosomes/sEVs in malignancies have been extensively studied; however, the majority of exosome studies of renal cell carcinoma (RCC) have focused on miRNAs [112]. Proteomic profiling of urinary exosomes/sEVs from RCC patients showed distinct protein profiles compared to urinary exosomes/sEVs from healthy controls. The urinary exosomal proteomic analysis of RCC patients showed enhanced expression of podocalyxin, ceruloplasmin, matrix metalloproteinase 9, carbonic anhydrase IX, and dickkopf-related protein 4. In contrast, the extracellular matrix metalloproteinase inducers aquaporin-1, dipeptidase-1, syntenin-1, and neprilysin (CD10) exhibited significantly reduced expression. From protein profiling and validation, urinary exosomes/sEVs manifested some portions of cancer protein profiles and might be involved in RCC progression [113]. Plasma EVs purified by using chemical affinity beads were also investigated for kidney cancer. In the study, cardiomyopathy-associated protein 5 (CMYA5), phosphorylated Crk-like protein (CRKL), and phosphorylated LYRIC (MTDH) were found to be higher in the kidney cancer group [20].

4.6. Other biological findings from uEVs for kidney function and disease

Recently, a large-scale animal study showed that the abundances of proteins in urine EVs (uEVs) are highly correlated with proteins in respective kidney tissues. Positive correlations were found for approximately 1000 proteins from uEVs and kidney tissues, which reveals that investigating uEV proteins is able to monitor particular (patho) physiological changes even without using renal biopsy [114]. More clinical applications can be found when using uEV or exosome as the keyword in combination with specific kidney diseases. For example, Gonzales et al. investigated proteomics and phosphoproteomics for human urine exosomes. They successfully identified 1132 unambiguous proteins, including solute and water transporters, vacuolar H^+-ATPase subunits, and 34 proteins related to renal diseases. Through phosphoproteomics investigation, serine-256-phosphorylated AQP2 in uEVs was identified as a potential marker for monitoring the state of vasopressin activation. The absence of sodium-potassium-chloride cotransporter 2 (NKCC2) in urine exosomes from patients with Bartter syndrome was also reported in this study [115]. In addition, Raimondo et al. investigated the proteome of uEVs and set up a panel of 5 proteins, including carbonic anhydrase (CA2), vacuolar-type ATPase B subunit 1 (VATB1), Annexin A2 (ANXA2), sodium chloride cotransporter (NCC), and NKCC2, which can differentiate Gitelman and Bartter syndromes (salt-losing tubulopathies) [116]. Sung et al. also found that NCC and phosphorylated-NCC were decreased in uEVs from patients with Gitelman syndrome, while Na^+-hydrogen exchanger 3 (NHE3), epithelial Na^+ channel β (ENaCβ), and pendrin were increased [117]. By investigating urinary microvesicles, Rood et al. found the increase of lysosome membrane protein 2 (LIMP-2) from patients with idiopathic membranous nephropathy, and the upregulation of LIMP-2 was identified in renal biopsies that the protein was co-localized with IgG along the glomerular basement membrane [118]. All the studies showed the potential of investigating uEVs for kidney disease research.

5. Summary and perspective

For kidney-related diseases, urinary exosomes/sEVs serve as a noninvasive biopsy and provide bulk information on kidney-related diseases. Some studies also investigated plasma exosomes/sEVs or those from primary culture conditioned medium for pathological and biomarker discovery. Despite the diverse methods of urinary exosome/sEV isolation, ultracentrifugation was currently found to be the most popular tool. Characterization based on the representative protein markers, size distribution, and morphology observation is required to ensure the precision of downstream analyses. With the necessity and the emergence of novel techniques, exosomal proteomic analyses of clinical samples will enable the elucidation of disease-related molecular mechanisms and enhance our understanding of kidney-related diseases.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgments

The authors acknowledge the academic and science graphic illustration service provided by the TMU Office of Research and Development. This work was financially supported by the Higher Education Sprout Project of the Ministry of Education (MOE) in Taiwan (DP2-110-21121-01-T-03-03).

References

[1] Kalluri R, LeBleu VS. The biology, function, and biomedical applications of exosomes. Science 2020;367:1–15.
[2] Trams EG, Lauter CJ, Norman Jr S, Heine U. Exfoliation of membrane ecto-enzymes in the form of micro-vesicles. Biochim Biophys Acta Biomembr 1981;645:63–70.
[3] Pan BT, Johnstone RM. Fate of the transferrin receptor during maturation of sheep reticulocytes *in vitro:* Selective externalization of the receptor. Cell 1983;33: 967–78.
[4] Harding C, Heuser J, Stahl P. Receptor-mediated endocytosis of transferrin and recycling of the transferrin receptor in rat reticulocytes. J Cell Biol 1983;97:329–39.
[5] Kassis S, Lauter CJ, Stojanov M, Salem Jr N. Exfoliation of the beta-adrenergic receptor and the regulatory components of adenylate cyclase by cultured rat glioma C6 cells. Biochim Biophys Acta 1986;886:474–82.
[6] Johnstone RM, Adam M, Hammond JR, Orr L, Turbide C. Vesicle formation during reticulocyte maturation. Association of plasma membrane activities with released vesicles (exosomes). J Biol Chem 1987;262:9412–20.
[7] Naslavsky N, Caplan S. The enigmatic endosome – sorting the ins and outs of endocytic trafficking. J Cell Sci 2018;131: 1–14.
[8] Klumperman J, Raposo G. The complex ultrastructure of the endolysosomal system. Cold Spring Harbor Perspect Biol 2014;6:1–22.
[9] Théry C, Witwer KW, Aikawa E, Alcaraz MJ, Anderson JD, Andriantsitohaina R, et al. Minimal information for studies of extracellular vesicles 2018 (MISEV2018): A position statement of the International Society for Extracellular Vesicles and update of the MISEV2014 guidelines. J Extracell Vesicles 2018;7:1–43.
[10] Kang YT, Purcell E, Palacios Rolston C, Lo TW, Ramnath N, Jolly S, et al. Isolation and profiling of circulating tumor-associated exosomes using extracellular vesicular lipid–protein binding affinity based microfluidic device. Small 2019;15:1–14.
[11] Stranska R, Gysbrechts L, Wouters J, Vermeersch P, Bloch K, Dierickx D, et al. Comparison of membrane affinity-based method with size-exclusion chromatography for isolation of exosome-like vesicles from human plasma. J Transl Med 2018;16:1–9.
[12] Zubiri I, Vivanco F, Alvarez-Llamas G. Proteomic analysis of urinary exosomes in cardiovascular and associated kidney diseases by two-dimensional electrophoresis and LC-MS/MS. Methods Mol Biol 2013;1000:209–20.
[13] Hogan MC, Johnson KL, Zenka RM, Charlesworth MC, Madden BJ, Mahoney DW, et al. Subfractionation, characterization, and in-depth proteomic analysis of glomerular membrane vesicles in human urine. Kidney Int 2014;85: 1225–37.
[14] Zubiri I, Posada-Ayala M, Sanz-Maroto A, Calvo E, Martin-Lorenzo M, Gonzalez-Calero L, et al. Diabetic nephropathy induces changes in the proteome of human urinary exosomes as revealed by label-free comparative analysis. J Proteonomics 2014;96:92–102.
[15] Zhou H, Pisitkun T, Aponte A, Yuen PST, Hoffert JD, Yasuda H, et al. Exosomal Fetuin-A identified by proteomics: A novel urinary biomarker for detecting acute kidney injury. Kidney Int 2006;70:1847–57.
[16] Hiemstra TF, Charles PD, Gracia T, Hester SS, Gatto L, Al-Lamki R, et al. Human urinary exosomes as innate immune effectors. J Am Soc Nephrol 2014;25:2017–27.
[17] Hiemstra TF, Charles PD, Hester SS, Karet FE, Lilley KS. Uromodulin exclusion list improves urinary exosomal protein identification. J Biomol Tech 2011;22:136–45.
[18] Witwer KW, Buzás EI, Bemis LT, Bora A, Lässer C, Lötvall J, et al. Standardization of sample collection, isolation and analysis methods in extracellular vesicle research. J Extracell Vesicles 2013;2:1–25.
[19] Lobb RJ, Becker M, Wen Wen S, Wong CSF, Wiegmans AP, Leimgruber A, et al. Optimized exosome isolation protocol for cell culture supernatant and human plasma. J Extracell Vesicles 2015;4:1–11.
[20] Iliuk A, Wu X, Li L, Sun J, Hadisurya M, Boris RS, et al. Plasma-derived extracellular vesicle phosphoproteomics through chemical affinity purification. J Proteome Res 2020; 19:2563–74.
[21] Gardiner C, Vizio DD, Sahoo S, Théry C, Witwer KW, Wauben M, et al. Techniques used for the isolation and characterization of extracellular vesicles: Results of a worldwide survey. J Extracell Vesicles 2016;5:1–6.
[22] Sidhom K, Obi PO, Saleem A. A review of exosomal isolation methods: Is size-exclusion chromatography the best option? Int J Mol Sci 2020;21:1–19.
[23] Brennan K, Martin K, FitzGerald SP, O'Sullivan J, Wu Y, Blanco A, et al. A comparison of methods for the isolation and separation of extracellular vesicles from protein and lipid particles in human serum. Sci Rep 2020;10:1–13.
[24] Chen BY, Sung CWH, Chen C, Cheng CM, Lin DPC, Huang CT, et al. Advances in exosomes technology. Clin Chim Acta 2019;493:14–9.
[25] Guan S, Yu H, Yan G, Gao M, Sun W, Zhang X. Characterization of urinary exosomes purified with size-exclusion chromatography and ultracentrifugation. J Proteome Res 2020;19:2217–25.
[26] Guerreiro EM, Vestad B, Steffensen LA, Aass HCD, Saeed M, Øvstebø R, et al. Efficient extracellular vesicle isolation by combining cell media modifications, ultrafiltration, and size-exclusion chromatography. PLoS One 2018;13:1–17.

[27] Böing AN, van der Pol E, Grootemaat AE, Coumans FAW, Sturk A, Nieuwland R. Single-step isolation of extracellular vesicles by size-exclusion chromatography. J Extracell Vesicles 2014;3:1−11.
[28] Pedersen KW, Kierulf B, Neurauter A. Specific and generic isolation of extracellular vesicles with magnetic beads. In: Kuo WP, Jia S, editors. Extracellular vesicles: Methods and protocols. New York: Springer; 2017. p. 65−87.
[29] Nakai W, Yoshida T, Diez D, Miyatake Y, Nishibu T, Imawaka N, et al. A novel affinity-based method for the isolation of highly purified extracellular vesicles. Sci Rep 2016;6:1−11.
[30] Ishida T, Hashimoto T, Masaki K, Funabashi H, Hirota R, Ikeda T, et al. Application of peptides with an affinity for phospholipid membranes during the automated - purification of extracellular vesicles. Sci Rep 2020;10:1−12.
[31] Zhang H, Freitas D, Kim HS, Fabijanic K, Li Z, Chen H, et al. Identification of distinct nanoparticles and subsets of extracellular vesicles by asymmetric flow field-flow fractionation. Nat Cell Biol 2018;20:332−43.
[32] Rood IM, Deegens JK, Merchant ML, Tamboer WP, Wilkey DW, Wetzels JF, et al. Comparison of three methods for isolation of urinary microvesicles to identify biomarkers of nephrotic syndrome. Kidney Int 2010;78:810−6.
[33] Chuo STY, Chien JCY, Lai CPK. Imaging extracellular vesicles: Current and emerging methods. J Biomed Sci 2018; 25:1−10.
[34] Wu Y, Deng W, Klinke DJ, II. Exosomes: Improved methods to characterize their morphology, RNA content, and surface protein biomarkers. Analyst 2015;140:6631−42.
[35] Musante L, Bontha SV, La Salvia S, Fernandez-Piñeros A, Lannigan J, Le TH, et al. Rigorous characterization of urinary extracellular vesicles (uEVs) in the low centrifugation pellet - a neglected source for uEVs. Sci Rep 2020;10:1−14.
[36] Jung MK, Mun JY. Sample preparation and imaging of exosomes by transmission electron microscopy. JoVE 2018: 1−5.
[37] Zhang M, Jin K, Gao L, Zhang Z, Li F, Zhou F, et al. Methods and technologies for exosome isolation and characterization. Small Methods 2018;2:1−10.
[38] Bruschi M, Granata S, Santucci L, Candiano G, Fabris A, Antonucci N, et al. Proteomic analysis of urinary microvesicles and exosomes in medullary sponge kidney disease and autosomal dominant polycystic kidney disease. Clin J Am Soc Nephrol : CJASN 2019;14:834−43.
[39] Soares Martins T, Catita J, Martins Rosa I, da Cruz e Silva AB, Henriques AG. Exosome isolation from distinct biofluids using precipitation and column-based approaches. PLoS One 2018;13:1−16.
[40] Rosa-Fernandes L, Rocha VB, Carregari VC, Urbani A, Palmisano G. A perspective on extracellular vesicles proteomics. Front Chem 2017;5:1−19.
[41] Rogers JC, Bomgarden RD. Sample preparation for mass spectrometry-based proteomics; from proteomes to peptides. In: Mirzaei H, Carrasco M, editors. Modern proteomics−sample preparation, analysis and practical applications. Cham: Springer International Publishing; 2016. p. 43−62.
[42] Suder P, Novák P, Havlíček V, Bodzoń-Kułakowska A. General strategies for proteomic sample preparation. In: Ciborowski P, Silberring J, editors. Proteomic profiling and analytical chemistry. 2nd ed. Boston: Elsevier; 2016. p. 25−49.
[43] Li J, He X, Deng Y, Yang C. An update on isolation methods for proteomic studies of extracellular vesicles in biofluids. Molecules 2019;24:1−24.
[44] Wiśniewski JR, Zougman A, Nagaraj N, Mann M. Universal sample preparation method for proteome analysis. Nat Methods 2009;6:359−62.
[45] Wiśniewski JR. Chapter two- Filter-aided sample preparation: The versatile and efficient method for proteomic analysis. In: Shukla AK, editor. Methods enzymol. Vols. 585. New york. Academic Press; 2017. p. 15−27.
[46] Tremblay T-L, Hill JJ. Adding polyvinylpyrrolidone to low level protein samples significantly improves peptide recovery in FASP digests: An inexpensive and simple modification to the FASP protocol. J Proteonomics 2021;230: 104000.
[47] Doellinger J, Schneider A, Hoeller M, Lasch P. Sample preparation by easy extraction and digestion (SPEED)-A universal, rapid, and detergent-free protocol for proteomics based on acid extraction. Mol Cell Proteomics 2020;19: 209−22.
[48] Guo X, Trudgian DC, Lemoff A, Yadavalli S, Mirzaei H. Confetti: A multiprotease map of the HeLa proteome for comprehensive proteomics. Mol Cell Proteomics : MCP 2014;13:1573−84.
[49] Giansanti P, Tsiatsiani L, Low TY, Heck AJR. Six alternative proteases for mass spectrometry−based proteomics beyond trypsin. Nat Protoc 2016;11:993−1006.
[50] Swaney DL, Wenger CD, Coon JJ. Value of using multiple proteases for large-scale mass spectrometry-based proteomics. J Proteome Res 2010;9:1323−9.
[51] Matthiesen R, Bunkenborg J. Introduction to mass spectrometry-based proteomics. In: Matthiesen R, editor. Mass spectrometry data analysis in proteomics. Totowa: Humana Press; 2013. p. 1−45.
[52] Gillet LC, Leitner A, Aebersold R. Mass spectrometry applied to bottom-up proteomics: Entering the high-throughput era for hypothesis testing. Annu Rev Anal Chem 2016;9:449−72.
[53] Wilson SR, Vehus T, Berg HS, Lundanes E. Nano-LC in proteomics: Recent advances and approaches. Bioanalysis 2015;7:1799−815.
[54] Yates JR, Ruse CI, Nakorchevsky A. Proteomics by mass spectrometry: Approaches, advances, and applications. Annu Rev Biomed Eng 2009;11:49−79.
[55] Guan S, Taylor PP, Han Z, Moran MF, Ma B. Data dependent−independent acquisition (DDIA) proteomics. J Proteome Res 2020;19:3230−7.
[56] Hu A, Noble WS, Wolf-Yadlin A. Technical advances in proteomics: New developments in data-independent acquisition. F1000Res 2016;5:1−12.
[57] Fernández-Costa C, Martínez-Bartolomé S, McClatchy DB, Saviola AJ, Yu NK, Yates JR. Impact of the identification strategy on the reproducibility of the DDA and DIA results. J Proteome Res 2020;19:3153−61.
[58] Suna G, Mayr M. Proteomics. In: Vasan RS, Sawyer DB, editors. Encyclopedia of cardiovascular research and medicine. Oxford: Elsevier; 2018. p. 166−80.
[59] Papadopoulos T, Krochmal M, Cisek K, Fernandes M, Husi H, Stevens R, et al. Omics databases on kidney disease: Where they can be found and how to benefit from them. Clin Kidney J 2016;9:343−52.
[60] Perez-Riverol Y, Csordas A, Bai J, Bernal-Llinares M, Hewapathirana S, Kundu DJ, et al. The PRIDE database and related tools and resources in 2019: improving support for quantification data. Nucleic Acids Res 2019;47: 442−50.
[61] Thul PJ, Åkesson L, Wiking M, Mahdessian D, Geladaki A, Ait Blal H, et al. A subcellular map of the human proteome. Science 2017;356:1−22.
[62] The UniProt C. UniProt: The universal protein knowledgebase in 2021. Nucleic Acids Res 2021;49:D480−9.
[63] Shao C. Urinary protein biomarker database 2.0: A literature-curated database for protein biomarkers in urine. In: Gao Y, editor. Urine: promising biomarker source for early disease detection. Singapore: Springer Singapore; 2019. p. 65−72.
[64] Keerthikumar S, Chisanga D, Ariyaratne D, Al Saffar H, Anand S, Zhao K, et al. ExoCarta: A web-based compendium of exosomal cargo. J Mol Biol 2016;428:688−92.
[65] Huang DW, Sherman BT, Lempicki RA. Bioinformatics enrichment tools: Paths toward the comprehensive functional analysis of large gene lists. Nucleic Acids Res 2009;37: 1−13.

[66] Jassal B, Matthews L, Viteri G, Gong C, Lorente P, Fabregat A, et al. The reactome pathway knowledgebase. Nucleic Acids Res 2020;48:D498–503.
[67] Erdbrügger U, Blijdorp CJ, Bijnsdorp IV, Borràs FE, Burger D, Bussolati B, et al. Urinary extracellular vesicles: A position paper by the urine task force of the international society for extracellular vesicles. J Extracell Vesicles 2021;10: e12093.
[68] Blijdorp CJ, Tutakhel OAZ, Hartjes TA, van den Bosch TPP, van Heugten MH, Rigalli JP, et al. Comparing approaches to normalize, quantify, and characterize urinary extracellular vesicles. J Am Soc Nephrol 2021;32:1210–26.
[69] Musante L, Saraswat M, Ravidà A, Byrne B, Holthofer H. Recovery of urinary nanovesicles from ultracentrifugation supernatants. Nephrol Dial Transplant 2013;28:1425–33.
[70] Makris K, Spanou L. Acute kidney injury: Definition, pathophysiology and clinical phenotypes. Clin Biochem Rev 2016;37:85–98.
[71] Kellum JA, Romagnani P, Ashuntantang G, Ronco C, Zarbock A, Anders HJ. Acute kidney injury. Nat Rev Dis Prim 2021;7:52.
[72] Ronco C, Bellomo R, Kellum JA. Acute kidney injury. Lancet 2019;394:1949–64.
[73] Sales GTM, Foresto RD. Drug-induced nephrotoxicity. 2020 Rev Assoc Med Bras 1992;66(Suppl 1):s82–90.
[74] Zhou H, Cheruvanky A, Hu X, Matsumoto T, Hiramatsu N, Cho ME, et al. Urinary exosomal transcription factors, a new class of biomarkers for renal disease. Kidney Int 2008; 74:613–21.
[75] Awdishu L, Le A, Amato J, Jani V, Bal S, Mills RH, et al. Urinary exosomes identify inflammatory pathways in vancomycin associated acute kidney injury. Int J Mol Sci 2021: 22.
[76] Dominguez 2nd JM, Dominguez JH, Xie D, Kelly KJ. Human extracellular microvesicles from renal tubules reverse kidney ischemia-reperfusion injury in rats. PLoS One 2018;13:1–16.
[77] Ammirati AL. Chronic kidney disease. Rev Assoc Med Bras (1992) 2020;66:s3–9.
[78] Glassock RJ, Warnock DG, Delanaye P. The global burden of chronic kidney disease: Estimates, variability and pitfalls. Nat Rev Nephrol 2017;13:104–14.
[79] Madaio MP, Harrington JT. The diagnosis of glomerular diseases: Acute glomerulonephritis and the nephrotic syndrome. Arch Intern Med 2001;161:25–34.
[80] Khanna R. Clinical presentation & management of glomerular diseases: Hematuria, nephritic & nephrotic syndrome. Mo Med 2011;108:33–6.
[81] Hebert LA, Parikh S, Prosek J, Nadasdy T, Rovin BH. Differential diagnosis of glomerular disease: A systematic and inclusive approach. Am J Nephrol 2013;38:253–66.
[82] Cavanaugh C, Okusa MD. The evolving role of novel biomarkers in glomerular disease: A review. Am J Kidney Dis 2021;77:122–31.
[83] Moon PG, Lee JE, You S, Kim TK, Cho JH, Kim IS, et al. Proteomic analysis of urinary exosomes from patients of early IgA nephropathy and thin basement membrane nephropathy. Proteomics 2011;11:2459–75.
[84] Monhart V. Hypertension and chronic kidney diseases. Cor Vasa 2013;55:e397–402.
[85] Gonzalez-Calero L, Martínez PJ, Martin-Lorenzo M, Baldan-Martin M, Ruiz-Hurtado G, de la Cuesta F, et al. Urinary exosomes reveal protein signatures in hypertensive patients with albuminuria. Oncotarget 2017;8: 44217–31.
[86] Selby NM, Taal MW. An updated overview of diabetic nephropathy: Diagnosis, prognosis, treatment goals and latest guidelines. Diabetes Obes Metabol 2020;22:3–15.
[87] Sulaiman MK. Diabetic nephropathy: Recent advances in pathophysiology and challenges in dietary management. Diabetol Metab Syndrome 2019;11:7.
[88] Zubiri I, Posada-Ayala M, Benito-Martin A, Maroto AS, Martin-Lorenzo M, Cannata-Ortiz P, et al. Kidney tissue proteomics reveals regucalcin downregulation in response to diabetic nephropathy with reflection in urinary exosomes. Transl Res 2015;166:474–84.
[89] Picken MM. The pathology of amyloidosis in classification: A review. Acta Haematol 2020;143:322–34.
[90] Ramirez-Alvarado M, Ward CJ, Huang BQ, Gong X, Hogan MC, Madden BJ, et al. Differences in immunoglobulin light chain species found in urinary exosomes in light chain amyloidosis (Al). PLoS One 2012;7:1–11.
[91] Ramirez-Alvarado M, Barnidge DR, Murray DL, Dispenzieri A, Marin-Argany M, Dick CJ, et al. Assessment of renal response with urinary exosomes in patients with AL amyloidosis: A proof of concept. Am J Hematol 2017;92: 536–41.
[92] Agarwal R. Defining end-stage renal disease in clinical trials: A framework for adjudication. Nephrol Dial Transplant 2016;31:864–7.
[93] Pearson LJ, Klaharn IY, Thongsawang B, Manuprasert W, Saejew T, Somparn P, et al. Multiple extracellular vesicle types in peritoneal dialysis effluent are prominent and contain known biomarkers. PLoS One 2017;12:1–18.
[94] Carreras-Planella L, Soler-Majoral J, Rubio-Esteve C, Morón-Font M, Franquesa M, Bonal J, et al. Proteomic profiling of peritoneal dialysis effluent-derived extracellular vesicles: A longitudinal study. J Nephrol 2019;32:1021–31.
[95] Pisitkun T, Gandolfo MT, Das S, Knepper MA, Bagnasco SM. Application of systems biology principles to protein biomarker discovery: Urinary exosomal proteome in renal transplantation. Proteonomics Clin Appl 2012;6:268–78.
[96] Dieudé M, Bell C, Turgeon J, Beillevaire D, Pomerleau L, Yang B, et al. The 20S proteasome core, active within apoptotic exosome-like vesicles, induces autoantibody production and accelerates rejection. Sci Transl Med 2015;7: 318ra200.
[97] Lim JH, Lee CH, Kim KY, Jung HY, Choi JY, Cho JH, et al. Novel urinary exosomal biomarkers of acute T cell-mediated rejection in kidney transplant recipients: A cross-sectional study. PLoS One 2018;13:1–17.
[98] Flechner SM, Kobashigawa J, Klintmalm G. Calcineurin inhibitor-sparing regimens in solid organ transplantation: Focus on improving renal function and nephrotoxicity. Clin Transplant 2008;22:1–15.
[99] Carreras-Planella L, Juega J, Taco O, Cañas L, Franquesa M, Lauzurica R, et al. Proteomic characterization of urinary extracellular vesicles from kidney-transplanted patients treated with calcineurin inhibitors. Int J Mol Sci 2020;21:1–14.
[100] Choi DS. Urinary extracellular vesicles for biomarker source to monitor polycystic kidney disease. Proteonomics Clin Appl 2015;9:447–8.
[101] Staubach S, Wenzel A, Beck BB, Rinschen MM, Müller S, Hanisch FG. Autosomal tubulointerstitial kidney disease-MUC1 type: Differential proteomics suggests that mutated MUC1 (insC) affects vesicular transport in renal epithelial cells. Proteomics 2018;18:e1700456.
[102] Hogan MC, Manganelli L, Woollard JR, Masyuk AI, Masyuk TV, Tammachote R, et al. Characterization of PKD protein-positive exosome-like vesicles. J Am Soc Nephrol 2009;20:278–88.
[103] Lea WA, McGreal K, Sharma M, Parnell SC, Zelenchuk L, Charlesworth MC, et al. Analysis of the polycystin complex (PCC) in human urinary exosome-like vesicles (ELVs). Sci Rep 2020;10:1500.
[104] Hogan MC, Bakeberg JL, Gainullin VG, Irazabal MV, Harmon AJ, Lieske JC, et al. Identification of biomarkers for PKD1 using urinary exosomes. J Am Soc Nephrol 2015;26: 1661–70.
[105] Salih M, Demmers JA, Bezstarosti K, Leonhard WN, Losekoot M, van Kooten C, et al. Proteomics of urinary vesicles links plakins and complement to polycystic kidney disease. J Am Soc Nephrol 2016;27:3079–92.
[106] Bruschi M, Granata S, Candiano G, Fabris A, Petretto A, Ghiggeri GM, et al. Proteomic analysis of urinary extracellular vesicles reveals a role for the complement system in

medullary sponge kidney disease. Int J Mol Sci 2019;20: 1–15.
[107] Jackson L, Woodward M, Coward RJ. The molecular biology of pelvi-ureteric junction obstruction. Pediatr Nephrol 2018;33:553–71.
[108] Liu R, Zhang W, Luo M, Qin X, Yang F, Wei Q. iTRAQ-based proteomics and in vitro experiments reveals essential roles of ACE and AP-N in the renin-angiotensin system-mediated congenital ureteropelvic junction obstruction. Exp Cell Res 2020;393:112086.
[109] Staubach S, Pekmez M, Hanisch FG. Differential proteomics of urinary exovesicles from classical galactosemic patients reveals subclinical kidney insufficiency. J Proteome Res 2016;15:1754–61.
[110] Claes DJ, Jackson E. Cystinuria: Mechanisms and management. Pediatr Nephrol 2012;27:2031–8.
[111] Bourderioux M, Nguyen-Khoa T, Chhuon C, Jeanson L, Tondelier D, Walczak M, et al. A new workflow for proteomic analysis of urinary exosomes and assessment in cystinuria patients. J Proteome Res 2015;14:567–77.
[112] Thongboonkerd V. Roles for exosome in various kidney diseases and disorders. Front Pharmacol 2020;10:1–14.
[113] Raimondo F, Morosi L, Corbetta S, Chinello C, Brambilla P, Della Mina P, et al. Differential protein profiling of renal cell carcinoma urinary exosomes. Mol Biosyst 2013;9: 1220–33.
[114] Wu Q, Poulsen SB, Murali SK, Grimm PR, Su X-T, Delpire E, et al. Large-scale proteomic assessment of urinary extracellular vesicles highlights their reliability in reflecting protein changes in the kidney. J Am Soc Nephrol 2021;32:2195–209.
[115] Gonzales PA, Pisitkun T, Hoffert JD, Tchapyjnikov D, Star RA, Kleta R, et al. Large-scale proteomics and phosphoproteomics of urinary exosomes. J Am Soc Nephrol : JASN (J Am Soc Nephrol) 2009;20:363–79.
[116] Raimondo F, Chinello C, Porcaro L, Magni F, Pitto M. Urinary extracellular vesicles and salt-losing tubulopathies: A proteomic approach. Proteomes 2020;8.
[117] Sung CC, Chen MH, Lin YC, Lin YC, Lin YJ, Yang SS, et al. Urinary extracellular vesicles for renal tubular transporters expression in patients with gitelman syndrome. Front Med 2021;8:679171.
[118] Rood IM, Merchant ML, Wilkey DW, Zhang T, Zabrouskov V, van der Vlag J, et al. Increased expression of lysosome membrane protein 2 in glomeruli of patients with idiopathic membranous nephropathy. Proteomics 2015;15: 3722–30.