Proteome of Human Urinary Exosomes in Diabetic Nephropathy

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Abstract

Diabetic kidney disease (DKD) is the major complication in diabetic patients, the leading cause of end-stage renal disease (ESRD), and main risk factor for cardiovascular disease (CVD). Its silent development, together with the lack of specific and early accessible indicators of renal damage, often results in a late diagnosis when kidney damage is irreversible. Omics approaches (genomics, proteomics, metabolomics) account with the advantage of investigating the molecular milieu as a whole, without preselection of potential targets. The complexity and wide range of concentration levels of biological fluids as plasma, serum, or urine makes difficult

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the discovery of novel markers of kidney disease progression, other than already known high-abundance molecules (e.g., albumin). Exosomes are microvesicles derived from kidney cells in contact with the urinary space with proven roles in RNA and protein transfer and cell–cell communication. Exosomes may directly reflect pathophysiological changes taking place in the damaged kidney, constituting a feasible alternative to the invasive biopsy. Once released into urine or plasma, exosomes can be isolated and thus represent a sub-proteome where molecular messengers are enriched. This chapter overviews the current panorama in the potential use of exosomes as a novel source of biomarkers able to improve DKD current diagnosis, patients' risk stratification, and prognosis prediction.

Keywords

Kidney disease • Diabetic nephropathy • Exosomes • Proteomics • Cardiovascular disease • Urine • Plasma

Abbreviations

BP	Blood pressure
CE	Capillary electrophoresis
CKD	Chronic kidney disease
CVD	Cardiovascular disease
DIGE	Difference gel electrophoresis
DN	Diabetic nephropathy
DKD	Diabetic kidney disease
ESRD	End-stage renal disease
GC	Gas chromatography
GFR	Glomerular filtration rate
iTRAQ	Isobaric tags for relative and absolute quantitation
LC	Liquid chromatography
MALDI	Matrix-assisted laser desorption/ionization
MS	Mass spectrometry
MVB	Multivesicular bodies
NTA	Nanoparticle tracking analysis
RAS	Renin–angiotensin system
SEC	Size exclusion chromatography
SELDI-TOF	Surface-enhanced laser desorption/ionization time-of-flight
TEM	Transmission electron microscopy
THP	Tamm–Horsfall protein
UAER	Urinary albumin excretion rate
UC	Ultracentrifugation
WB	Western blotting

Key Facts of Exosomes

- Exosomes are 40–100 nm vesicles with density values in the range of 1.13–1.19 g/mL.

- Exosomes derive from kidney cells in contact with the urinary space and have proven roles in intercellular communication.
- Exosomes are direct messengers of what is happening in the kidney, both in acute and chronic damage, and carry molecular markers of renal dysfunction and structural injury.
- Several methodologies have been described for isolating exosomes from urine, paying specific attention to the purity and recovery of the isolated fraction.
- In kidney disease patients, albumin can seriously interfere when being co-isolated from urine with the exosomal fraction.
- A few exosomal markers of diabetic kidney disease and other renal disorders have been found by proteomics approaches.

Definitions of Words and Terms

Albuminuria Abnormally increased amount of protein (albumin) detected in the urine.

Biomarker A characteristic (molecule) that is objectively measured and whose levels are evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacological responses to therapeutic intervention.

Chronic kidney disease Progressive and permanent kidney damage, classified in five different stages depending on severity of renal dysfunction.

Diabetic kidney disease Chronic renal disease affecting patients with type1 or type2 diabetes.

End-stage renal disease Kidney failure which requires dialysis or kidney transplantation. ESRD is the last and more severe stage in chronic kidney disease.

Exosome 40–100 nm microvesicles of endocytic origin secreted by most cell types.

Proteomics Study of the whole set of proteins present in a cell, organ, or biological fluid in a certain moment.

Introduction: Diabetic Kidney Disease (DKD) – Diagnosis, Treatment, and Challenges

Diabetes prevalence is globally increasing, and diabetes major complication is a renal disease, commonly named diabetic nephropathy (DN) and more recently referred to as diabetic kidney disease (DKD). Chronic kidney disease (CKD) is the major clinical outcome of diabetic patients with DKD being the leading cause of end-stage renal disease (ESRD) and a risk factor for cardiovascular disease (CVD), i.e., if diabetes is present, ESRD patients mainly die from CVD. Unfortunately, the disease courses silently, diagnosis is not straightforward, and kidney damage is irreversible. In many

Table 1 Clinical indicators of diabetic kidney disease (DKD) (Caramori et al. 2006; Jha et al. 2014)

Initiators
Hyperglycemia
Genetics/epigenetics
Defining parameters
Albuminuria or ACR: normo (<30 mg/g), micro (30–300 mg/g), macro (≥300 mg/g)
GFR changes
Progression factors
Albumin
GFR changes
Glucose (HbA_{1C})
↑ BP
Lipids (cholesterol, triglycerides)
Uric acid
Novel indicators of kidney injury (pending confirmation)
Glomerular (adiponectin, ceruloplasmin, laminin)
Tubular (NGAL, KIM-1, α1- and β2-microglobulin, L-FABP, cystatin C)
Fibrosis (collagen type IV, TGF-β1-to-BMP-7 ratio)
Inflammation (TNF-α, TNFR1)

ACR albumin/creatinine ratio, *GFR* glomerular filtration rate, *BP* blood pressure

cases, initial diagnosis is not made by nephrologist, to whom the patient may be often lately referred, resulting in an increase in mortality rates as no early management to prevent disease progression has been attempted. Diagnosis is based on several clinical manifestations with different interpretation depending on, e.g., patient with type 1 or type 2 diabetes (Park 2014). Urinary albumin excretion rate (UAER or AER) and rising blood pressure (BP) are the most commonly considered indicators together with histological manifestations if biopsy material is available, which mainly happens if atypical clinical course for diabetic nephropathy individuals is observed. In diabetic patients, microalbuminuria is an indicator of nephropathy and a marker of vascular damage and higher cardiovascular risk. Microalbuminuria reflects an abnormality in glomerular capillary permeability to proteins and is also dependent on the tubular capacity to reabsorb filtered albumin. Clinically, it is defined in the range 20–199 mg albumin/g creatinine in males and 30–299 mg albumin/g creatinine in females. Higher values are defined as macroalbuminuria or proteinuria and indicate a decline in the renal function. AER as predictor for DKD accounts with several limitations. It may be the case that healthy subjects with normal renal function show increased AER or that diabetic patients with high risk of developing proteinuria are normoalbuminuric in an early screening. In some cases, albuminuric patients revert to normoalbuminuria in some years without treatment, and, in the opposite case, normoalbuminuric individuals may develop DKD and progress to kidney failure (Molitch et al. 2010; Kramer et al. 2003). Table 1 summarizes most commonly used initiators, defining parameters, and progression factors of DKD.

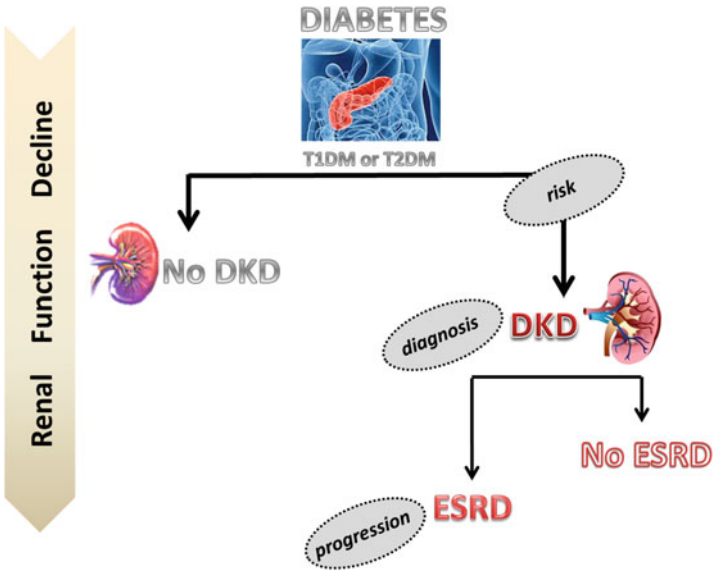

Fig. 1 Kidney disease progression in diabetic patients. Type 1 or 2 diabetic patients may or may not develop diabetic kidney disease (*DKD*). Novel markers able to predict individuals at risk for DKD are pursued. Once renal function starts declining, early diagnosis is mandatory together with the ability to predict whose patients will progress to end-stage renal disease (*ESRD*)

When facing individuals with diabetes, the main questions are as follows: Are they going to develop DKD? And if so, will they progress to ESRD (Fig. 1)? Current risk markers for DKD are albuminuria and decline in glomerular filtration rate (GFR) with cutoff stratification values depending on age, serum uric acid, and serum soluble 1 and 2 TNF receptors, among others (MacIsaac et al. 2014), together with family history, smoking habits, and ambulatory BP and lipids (Caramori et al. 2006; Gray and Cooper 2011). No cure is available. The best approach would be preventing microalbuminuria development and CVD in diabetic patients, e.g., by means of tight BP control and renin–angiotensin system (RAS) suppression (Ruilope et al. 2010) or attempting to stop progression once DKD is diagnosed (Fernandez Fernandez et al. 2012). Precise glycemic control, BP reduction, cholesterol management, and lifestyle improvement compose the current therapy which should be defined as personalized and multitargeted (Bakris 2011).

Despite of all efforts, DKD prevalence remains stable (de Boer et al. 2011), pointing to an urgent need of novel early markers of disease, markers of patient's risk, and predictors of patient's prognosis once DKD is diagnosed in early stages.

Urinary and Plasma Markers of DKD

Limitations of current clinical makers of renal damage in diabetic patients prompt further research aimed to discover novel indicators easily accessible (i.e., able to be monitored in biological fluids as urine or serum/plasma). The ultimate goal is

twofold: (1) achieving early diagnosis of diabetic kidney disease, particularly in those patients with apparent normal renal function (normoalbuminuric), and (2) successful individual stratification of CV risk and renal damage progression.

Classical approaches focus to one or various molecules for which there is evidence or proved connection with the disease under research. Preselection of potential targets implies an enormous limitation in view of the complexity of the interactions and underlying mechanisms operating in the cross talk among the different organs, tissues, and cells. The advantage of the omics strategy is that no potential marker and no key target are preselected, but all the protein and/or metabolite sets are investigated as a whole in the search for significant differences. Thus, not only particular pathways or responding molecules commonly measured in routine biochemical patient's analysis or previously discovered are being investigated but also those whose relationship with the pathophysiological processes taking place is still unknown. A validation (confirmation) phase should then follow to further investigate candidate makers discovered, to set valid conclusions for the clinical practice.

A systematic review of DKD markers is out of the scope of this chapter. Representative studies are referred in Table 2, showing main urinary and plasma/serum markers of DKD found by omics approaches mainly as responders to diabetic condition itself, diabetic nephropathy, albuminuria development or progression, and renal function decline over time or stable. Very different and complementary approaches have been used, i.e., gas chromatography (GC) or liquid chromatography (LC) or capillary electrophoresis (CE) coupled with mass spectrometry (GC-MS or LC-MS or CE-MS), differential gel electrophoresis (DIGE) analysis, MALDI-TOF-MS, SELDI-TOF-MS, and label-free or isotopic labeling (iTRAQ)-LC-MS/MS.

Exosomes: A Novel Source of Research in Kidney Disease

Urine exosomes are 40–100 nm vesicles coated with lipid bilayer membranes with density values in the range of 1.13–1.19 g/mL, derived from all types of kidney cells in contact with the urinary space, including renal tubule cells and podocytes. Exosomes have proven roles in regulating immune response, antigen presentation, RNA and protein transfer, and cell–cell interaction/signaling (Mathivanan et al. 2010; Camussi et al. 2010; Van Balkom et al. 2011). These microvesicles have an endosomal origin. They are formed by the fusion of multivesicular bodies with the plasma membrane and release of their intraluminal vesicles, which are then termed exosomes once in the extracellular space. Exosomes thus contain membrane and cytosolic cellular proteins and are considered a mechanism of nonclassical secretion of proteins, representing 3 % of the whole urine proteome. ExoCarta is a protein, lipid, and RNA exosomal database providing with the contents of exosomes which have been identified in multiple organisms, cells, and fluids.

The use of urinary exosomes as starting material for biomarker discovery has shown to be advantageous. They constitute a sub-proteome of the whole urinary proteome with minor complexity and reduced protein dynamic concentration range, which represents a better alternative for detection of low-abundance proteins that

Table 2 Representative proteomics studies to approach diabetic nephropathy and discover novel markers of disease

Clinical groups	Biological source	Technical approach	Main findings	References
T1DM ($n = 122$): Normo, micro, macro	Plasma peptidome	RPC18, wCX, MALDI-TOF	C3f, apolipoprotein C-I (markers of DN)	(Hansen et al. 2010)
T2DM ($n = 6$) Control ($n = 6$)	Plasma glycoproteins	PAGE+LC-MS/MS	Lumican, vasorin, RBP4	(Ahn et al. 2010)
T2DM ($n = 90$) Normo–micro, micro–macro HTN ($n = 150$)	Plasma and urine metabolome	LC-MS/MS	Plasma: histidine, butenoylcarnitine (T2DM vs. control) Urine: hexose, glutamine, tyrosine (risk predictors of albuminuria evolution)	(Pena et al. 2014)
DM DN ($n = 150$)	Plasma	GC-MS	NEFAs, EFAs	(Han et al. 2011)
T2DM ($n = 30$) DN ($n = 52$) Control ($n = 30$)	Plasma	UPLC-MS/MS	Phospholipids PI C18:0/22:6 SM dC18:0/20:2	(Zhu et al. 2011)
T1DM+micro (stable or declined renal function)	Plasma peptides	LC-MALDI-TOF	Kininogen	(Merchant et al. 2013)
DN ($n = 66$) T2DM ($n = 82$)	Urine peptides	CE-MS	Collagen fragments	(Alkhalaf et al. 2010)
T2DM: normo ($n = 43$) Micro ($n = 43$)	Urine	iTRAQ	Alpha-1-antitrypsin Alpha-1-acid glycoprotein 1 Prostate stem cell antigen	(Jin et al. 2012)
T1DM: normo ($n = 52$) (progressed ($n = 26$) or stable ($n = 26$))	Urine	GC-MS, LC-MS	Metabolite panel	(van der Kloet et al. 2012)
T1DM (normo and macro)	Urine	LC-MS/MS	Vanin-1	(Fugmann et al. 2011)
1. Control ($n = 20$), normo ($n = 20$), micro ($n = 18$) + T2DM 2. DN ($n = 65$), T2DM +ndCKD ($n = 10$), nDM +CKD ($n = 57$)	Urine	SELDI-TOF	Ubiquitin B2-microglobulin	(Papale et al. 2010)
T1DM+micro (normal renal function): declined renal function ($n = 21$) and stable ($n = 40$)	Urine	LC-MALDI-TOF	α1(IV) collagen α1(V) collagen Tenascin-X Inositol pentakis phosphate 2-Kinase	(Merchant et al. 2009)

(*continued*)

Table 2 (continued)

Clinical groups	Biological source	Technical approach	Main findings	References
DM+albuminuria ($n = 38$) DM w/o albuminuria ($n = 45$) noDM+albuminuria ($n = 34$) Control ($n = 45$)	Urine	SELDI-TOF	UbA52	(Dihazi et al. 2007)

DM diabetes mellitus, *T1DM* type 1 diabetes mellitus, *T2DM* type 2 diabetes mellitus, *DN* diabetic nephropathy, *CKD* chronic kidney disease, *ndCKD* nondiabetic CKD, *HTN* hypertension

otherwise could be masked by major proteins. As a consequence of their endocytic origin, urinary exosomes contain proteins characteristic of every renal tubule epithelial cell type and from the urinary collecting system, including proteins that are characteristic of the membrane and cytoplasm of the cells in which they have been generated. In particular, exosomes can be released in the kidney by cells as podocytes, pass through the renal tubule, and either be untaken by recipient epithelial cells of the collecting duct or influence them through secretion of their content (Street et al. 2011). In this sense, more than a way of exocytic cell waste elimination, exosomes should be considered as messengers, transferring information between renal and nonrenal cells and carrying molecular markers of renal dysfunction and structural injury (Salih 2014; Zhou et al. 2008). This role of exosomes as messengers between cells and tissues gains particular importance in complex scenarios where multi-organ cross talk takes place. That is the case of the cardiorenal syndrome, defined (although not fully understood) by proved evidence that an acute/chronic worsening of kidney function influences an acute/chronic cardiac dysfunction and vice versa. In cardiovascular disease, exosomes have gained increasing interest (Cosme et al. 2013) although their specific role in atherosclerosis development still constitutes an underexplored field (Gonzalez-Calero et al. 2014).

Exosomal Isolation from Urine

Independent of the methodological approach to be used in the study of exosomal molecular content, key aspects should be taken into account, which may strongly influence the purity and recovery of the exosomal isolated fraction. Collection and storage of urine samples influence in a high degree the quality of the recovered exosomal fraction, and general guidelines have been published, including the use of protease inhibitors at collection time, sample storage at -80 °C, and extensive vortexing of urine samples after thawing as mandatory steps (Zhou et al. 2006a). Exosomal isolation (purification) from urine is not straightforward. High abundant urinary proteins as Tamm–Horsfall protein (THP or uromodulin) and albumin when renal function is compromised are co-isolated together with the exosomes. This contamination source can be overcome using different methodological approaches, as detailed below.

In the last decade, different methods have been proposed for the isolation of exosomes from diverse biological fluids, and there is no consensus on the best method to obtain a pure and well-characterized exosomal fraction from urine. Despite the lack of agreement, most commonly used approaches are based on (differential) ultracentrifugation (UC) (Pisitkun et al. 2004) (density gradient- or cushion-based UC) (Raj et al. 2012) and based on the use of a nanomembrane concentrator (Cheruvanky et al. 2007), immunoaffinity (Sun et al. 2012), or microfluidic technology (He et al. 2014). There are also new commercial methods such as the Total Exosome Isolation™ precipitation solution (Invitrogen), immunobeads or immunoplates (HansaBioMed LLC), or ExoQuick™ precipitation reagent suitable for the isolation of these microvesicles from urine, serum, and plasma.

Differential centrifugation and UC isolation method is recommended by the Human Kidney and Urine Proteome Project (http://www.hkupp.org/Exosome%20Preparation.htm). Currently, this is the most frequently used methodology for the isolation of exosomes from urine (Fig. 2). In brief, urine samples are centrifuged at 17,000 × g in order to remove the whole cells, large membrane fragments, and debris and recover the supernatant, which is then ultracentrifuged (200,000 × g, 1 h, 4 °C). Exosomes are recovered in the pellet. Particular attention should be paid by

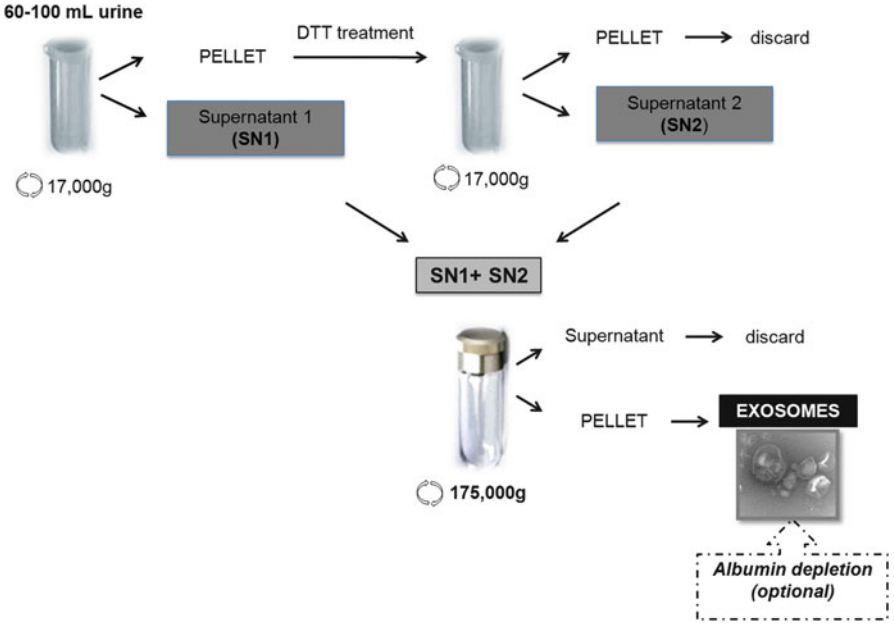

Fig. 2 Schematic view of isolation protocol of exosomes from urine. Ultracentrifugation based is one of the most commonly used methodologies for isolating urinary exosomes. Serial centrifugation steps and enrichment of the exosomal pellet by DTT treatment are applied to maximize purity and recovery of the exosomal fraction. An extra albumin depletion step is recommended when analyzing urine samples from kidney disease patients diagnosed with macroalbuminuria

the partial entrapment of exosomes by the polymerized THP network, thus reducing exosomal recovery. This drawback can be overcome by treatment of the first (low-speed centrifugation) pellet with reducing agents (e.g., DTT) and heat. Following centrifugation again, the supernatant is then collected and pulled together with the supernatant obtained in the first low-speed centrifugation to proceed with ultracentrifugation (Fernández-Llama et al. 2010). The final pellet can still contain important amounts of THP polymers coprecipitating with the exosomes which can be treated again with reducing agents and heat to dissolve the aggregates and ultracentrifugated again to obtain a final clean exosome pellet (Gonzales et al. 2010). Another option to minimize THP interference and further purify the exosomal fraction is to perform extra steps of UC using sucrose gradient or 30 % sucrose cushion.

Nanomembrane concentration is an alternative to ultracentrifugation, which is time consuming and requires instrumentation not always available. This approach is fast and simple and is based on the use of nanomembranes with a uniform pore size of 13 mm. However, protein recovery is generally not uniform nor pure, and for comparative proteomic analysis, this variation needs to be taken into account. Exoquick® is a commercial reagent designed for specific isolation of exosome by precipitation, but obtaining enough exosomal recovery from control urine samples and high purity of the isolated fraction from albuminuric samples is not guaranteed, as urine-contaminating proteins can be co-isolated in proteinuric conditions [unpublished data]. A modified Exoquick® protocol has been described with improved results (Alvarez et al. 2012). Six different protocols were compared concluding that ultracentrifugation methods result in the purest exosomal protein yield, and the fast and simple modified Exoquick® protocol proved to be the most effective alternative, particularly when analyzing exosomal mRNA and miRNA.

Exosomal isolations from plasma or secreted by B cell have been accomplished by immunoaffinity based or by microfluidic isolation technology which separates microvesicles as a function of diameter from heterogeneous populations of cancer-cell-derived extracellular shed vesicles (Santana et al. 2014). These methods will be probably adapted to the isolation of microvesicles from urine in the near future.

Characterization of the Isolated Exosomal Fraction

There are numerous techniques able to confirm the presence and purity of exosomes obtained by any of the above-described isolation methods. Transmission electron microscopy (TEM), Western blotting (WB), and most recently NanoSight technique are the approaches most commonly used. TEM requires sample fixation with 4 % paraformaldehyde to be later deposited on Formvar carbon-coated nickel grids and stained with uranyl acetate to obtain images of the exosomes that can allow the user to determine the size and shape (cup shaped) of these microvesicles under the microscope. Immuno-electron microscopy (IEM) allows the immune detection and direct imaging of exosomes. For WB characterization, specific well-known exosomal proteins are detected. ALIX, TSG101, and clathrin are involved in the

maturation of MVB and are known to be present in the human urinary exosome membrane. Exosomes are also rich in tetraspanins like CD9, CD63, and CD81 and heat shock proteins like HSP60, HSP70, and HSPA5. All these specific markers are independent from the origin of the exosomes and can be used to characterize exosomes from urine as well as from other sources. In urine, exosomes originate from podocytes and epithelial cells, and it is possible to detect the presence of proteins that are segment specific such as aquaporin 2 (AQP2, collecting duct), sodium proton exchanger 3 (NHE-3, proximal tubule), or podocalyxin (PODXL) found in podocytes. Apart from WB, enzyme-linked immunosorbent assays (ELISAs) or flow cytometry can be used for detection of specific exosomal markers. Because of their small size, exosomes can only be analyzed in a flow cytometer after linkage to larger particles of known size. Exosomes can be adsorbed to solid latex microspheres, and microspheres/exosomes can be later incubated with specific antibodies and analyzed on a flow cytometer (Benito-Martin et al. 2013). A novel tool that has proven efficiency in the characterization of exosomes is the nanoparticle tracking analysis (NTA) using the NanoSight which allows specific exosomes and microvesicles in the range of 50–1,000 nm in liquid suspension to be directly and individually visualized and counted in real time.

Albumin Potential Interference in the Study of Kidney Diseases by Proteomics

The albuminuric condition may condition the purity of the exosomal isolated fraction. Albumin overload in urine can represent an important problem when, e. g., approaching a proteomics study of kidney diseases characterized by an abnormally high content of this protein in urine (Martin-Lorenzo et al. 2014). Unspecific co-isolation of albumin in the exosomal fraction may diminish reproducibility, condition the robustness of the methodology with comparative purposes, and reduce the possibility to detect low-abundance proteins, making more challenging the comparison between healthy and disease condition. Total protein quantification of exosomal fractions can vary substantially between control and disease samples, resulting in a significantly higher total "exosomal protein content" in patient samples due to albumin and thus causing underestimation of the low-abundance exosomal proteins. Depletion of major soluble urine protein contaminants is therefore advisable to broaden our understanding of exosomal proteome changes apart from albumin content in proteinuric kidney disease. An isolation methodology by serial (ultra)centrifugation steps followed by depletion of the major proteins present in the exosome fraction was described based on ProteoPrep® Immunoaffinity Albumin & IgG Depletion Kit (Sigma-Aldrich) originally developed for plasma samples but adapted to urinary exosomal fraction. This method proved to be useful and simple, allowing an increase up to 60 % in the number of identified proteins when using LC-MS/MS techniques to investigate candidate exosomal markers of diabetic nephropathy in human samples (Zubiri et al. 2013, 2014). The efficiency of isolation methods in patients with nephrotic-range proteinuria was investigated by

comparison of three techniques: nanomembrane ultrafiltration, ultracentrifugation, and ultracentrifugation followed by size exclusion chromatography (UC-SEC). They demonstrate that highly abundant urinary proteins were still present in sufficient quantity after ultrafiltration and ultracentrifugation and were able to overcome this problem when using UC-SEC (Rood et al. 2010).

These two methods represent an improvement in the available exosomal isolation methods, particularly challenging when dealing with nephropathy urine samples.

Exosomal Markers of Diabetic Kidney Disease

Since 2004 when the presence of exosomes in urine was reported (Sun et al. 2012), a growing number of studies have been published aimed to the search of novel biomarkers of disease in these microvesicles. Protein and RNA biomarker candidates have been postulated for a variety of bladder, prostate, urinary tract diseases and kidney diseases including DKD. In this specific context, several promising biomarkers have been described in urinary exosomes from patients and animal models (Table 3). The activity of dipeptidyl peptidase IV (DPP IV) in urine microvesicles measured by ELISA positively correlated with the progression of proteinuria in type 2 diabetic nephropathy patients, being a good candidate to represent an early biomarker of renal damage before onset of albuminuria. Podocyte injury contributes to the initiation and decline of kidney function in diabetic nephropathy (Wolf et al. 2005), and podocyte apoptosis is an early mechanism leading to diabetic nephropathy (Susztak et al. 2006). Measuring podocyte protein expression changes in a noninvasive manner was possible after isolating urine exosomes. Expression of Wilms' tumor 1 (WT1) protein, a transcription factor and podocyte marker, was measured in urine exosomes from 48 type 1 diabetic patients and 25 healthy controls, showing for the first time a predominant expression of *WT1* protein in urinary exosome in type 1 diabetic patients. This protein was not present in healthy age-matched controls, and higher levels of this marker were found in exosomes from patients with proteinuria. The strong correlation found between the expression of WT1 and the increase in urine protein excretion suggests a considerable predictive value of this protein as an early biomarker of DN (Kalani et al. 2013).

Omics approaches account with the advantage of generating data which are not individual, referred to a unique molecule (protein, metabolite), but global, describing hundreds or thousands of compounds altered simultaneously in response to a certain disease or stimulus. This is possible due to the ability of a wide range of available techniques to characterize thousands of molecular species in each run, thus generating profiles or data sets which reflect the general situation of the sample (cell, tissue, biopsy, serum, urine, etc.). Different methodological approaches currently available can be applied to investigate the exosomal proteome, making the choice mainly dependent on (1) the nature of the analytes to investigate (i.e., peptides, proteins, metabolites, lipids); (2) the performance in terms of sensitivity, selectivity, specificity, and throughput; and (3) the step in the, e.g., biomarker research pipeline to approach (discovery or validation) which may require a targeted (e.g., SRM or

Table 3 Studies showing the potential of urinary exosomes in the search for biomarkers of kidney diseases

Disease	Biomarker candidate	Main technique	Isolation method	References
DKD	miR-145	RT-QPCR	UC	(Barutta et al. 2013)
DKD	AMBP	LC-MS/MS	UC	(Wolf et al. 2005)
	MLL3			
	VDAC1			
DKD	13 mitochondrial metabolite panels	GC-electron impact MS	Volume exclusion	(Sharma et al. 2013)
DKD	Xaa-Pro dipeptidase	LC-MS/MS	UC	(Raimondo et al. 2013a)
	Major urinary protein 1			
	Neprilysin			
DKD	DPP IV	ELISA	Immunoaffinity isolation	(Gonzales et al. 2010)
DKD	WT1	Immunoblotting	UC	(Zhou et al. 2013)
Podocyte injury				
CKD	OPG	Immunoblotting	UC	(Benito-Martin et al. 2013)
		ELISA		
Kidney fibrosis	mRNA of CD2AP	RT-QPCR	UC	(Lv et al. 2014)
Cystinuria	38 protein panels	IEF LC-MS/MS	UC	(Bourderioux et al. 2015)
AKI	ATF3 RNA	RT-QPCR	UC	(Chen et al. 2014)

(*continued*)

Table 3 (continued)

Disease	Biomarker candidate	Main technique	Isolation method	References
AKI	ATF3	Immunoblotting	UC	(Zhou et al. 2008)
	WT1	IHC		
AKI	Fetuin-A	2D-DIGE	UC	(Zhou et al. 2006b)
Renal carcinoma	10 protein panels	LC-MS/MS	UC	(Raimondo et al. 2013b)
Renal transplantation	NGAL	Immunoblotting	UC	(Alvarez et al. 2013)
IgA nephropathy	Aminopeptidase N vasorin	LC-MS/MS	UC	(Moon et al. 2011b)
Thin basement membrane nephropathy	α-1-Antitrypsin, ceruloplasmin			
Renal ischemia–reperfusion injury	AQP1	Immunoblotting	UC	(Sonoda et al. 2009)
Bartter syndrome type I	INKCC2	LC-MS/MS	UC	(Gonzales et al. 2009)
		Immunoblotting		
Autoimmune glomerulonephritis	miR-26a	RT-QPCR	UC	(Ichii et al. 2014)
Polycystic kidney disease	PC1/TMEM2 PC2/TMEM2	LC-MS/MS	Sucrose gradient	(Hogan et al. 2015)
Obstructive nephropathy	E-cadherin	Immunoblotting	UC	(Trnka et al. 2012)
	N-cadherin			
	TGFB			
	L1CAM			

DKD diabetic kidney disease, *AKI* acute kidney injury, *UC* ultracentrifugation

MRM) or a wider approach (e.g., label-free or (iTRAQ)-LC-MS/MS, CE-MS, SELDI-TOF-MS). Proteomics analysis has been applied in the search for potential markers of disease in isolated exosomes (Choi et al. 2013; Moon et al. 2011a; Simpson et al. 2009). By label-free LC-MS/MS quantitative analysis of exosomes isolated from urine of Zucker diabetic fatty (ZDF) rats as a model of type 2 DN, 286 proteins were identified and quantified. Confirmed by immunoblotting, increased Xaa-Pro dipeptidase and decreased urinary protein 1 were shown. In a similar study carried out in humans, spectral counting analysis revealed a total of 562 proteins identified (207 had been previously identified in urinary exosomes, 108 had been identified in exosomes from different origin, and 244 were identified as exosomal proteins for the first time). Among those, a panel of 25 proteins significantly changed in diabetic nephropathy. Confirmed by selected reaction monitoring (SRM) mass spectrometry technique, three protein candidate markers of DN in exosomes were postulated, alpha-1-microglobulin/bikunin precursor (AMBP), voltage-dependent anion-selective channel protein 1 (VDAC1), and isoform 1 of histone-lysine N-methyltransferase (MLL3), opening a new possibility to monitor DN by analyzing urinary exosomes (Wolf et al. 2005).

The metabolome represents the downstream changes in the genome, transcriptome, and proteome as a reflection of real-time processes occurring in living organisms. Compared to more than ten million proteins in the proteome, a few thousand metabolites present in an organism imply a considerable reduction in complexity. Urine metabolomics is another important field for the study of diabetic complications. By GC-MS 94 urine metabolites were quantified in cohorts of patients with diabetes mellitus with and without kidney disease and in healthy controls. Thirteen metabolites were found significantly reduced in the diabetic nephropathy cohorts, related to the mitochondrial metabolism, indicating a suppression of mitochondrial activity in diabetic kidney disease. A consequence of this dysregulation was also detectable in urine exosomes as they showed that urine exosomes from patients contain a lower amount of mitochondrial DNA. This founding was consistent with later gene expression measurements performed in the kidney tissue, where a lower expression of PGC1α, a master regulator gene of mitochondrial biogenesis, was observed.

The majority of the studies based in urine exosomes in the search of biomarkers for DN are focused on the analysis of the proteomic composition of these microvesicles in healthy and disease stages. The potential of the exosomal RNA as source of kidney disease markers has also been reported (Miranda et al. 2010). RNA present in urine tends to be easily degraded and can be originated in apoptotic or necrotic cells not being representative of the transcriptional profile (Wang and Szeto 2007). RNAs protected by the lipid membrane of the exosomes are more stable and can be recovered and analyzed through the isolation of the exosomal fraction. RNAs contained in exosomes are produced in viable cells; thus they can provide a key insight of the physiopathological processes taking place in the kidney (van Balkom et al. 2011). Exosomes contain microRNA (miRNA), a class of small nonprotein-

encoding RNAs that regulate gene expression via suppression of target mRNAs. miRNA expression was analyzed in urinary exosomes from type 1 diabetic patients with and without diabetic nephropathy. Two hundred twenty-six miRNAs were detected in the normoalbuminuric patient urinary exosomes, and 22 miRNAs showed differential expression between normoalbuminuric and microalbuminuric patients. In the validation phase, miR-145 was found enriched in urinary exosomes from microalbuminuric patients, a glomerular marker of mesangial cells (Harvey et al. 2008) induced by TGF-β1 in this cell type (Denby et al. 2011). The expression of miR-145 was explored in both streptozotocin-induced diabetic mice and cultured mesangial cells. An upregulation in miR-130a was observed in type 1 diabetic patients. On the contrary, miR-155 and miR-424 were downregulated, and this effect was observed specifically in those patients with incipient diabetic nephropathy. In conclusion, miR-145 was identified as a new potential player in diabetic glomerulopathy, and the feasibility of the study of urinary exosomal miRNA as a source for candidate biomarker discovery in diabetic and other renal diseases was demonstrated here.

The global results of these studies evidence the potential use of the urine exosomes to monitor changes occurring in the kidney, opening an interesting alternative to the invasive kidney biopsies used nowadays to diagnose patients and follow progression. Further exosomal studies will follow to expand current knowledge of underlying operating mechanisms in DKD, which ultimately end in the discovery of novel therapeutic targets, and key molecules able to (a) diagnose diabetic patients in asymptomatic stages, (b) predict who of them will or not further progress to DKD or ESRD, and (c) stratify individual cardiovascular risk.

Potential Applications to Prognosis, Other Diseases, or Conditions

This chapter shows the applicability of exosomes in the study of kidney diseases and diabetic nephropathy in particular. The silent progression, asymptomatic at early stages, and irreversible damage of kidney functionality prompt the application of novel strategies in the search for novel markers, and exosomes arise as a powerful underexplored source. Markers can be classified according to their utility in (1) "risk assessment" (markers responding to disease susceptibility), (2) "screening" (markers able to discriminate between healthy and asymptomatic diseases in large populations), (3) "prognosis" (markers able to predict probable course of disease or aggressiveness of therapy), (4) "stratification" (envisage responders and non-responders to drug), and (5) "therapy monitoring" (indicators of the efficacy of treatment once the responder status is established). Depending on the ultimate goal, the experimental study should be carefully designed and patients' cohorts, properly matched with a healthy control group, carefully chosen (Fig. 3). Apart from these general rules, all technical and methodological improvements focused to efficiently isolate, characterize, and analyze the exosomal fraction from biological fluids can be implemented to the study of this and other diseases. This is an open

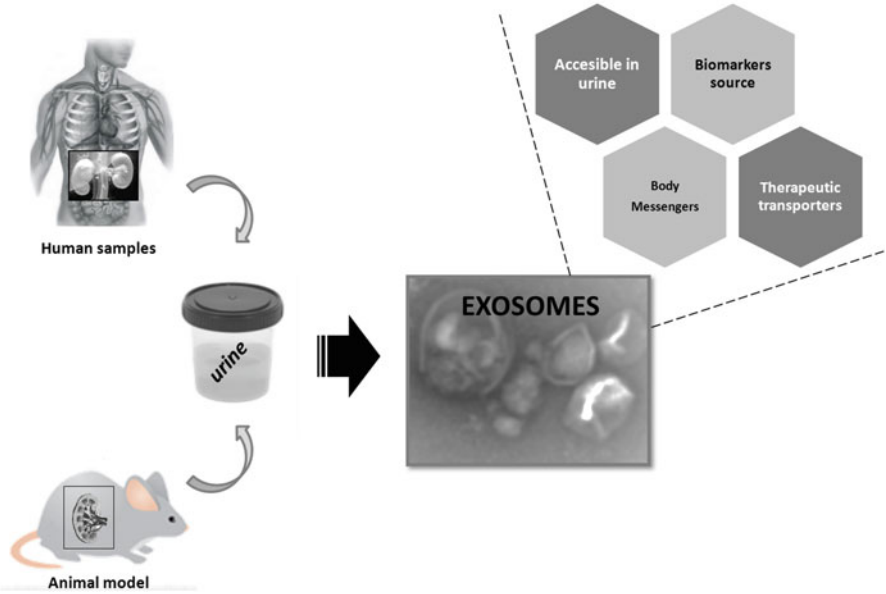

Fig. 3 Schematic workflow. Exosomal proteome is a powerful tool in the search for novel markers of disease. Isolated from urine, they constitute an enriched sub-proteome which directly reflects changes taking place in the kidney

field of research, which, although not fully mature in methodology, already accounts with proven applicability in the clinical proteomics field.

Summary Points

- Diabetic kidney disease is the major complication in diabetes and main risk factor for cardiovascular disease.
- Diabetic kidney disease develops silently, it is asymptomatic at early stages, and it is often diagnosed once the renal damage is irreversible.
- Proteomics arises as a powerful approach in the search for novel markers of diabetic nephropathy, once the challenge of the proteome dynamic range in the biological fluids is overcome.
- Exosomes are microvesicles released into urine which act as messengers of changes taking place in the kidney.
- Exosomal isolation from biological fluid is challenging, and ultracentrifugation-based method is one of the most efficient approaches. Particular care has to be taken with co-isolation of albumin from urine of renal patients.
- Exosomes constitute a novel and enriched source of biomarkers of kidney diseases and diabetic nephropathy, in particular.

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