

## Urinary Extracellular Vesicles: A Position Paper by the Urine Task Force of the International Society for Extracellular Vesicles

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## Abstract

Urine is commonly used for clinical diagnosis and biomedical research. The discovery of extracellular vesicles (EV) in urine opened a new fast-growing scientific field. In the last decade urinary extracellular vesicles (uEVs) were shown to mirror molecular processes as well as physiological and pathological conditions in kidney, urothelial and prostate tissue. Therefore, several methods to isolate and characterize uEVs have been developed. However, methodological aspects of EV separation and analysis, including normalization of results, need further optimization and standardization to foster scientific advances in uEV research and a subsequent successful translation into clinical practice. This *position paper* is written by the Urine Task Force of the Rigor and Standardization Subcommittee of ISEV consisting of nephrologists, urologists, cardiologists and biologists with active experience in uEV research. Our aim is to present the *state of the art* and identify challenges and gaps in current uEV-based analyses for clinical applications. Finally, recommendations for improved rigor, reproducibility and interoperability in uEV research are provided in order to facilitate advances in the field.

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## Introduction

Urinalysis has been part of standard clinical practice since antiquity (Magiorakis and Diamantis 2015). Today, urine is the second most commonly used biofluid for clinical diagnostics after blood. Urine is produced by the kidneys to eliminate waste products (*e.g.*, urea, metabolites) from the body and to maintain the homeostasis of water, ions, and pH in blood. Humans normally generate approximately 1-2 liters of urine per day, which is released via the urinary tract (ureters, urinary bladder, and urethra). In addition to soluble components like organic and inorganic molecules, urine typically contains some epithelial and blood cells, bacteria, viruses and importantly also extracellular vesicles (EVs) (Pisitkun, Shen *et al.* 2004, Santiago-Rodriguez, Ly *et al.* 2015). One key advantage of working with urine compared to other biofluids is that it can be easily and frequently collected in large quantities in a noninvasive manner (Decramer, Gonzalez de Peredo *et al.* 2008, Harpole, Davis *et al.* 2016). However, urinary concentration and contents are highly variable and of dynamic nature due to differences in fluid intake, time of collection, diet and exercise, age, gender, medications and health status. These well recognized factors can complicate data interpretation and the use of urine in diagnostics, particularly when reference normality ranges are to be set (Parolini, Federici *et al.* 2009, Molina, Salvetat *et al.* 2011, Nagaraj and Mann 2011, Guo, Zhang *et al.* 2015). These variables may be equally relevant for uEV analyses, and hence lessons from other fields employing urine analysis are likely to be important and applicable for uEV research.

The presence of EVs in urine was first documented by electron microscopy images in 1986 when Wiggins *et al.* investigated the procoagulant activity of pelletable material (100,000 x g ultracentrifugation) in normal urine (Wiggins, Glatfelter *et al.* 1986). Representative examples for images of EVs including electron microscopy are shown in Figure 1. Several years later, membrane vesicles of tubular (100,000 x g pellet) (Scherberich 1989) and podocyte (200,000 x g pellet) (Pascual, Steiger *et al.* 1994) origin were described in urine from patients with glomerulonephritis. However, uEVs caught wider attention in 2004 when Pisitkun *et al.* provided a thorough characterization of uEVs pelleted by ultracentrifugation of urine at 200,000 x g (Pisitkun, Shen *et al.* 2004). In this pioneering mass spectrometry analysis, the authors identified 295 proteins including typical proteins originating from nephron epithelial cells and urothelial cells, as well as proteins involved in the formation of multivesicular bodies. This initial overview of the proteome of uEVs and the evident alteration of the molecular composition of uEVs in pathological conditions opened a new frontier of biomarker discovery, sparking an exponential growth in uEV research and providing new possibilities for the use of urine in noninvasive clinical diagnostics. Urinary EV isolates enabled the detection of molecules that were not previously identified in urine because of their low concentration in the bulk fluid or because of their location inside EVs. Importantly, many of these low concentration proteins are connected to specific cells and/or organs (Gonzales, Pisitkun *et al.* 2009, Santucci, Candiano *et al.* 2015).

Urinary EVs have generally been considered to originate from cells of the urogenital tract and the residing bacteria and may be mixed with similarly-sized viruses (Figure 2). Therefore, uEVs constitute a source of potential molecular biomarkers for diseases of the kidneys, bladder and urogenital tract (prostate, uterus/vagina), and likely play a functional role in the physiology and pathology of these organs (Erdrugger and Le 2016, Karpman, Stahl *et al.* 2017, Merchant, Rood *et al.* 2017).

Importantly, however, proteins arising from other distant anatomical sites in the body have also been identified in uEVs. For example, uEVs have been proposed as a source of biomarkers for diseases such as Parkinson's disease and lung cancer (Li, Zhang *et al.* 2011, Fraser, Rawlins *et al.* 2016). Nevertheless, analysis of uEVs may open a window into the EV-repertoire of the circulation and provide a systemic readout of disease states from a non-invasive sample.

Both standard analytical methods and high-throughput omics technologies have been applied in (urinary) EV biomarker research, leading to the discovery of numerous potential EV-based biomarkers for a range of diseases. Early studies focused mainly on cancers related to the urogenital system and led to the identification of protein, mRNA, miRNA, lipid and metabolite biomarkers for prostate, bladder, and renal cancers (Mitchell, Welton *et al.* 2009, Nilsson, Skog *et al.* 2009, Chen, Lai *et al.* 2012, Del Boccio, Raimondo *et al.* 2012, Bijnsdorp, Geldof *et al.* 2013, Raimondo, Morosi *et al.* 2013, Øverbye, Skotland *et al.* 2015, Koppers-Lalic, Hackenberg *et al.* 2016, Fujita, Kume *et al.* 2017, Leiblich 2017, Rodriguez, Bajo-Santos *et al.* 2017, Sequeiros, Rigau *et al.* 2017, Skotland, Ekroos *et al.* 2017, Clos-Garcia, Loizaga-Iriarte *et al.* 2018, Dhondt, Van Deun *et al.* 2018, Lee, McKinney *et al.* 2018, Zhan, Du *et al.* 2018). In particular, two prostate-associated RNAs, PCA3 and TMPRSS2:ERG, were identified in urinary extracellular vesicles by Nilsson *et al.* in 2019 (Nilsson, Skog *et al.* 2009). These results were the foundation for a prostate cancer diagnostic test that has been extensively validated in two prospective multi-center US studies (McKiernan, Donovan *et al.* 2016, McKiernan, Donovan *et al.* 2018). Altogether, these promising results inspired the search for uEV-based biomarkers for other urogenital tract pathologies such as polycystic kidney disease, cystinuria, diabetic nephropathy, acute kidney injury/ renal ischemia-reperfusion injury, glomerulonephritis, renal interstitial fibrosis/ chronic kidney disease, lupus nephritis, nephronophthisis-related ciliopathies, tubulopathies and primary and secondary hypertension (Sonoda, Yokota-Ikeda *et al.* 2009, Zubiri, Posada-Ayala *et al.* 2014, Bourderioux, Nguyen-Khoa *et al.* 2015, Raimondo, Cerra *et al.* 2016, Salih, Demmers *et al.* 2016, Abe, Sakurai *et al.* 2018), (Corbetta, Raimondo *et al.* 2015, Chun-Yan, Zi-Yi *et al.* 2018, Morikawa, Takahashi *et al.* 2018, Tangtanatakul, Klinchanhom *et al.* 2018, Stokman, Bijnsdorp *et al.* 2019, Raimondo, Chinello *et al.* 2020, Williams, Bastos *et al.* 2020), (van der Lubbe, Jansen *et al.* 2012, Qi, Wang *et al.* 2016, Gonzalez-Calero, Martinez *et al.* 2017, Kwon, Woollard *et al.* 2017, Wolley, Wu *et al.* 2017, Salih, Bovee *et al.* 2018, La Salvia, Gunasekaran *et al.* 2020). Many of the newly identified candidate biomarkers have not yet been validated in large independent cohorts or in additional laboratories, but nevertheless these examples highlight the enormous potential for uEV analyses as readouts for pathophysiological alterations within the urogenital and other systems.

The diverse origins and dynamic molecular composition of uEVs present an enormous analytical challenge. It is therefore unlikely that a single standardized approach for urine collection, uEV isolation and measurement will effectively cover all disease scenarios and questions. Nevertheless, arriving at a consensus on best methodological practices is of particular importance in preclinical and clinical uEV studies addressing biomarker discovery and validation, where new understanding would ultimately be applied to inform clinical decisions. Herein, we give a brief overview of the state of the art in uEV research and identify the critical knowledge gaps. We also provide recommendations regarding biospecimen handling, processing and reporting requirements to improve experimental

reproducibility and interoperability. This is of utmost importance for the development of high quality, multi-site studies and realization of the true potential of uEVs in varied clinical settings.

## Biology of urinary EVs

### 2.1. Origins of uEVs

Urine contains a mixture of EVs that originate from several parts of the urogenital tract, including the kidneys, bladder, prostate (males), and utero-vaginal tract (females) (Table 1 and Figure 2) (Pisitkun, Shen et al. 2004, Gonzales, Pisitkun et al. 2009, Zaichick 2014). The biogenesis of this heterogeneous EV population including exosomes, microvesicles, apoptotic bodies, is illustrated in Figure 3 and discussed in detail in other review papers (van Niel, D'Angelo et al. 2018, Kalluri and LeBleu 2020). The relative contributions of each part of the urogenital tract to the total population of uEVs has not yet been determined, but it has been shown that specific subpopulations of uEVs in urine can be enriched by particular interventions, *e.g.* the collection of urine after digital rectal examination (DRE) increases the amount of prostatic fluid in urine and subsequently the quantity of EVs originating from prostatic luminal epithelium cells (Duijvesz, Versluis et al. 2015, Hendriks, Dijkstra et al. 2016). Hence, it is possible to manipulate the uEV composition in this and perhaps other ways, in order to facilitate the detection of specific uEV-associated molecules.

Apart from being produced by different cell types in the urogenital tract, uEVs can also originate from residing immune cells, bacteria and yeast, while enveloped viruses, themselves a type of EV, may also be present (Hiemstra, Charles et al. 2014, Nolte-'t Hoen, Cremer et al. 2016, Salih, Demmers et al. 2016, van Dongen, Masoumi et al. 2016). In addition, some reports suggest that a subset of uEVs enters the urine from the circulation and contain many immunity-related proteins (Oosthuyzen, Scullion et al. 2016, Erozenski, Bottger et al. 2019). It is unclear how these EVs reach the urine (Cheng, Wang et al. 2012, Erozenski, Bottger et al. 2019). In order to pass the glomerular filtration barrier (GFB) and basement membrane of the kidney the EVs would have to be smaller than the membrane-pores (6 nm in the healthy state), or the integrity of the membrane-pores would need to be perturbed (something seen in various pathological states), allowing passage of larger structures like EVs from the circulation into the urinary space (Patrakka, Lahdenkari et al. 2002, Longmire, Choyke et al. 2008). Larger pores of the slit diaphragm of up to 70nm in size are found in minimal change disease, an example of a proteinuric disease state with podocyte damage. Small EVs are likely able to move through this barrier in this disease state. In addition, the endothelial barrier of the GFB might also be penetrated as it has fenestrae of up to 100nm in size which can also allow EVs to move through the GFB (Ndisang 2018). Alternatively, it is possible that uEVs preparations include non-vesicular circulating proteins. It is likely that these are endocytosed from the blood by renal tubular cells as it has been demonstrated for modified circulating albumin molecules in diabetes (Londono and Bendayan 2005). The proteins are then released into the urinary space within EVs. This is supported also by proteomic data, in the case of albumin it is shown that uEVs contain this protein (Musante, Bontha et al. 2020). Similar mechanisms have been described as early as 1989 suggesting that EVs might be transported by transcytosis through podocytes and secreted into the luminal side as 'waste' (Kerjaschki, Schulze et al. 1989).



An additional enigmatic particle type, known as nanobacteria or calcifying nanoparticles (Yaghobee, Bayani *et al.* 2015) is discussed controversially. These entities are composed of crystalline minerals, nucleic acids, and other organic material and appear to be replication competent, albeit through ill-defined processes. Nanobacteria have been associated with various diseases like nephrolithiasis, polycystic kidney diseases, chronic prostatitis, and pelvic pain syndrome (Ciftcioglu, Bjorklund *et al.* 1999, Hjelle, Miller-Hjelle *et al.* 2000, Shoskes, Thomas *et al.* 2005). It remains unclear to what extent these structures contribute to the uEV pool. Further, EVs from both Gram-positive and Gram-negative microorganisms, along with viruses inhabiting the urinary system, are also readily detectable in urine and can be indicative of metabolic or pathological microbial activity (Kang, Ban *et al.* 2013, Yoo, Rho *et al.* 2016, Lee, Park *et al.* 2017).

## 2.2. Molecular composition of uEVs

Urinary EVs contain proteins, nucleic acids, lipids and metabolites. In recent years, the Vesiclepedia repository (V4.1; [microvesicles.org](http://microvesicles.org), accessed 17 July 2020) (Kalra, Simpson *et al.* 2012) has expanded exponentially and, at time of this writing, contains data from 1254 EV studies, including 38 146 RNA entries, 349 988 protein entries and 639 lipid/metabolite entries. From this list, 89 studies (7%) used urine as the EV sample source.

The protein composition of EVs pelleted at 100,000 - 200,000 x g from urine of healthy individuals has been extensively investigated. In these conditions, approximately 0.6 - 3% of the protein in urine is associated with this EV fraction (Zhou, Yuen *et al.* 2006, Bryzgunova, Zaripov *et al.* 2016). The first mass spectrometry study of uEVs in 2004 (200,000 x g pellet) detected 295 proteins (Pisitkun, Shen *et al.* 2004, Gonzales, Pisitkun *et al.* 2009). By 2009 the number of identified proteins reached 1132 (Pisitkun, Shen *et al.* 2004, Gonzales, Pisitkun *et al.* 2009), likely due to improvements in mass spectrometric techniques. The use of newer generation mass spectrometry instrumentation has expanded the uEV proteome to over 3,000 proteins, enabling deeper analysis of EV biology and identification of additional biomarker candidates (Bijnsdorp, Maxouri *et al.* 2017, Fujita, Kume *et al.* 2017, Stokman, Bijnsdorp *et al.* 2019, Dhondt, Geurickx *et al.* 2020). Proteins identified in uEVs include membrane trafficking components, cytoskeletal proteins, motor proteins, membrane transporters and glycosylphosphatidylinositol-linked proteins (Zhou, Yuen *et al.* 2006). In agreement with the idea of uEVs having diverse cellular origins, characteristic proteins of the different organs of the urogenital system, *i.e.* the kidneys (glomeruli, proximal tubule and distal tubule), the bladder and the prostate, have been detected in uEVs (Pisitkun, Shen *et al.* 2004, Gonzales, Pisitkun *et al.* 2009, Street, Koritzinsky *et al.* 2017) (Table 1 and Figure 2). For comprehensive discussions of the proteomic analysis of uEVs we refer the reader to review papers on this topic (Merchant, Rood *et al.* 2017, Eroze, Böttger *et al.* 2019). The analysis of uEV surface markers by flow cytometry and Western blotting has confirmed the presence of uEVs derived from the cells lining all nephron segments (Table 1) (Gamez-Valero, Lozano-Ramos *et al.* 2015). The presence of podocin, podocalyxin or nephrin indicate uEVs from glomerular podocytes, whereas the presence of megalin, cubilin, aminopeptidase or aquaporin-1 (AQP1) indicate uEVs from proximal tubular cells. Uromodulin (UMOD, also known as Tamm-Horsfall protein (THP), CD9, and type 2 Na-K-2Cl cotransporter (NKCC2) mark uEVs from the cells of Henle's loop and aquaporin-2 (AQP2) marks uEVs

from collecting ducts. CD133 identifies uEVs from proliferating/progenitor tubular cells (Dimuccio, Ranghino *et al.* 2014). Finally, bladder derived uEVs contain uroplakin (Pisitkun, Shen *et al.* 2004).

**Lipids and different metabolites** are also components of uEVs, but only a few studies have focused on these molecules (Del Boccio, Raimondo *et al.* 2012, Skotland, Ekroos *et al.* 2017, Clos-Garcia, Loizaga-Iriarte *et al.* 2018). A recent lipidomic study identified over 100 lipid species by mass spectrometry in uEVs (100,000 x g pellet). These EVs showed a remarkably high content of cholesterol (63%), with phosphatidyl serine 18:0/18:1 being the next most abundant lipid species (Skotland, Ekroos *et al.* 2017). In addition, uEVs have a higher cholesterol content compared to plasma derived EVs (Skotland, Hessvik *et al.* 2019). Another recent study using targeted ultra-performance liquid chromatography-tandem mass spectrometry identified metabolites from five main categories of metabolites in uEVs (organic acids and their derivatives, nucleotides, sugars and derivatives, carnitines, vitamin B/related metabolites and amines). The most abundant metabolites detected were ornithine, creatinine, D-ribose 5-phosphate, L-cystathionine, alanine and serine (Puhka, Takatalo *et al.* 2017).

The membrane of uEVs is highly decorated with a variety of **glycans** linked directly to the proteins and lipids of the EV membrane. The abundance of different glycosylations adds to the biomolecular complexity of uEVs and it has been shown that these integral structural and functional components play a role in EV uptake (Williams, Pazos *et al.* 2019). Analysis of uEV carbohydrate content by mass spectrometry and lectin arrays demonstrated that uEVs are highly enriched in complex type N-glycans, with terminal modification consisting of mannose and fucose residues (Saraswat, Joenvaara *et al.* 2015, Kalluri and LeBleu 2020). For a detailed review of EV glycosylation see Williams *et al.* (Williams, Royo *et al.* 2018).

The presence of **RNA** in EVs was discovered in 2006 and the first reports of mRNAs and miRNAs in uEVs followed soon after (Baj-Krzyworzeka, Szatanek *et al.* 2006, Ratajczak, Miekus *et al.* 2006, Valadi, Ekstrom *et al.* 2007, Nilsson, Skog *et al.* 2009, Miranda, Bond *et al.* 2010, Palanisamy, Sharma *et al.* 2010, Bryant, Pawlowski *et al.* 2012). So far, most studies of small noncoding RNAs in uEVs have focused on miRNAs, but other noncoding RNAs such as small nuclear RNAs, small nucleolar RNAs, tRNAs and lncRNAs or fragments thereof have also been found in pelleted uEVs (Barutta, Tricarico *et al.* 2013, Cheng, Sun *et al.* 2014, Delic, Eisele *et al.* 2016, Ghai, Wu *et al.* 2018, Srinivasan, Yeri *et al.* 2019) and in SEC-enriched uEVs (Lozano-Ramos, Bancu *et al.* 2018). Non-coding RNAs were found to be the predominant nucleic acid cargo in the deep sequencing study of uEVs by Miranda *et al.* (Miranda, Bond *et al.* 2014). However, more than 13,000 protein coding genes were detected as well, along with abundantly present rRNA transcripts. A total RNA sequencing approach by Everaert *et al.* and a poly-A based RNA sequencing approach targeting mRNAs by Barreiro *et al.*, confirmed this vast representation, as they reproducibly detected transcripts from over 10,000 genes in uEVs, which was found to be the highest number of all evaluated biofluids (Everaert, Helmoortel *et al.* 2019, Barreiro, Dwivedi *et al.* 2020). Interestingly, uEVs were also shown to be a good source of novel RNA species, such as circular RNAs. In conclusion, many studies have shown the association of RNA and uEVs. However, since RNA can also be found in other molecular structures than EVs, it is recommended to show that the EV-RNAs resist mild degradation by proteinases and nucleases (Mateescu, Kowal *et al.* 2017, They, Witwer *et al.* 2018, Veziroglu and Mias 2020). It is not yet clear

whether **DNA** is present in the lumen of uEVs, but DNA may be found on their exterior (Miranda, Bond *et al.* 2010, Bryzgunova, Zaripov *et al.* 2016). Concerning DNA in the uEV lumen, a study showed that no large differences were observed when comparing the read distribution of the uEV inner nucleic acid cargo with and without DNase I digestion following deep sequencing (Miranda, Bond *et al.* 2014).

These and many other studies have given us an overview of the molecular composition of uEVs. Nevertheless, it is generally recognized that the different EV isolation methods do not entirely remove all non-vesicular material and that the methods separate distinct EV populations to a different extent (Thery, Witwer *et al.* 2018). Hence when reviewing such data, care and caution are needed, as some of the identified molecules may not represent genuine EV-related components and/or the specific EV population that is being investigated.

### **2.3. Physiological functions of uEVs**

Increasing evidence indicates that EVs released into the urine can be internalized by other cells and can modulate their function, suggesting the presence of intra-nephron communication along the urinary lumen (Gildea, Seaton *et al.* 2014). By electron microscopy studies, EVs were shown to be internalized by proximal tubular epithelial cells through cilia *in vitro* (Hogan, Manganelli *et al.* 2009). Moreover, other *in vitro* studies showed that collecting duct-derived EVs could be internalized by tubular cells, transferring AQP2 (Street, Birkhoff *et al.* 2011). Treatment of cultured tubular epithelial cells with podocyte-derived EVs induced a profibrotic phenotype, potentially identifying a novel form of glomerular-tubular communication (Munkonda, Akbari *et al.* 2018). Studies have also identified a role for uEVs in innate immunity (Hiemstra, Charles *et al.* 2014).

In addition, the accumulation of a diverse mixture of uEVs in the bladder followed by their expulsion from the body through urination strongly suggests a principal role for uEVs as a route of elimination. It remains undetermined if excretion through urine is the primary mode for eliminating EVs in general including circulating ones or whether this is mostly related to EVs of the genitourinary system. The study of the physiological functions of uEVs is still in its infancy.

## **3. Current state of the art of urinary EV research**

### **3.1. Collection, processing, and storage of urine for uEV research**

Urine collection, processing and storage are important topics that should be carefully considered in uEV studies because they are major sources of data variability and can limit reproducibility (Zhou, Yuen *et al.* 2006, Dhondt, Van Deun *et al.* 2018, Clayton, Boilard *et al.* 2019). Currently, only general guidelines like the Biospecimen Reporting for Improved Study Quality (BRISQ), including urinalysis and standards (ISO 20387:2018) are established for best practices in urine biobanking (Rabinovitch, Arzoumanian *et al.* 2009, Moore, Kelly *et al.* 2011). Studies addressing collection, processing and storage of urine specifically for uEV research are very limited. The data can be profoundly influenced by the up-front pre-analytical variables, where biospecimen handling is subject to different methods, *e.g.* in collection times, preservatives or centrifugation (Table 3). These differences can lead to selective and variable inclusion of EV subpopulations and non-EV contaminants such as cells or their fragments,

uromodulin networks and protein aggregates. Therefore, it is of utmost importance that the modality of urine handling is consistent within any study. In addition, for interoperability, it is essential that the reporting of such methods is also harmonized across research teams. The EV field would highly benefit from proficiency testing trials that could ideally be conducted in collaboration with biobanks (*e.g.* [www.ibbl.lu/ibbl-bioservices/biospecimen-proficiency-testing/](http://www.ibbl.lu/ibbl-bioservices/biospecimen-proficiency-testing/)). Within ongoing and future urine biobanking studies, we consider that special focus should be put on method validation/consistency and particularly on identifying the most and the least variable preanalytical parameters that affect EV research (Table 2).

Individual research studies have typically employed different urine collection and storage approaches. This is often a result of study-specific protocols and/or logistic restrictions. Large professional biobanks are designed to allow measurement of a wide variety of urine analysis parameters, meaning that the sample collection and storage protocols used might be sub-optimal for uEVs. Therefore, it is unlikely that a universal pre-analytical procedure will be adopted for all uEV studies. Instead, it is more likely that different best practice protocols will be established depending on the molecular component of interest, the choice of analytical platform(s) and the investigated health condition or disorder. As long as standard operating procedures for collection and storage of uEVs are not established by the community, it is safest to report all available pre-analytical information related to the studies in the EV-TRACK knowledgebase, in accordance with the Minimal Information for Studies of Extracellular Vesicles 2018 (MISEV2018) and other ISEV rigor initiatives as well as other suited guidelines developed particularly for preanalytical variables of fluid samples (Betsou, Lehmann *et al.* 2010, Lehmann, Guadagni *et al.* 2012, Nanni, Betsou *et al.* 2012, Witwer, Buzas *et al.* 2013, Lotvall, Hill *et al.* 2014, Consortium, Van Deun *et al.* 2017, Thery, Witwer *et al.* 2018). This will enable a better understanding of the impact of these variables and ideally enable more meaningful comparisons between different studies. In the future, the evaluation of pre-analytical conditions could be used to establish case-specific “Best Practice” protocols. Below we provide the current state of the art of uEV research which also includes common practices. This will be followed by consensus recommendations and an indication of knowledge gaps in the field of uEV research.

### 3.1.1. Patient information

Demographic and clinical parameters including gender, age, ethnic background, weight, height, fluid intake, diet, time of urine collection, laboratory measurements and medication *etc.* should be recorded to identify potential sources of variability, confounders and introduction of unintended bias through the selection of inappropriate members in these cohorts (Ransohoff and Gourlay 2010). When possible, particular attention should be paid to clinical information about kidney function (*e.g.* glomerular filtration rate, albuminuria) as a pathological condition of the kidney has a major effect on the urine and uEV composition (Simeone, Bologna *et al.* 2020). Kidney pathology may also affect uEV excretion, potentially biasing normalization at a later stage (see below). A good example of a study in which careful clinical characterization was done and kidney disease was ruled out as a confounder is a recent examination of uEV cargo as markers for neurological disorders (Wang, Kojima *et al.* 2019). It is also important to record a patient’s use of diuretics or other drugs which may drastically affect urine composition and pH. pH has been reported to affect uEV physiology and isolation (Parolini, Federici *et al.* 2009, Zhao, Chen *et al.* 2017). In addition, urinary pH is highly

influenced by diet, i.e. vegetarian diet causes a high alkaline load (Trilok and Draper 1989, Trilok and Draper 1989). Therefore, reporting general dietary information may improve interpretation of results. Guidelines for appropriate biospecimen reporting for initiation of studies have been developed by several organizations, and some offer online tools to assist with this (Cheah, Dee *et al.* 2012). Nonetheless, detailed information about the patient population under investigation is an aspect that is notoriously under-reported in the literature, a recognized general failing of biomarker studies (Moore, Kelly *et al.* 2011).

### 3.1.2. Urine collection types and variables

#### 3.1.2.1. Instructions and donors

Urine collection is typically performed by the donors themselves. Thus, before the collection, clear and concise instructions on the sample collection process including appropriate hygiene should be given, ideally in both spoken and written forms. As the collection methods may be quite complex or laborious and instructions as well as donors differ greatly, highly standardized collections are difficult to achieve (Fisher, Johnson *et al.* 1977).

#### 3.1.2.2. Time and void

Urine can be collected during a single voiding episode (“spot urine collection”) or can be collected across several voiding episodes during a fixed time period (“timed urine collection”). Spot urine collections can be done at a random time (“random” spot urine) or standardized to the first or second morning urine. Timed urine collections can be over the course of hours or a day (called “24-hour urine”). The volume of urine collection can be “full void” or “midstream urine” (*e.g.* without collecting the earliest portion of the voided urine). Relatively little is known about the impact of different collection types on uEV measurements.

The first morning urine is generally more concentrated than a random spot urine (Thomas, Sexton *et al.* 2010), possibly resulting in a higher uEV concentration in the first morning urine. Zhou *et al.* found only minor differences between first and second morning urine with respect to total protein in uEVs or exosome-associated proteins (Zhou, Yuen *et al.* 2006). Another study of uEVs from first and second morning voids in three control males showed that only 4 % of the identified proteins by mass spectrometry were significantly altered in abundance between the two conditions (Øverbye, Skotland *et al.* 2015). Nevertheless, specific uEV biomarkers may fall within this fraction, and it is therefore recommended to determine the stability of identified biomarkers in relation to pre-analytical variables. In addition, physiological processes in the kidney and some kidney bio-markers follow a circadian rhythm (Firsov and Bonny 2018). It is currently unknown whether the release of uEVs or the composition of their cargo demonstrate a circadian rhythm in humans, although one study has examined these questions in rodents (Koritzinsky, Street *et al.* 2019). Periodicity would be discovered only by analyzing timed urine collections, ideally gathered in fractions over 24 hours (Firsov and Bonny 2018).

In the case of a timed collection, documenting and reporting the time between the last uncollected and first collected void would help with assessing urine transition time in the bladder and may be of additional value for normalization. For example, uEV protein content could be related to a time period of 4 or 6 hours, which might be easier to collect than 24-hour urines. In many prostate cancer studies, urine samples are collected after a DRE by the urologist. Collection at this time point can greatly increase the amount of prostatic fluid in the urine and consequently enriches the sample for prostate-derived EVs (Duijvesz, Versluis *et al.* 2015, Hendriks, Dijkstra *et al.* 2016, Fujita and Nonomura 2018).

The collected urine void impacts the availability or enrichment of specific EVs and other urine components. First void after DRE has been shown to increase the chance of finding prostate cancer associated EVs (McKiernan, Donovan *et al.* 2016, Fujita and Nonomura 2018). However, first void also contains more cells and bacteria than the mid-stream void, leading to 36% of urine samples to exceed health related upper reference limit vs 10% of mid voids (Manoni, Gessoni *et al.* 2011). It is unclear which urine collection is the “cleanest” without significant contamination by cells or bacteria. Reduction of microbe content requires attention to the entire uEV workflow (Tataruch-Weinert, Musante *et al.* 2016). Another point to be addressed is the need to establish an optimal workflow that addresses the presence of bacterial outer membrane vesicles (OMVs) in urine (derived from either normal or pathogenic urinary tract microbiota) (Yoo, Rho *et al.* 2016, Barreiro and Holthofer 2017, Lee, Park *et al.* 2017). Another mechanism that may influence EV secretion rate includes urinary flow; *i.e.* kidney tubule cells have cilia that may be activated by flow and have an important role in EV secretion (Wang and Barr 2016). However, the *in vivo* implications have not been studied.

Little is known about the inter-day variation of uEVs. For example, Wang and others investigated the variability of the uEV proteome in morning urine from two healthy volunteers over a two-month period (Oeyen, Willems *et al.* 2019, Wang, Kojima *et al.* 2019). They showed that approximately 50% or hundreds of uEV proteins were stable at the inter-day and intra-individual level. As expected, most variation was found within the low abundance proteins. Some of the stable proteins could be classified as housekeeping, including numerous heat shock proteins, actin and annexin A4. On the RNA level, Murakami *et al.* (Murakami, Oakes *et al.* 2014) have found that the expression of some uEV mRNAs from different parts of the kidney were stable on the intra-individual level over a two-week period. On the other hand, larger inter-individual differences were found. While the authors could confirm the stability of five mRNAs among the subjects, further studies are needed for discovery and validation of truly stable control uEV RNAs.

### **3.1.2.3. Collection containers and devices**

Urine collection containers are typically made of plastics, such as high-density polyethylene or polypropylene, can be sterile or unsterile, open or closed, anatomically compatible or have tube transfer systems. Some even have a urine temperature thermometer affixed to the outside of the cup. There are no studies known to have tested the impact of different containers on uEV collection. However, it is important to ascertain that containers should not bind uEVs or shed (plastic micro-) particles. Models with a lid are preferable to prevent the introduction of external EVs. Sterile tubes

may be especially important for studying microbial uEVs. Specialized collection devices might be needed (*e.g.* urine bag for infants) or part of a protocol for standardized collection of different voids (*e.g.* first 20 mL void using a Colli-Pee device, Novosanis, Belgium).

#### **3.1.2.4. Preservation: storage before freezing**

Unprocessed urine should be kept at 0-4 °C and processed within to 8 hours to avoid bacterial growth, cell lysis, molecular degradation of RNA and protein, and formation of sediments (Saetun, Semangoen *et al.* 2009, Moatamed 2019, Barreiro, Huber *et al.* 2020). However, it may not be universally recommended to keep urine cold. Armstrong *et al.* found that miRNA and other small RNA contents of uEVs declined during 4-24 hours of storage after collection, and the decline was greater when samples were kept at 2-4°C rather than at room temperature (RT) (Armstrong, Dessaint *et al.* 2018). The authors discussed that the decline could be due to cold induced precipitation and that it could be rescued by warming the urine sample for 5 min at 37°C. Indeed, heating increased RNA yields from frozen samples that had formed precipitates. However, this could also be related to the formation of uromodulin polymers that form when urine is kept cold, *e.g.* below 4 °C (Wachalska, Koppers-Lalic *et al.* 2016). These polymers can trap EVs to some extent, which are subsequently removed from the sample after low-speed centrifugation (Wachalska, Koppers-Lalic *et al.* 2016).

With longer timed collections, such as 24h collections, fast processing cannot be achieved, and studies of the possible effect of repeated warming (37°C) and cooling (either to RT or +4°C) of the urine specimen during collection are lacking. However, generally, if long urine collection times are required, the addition of preservatives such as azide should be considered to avoid microbial overgrowth, at least when the preservative is compatible with further uEV processing steps (Thongboonkerd and Saetun 2007, Havanapan and Thongboonkerd 2009). Effects of RNase inhibitor addition have not been investigated systematically, even though RNases are present in urine.

Several studies have investigated whether protease inhibitors should be added to urine to avoid uEV protein degradation (Zhou, Yuen *et al.* 2006, Mitchell, Welton *et al.* 2009). Although this may preserve some specific uEV proteins such as NKCC2, analysis of CD9 and TSG101 showed that not all EV proteins are prone to proteolysis in urine (Zhou, Yuen *et al.* 2006, Mitchell, Welton *et al.* 2009). It is important to address this issue more conclusively because urine samples in biobanks are not typically collected with protease inhibitors because the use of protease inhibitors would increase costs considerably, especially in large sample studies. Similarly, when analyzing phosphorylated proteins, the use of phosphatase inhibitors should be considered although it has not been thoroughly studied.

#### **3.1.2.5. Urine quality control**

Commercially available dipsticks can be used as a form of rapid quality control by measuring urine pH and various contents (*e.g.* leukocytes, erythrocytes, protein, glucose, nitrate, ketones, blood, bilirubin, urobilinogen) (Welton, Khanna *et al.* 2010, Øverbye, Skotland *et al.* 2015, Royo, Zuniga-Garcia *et al.* 2016). Information obtained by these rapid, simple procedures identify patient status

and allow exclusion of deviating samples, such as those heavily contaminated by microbial infection or blood. However, dipstick use for inclusion/exclusion in uEV studies has been rather arbitrary to date: there is no consensus in defining which dipstick test is most suitable, or on where to set inclusion/exclusion criteria.

### 3.1.2.6. Clearing before freezing

Frozen, precleared urine is used in many uEV studies. Preclearing usually involves centrifugation to remove cells, large cell debris and often also the bulk of uromodulin, and it is done so that these materials do not contaminate uEV preparations with artifactual, similarly sized particles during freeze-thaw cycles. Interestingly, however, one study found that uEV miRNA/small RNA correlated highly when comparing urine aliquots that had been centrifuged alternatively before freezing or after a freeze-thaw (Armstrong, Dessaint *et al.* 2018).

Several urine processing protocols are currently available from uEV researchers and biobanks *e.g.* the European Association of Urology Standard Operating Procedures in UroWeb ([uroweb.org/research/how-we-work/](http://uroweb.org/research/how-we-work/)) and the MMI Guidelines for Standardized Biobanking ([crdi.ie/resources/biobanking-guidelines/](http://crdi.ie/resources/biobanking-guidelines/)) from the Clinical Research Development Ireland. According to these sources as well as other uEV literature, centrifugation parameters used for preclearing vary widely. For example, the centrifugation speed used ranges from roughly 1-20,000 x g, centrifugation time varies between 0-30 min, and both one- and two-spin approaches are used. Centrifugation volumes, use of a brake and supernatant removal methods also vary between studies, although these parameters are rarely specified in publications. Low speed centrifugation (<1,000 x g) is generally used to remove whole cells and large cell debris, but data has shown that lower speeds may not suffice for this task. A single 400 x g step for 5 minutes results in inadequate removal of cells while efficient cell pelleting was achieved by centrifugation of 10 ml volumes at 1358 x g for 10 minutes in round bottom tubes (Bunjevac, Gabaj *et al.* 2018).

Uromodulin, also known as Tamm-Horsfall protein, is the most abundant protein excreted into urine (Micanovic, Khan *et al.* 2018). Most uromodulin can be sedimented with a 2,000 x g spin for 30 minutes without a gross loss of uEVs, whereas speeds  $\geq 10,000$  x g result in pelleting of uromodulin and EVs (Fernandez-Llama, Khositseth *et al.* 2010, Musante, Tataruch *et al.* 2014, Puhka, Nordberg *et al.* 2017, Musante, Bontha *et al.* 2020). Some studies have combined first a lower speed spin, *e.g.* 300 x g, with a second higher speed spin, *e.g.* 2,000 x g (Mussack, Wittmann *et al.* 2019), to deplete the larger and smaller contaminants consecutively. While this method might be more effective than a single spin, it also adds to the handling time and steps, which can be limiting for large sample numbers. Loss of EVs due to binding to polymeric uromodulin can be reduced or eliminated through use of reducing agents that depolymerize uromodulin by breaking disulfide bridges between individual uromodulin monomers (Fernandez-Llama, Khositseth *et al.* 2010).

The choice of preclearing parameters usually depends on the study goal. Ammerlaan *et al.* optimized urine processing (centrifugation speed, time, temperature and brake) for reproducibility in proteomic and metabolomics studies with the criteria that the urine supernatant should still contain the EV component (Ammerlaan, Trezzi *et al.* 2014). As depletion of larger EVs (microparticles) was



preferred in the original study, the best protocol with low microparticle counts in the recovered supernatant was a 20 min, 12,000 x g centrifugation at 4°C with a hard brake. An optimal pre-clearing/pre-freezing protocol might thus be EV subtype-specific, but in practice, compromises may be necessary when using biobanked samples, because these resources are designed to provide urine samples for a variety of uses.

### **3.1.2.7. Collection volume and freezing aliquots**

The volume of urine required for uEV analysis depends on the yield of the method used to isolate EVs and the sensitivity of the analytical method, but 10-30 ml of urine is sufficient for many purposes, for example RNA sequencing or proteomics (Musante, Bontha *et al.* 2020). It is advisable to collect and store processed urine in aliquots (as a backup or for use in different analyses). Most urine collection containers collect sufficient volume to allow division into multiple aliquots of suitable size (e.g. 1 to 30 ml) which speeds up the freeze and thaw processes and avoids unnecessary pooling of aliquots or multiple freeze/thaw cycles. Whenever possible, it is recommended to preserve the cellular pellet or the low-speed centrifugation pellet, which may also contain uEVs (Musante, Bontha *et al.* 2020), as well as aliquots of whole urine for monitoring of the purification process, for comparative analyses or as controls.

### **3.1.2.8. Freezing temperature and storage time**

Freezing and storage at -70°C or lower temperature is preferred. Zhou *et al.* showed that storage at -20°C caused more than 50 % loss of EVs compared with storage at -80°C where EV loss was 14 % (Zhou, Yuen *et al.* 2006). Partially supporting these findings, Oosthuyzen *et al.* measured particle count by nanoparticle tracking analysis (NTA) in urine samples stored at room temperature, at +4°C, or frozen for 2h to 1 week (Oosthuyzen, Sime *et al.* 2013). Particle counts were lower in the samples stored at -20°C compared with -80°C or other temperatures. Protease inhibitors in this study also had a positive effect increasing the recovery of particles from less than 40% to over 80% from the original counts. A later study reported that concentration and particle size remain similar after freezing at different temperatures, *i.e.* -20°C, -80°C or -196°C without gross changes in uEVs morphology as observed by TEM. Particle concentration analysis by NTA showed approximately 2-fold increase and similar decrease as measured by resistive pulse sensing (RPS), in comparison with fresh samples. (Yuana, Boing *et al.* 2015). Particle mode size increased by 17% during 1 year of storage at -80°C. Overall, uEVs were found to be more stable during 1-year storage at -80°C as compared with EVs from other body fluids. Thus, most evidence for uEVs storage temperature is in line with the recommendations for EVs from other body fluids and storage at -70°C or colder is recommended (Cheng, Zeng *et al.* 2019). Regarding antigenicity after freezing and long-term storage, it is of importance to note that the uEVs proteome includes thousands of proteins, therefore it cannot be excluded that some proteins can be more prone than others to lose antigenicity after long term storage. For example, in The Finnish Diabetic Nephropathy Study ([www.finndiane.fi](http://www.finndiane.fi)), a well-established cohort of urine samples with different levels of albuminuria, many isolated uEVs were associated with antigens including proteases, protease inhibitors and ubiquitin (Musante, Tataruch *et al.* 2015). These proteins might lead to loss of antigenicity in different cohorts after thawing, but the process of freezing and thawing by itself could affect the

antigenicity. Only dedicated studies of uEVs can establish the best condition for freezing temperature and storage time for the protein(s) under investigation.

Frozen uEVs might interact with cryoprecipitates, mainly calcium oxalate dehydrate and amorphous calcium or polymerized proteins, leading to uEVs entrapment and apparent loss unless released by measures such as vortexing, dilution, lowering of ionic strength or depolymerization of proteins (Saetun, Semangoen *et al.* 2009, Puhka, Nordberg *et al.* 2017). Early studies reported that vortexing after thawing can considerably increase uEV recovery from urine frozen either at  $-20^{\circ}\text{C}$  (87% recovery) or  $-80^{\circ}\text{C}$  (to 100% recovery), even after 7 months of storage (Zhou, Yuen *et al.* 2006). However, it is not known if vortexing damages vesicles or if it leads to loss of luminal content and other studies did not observe significant effect of post-thaw vortexing (Oosthuyzen, Sime *et al.* 2013). Additional work is needed to investigate in a comprehensive manner to which extend the size, number and molecular composition of uEVs is affected by freezing temperature and storage time.

### 3.2. uEV separation

Several EV separation methods that show specific advantages and disadvantages have been developed (Coumans, Brisson *et al.* 2017, Konoshenko, Lekchnov *et al.* 2018). Moreover, the selected isolation method may affect the characteristics and analysis of both isolated EVs and contaminants (Alvarez, Khosroheidari *et al.* 2012, Royo, Diwan *et al.* 2016, Royo, Zuniga-Garcia *et al.* 2016, Merchant, Rood *et al.* 2017, Mussack, Wittmann *et al.* 2019). A main focus has been on purity and yield of uEVs and usually one improves at the expense of the other. In addition to yield and purity, emphasis should also be given to practical considerations such as speed, scalability and throughput, as any high-impact clinical research, and biomarker research in particular, requires validation of the results in hundreds to thousands of samples. Further, all isolation techniques yield only a subset of uEVs, which does not necessarily contain all uEVs of interest. However, in some cases, specific enrichment of a subset may be advantageous and improve the detection of some markers.

Traditionally, uEVs have been separated by ultracentrifugation. However, “ultracentrifugation” is not one technique, and there are a host of protocol variants and specifics across studies contributing to variable results within this category of separation modality (EV-TRACK, (Consortium, Van Deun *et al.* 2017)). Sequential centrifugation is more commonly used and involves low speed centrifugation to remove cells and debris, followed by the subsequent consecutive collection of large and small EVs at increasing centrifugation speed (in general  $10,000 - 20,000 \times g$  for 20-30 minutes for large EVs, and  $100,000 - 200,000 \times g$  for 1-2 hours for smaller EVs) (Gonzales, Zhou *et al.* 2010). However, it has been reported that ultracentrifugation (UC) can have poor efficiency, with up to 40% of small uEVs retained in the supernatant after UC at  $200,000 \times g$  (Musante, Saraswat *et al.* 2013).

A major challenge to effective EV separation is the highly abundant urinary protein uromodulin, which forms long polymers that can entrap small EVs (Pisitkun, Shen *et al.* 2004, Musante, Bontha *et al.* 2020). Trapped EVs will then co-pellet with uromodulin at low centrifugation speeds and may reduce the recovery of small uEVs isolated by sequential centrifugation (Figure 1A). Several

approaches have been shown to release entrapped vesicles such as addition of the reducing agent dithiothreitol (DTT) Tris (2-carboxyethyl) phosphine hydrochloride (TCEP-HCl), the detergent 3-[[3-Cholamidopropyl] dimethylammonio]-1-propanesulfonate (CHAPS) or alkaline buffers (Fernandez-Llama, Khositseth et al. 2010, Musante, Saraswat et al. 2012, Puhka, Nordberg et al. 2017, Musante, Bontha et al. 2020). Some groups have reported that DTT only slightly improves the yield (Cheng, Sun et al. 2014). A recent study demonstrated that removal of uromodulin using TCEP-HCl does not affect particle counting with NTA/Tunable Resistive Pulse Sensing (NTA/TRPS), or results of flow cytometry or, qPCR, but did influence Western blotting and mass spectrometry results (Musante, Bontha et al. 2020). Of note, detection of antigens depends on the analytical technique in use. SDS-PAGE followed by western blot is usually performed in reducing conditions without affecting the detection of the antigen of interest. However, there are exceptions depending on the type of antibody used and the nature of the antigen studied. For example, detection of tetraspanin in WB seems to be favored when the sample is solubilized without any reducing antigen (Musante, Tataruch-Weinert et al. 2017). Nevertheless, this evidence originates from very few examples and it would not be correct to extend this conclusion to the whole uEVs proteome which accounts for more than a thousand proteins. In addition, the use of reducing agents does not seem to affect the integrity of uEVs as reported by electron microscopy pictures of several studies which included either the use of DTT and / or TCEP (Fernandez-Llama, Khositseth et al. 2010, Musante, Bontha et al. 2020). A heterogeneous population of EVs was found in the pellet with size and morphology not dissimilar from the fraction before treatment including multi-lamellar or composite structure with smaller EVs enclosed in larger ones. These findings reaffirm the importance of depleting uromodulin for certain downstream uEV analyses.

Disease-related changes in urine content, such as proteinuria, can also complicate EV isolation. In particular, albumin (and other proteins) that leak into the urine in glomerular disease can bind to the surface of EVs or co-elute as a protein complex (Merchant, Rood et al. 2017, Santucci, Bruschi et al. 2019). This can impair certain ultrafiltration-based approaches and interfere with protein-based characterization, e.g. mass spectrometry or Western blot following ultracentrifugation (Rood, Deegens et al. 2010). Coupling ultracentrifugation with size exclusion chromatography, the use of sucrose or other density gradients, or the isolation via filtration dialysis have been shown to reduce albumin and other proteins in EV isolates (Musante, Tataruch et al. 2014, Santucci, Bruschi et al. 2019). Another consideration is that proteinuria can alter urine viscosity which is a critical determinant of EV recovery in centrifugation-based approaches (Momen-Heravi, Balaj et al. 2012, Inman, Etienne et al. 2013). However, the impact of changes in urine viscosity in proteinuria on EV recovery is not known. The presence of red blood cells in urine samples (hematuria) can also alter the purity of EV isolates. A trypsin treatment performed before uEV isolation was recently described to prevent hematuria-related proteomic alterations (Raimondo, Chinello et al. 2018).

Many of the new methodologies developed for EV separation have been applied to uEVs, including filtration, precipitation, hydrostatic dialysis, ultrafiltration combined with size exclusion chromatography, acoustic trapping and immunocapture (Cheruvanky, Zhou et al. 2007, Musante, Tataruch et al. 2014, Wang and Sun 2014, Huebner, Somparn et al. 2015, Lozano-Ramos, Bancu et al. 2015, Consortium, Van Deun et al. 2017, Merchant, Rood et al. 2017, Ku, Lim et al. 2018, Oeyen, Van Mol et al. 2018, Dhondt, Lumen et al. 2020, Svenningsen, Sabaratnam et al. 2020). The efficacy and

yield, and potential contaminants of these EV isolation techniques still need to be evaluated. Multiple studies have shown that the choice of isolation method can have a significant effect on measured EV molecular content (Freitas, Balmana *et al.* 2019, Srinivasan, Yeri *et al.* 2019). Co-isolation of abundant proteins in urine with uEVs hampers the detection of less abundant proteins in uEVs. One strategy to account for high-abundance uromodulin contamination in mass-spectrometry proteomics analysis of uEVs is the use of an exclusion list of uromodulin peptides (Hiemstra, Charles *et al.* 2011). However, it should be noted that highly abundant proteins will still influence the identification and quantification of low abundant peptides, even with computational filtering after spectrum acquisition.

Finally, it is important to investigate to which extend the different EV isolation methods remove potential molecules/structures that may contaminate the uEV pellet. Main contaminants of uEV pellets can be bacteria, blood cells and lymphocytes, uromodulin and albumin. In normal conditions the main contaminant of the uEV fraction is considered to be uromodulin. Urine test strips can be used to detect abnormal levels of bacteria and protein (albumin), and the presence of blood cells and lymphocytes in the urine samples. A gel electrophoresis can also show the protein pattern of each urine sample before and after uEV enrichment. This information can be considered during sample inclusion as well as when analyzing the uEV fraction. Electron microscopy can be used to detect the presence of abnormal vesicle morphology or other structures in the uEV sample, such as uromodulin (precipitates). Western blot can be used to detect specific co-isolating proteins that may be present in the EV sample such as uromodulin or albumin. Proteomics analysis is also useful as it allows to compare the abundance of these and other co-isolating proteins in relation to uEV proteins.

In conclusion, we note limited consistency and coherence between uEV separation methods. These limitations also apply to methods for characterization and analysis of EVs (addressed in the next section).

### **3.3. uEV characterization**

#### **3.3.1 Post-separation characterization and analysis of enriched uEVs**

Authors reporting uEV characterization should be guided by MISEV reporting requirements (They, Witwer *et al.* 2018). There are additional specific considerations for uEVs since urine is a particularly dynamic body fluid that contains EVs derived from a variety of cells. Parameters that vary in urine include concentration, osmolality, electrolytes, pH level, excreted/secreted proteins as well as cellular, bacterial, and viral quantity and content. There is no single technique that can characterize uEV heterogeneity by describing EV morphology, size, count and content. Each post-isolation characterization is affected by the EV separation method used (see section 3.3.2 for further discussion of this topic). In many cases (*i.e.* animal work, archived random time spot urine) the amount of urine available may limit the number of complementary analyses that can be conducted (Musante, Bontha *et al.* 2020).

The **morphology of uEVs** has been described by transmission electron microscopy (TEM), cryogenic electron microscopy (cryo-EM), atomic force microscopy (AFM) and super resolution fluorescence microscopy (Figure 1). In particular, TEM and cryo-EM show a heterogeneous group of EVs of different sizes and shapes, and cryo-EM also allows the visualization of intraluminal structures (Musante, Bontha *et al.* 2020) (Figure 1A). In addition to providing information about uEV size distribution, EM can also be used to assess sample purity, as gross protein aggregates, major vault proteins and other structural contaminants can be visualized and distinguished from EVs. See for example the presence of uromodulin in uEV samples in Figure 1. Cryo-EM preserves EV morphology and shows the lipid bilayers at high resolution, making it well suited for structural characterization of the EVs. However, performing systematic quantification of these parameters by cryo-EM is time consuming and thus low throughput. Additionally, cryo-EM requires costly equipment and specialized technical staff, which limits its accessibility and makes broader adoption of this approach to quality control unlikely. TEM (Figure 1B) also requires specialized facilities, but is generally more accessible. TEM negative staining protocols are straightforward, allowing visualization and sizing of EVs, and rough estimation of their purity in a large number of samples relatively quickly. TEM can also show EVs heterogeneity by differential staining densities to highlight morphological characteristics and surface features. Recently, super-resolution microscopy has been used to directly visualize fluorescently labeled molecules within vesicles with 20nm resolution, revealing the biomarker distribution and expression levels on single vesicles (Saliba, Cespedes-Donoso *et al.* 2019). Many investigators have found SDS-PAGE gel electrophoresis of the uEV sample to be useful as a general profiling tool to explore the protein pattern and detect potential protein degradation or protein contaminants, such as uromodulin (Zhou, Yuen *et al.* 2006, Musante, Tataruch *et al.* 2014, Musante, Bontha *et al.* 2020). Of note, uromodulin is rarely fully eliminated from uEV preparations, regardless of the separation method used, because there is a GPI-anchored, membrane-associated form of uromodulin which may be a normal constituent of tubular cell-derived EVs (Rindler, Naik *et al.* 1990, Musante, Bontha *et al.* 2020).

**uEV size distribution and counts** can be measured with commercially available particle analyzers including NTA, based on Brownian motion and tunable resistive pulse sensing (TRPS), based on the Coulter principle (Oosthuyzen, Sime *et al.* 2013, Musante, Bontha *et al.* 2020.). Both methods are discussed below, however there is only limited data comparing these methods.

Several technologies are utilized to study **EV content** (*e.g.* proteins, RNA, lipids, glycans). Western blot or ELISA techniques are based on bulk analysis of uEV content, whereas flow cytometry offers high-throughput single-EV surface protein analysis but requires advanced instrumental setup and experience to obtain sufficient resolution (Welsh, Van Der Pol *et al.* 2020). In addition, specialized cytometers with higher scatter sensitivity to measure small particles are not widely available (high-resolution flow cytometry). As an alternative, bead-based cytofluorimetric analysis can provide semi-quantitative analyses of EV surface markers (Suarez, Gamez-Valero *et al.* 2017, Monguio-Tortajada, Moron-Font *et al.* 2019). Recently, a bead-based commercial kit detected up to 37 surface markers of EVs captured by CD63, CD9 and CD81-coated beads (Wiklander, Bostancioglu *et al.* 2018). For uEVs, CD24 and CD133 might be of interest as markers of kidney function whereas other markers may identify kidney infiltrating cells (Dimuccio, Peruzzi *et al.* 2020). Numerous omics analyses have also been performed to define the molecular content of uEVs and identify novel biomarkers for

several diseases. Such studies include (small) RNA-Seq and other transcriptomics analyses as well as mass spectrometry-based proteomics (Cheng, Sun et al. 2014, Salih, Demmers et al. 2016, Thomas, Hao et al. 2016, Rodriguez, Bajo-Santos et al. 2017, Erozezi, Bottger et al. 2019, Everaert, Helmsmoortel et al. 2019, Srinivasan, Yeri et al. 2019, Stokman, Bijnsdorp et al. 2019, Carreras-Planella, Cucchiari et al. 2020, Carreras-Planella, Juega et al. 2020, Park, Lee et al. 2020). Metabolomic and lipidomic studies of uEVs are also under development, but remain rather complicated, with workflows requiring specialized instrumentation and expertise (Clos-Garcia, Loizaga-Iriarte et al. 2018, Williams, Palviainen et al. 2019).

Recently, a systematic comparison of 10 different isolation methods for small RNA EV-cargo across 5 biofluids revealed marked differences in the complexity and reproducibility of the resulting small RNA-Seq and mRNA-fragment profiles with the type of the RNA (*i.e.* miRNA, tRNA or mRNA fragments) being a major factor in the choice of isolation method. An interactive web-based application (miRDaR) with incorporated comparative statistics was also developed to help investigators select the optimal RNA isolation method for their studies (Srinivasan, Yeri et al. 2019). Results for uEVs demonstrated that when miRNAs are the RNA type under investigation, none of the tested methods has both high reproducibility and high sample complexity, suggesting that choice of (small) RNA extraction method should be driven by the overall small RNA-Seq data quality metrics to be applied. Interestingly, uEV small RNAs were almost entirely comprised of tRNA fragments (tRFs), and tRF profiles grouped in 2 clusters based on separation method, suggesting the presence of two major uEV subclasses that carry these small RNAs. For mRNA fragments present in the sequencing libraries, a clear separation of samples based on both sex and type of isolation method was observed, suggesting that gender should be taken into consideration early in study design (Srinivasan, Yeri et al. 2019).

Capture of uEVs, followed by direct RNA isolation with an optional uEV purification step in-between and followed by next generation sequencing is common for uEV-RNA analysis (Mussack, Wittmann et al. 2019, Park, Lee et al. 2020). Such approaches can be utilized to minimize sample handling and maximize EV recovery, both of which are attractive for clinical utilization. Acoustic trapping of uEVs followed by RNA isolation and next generation sequencing is another recent example (Ku, Lim et al. 2018, Ku, Ravi et al. 2019). Importantly, a recent study comparing a variety of EV separation methods clearly demonstrates that some widely used methods are not suitable for small and long RNA sequencing, particularly those that combine uEV isolation/separation and RNA isolation (Karina, Prakash et al. 2020). Thus, it is highly recommended that appropriate pilot studies are performed to assess key performance characteristics of the planned RNA sequencing methods, especially when newly available commercial isolation kits are used. This is particularly vital in studies where small and long RNA sequencing are equally important targets.

A pipeline application for proteomic analysis, including a heat-shock protein-based EV capture (Vn96-peptide ligand) and a subsequent protein fractionation step followed by mass spectrometry was recently described and applied for biomarker discovery in nephronophthisis-related ciliopathies (Ghosh, Davey et al. 2014, Knol, de Reus et al. 2016, Bijnsdorp, Maxouri et al. 2017, Stokman, Bijnsdorp et al. 2019). A variety of ELISA immunoassay methods exploit unique biophysical features of EVs to facilitate large-scale and high-throughput screening of uEVs for clinical applications

(Reviewed in (Salih, Fenton *et al.* 2016)). Microfluidic devices such as nanoscale lateral displacement arrays on a chip (Nano-DLD arrays), double filtration microfluidic system on a microchip, microfluidic nanowires followed by *in situ* RNA extraction, centrifugal lab-on-a-disc nanofilters, and nanoparticle-based time resolved fluorescence immunoassay (NP-TRFIA) are prototypes showing the feasibility of isolating and analyzing uEVs directly from cell-free urine (Duijvesz, Versluis *et al.* 2015, Liang, Kong *et al.* 2017, Woo, Sunkara *et al.* 2017, Yasui, Yanagida *et al.* 2017, Smith, Wunsch *et al.* 2018, Islam, Syed *et al.* 2019). Important developments allow multiplexing and enable the detection of combinations of markers on the EV surface (Burbidge, Zwickelmaier *et al.* 2020). In line with this, a single particle interferometric reflectance imaging sensor platform (SP-IRIS) is now commercially available (Daaboul, Gagni *et al.* 2016). A capture chip based on a tetraspanin (CD63, CD9 and CD81) can obtain particle size distribution, images of vesicles and detect up to four different protein markers per EV. These are just few examples applied to the analysis of urine and more information can be found in (Chiriaco, Bianco *et al.* 2018, Hartjes, Mytnyk *et al.* 2019, Iliescu, Vrtacnik *et al.* 2019). Such technologies are advancing at pace, but none of these have become a consensus standard approach within the community. Uncertainty remains regarding which technology would be the most the optimal system for developing a uEV assay that is truly fit for purpose in clinical diagnostic laboratories.

### 3.3.2. Direct quantification and characterization of uEVs in cell-free urine

Reliability of EV separation techniques often correlates with investment of time and money. As stated earlier, all isolation techniques yield a subset of uEVs, which does not necessarily contain all uEVs of interest. Therefore, ideally analysis and assessment of uEVs should be performed on cell-depleted urine (urine supernatant). Overall, urine analytes are relatively dilute and few platforms are sensitive enough to perform analysis without any pre-enrichment processing, but quantification and characterization techniques developed for the analysis of cell-depleted urine are making important progress, and might someday facilitate clinical application of uEVs.

One of the best-defined techniques for direct quantification and characterization of uEVs is NTA, which can measure particle size distribution and concentration in biofluids (Oosthuyzen, Sime *et al.* 2013). However, NTA (Patrick Hole, J Nanopart Res 2013) is prone to user and equipment/software bias, which can further complicate the comparison of multiple datasets. NTA measures all particles present in urine, including protein aggregates, *e.g.* uromodulin or human serum albumin (HSA) aggregates in patients with proteinuria or albuminuria. This can distort the quantification and characterization of uEVs (McNicholas, Li *et al.* 2017, Gleadle, McNicholas *et al.* 2018). Conversely, NTA has a lower size detection limit for particles in urine of less than 70 nm in diameter in scatter mode (Oosthuyzen, Sime *et al.* 2013). Thus, NTA may not detect smaller uEVs, which are thought to be the majority (Pisitkun, Shen *et al.* 2004), resulting in an under representation of uEVs. Appropriate resuspension and dilution are necessary since NTA measures clumped particles as a single particle.

Other techniques used to detect and characterize uEVs in cell-free urine include specialized flow cytometry and TRPS (Coumans, van der Pol *et al.* 2014). EV flow cytometry uses specific antibodies and/or ligands to either enrich uEVs or exploit the signal of a fluorescent tag linked to the antibody.

An example is the use of anti-tetraspanin coated magnetic beads when analyzing EVs with conventional flow cytometry, which offers combined isolation and analysis of uEVs (Campos-Silva, Suarez et al. 2019, Welsh, Van Der Pol et al. 2020). Further developments of flow cytometric based analysis of EVs include use of imaging flow cytometry (Musante, Bontha et al. 2020) and nano-flow cytometry (Tian, Gong et al. 2020) for direct uEV analysis in cell-free urine. Whilst relatively new techniques, both of these offer the potential for analysis of individual EVs. Another newly developed assay for the quantification of EVs and detection of multiple biomarkers on the EV surface, without the bias induced by marker dependent EV capture, is EVQuant (Hartjes, Slotman et al. 2020). In EVQuant, in-gel immobilization of fluorescently labeled EVs allows high throughput detection of individual EVs and the detection of EV subpopulations and their size distribution (Blijdorp, Tutakhel et al. 2021). Another recent technique that could assess the global composition of uEVs at the single particle level or in a limited group of EVs is Raman Tweezers microspectroscopy (RTM) which could help to determine the percentage of different EV subpopulations and contaminants present in the preparation (Kruglik, Royo *et al.* 2019).

In general, all EV analysis approaches and assays currently developed are hampered by the small size and large heterogeneity of EVs in bio-fluids. Improvements in sensitivity and specificity are needed to truly access the whole range of EVs and EV subpopulations in both research and clinical applications.

### 3.4. Normalization

In order to effectively maintain water and salt homeostasis, urine production can be highly variable. Consequently, the concentration of EVs in urine may vary more than in blood and other body fluids. In addition, uEV processing protocols invariably induce additional variation that may need to be corrected for (Yuana, Bertina *et al.* 2011, Momen-Heravi, Balaj *et al.* 2012). Thus, a major challenge of uEV research is the lack of robust methods to normalize uEV content to adjust for confounding factors such as excretion rate and uEV-processing-related variation (Blijdorp and Hoorn 2019). Normalization approaches for urine biomarkers can be broadly classified as calculating an absolute or relative excretion rate. **Relative excretion rate** defines the abundance of the uEV marker in relation to another marker, such as uEV number, a protein or RNA marker, or total EV protein, RNA or lipid amount (Table 4). This is most commonly applied in urologic and in proteomics studies but also used in kidney-related studies (Pisitkun, Shen et al. 2004, Gonzales, Pisitkun et al. 2009, Chen, Lai et al. 2012, Oosthuyzen, Sime et al. 2013, Dijkstra, Birker et al. 2014, Samsonov, Shtam et al. 2016, Silvers, Liu et al. 2016, Bijnsdorp, Maxouri et al. 2017, Dhondt, Geurickx et al. 2020). **Absolute excretion rate** defines the rate (per unit of time) in which a uEV marker is excreted. This can be measured using a timed collection, or may be approximated by normalization to urine osmolality or creatinine in a spot urine (Nisell, Trygg *et al.* 2006, Adedeji, Pourmohamad *et al.* 2019). This is mostly used in kidney-related research, and is of particular importance in physiological studies (Salih, Fenton et al. 2016) Blijdorp, Tutakhel, JASN 2021).



### 3.4.1. Relative excretion rate

A commonly used normalization strategy for proteomic analyses is to start with a reproducible method to enrich for uEVs and conduct the experiment with the same **amount of total protein** (for example 20 µg) per sample. After acquisition, protein data can be processed using quantile normalization, which assumes that the majority of proteins present in the sample are stable. Protein variation in uEVs has been recently determined by Oeyen, *et al.* (Oeyen, Van Mol *et al.* 2018). Such global normalization approaches (*e.g.* Linear scaling to Counts Per Million) are also applicable to transcriptomics studies and were recently demonstrated for small RNA-Seq data generated from EVs in different biofluids, including uEVs (Srinivasan, Yeri *et al.* 2019). Nevertheless, the effect of different normalization approaches, in particular for long transcripts, remains to be systematically evaluated. Importantly, in urine, uromodulin and albumin are known to be overrepresented in protein content after uEV enrichment protocols (Fernandez-Llama, Khositseth *et al.* 2010, Musante, Saraswat *et al.* 2012, Xu, Barreiro *et al.* 2019).

Expression of uEV-biomarkers as a ratio to **uEV number** or to a **uEV-biomarker** (*e.g.* a housekeeping control transcript or a protein, present in uEVs) that is considered to be stable in the studied condition has been also proposed (Colombo, Moita *et al.* 2013, Rodriguez, Bajo-Santos *et al.* 2017). However, such ratios can be affected by the quality of the chosen control(s) in terms of expression stability. In addition, common EV-markers such as CD9 or CD63 may be differentially expressed throughout the urogenital system, and therefore not be generally applicable on urine samples (Blijdorp Tutakhel JASN 2021). External factors such as an undetected infection or damage/injury/inflammation in any part of the urogenital system affect the total excretion of uEVs and the composition of the uEV pool (Duijvesz, Versluis *et al.* 2015, Hendriks, Dijkstra *et al.* 2016).

### 3.4.2. Absolute excretion rate

**Timed collection**, and in particular 24-hour collection (*i.e.* during an exact 24-hour time-course discarding first morning void and including first morning void of following day) is considered the gold standard to determine excretion rate of general urinary biomarkers such as albumin, because it is less sensitive to fluctuations due to circadian rhythm (Koopman, Krediet *et al.* 1989). However, 24-hour urine collections are time consuming and impractical for the patient and can lead to collection errors (Boyd, Wood *et al.* 2018). Moreover, prolonged collection of uEVs may accelerate their degradation (Oosthuyzen, Sime *et al.* 2013), although this remains under debate (Mitchell, Welton *et al.* 2009). The measurement of an absolute excretion rate of a urine biomarker using a timed collection can be approximated in a spot urine measurement by a **ratio to urinary creatinine** (Gunasekaran, Luther *et al.* 2019), which has been shown to be highly effective for both intra-individual comparison (96% of uEV variation explained by creatinine concentration) and inter-individual comparison (47-82%) (Blijdorp, Tutakhel, JASN 2021). Creatinine is a waste product of muscle catabolism. In the healthy kidney, the excretion rate of creatinine is constant when the glomerular filtration rate, secretion by organic cation transporters, and body muscle mass do not change. Thus, the ratio to creatinine should be validated in acute kidney injury or different stages of chronic kidney disease (Waikar, Sabbiseti *et al.* 2010, Forni Ogna, Ogna *et al.* 2015). In addition, comparing individuals may need correction for creatinine excretion or muscle mass. **Urine**

**osmolality** has also been applied as an alternative urine normalization factor in targeted metabolomics (Khamis, Holt *et al.* 2018). Urine osmolality assumes there is constant excretion of osmoles in steady state, which was shown not to be the case during water loading (Blijdorp Tutakhel JASN 2021). Ginsberg, *et al.* (Ginsberg, Chang *et al.* 1983) show that the protein/creatinine ratio of single void urine collected after the first voided morning specimen and before bedtime best correlates with the quantity of protein excreted during 24 hours.

### 3.4.3. Normalization to organ-specific biomarkers

In some cases, organ-related biomarkers can be utilized for normalization. For example, in studies addressing prostate-derived EVs, the **urinary Prostate Specific Antigen** (uPSA) can be used as a measure of the amount of prostatic fluid released in the urine and as a surrogate marker and normalization factor for the number of prostate-derived uEVs (Ploussard and de la Taille 2010, Duijvesz, Versluis *et al.* 2015, Minciacchi, Zijlstra *et al.* 2017). CD24, a kidney-specific uEV marker, could possibly be used as a reference for kidney-derived EVs (Keller, Rupp *et al.* 2007). While normalization to GFR or nephron mass has not been used in the literature, it may improve results of studies concerning the kidney.

## Recommendations and Considerations

### 4.1. Urine collection and biobanking for uEV research

Biobanking of urine is crucial for future biomarker studies. Academic institutions, hospitals and professional biobanks worldwide often share biobanking protocols. However, collection, processing and storage methods as well as the extent of gathered sample/donor information differ greatly between sites. As specific biobank guidelines covering all uEV research have not been established and EV-dedicated biobanks/collections are rare, it is recommended to follow the general recommendations related to the collection, storage, preprocessing and transportation of the urine samples by the authorities in the urine analysis field, including the Clinical Laboratories and Standard Institute (CLSI) (Rabinovitch, Arzoumanian *et al.* 2009). It is important to be aware of the preanalytical variables and follow as much as possible, the recommendations for their reporting summarized in (Table 2).

Based on the expertise existing among the actual members of the ISEV Urine Task Force some recommendations for uEV research can be given. However, it should be clarified that this is a rapidly evolving field and that the recommendations are part of an ongoing work. Moreover, at this stage these recommendations do not represent the view of all the uEV researchers.

- When starting research with **existing biobank** samples collections, gather all available sample and donor related data for reporting and analysis purposes.
  - Dipstick data can be gathered after thawing to indicate the presence of interfering or abnormal components.
- When **starting a new urine collection or a biobank**, consider and record the parameters in the whole logistics chain from donor recruitment to data management

and urine collection, transport, preprocessing, aliquoting and storage. It is safest to consider the broadest possible future uses of the urine and uEV samples.

- Keep collection, processing, and storage procedures the same throughout the study. If this is not possible, perform controls to identify the possible effect of the varied step.
- Aim for fast processing (hours), keep samples cold (+4°C, ice or equivalent) and consider additives (*e.g.* azide, protease inhibitors, EDTA) to avoid microbial growth and maximize preservation of the EVs.
- Gather dipstick data to indicate the presence of interfering or abnormal components.
- Centrifuge urine before freezing to remove cells that could be disrupted during freezing. Aliquot samples according to future use and available space. Freeze at -70°C or colder.
- Use only hygienic collection devices, containers and plastics that resist urine pH and do not bind uEVs (lipids/proteins) or shed particles.

It is also important to collect and report low evidence level items to improve our understanding of the impact of these factors and reduce current uncertainties. These may include:

- Need for light protection or for some sample protecting agents, such as RNase inhibitors or cryoprotectants.
- Freezing speed. Quick freezing appears to work, but tests for a range of freezing speeds are lacking.
- Defrosting temperature.

#### 4.2. Downstream analysis of uEVs

As with most body fluids, urine contains EVs from a plethora of different organs, tissues and cell types from the urinary tract (Figure 2). Together with the wide variety of analytical parameters that can be obtained from EVs, this results in several important considerations for the analysis of uEVs (Figure 4).

The first consideration is the **type of analytical parameter** that is going to be studied. uEV analysis can be focused on physical parameters (*e.g.* concentration, size distribution, morphology) and/or the biochemical content of uEVs (*e.g.* proteins, nucleic acids, lipids and metabolites). This is reflected by the wealth of state of the art and newly emerging EV assays and analysis technologies available (Hartjes, Mytnyk et al. 2019, Williams, Palviainen et al. 2019, Nazarenko 2020, Paisrisarn, Yasui et al. 2020, Skotland, Sagini et al. 2020, Soekmadji, Li et al. 2020).

However, there is no single consensus protocol for pre-processing EVs, or analytical technology that suites most or all analytical parameters. Importantly, the MISEV2018 and EV-TRACK guidelines

recommend to report on several complementary analytical parameters (*e.g.* concentration, size distribution, morphology, EV markers) to confirm the presence of EVs (Consortium, Van Deun *et al.* 2017, Thery, Witwer *et al.* 2018).

The requirement of pre-analysis **separation and purification** of uEVs is essential for many of the (biochemical) analyses to avoid interference of non-EV contaminants in urine, but might be nonessential or maybe even disadvantageous for other analyses as any isolation or purification protocol unavoidably leads to significant loss of EVs and EV material. In addition, isolation procedures are generally biased towards certain EV size and density ranges. It is therefore recommended to avoid EV isolation or purification protocols as much as possible (except for the pre-freezing clearing as described in section 3.1.2.6) and only implement extensive EV isolation and purification when needed due to interference by other components of urine (Wachalska, Koppers-Lalic *et al.* 2016, Vergauwen, Dhondt *et al.* 2017, Xu, Barreiro *et al.* 2019). Direct analysis of uEVs without time-consuming and costly extensive pre-processing would be highly beneficial for clinical implementation. However, when EV isolation is required, different approaches (*e.g.* ultracentrifugation and precipitation) should be evaluated for urine as specific biofluid and the analytical parameter of choice (Wang and Sun 2014, Royo, Zuniga-Garcia *et al.* 2016, Mussack, Wittmann *et al.* 2019, Oeyen, Willems *et al.* 2019, Coughlan, Bruce *et al.* 2020, Paisrisarn, Yasui *et al.* 2020). Regarding estimates of size and concentration, different techniques can be applied. While NTA and TRPS offer particle counting and sizing including non-EV particles, flow cytometry for example can offer single EV detection and might be more precise. To our knowledge studies are needed to understand if counting with these techniques are suitable for normalization.

A third consideration is a result of the wide variety of organs, tissues and cell types that contribute to the uEV pool. Depending on the scientific or medical question being asked, **initial (on-assay) capture of specific uEVs of interest** (*e.g.* uEVs derived from specific organs or diseased tissue) can enhance the specificity and sensitivity of the analysis. Such capture within the analytical assay relies on the availability of suitable capture targets on the EV surface and the efficiency of capture. Moreover, the yield of specific uEVs in these capture approaches could be a concern. Capture based assays often use the (so called) general and abundant EV surface markers CD9, CD63 and CD81 for capture, for example time-resolved fluoroimmunoassay (TR-FIA), surface plasmon resonance imaging (SPRI), ExoView® (Duijvesz, Versluis *et al.* 2015, Daaboul, Gagni *et al.* 2016, Rikkert, de Rond *et al.* 2020). However, it has become increasingly apparent that only fractions of EVs carry these 'general' EV markers, and that expression of these markers is largely dependent on the cells of origin (Kowal, Arras *et al.* 2016, Salih, Fenton *et al.* 2016).

The need for capture of tissue or disease specific EVs can be overcome by analysis of **individual uEVs rather than bulk analysis**. The analysis of individual EVs allows the identification and subsequent characterization of specific uEV subtypes without the need for specific isolation. For example, multiplexing strategies allow the analysis of multiple EV surface markers on individual EVs (Headland, Jones *et al.* 2014, Hartjes, Slotman *et al.* 2020), sometimes after capture of the EVs (Daaboul, Gagni *et al.* 2016, Koliha, Wiencek *et al.* 2016, Wiklander, Bostancioglu *et al.* 2018). This is again dependent on the availability of specific EV (surface) markers that can be used for detection. Moreover, the analysis of individual EVs is currently restricted to measuring physical parameters like

concentration, size and morphology, as well as proteins on the EV surface and lumen. Super-resolution imaging for instance may enable visualization of structure, biomarker distribution, and relative abundance of each biomarker on single EVs. Technologies to analyze RNAs, DNA, lipids and metabolites in individual EVs are not yet available.

The level of EV analysis varies from global, discovery based approaches using the 'omics' family of technologies (*e.g.* proteomics, transcriptomics, genomics, lipidomics and metabolomics) (Rigau, Olivan *et al.* 2013, Cheng, Sun *et al.* 2014, Lee, McKinney *et al.* 2018, Park, Lee *et al.* 2020, Wang, Shi *et al.* 2020), to more targeted analysis of specific EV contents using immune detection or PCR-like approaches to measure specific proteins or RNAs of interest (Sun, Deng *et al.* 2012, Samsonov, Shtam *et al.* 2016, Yamamoto, Murakami *et al.* 2018, Sole, Moline *et al.* 2019). The latter is more present in target-specific EV assays and is more suitable for clinical implementation. Analytical technologies and assays for these two levels of uEV analysis differ and require different levels of pre-processing and purification.

The last consideration is the requirement of **scalability**. Many current technologies for the analysis of (individual) EVs require individual samples be measured independently. Large-scale experiments and studies on larger cohorts of uEV samples will require more high/medium throughput technologies. To support the scalability of uEV analysis, several technologies are being developed that enable higher throughput using automation and miniaturization of assays in (microfluidic) devices. Related to scalability is standardization. At this moment, many of the analytical assays for EVs are highly dependent on details in the protocols and settings. It is therefore pivotal to introduce optimal levels of standardization and reporting in the analysis of uEVs to improve reproducibility (Consortium, Van Deun *et al.* 2017, Thery, Witwer *et al.* 2018).

#### 4.2.1 Analysis of the uEV proteome

Many of the potential challenges of working with uEVs highlighted elsewhere in this manuscript also apply to proteomic analysis of uEVs, especially those relating to vesicle isolation and purity (section 3.2). Abundant proteins in urine such as uromodulin, previously reported to be present in uEVs (Pisitkun, Shen *et al.* 2004), may in fact be co-isolated or partially related to EVs that have been co-isolated with uEVs (Musante, Bontha *et al.* 2020). Moreover, problems associated with a high abundance of soluble proteins are exacerbated in various clinical scenarios such as proteinuria, hematuria, and other conditions. Therefore, one must be careful when analyzing complex data sets from broad proteomic studies of uEVs. Whilst additional techniques can be used to remove soluble proteins from the sample, it remains a challenge to distinguish proteins that are genuinely uEV-associated from soluble contaminants. Furthermore, issues with protein contaminants make normalization based on vesicular proteins extremely difficult. An alternative approach is to normalize sample inputs based on vesicle count. The challenges associated with either approach are summarized in section 3.6. There have been several advances in technologies for focused analysis of the uEV proteome. Technologies such as aptamers or proximity extension assays (PEA) have been utilized for analysis of EV proteins (Welton, Brennan *et al.* 2016, Larssen, Wik *et al.* 2017, Zhu, Li *et al.* 2020). Such techniques offer greater sensitivity and limit the background noise which may accompany traditional mass spectrometric approaches, but the breadth of analytes assessed is

limited. Additional approaches utilizing immuno-based capture and detection of proteins can also be used for assessment of selected uEV proteins. Low density array (LDA) profiling can be adapted for the study of vesicular proteins (Cha, Shin *et al.* 2018, Mata Forsberg, Bjorkander *et al.* 2019). Whilst such arrays are limited in their coverage, they do not require access to specialized equipment. In addition, there are several commercially available platforms for assessment of multiple uEV surface markers in plate- or chip-based formats (Musante, Tataruch-Weinert *et al.* 2017, Gori, Romanato *et al.* 2020). However, such immuno-affinity assays are susceptible to soluble protein contaminants that can interfere with uEV capture and detection. A comparison of techniques for uEV protein analysis described above is shown in Table 4.

#### 4.2.2. Analysis of the uEV transcriptome

RNAs carried by uEVs are biologically active, can reflect the physiological status of cells of origin, and have been intensely studied in the search for biomarkers (Valadi, Ekstrom *et al.* 2007, Peinado, Aleckovic *et al.* 2012). Characterization of the RNA species in uEVs depends on the preanalytical and analytical conditions. The RNA yield from uEVs is related to the uEV separation technique used (*e.g.*, 2.6 - 50 pg/ml for uEVs isolated by ultracentrifugation (UC) followed by 0.1  $\mu\text{m}$  filtration) (Bryzgunova, Zaripov *et al.* 2016), and 17 – 46 pg total RNA per million uEVs obtained by UC alone (Royo, Zuniga-Garcia *et al.* 2016). An extensive description of analytical conditions for RNA analysis was recently reviewed (Everaert, Helmoortel *et al.* 2019). Furthermore, microfluidic techniques have been developed to reduce bias introduced by high manipulation of the sample for targeted detection (Yasui, Yanagida *et al.* 2017). A comparison of techniques for uEV RNA analysis is shown in Table 5.

#### 4.2.3. Analysis of the uEV lipidome

Preanalytical and analytical parameters can affect outcomes of EV lipid analyses and should be reported (Avela and Siren 2020, Gori, Romanato *et al.* 2020, Wu, Bagarolo *et al.* 2020). Protocols for sample preparation, lipid extraction, and separation must be reproducible. For example, it is not clear yet to which extent uEV lipids can be degraded under different conditions. Moreover, the presence of lipoparticles in EV samples can affect lipid analysis, which should be considered in studies of conditions that can lead to an increased lipid concentration in urine. Recent studies of the EV lipidome have often used mass spectrometry. Because of the high molecular diversity of lipids, overlaps of mass spectrometric ions of lipid species frequently occur. Therefore, using high-resolution MS is recommended for analysis of the uEV lipidome (Zullig and Kofeler 2020). In addition, proper internal standards, normalization and/or labelling are crucially required for precise quantitative lipidomics of uEVs (Tiphthara and Thongboonkerd 2016, Glover, Nouri *et al.* 2019, Avela and Siren 2020, Wu, Bagarolo *et al.* 2020, Zullig and Kofeler 2020).

#### 4.2.4. Analysis of the uEVs metabolome

uEVs carry different types of metabolites such as many organic acids involved in the TCA cycle, bile acids, amino acids, nucleotides and steroid hormones pointing to these vesicles as indicators of the metabolic status of tumor tissue (Royo, Zuniga-Garcia *et al.* 2016, Puhka, Takatalo *et al.* 2017, Clos-

Garcia, Loizaga-Iriarte et al. 2018). However, several issues exist with the analysis of EVs by MS-based metabolomics. The technique is very sensitive, and it is likely that some non-EV metabolites will be retained by most EV separation methods. Background metabolites can be assessed easily for cell culture conditions by analyzing unconditioned medium (Palomo, Casal *et al.* 2014, Royo, Gil-Carton *et al.* 2019), but it is not as easy to judge background metabolites for urine. Therefore, it is recommended to study multiple biological replicates and take into consideration only those metabolites that are consistently detected among technical replicates and samples (Clos-Garcia, Loizaga-Iriarte *et al.* 2018). Another aspect to consider is that a minimum amount of uEVs will be required to obtain reliable measurements, for example 50 micrograms of total uEV protein. Finally, the varied chemical nature of the metabolites in uEVs means that there is no single method capable to analyze all uEV metabolites at once. A combination of different extraction methods chromatographic parameters and mass spectrometric conditions are likely needed to construct a complete picture of the uEV metabolome.

#### 4.3. Normalization of uEV data

Normalization approaches for urine biomarkers can be broadly categorized as absolute or relative excretion rates. The **relative excretion rate**, is generally applicable as a normalization method for uEV samples subjected to any isolation protocol while the **absolute excretion rate** is ideally used with techniques that characterize uEVs directly in cell-depleted urine. Without a universal approach to normalize uEV samples, we list here current normalization methods in use:

- Timed collection (gold standard: 24-hour collection) – absolute excretion rate
- Creatinine/osmolality normalization – estimate of absolute excretion rate using spot urine
- Constitutively expressed uEV marker – relative excretion rate
- Specific marker ratio (e.g. organ specific proteins) – relative excretion rate
- Relation to total uEV count – relative excretion rate
- Z-normalization (RNAseq / MassSpec) - relative excretion rate
- To GFR (or nephron number) – relative excretion rate (organ-related: kidney)
- Relation to PSA (e.g. after DRE) – relative excretion rate (organ-related: prostate)

The strengths and limitations of each normalization method are mentioned in Table 3.

Important criteria for developing new normalization tools are:

- Decreases variation within normal or expected range
- Widespread availability and feasibility
- Can be validated internally and across testing sites, ideally with (shared) external standards
- Compatibility with commonly used isolation and/or analysis methods.

## 4.4. Functional studies of uEVs

### 4.4.1. General recommendations for uEV functional studies

Common issues and general recommendations to be considered for attributing a functional activity to EVs are extensively detailed in MISEV 2018 (Thery, Witwer et al. 2018). Therefore, refer to the MISEV 2018 guidelines for the design of experiments evaluating functional activities of uEVs or uEV subtypes. Here, we briefly summarize the most relevant points of interest:

- Possible artifacts due to EV contaminants should be excluded. This can be achieved by comparative evaluation of the effect of the biofluid of interest before and after EV removal, together with that of the isolated EVs; when possible, the main contaminants must be isolated and their effect tested as well. Moreover, the role of co-isolated non-EV material should be studied using (combined) enzymatic degradation of proteins or RNA/DNA species to allow investigations addressing the “EV-corona” (Palviainen, Saraswat et al. 2020). In particular, low dose trypsin, proteinases, RNAses and DNAses might be useful. Appropriate protocols should be optimized in order to avoid EV disruption or degradation in the same time.
- Isolation of the crude EV population and, when of interest, of the different EV fractions, should be achieved using multiple and accurate methods. To ascribe a functional property to specific fractions, side-by-side analysis of all fractions is recommended.
- Appropriate controls should be included such as unrelated EV sources and disease EV controls such as healthy, untreated or otherwise matched donors,
- Functional activity should be quantitatively related to the amount of EVs or of a specific EV component; this can be achieved by EV normalization strategy supporting comparison of different EVs, fractions and active cargo and possibly by the evaluation of dose response effects.

### 4.4.2. Specific considerations for uEV functional studies

While the fundamental practical considerations detailed above must be applied to all functional analysis studies (regardless of the source of EVs), urine presents certain specific challenges that must be considered when evaluating the functional activity of urine EVs. As detailed earlier, the timing and type of collection method may lead to dramatically different levels of cellular elements, including EVs in urine samples. Thus, when possible, the same collection method should be used for any comparative analysis. In addition, uromodulin can entrap small EVs in polymer “nets” and reduce recovery. Releasing EVs from uromodulin is therefore necessary to avoid a biased functional analysis which focuses on a small subset of urinary EVs. However, complicating this is the fact that procedures which disrupt the uromodulin network and release EVs (*i.e.* DTT (Fernandez-Llama, Khositseth *et al.* 2010)) may also lead to co-elution of uromodulin in the EV pellet. As uromodulin is well known to modulate a diverse array of processes (*i.e.* immune function, sodium handling, complement system (Olinger, Lake *et al.* 2019)) one must consider whether co-eluted uromodulin is responsible for any effects attributed to EVs. Similarly, as bacteria may also co-elute in EV isolation



procedures, one must consider the possibility of bacterial contamination in urine samples. This could also lead to biological activity that is incorrectly attributed to EVs. One strategy to address this may be to assess contamination after collection and discard contaminated samples (Hogan, Lieske *et al.* 2015), however this is not practical for all applications. Ultimately the task force recognizes that functional analysis of uEVs is very early in its evolution and identification of strategies to address the above challenges should be a research priority.

## **Future perspectives**

### **5.1. Clinical challenges**

Use of uEVs as novel biomarkers for diagnosis, prognosis and guidance for treatment also has its challenges. The uEVs research community faces several gaps that should be overcome to systematically advance the field (Figure 5). Validation studies are needed to show superiority of uEV-shuttled biomarkers to direct measurement of the protein/RNA/lipid biomarker of interest in urine, *i.e.* is there a genuine advantage to concentrating uEVs. It is also important to note that a single standardized approach for urine collection, uEV separation and measurement has not yet been adopted and likely will not be. The impact of different pre-analytical variables on the nature and quality of uEV isolates has to be understood in order to design, optimize and escalate protocols towards real-world clinical applications. Use of uEVs from existing biobanks also represents a clinical challenge because the standardization necessary for many assays may be insufficient or different compared with what is needed for uEV assays. An additional challenge in the field relates to normalizing biomarker signals (Gunasekaran, Luther *et al.* 2019) because urine is one of the most dynamic biofluids. In order to move the field of uEV research forward, uEV reference standards are needed for many experimental purposes, including single EV analysis, *e.g.* for flow cytometry and particle analyzers for assessment of size and concentration or normalization to excretion rate and uEV processing-related variation.

Among the many issues mentioned herein is the overriding need for more cost effective and tractable assay approaches that can provide fast quantitative information in a standardized fashion. Currently, the technologies available for EV analysis are highly diverse and somewhat idiosyncratic. Many of these platforms have limited accessibility, residing within specialized laboratories or within companies providing analytical services based on their proprietary technologies. Although healthcare systems globally operate differently, development of uEV biomarker measurement technologies that can be broadly deployed to diagnostic centers, *e.g.* within hospitals, will be needed to fully realize the biomarker potential of uEVs (Rayyan, Zheutlin *et al.* 2018). These are not trivial issues and will require continuous collaborative discussions involving industry, regulatory bodies and standards agencies to ensure success.

### **5.2. Clinical potential of uEVs**

Currently the diagnosis of many diseases of the kidney and urinary tract are based on insensitive and non-specific biomarkers. For instance, changes in kidney function are still measured using changes in serum creatinine (SCr) – a late and nonspecific marker of kidney dysfunction (Thomas, Blaine *et al.*

2015). Despite years of intense research, there are only a few biomarkers approved for clinical use. Examples include tissue inhibitor of metalloproteinase 2 (TIMP2) and insulin-like growth factor-binding protein (IGFBP7), urinary biomarkers for acute kidney injury (AKI) incorporated in a commercial test (Nephrocheck) (Kashani, Al-Khafaji *et al.* 2013, Fan, Ankawi *et al.* 2018). Even this FDA-approved test is falsely positive in 50% of people without AKI (Gaffney 2014) pointing to a clear requirement for a new approach to identify and measure fit-for-purpose disease markers. Early identification of disease processes in the kidney and urinary tract is clearly needed to improve the specificity of diagnosis, facilitate earlier and better tailored interventions and ultimately for improved outcome for patients.

Urinary EVs hold excellent potential as a multiplex-biomarker source. They are easily accessible **non-invasively, available in large quantities, and amenable to frequent longitudinal sampling**. uEVs in part resemble the molecular content of the parent cells from which they are released (Bazzell, Rainey *et al.* 2018). They carry cell specific markers from every segment of the nephron and urogenital tract and therefore are ideal for sampling the health status of these systems. Moreover, reports of EVs arriving into the urinary system from distant sites such as in lung cancer (Li, Zhang *et al.* 2011, Fraser, Rawlins *et al.* 2016) are important, as they highlight the potential for identifying diseases in unrelated organ systems through urinary sampling. These are avenues ripe for future exploration and development, potentially establishing uEVs as the ultimate biomarker source.

It is also increasingly recognized that improvements in the diagnosis, prognosis and treatment of disease processes require a better understanding of distinct underlying cellular and molecular mechanisms. Therefore, researchers in this field are exploring **site-specific or disease-specific damage/injury markers and pathways** with the intent to combine them with functional testing and clinical information. This approach may facilitate an earlier diagnosis in kidney and genitourinary tract diseases and thereby provide a more accurate diagnosis and prognostic assessment, and potentially identify novel routes for intervention. Valuable biomarkers, including uEVs should be linked to mechanistic components of disease processes.

EV-based biomarkers in urine are currently investigated for an array of malignancies and other diseases such as polycystic kidney disease (Raimondo, Cerra *et al.* 2016, Salih, Demmers *et al.* 2016), cystinuria (Bourderioux, Nguyen-Khoa *et al.* 2015), diabetes (Zubiri, Posada-Ayala *et al.* 2014, Lytvyn, Xiao *et al.* 2017, Abe, Sakurai *et al.* 2018), renal ischemia-reperfusion injury (Sonoda, Yokota-Ikeda *et al.* 2009), glomerulonephritis (Morikawa, Takahashi *et al.* 2018), renal interstitial fibrosis (Chun-Yan, Zi-Yi *et al.* 2018, Carreras-Planella, Cucchiari *et al.* 2020), hypertension or lupus nephritis (Tangtanatakul, Klinchanhom *et al.* 2018) and in calcineurin inhibitor-induced nephrotoxicity (Carreras-Planella, Juega *et al.* 2020). However, many of the identified candidate biomarkers have not yet been validated in large independent cohorts or tested in more than one laboratory. An exception is the uEV biomarker test for prostate cancer based on PCA3 and ERG that reduces the number of unnecessary prostate biopsies performed (Donovan, Noerholm *et al.* 2015, McKiernan, Donovan *et al.* 2016, McKiernan, Donovan *et al.* 2018). Candidate uEV markers require more expansive, multicenter validation, that can provide the large datasets needed to support eventual clinical deployment.

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## Text box 1

### Characteristics specific to urine and uEVs that influence uEVs analysis

#### Biology:

- uEVs are (mostly) derived from epithelial cells
- uEVs are (mostly) derived from three major organs: kidney, urothelium, prostate
- Normally, urine does not contain platelets or lipid particles other than EVs
- Urine has variable contamination with microbiota
- Urine composition is highly variable (pH; osmolality, concentration) and influenced by certain medications and diet

#### Collection:

- Urine collection is minimally invasive
- Urine can be collected in large quantities
- Urine collection is sensitive to collection errors by the patients, i.e. mid-stream vs first void; incomplete timed collections, etc.
- Release of prostate EVs can be stimulated by digital rectal examination (DRE)
- Urine dipstick may be used as an easy quality control of urine
- Urine can contain cells that should (and can easily) be cleared before freezing

#### Separation / characterization:

- Uromodulin lowers yield of uEV separation techniques
- Kidney disease can cause proteinuria / albuminuria and interfere with molecular uEV analysis

#### Normalization:

- An absolute uEV excretion rate can be determined from timed urine collection

## Figure legends

**Figure 1 uEV microscopy.** **A:** Urinary EVs (uEVs) were isolated by centrifugation (20,000 x g pellet) and processed for cryoelectron microscopy (as described in (Musante, Bontha *et al.* 2020)). The left image shows a wide variety of EVs in size, density and shape. In addition, polymers of uromodulin are shown which seem to entrap uEVs (see arrows). The right image shows a higher magnification of uEVs demonstrating spike like structures emerging from the phospholipid layer which likely represents the glycocalyx of some uEVs. **B:** uEVs were isolated with ultracentrifugation (100,000 x g pellet) and processed for Transmission electron microscopy (TEM) using a negative staining protocol (as described in (Puhka, Takatalo *et al.* 2017)). To the left we see a lower magnification image displaying a large number and variety of uEVs in size, shape and density. The right image shows a higher magnification demonstrating the uEV heterogeneity with differential staining densities and some spike like surface features that can be visualized despite the cup shape morphology which is due the processing of TEM. **C:** Super-resolution images were obtained using a Nanoimager S Mark II microscope from ONI (Oxford Nanoimaging) equipped with 405 nm/150mW, 473 nm/1W, 560 nm/1W, 640 nm/1W lasers and dual emission channels split at 640nm. The figure shows uEVs stained for CD81 (cyan) and Klotho (magenta) using primary antibodies conjugated with Alexa Fluor 555 and 647 respectively. Representative images with zoomed in insets show the expression and nanoscale distribution of the peptide and tetraspanin on the surface of two representative EVs bound to the coverslip surface. Two-channel dSTORM data was acquired sequentially at 30 Hz in total internal reflection fluorescence (TIRF) mode. Single molecule data was filtered using NimOS (Version 1.7.1.10213, ONI) based on point spread function shape, photon count and localization precision to minimize background noise and remove low precision localizations.

## Figure 2 Origins of urinary EVs

**Figure 3 Biogenesis pathways of urinary extracellular vesicles (uEVs).** EVs are a highly heterogeneous group of membrane-bound particles released by both healthy and malignant cells. Biosynthesis of exosomes, a specific population of **small uEVs**, occurs via formation and maturation of **multivesicular endosomes (MVEs)**. Exosomes are formed as **Intraluminal vesicles (ILVs)** in the lumen of MVEs by inward budding of the endosomal membrane. Upon fusion with the cell membrane, exosomes are released into the intercellular space. **Microvesicles and ectosomes** represent both smaller and larger EVs and are formed by outward budding and scission of the plasma membrane. The process is associated with the accumulation of Ca<sup>2+</sup>-dependent enzymes that change the polarity of membrane phospholipids. This causes physical bending of the cellular membrane and rearrangements in the underlying cytoskeleton, leading to the formation of microvesicles. Once released by the cell, small uEVs formed at the PM and MVB-derived exosomes exhibit overlapping size and composition, which makes it difficult to establish their biosynthetic origin. **Apoptotic bodies** are formed during apoptosis (programed cell death) when cells undergo characteristic outward blebbing caused by breaks in the cytoskeleton. During this process the cellular membrane bulges outward and portions of the cytoplasm and its contents separate forming apoptotic bodies. **Secretory vesicles (SV)** are produced by the ER and Golgi apparatus. Most of them have specialized cargo such as hormones and neurotransmitters. SVs fuse with the cell membrane at specialized supramolecular structures (porosomes) to release their cargo in the extracellular space.

**Figure 4 Analytical method selection in uEV research.** Analytical methods used for the characterization of EVs explore their physical properties (gray) and/or molecular components (color). Commonly studied molecular components found in EVs are proteins, nucleic acids, lipids and metabolites. Localization of these molecular components largely defines the choice of an analytical approach. Proteins (purple) can be localized in the EV membrane or lumen. EV surface proteins can be assessed specifically by antibodies, both in bulk analysis, *e.g.* by a time-resolved fluoroimmunoassay (TR-FIA), Immunoblot, immuno-bead capture-based flow cytometry, or surface plasmon resonance imaging (SPRi) and with assays that analyze individual EVs such as fluorescent NTA, high-resolution flow cytometry and microscopy. Analysis of luminal proteins can be performed in bulk assays, *e.g.* immunoblot, ELISA and time-resolved TR-FIA after membrane permeabilization. Generally, labelling of luminal cargo can facilitate individual EV analysis through the use of membrane-permeable fluorescent dyes that label proteins or nucleic acids such as ExoGlow™ or Syto™13. Whilst such dyes lack the specificity of more targeted approaches, they enable analysis of EVs by fluorescent microscopy, fluorescent NTA, and high-resolution flow cytometry. Specific analyses of nucleic acids (blue) and metabolites (green), generally considered to be luminal, are usually achieved in bulk EV assays by either omics-based approaches, or by transcript-specific PCR based techniques. Lipids (yellow), are localized within the EV membrane and are commonly analyzed in bulk assays either by mass spectrometry or colorimetric reagents, like the sulfo-phospho-vanillin (SPV) lipid assay.

**Figure 5 Methodological and knowledge gaps in the current uEV work flow**

The urine EV task force of the International Society for Extracellular Vesicles is in the process of recruiting uEV researchers to perform collaborative studies of rigor and reproducibility to address the outlined knowledge gaps.

## Tables

**Table 1 List of uEV markers characterizing different structures of the urinary tract.** The markers were described in uEVs isolated from human urine and identified by Western blot and/or flow cytometric analyses

Organ	Structure/Cell of origin	EV marker	References
Kidney	Glomerulus (Podocytes)	Podocin	(Hogan, Johnson <i>et al.</i> 2014)
		Podocalyxin	(Hogan, Johnson <i>et al.</i> 2014)
		Wilms' tumor 1 (WT 1)	(Kalani, Mohan <i>et al.</i> 2013)
		Complement receptor 1 (CR1)	(Prunotto, Farina <i>et al.</i> 2013)
		Canonical transient receptor potential 6 (TRPC6)	(Hogan, Johnson <i>et al.</i> 2014)
		Nephrin	(Hogan, Johnson <i>et al.</i> 2014)
	Glomerulus/proximal tubules	Angiotensin-converting enzyme (ACE)	(Pisitkun, Shen <i>et al.</i> 2004)
	Proximal tubules	Megalin	(Pisitkun, Shen <i>et al.</i> 2004)
		Aminopeptidase N (APN)	(Pisitkun, Shen <i>et al.</i> 2004)
		Cubilin	(Hogan, Johnson <i>et al.</i> 2014)
		Sodium/glucose cotransporter 2 (SGLT 2)	(Øverbye, Skotland <i>et al.</i> 2015)
		Carbonic anhydrase (CA IV)	(Pisitkun, Shen <i>et al.</i> 2004)
		Na <sup>+</sup> /H <sup>+</sup> exchanger isoform 3 (NHE3)	(Zhou, Yuen <i>et al.</i> 2006)
	Renal progenitor cells	CD133 (Prominin 1)	(Dimuccio, Ranghino <i>et al.</i> 2014)
	Tubular epithelial cells	CD24	(Keller, Rupp <i>et al.</i> 2007)
	Proximal tubules/ Henle's loop	Aquaporin 1 (AQP1)	(Pisitkun, Shen <i>et al.</i> 2004)
	Henle's loop	Uromodulin (UMOD, Tamm-Horsfall Protein, THP)	(Pisitkun, Shen <i>et al.</i> 2004)
		Na-K-2Cl cotransporter (NKCC2)	(Pisitkun, Shen <i>et al.</i> 2004)
	Proximal/ distal	Klotho	(Grange, Papadimitriou

	tubules		<i>et al.</i> 2020)
	Distal tubules	Prominin 2	(Turco, Lam <i>et al.</i> 2016)
		Thiazide-sensitive Na-Cl cotransporter (NCC)	(Pisitkun, Shen <i>et al.</i> 2004)
	Distal tubules/collecting duct	Aquaporin 2 (AQP2)	(Pisitkun, Shen <i>et al.</i> 2004)
		Claudin 1	(Turco, Lam <i>et al.</i> 2016)
	Collecting duct	Mucin-1	(Pisitkun, Shen <i>et al.</i> 2004)
<b>Bladder</b>	Transitional epithelial cells	Uroplakin-1	(Pisitkun, Shen <i>et al.</i> 2004)
		Uroplakin-2	(Pisitkun, Shen <i>et al.</i> 2004)
		Mucin-1 (MUC-1)	(Pisitkun, Shen <i>et al.</i> 2004)
<b>Prostate</b>	Epithelial cells	Prostatic acid phosphatase (PPAP)	(Øverbye, Skotland <i>et al.</i> 2015)
		Prostate transglutaminase (TGM4)	(Sequeiros, Rigau <i>et al.</i> 2017)
		Prostate-specific membrane antigen (PSMA)	(Mitchell, Welton <i>et al.</i> 2009)

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**Table 2 Reporting on urine collection, processing and storage.** Reporting Priority Level is primarily meant to indicate the importance of recording a specific parameter in a biobank database. Not all of these parameters are relevant for publication reports. The Evidence Level is an expert consensus opinion of the current level of confidence that the parameter is a variable to consider during sample biobanking and data analysis and interpretation.

Parameters	Reporting Priority level	Evidence Level	What to report	Recommendation
Research subject information (demographical and clinical data)				
Species	Obligatory	High: There are clear species-specific differences that impact all of their characteristics	Species, subspecies	Record: Species and subspecies information
Gender/ Biological Sex	Obligatory	High: There are clear gender/sex differences between urine biomarkers (e.g. creatinine, prostate EVs)	Male, Female, Genderqueer	Make sure to gender-balance cohorts to be compared
Age	High/Obligatory	Medium: Based on mesenchymal stem cells and blood EVs (reviewed in (Boulestreau, Maumus <i>et al.</i> 2020))	Age in years	Make sure to age-match cohorts to be compared
Clinical Data, e.g. diseases, kidney function parameters, medication, comorbidities	High/Obligatory	High: Clinical parameters are essential for disease/condition/organ-related EV research	Clinical parameters in standard units	<ul style="list-style-type: none"> <li>- Utilize urine dipstick</li> <li>- Measure urine creatinine</li> <li>- Measure disease-specific markers (e.g. urinary PSA for prostate and albumin for kidney research)</li> <li>- Record all relevant clinical parameters</li> </ul>
Supporting information, e.g. BMI, ethnicity, diet, fluid intake, geographical information.	Medium	Medium-High: Certain supportive information is important to record as it might influence urine EVs	Supporting parameters in standard units	Determine relevant supporting information and record them: Based on the study goal, supporting information can

				be crucial
Urine collection				
Pretreatment	Obligatory	High: The most common pretreatment methods prior to urine collection (DRE, prostate massage, catheterization) can have an effect on the EV content of the sample (Duijvesz, Versluis <i>et al.</i> 2015)	DRE and/or prostate massage (yes/no, Number of strokes) Catheterization (yes/no)	Any manipulation which could affect the composition of the urine should be reported in detail
Ethical approvals	Obligatory	N/A	Approving authority, Informed consent forms, collection details (origin, type and number of samples)	All collected samples should be linked to designated ethical approval, applied for uEV research
Collection method	Obligatory	Medium: The information of the transition of urine through the urethra is important particularly for disease-related uEV studies	- Clean-catch - Sterile urine bag - Assisted (urethral catheterization, suprapubic aspiration, pediatric specimen - Animal collection cage	Details of the collection method <i>e.g.</i> use of syringe, possible transfer of the sample to container
Time and type	Obligatory	Medium: uEV concentration can vary depending on the urine transition time from the bladder	- Collection type (morning/random/spot) - Timed collection, <i>e.g.</i> 24 hour	Type of collection <i>e.g.</i> random/spot urine, first or second morning urine. Record: Time between the last uncollected and collected void
Volume and void	Obligatory	Medium: The collection of first void urine transitioning from the urethra may affect the uEV quantity/composition	- Void (first/mid/full) - Volume in ml	Collection of midstream urine is recommended to avoid microbial contamination
Collection device and	Medium	High-Medium: Certain containers and	- Brand - Sterile yes/no	The container should be clean,

container type		devices may have an effect on the uEV content; <i>e.g.</i> the material may bind EVs or contain microbial contaminants if not sterile	- Material - Open/closed	leak-proof, urine pH-range resistant and not shed plastic particles. Record: Material, manufacturer, lot number
Storage prior to processing				
Storage Time	Obligatory	High: Longer storage time may lead to microbial growth, cell debris and particularly to degradation of more labile biomolecules ( <i>e.g.</i> RNA)	Hours	Samples should be stored max. 8 hours before processing
Storage Temperature	Obligatory	High: Freshly collected urine samples should be cooled promptly to avoid microbial growth or biomolecule degradation	Degrees Celsius	Max 4°C is recommended
Light Protection	Medium	Low: Some urinary analytes may be light sensitive ( <i>e.g.</i> bilirubin, porphyrins); impact on uEVs unknown	Light protection (yes/no)	Use of amber-colored/dark collection tubes
Urine quality control				
Use of Dipstick	High	High: Presence of <i>e.g.</i> cells, microbes and high protein levels affects purity and composition of uEV population	- Yes/no - Brand - Deviating parameter(s)	Recommended for preliminary urine assessment (pH, protein level) and exclusion of deviating samples (blood, microbes)
Preprocessing				
Collection Container preparation	Medium	Medium: Preservative might be affected by time and storage in collection container	- Preservative already present in collection container (yes/no) - Preservative in container freshly prepared (yes/no)	- Keep the protease inhibitor cocktail on ice or at the manufacturer's recommended temperature at all times

				<ul style="list-style-type: none"> <li>- If protease inhibitors are used at collection time, it is recommended that sample containers are prepared by adding protease inhibitor cocktail and keep frozen at -20°C for max. 6 months until use</li> <li>-Alternatively, prepare fresh and use immediately</li> </ul>
Urine sample preprocessing	High	High: Freshly collected urine samples should be cooled promptly to avoid microbial growth or biomolecule degradation	<ul style="list-style-type: none"> <li>- Time</li> <li>- Temperature</li> </ul>	<ul style="list-style-type: none"> <li>-Process urine within 4-6 hours from sample collection</li> <li>-Consider addition of protease inhibitors or preservatives when fast processing (&gt;6 hours at 4°C) is not possible (see below)</li> </ul>
Urine Centrifugation	Obligatory	Medium: 800 g to sediment cells and debris without damaging urine cells	<ul style="list-style-type: none"> <li>- G-force</li> <li>- Volume/tubes</li> <li>- Temperature</li> <li>- Time</li> </ul>	<ul style="list-style-type: none"> <li>- Homogenize urine sample before centrifugation</li> <li>- G-force range 500 to 800 g</li> <li>- Centrifugation at 4°C</li> </ul>
Recovered Supernatant (method/volume)	High	Medium: Largely operator-dependent	<ul style="list-style-type: none"> <li>- Pipetting, decanting, pouring</li> <li>- Recovered volume</li> </ul>	<ul style="list-style-type: none"> <li>- Loose pellets (low speed centrifugation, e.g. &lt;1,000 x g): Pipetting without disturbing the pellet is</li> </ul>

				recommended to avoid pellet carry over - Tight pellets: uniform procedure for all samples
Other urine fractions	Low	Medium-High: To monitor the purification process of EVs	- Pellet - Whole Urine	- Less-used source of EVs - Collection for use as controls or exploration of EVs in these fractions is recommended
Collected aliquots of Supernatant	Obligatory	Medium: As samples may be used for several techniques/ isolation protocols, aliquots of different volume may be required to avoid repeated freeze/thawing and to optimize workflows and storage capacity	- Number of aliquots - Date - Volume (if different volumes are collected)	- Immediate freezing at -70°C or colder is recommended after aliquoting - Suggested volumes of aliquots: Large (up to 30 mL) Medium (5 - 10 mL) Small (1 - 2 mL)
<b>Storage</b>				
Storage container	High	Medium: Should resist pH range of urine and not shed any particles, low EV (protein or lipid) binding properties generally beneficial	- Brand - Volume	Use of ¾ of the maximum volume of the container is recommended to accommodate the expansion of the sample due to freezing
Temperature	Obligatory	Medium-High: EV yield may be lower from samples stored at -20°C	-Degrees Celsius	-70°C or colder is recommended
Method of freezing	High	Low: Quick freezing is generally recommended to preserve biological specimens, but tests and impact on uEVs of about speed of	- Snap freezing in liquid nitrogen - Freezing at a freezer - other if applicable, e.g. gradual freezing or use of cryoprotective	Freezing quickly at -70°C or colder or in liquid nitrogen is recommended

		freezing speed or cryoprotective agents in urine are lacking	agents	
<b>Defrosting</b>				
Temperature	Obligatory	Low: The effect of thawing temperature on uEVs has not been extensively studied, but might affect heat labile biomolecules or to sediment formation	Degrees Celsius	- Record: The temperature(s) at which the sample has been thawed
Method	Obligatory	N/A	Heating pad, water bath, incubator, room temperature, refrigerator	- If applicable, the model and type of the device used for the thawing - Defrosting should be done equally for all compared samples
Time	High	Medium: For longer thawing times preservatives may be needed	Minutes, hours	- Record: The time it takes to completely thaw the sample - Prolonged warming not recommended to avoid microbial growth
<b>Additives at time of collection:</b>				
- Protease inhibitors - RNase Inhibitors - Chemical preservatives, e.g. azide	Obligatory	Medium: Preservatives inhibit microbial growth and protease inhibitors preserve certain urine proteins (many proteins are not prone to proteolysis)	- Type - Name - Brand - Final concentration - Stage/time at which additive was used (to whole or pre-cleared urine)	- Relevant only for longer collection times (inhibiting microbial growth) or for specific downstream EV applications (e.g. surface antigen characterization).

				<ul style="list-style-type: none"> <li>- Preferably use preservatives targeting specific enzymes (<i>e.g.</i> RNase), as general (RNA) protecting agents likely affect EVs</li> <li>- Add selected preservatives immediately at the time of urine collection</li> </ul>
Sample transportation				
Temperature	Obligatory	Medium-High: EV quality and quantity diminish with long-term RT and by multiple freeze-thawing. Preservatives can prevent protein/RNA breakdown and bacterial outgrowth	<ul style="list-style-type: none"> <li>- Degrees Celsius at transport and degrees Celsius at arrival</li> <li>- Cooling system, when applicable (<i>e.g.</i> ice)</li> </ul>	Aliquot urine and freeze at -80°C to be transported frozen at -80°C. For non-aliquoted fresh urine ( <i>e.g.</i> home-testing), immediate transport at RT or 4°C can be considered, particularly when preservatives are added
Time and Method	High	Medium-High: EV quality and quantity diminish with long-term at RT. Container leakage could introduce contamination	<ul style="list-style-type: none"> <li>- Transport duration in hours</li> <li>- Container damage/leakage</li> </ul>	Record: Transport duration and container damage
Existing biobanks				
Existing urine sample collections	N/A	High: Existing urine biobanks with protocols not optimal for EV preservation are often used for research	N/A	<ul style="list-style-type: none"> <li>- Collect all above-mentioned parameters and determine appropriateness of the sample collection for your research</li> </ul>

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				<p>purpose - Perform tests to determine urine quality, number and characteristics of EVs as described in sections 3.3-3.4</p>
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**Table 3 Normalization methods**

Normalization method	Application	Strengths	Limitations
Constitutively expressed uEV biomarker	Relative excretion rate	<ul style="list-style-type: none"> <li>• Adjusts for isolation variability or incomplete THP depletion</li> <li>• Simple normalization rationale</li> <li>• Possible surrogate measure for EV number (requires further validation)</li> </ul>	<ul style="list-style-type: none"> <li>• Currently limited to proteins</li> <li>• Biomarker not always valid for the analyte of interest</li> <li>• Affected by changes in (external) excretion of biomarker from any part of the system (<i>e.g.</i> urothelial release when studying kidney disease)</li> <li>• Some EV biomarkers may not be as universal as originally believed</li> </ul>
Relation to total uEV quantity	Relative excretion rate	<ul style="list-style-type: none"> <li>• Adjusts for isolation variability or incomplete THP depletion</li> <li>• Simple normalization rationale</li> <li>• Adjusts for changes in general EV release</li> </ul>	<ul style="list-style-type: none"> <li>• Problematic if change in total excretion of uEVs is part of underlying pathology (<i>e.g.</i> after nephrectomy)</li> <li>• Highly dependent on the method of uEV characterization</li> <li>• Affected by change in (external/crossover) EV secretion from any part of the system (<i>e.g.</i> urothelial release when studying kidney disease)</li> </ul>
Specific biomarker ratio: ratio of two or more (disease) related biomarkers, ideally with a (known) similar source	Relative excretion rate	<ul style="list-style-type: none"> <li>• Adjusts for isolation variability or incomplete THP depletion</li> <li>• Can leverage mechanism of action of biomarkers, especially when they go in opposite directions</li> <li>• Less sensitive to external/crossover secretion of uEVs</li> </ul>	<ul style="list-style-type: none"> <li>• Depends on the existence of a biomarker ratio that steadily predicts an outcome</li> <li>• Often high variability</li> <li>• Each ratio should be independently validated</li> </ul>
MassSpec Proteomics; Z- or quantile normalization	Relative excretion rate	<ul style="list-style-type: none"> <li>• Adjusts for isolation variability</li> <li>• Uses all protein information available to normalize content – less sensitive to</li> </ul>	<ul style="list-style-type: none"> <li>• Albumin and/or THP can dominate the uEV proteome and can vary more than other uEV proteins</li> <li>• Affected by change in</li> </ul>

		external/crossover factors provided they are small	(external) EV secretion from any part of the system ( <i>e.g.</i> urothelial release when studying kidney disease)
RNAseq; Z- or quantile normalization	Relative excretion rate	<ul style="list-style-type: none"> <li>• Adjusts for isolation variability</li> <li>• Uses all RNA information available to normalize content – less sensitive to external/crossover factors provided they are small</li> </ul>	<ul style="list-style-type: none"> <li>• May be biased when comparing two different patient groups</li> <li>• Affected by change in (external) EV secretion from any part of the system (<i>e.g.</i> urothelial release when studying kidney disease)</li> </ul>
Timed collection (ideally 24 hours)	Absolute excretion rate	<ul style="list-style-type: none"> <li>• Compare intra- and inter-individual differences without further normalizations</li> <li>• Eliminates variability due to circadian rhythm</li> </ul>	<ul style="list-style-type: none"> <li>• Inconvenient</li> <li>• Often incomplete collections</li> <li>• Long processing time increases chances of sample degradation</li> <li>• Does not adjust for possible variability in uEV processing protocols</li> <li>• Consider longer cyclical variation periods (<i>e.g.</i> changes over several days or even weeks)</li> </ul>
Urine creatinine/osmolality	Measure of absolute excretion rate in random spot urine	<ul style="list-style-type: none"> <li>• Commonly used clinically</li> <li>• Easy and inexpensive to assay</li> <li>• May correct for circadian rhythm in GFR</li> </ul>	<ul style="list-style-type: none"> <li>• Differences or changes in muscle mass / creatinine excretion require correction</li> <li>• Does not adjust for possible variability in uEV processing protocols, or circadian rhythm in uEV release.</li> <li>• Requires further validation in uEVs</li> </ul>
GFR / nephron number	Excretion relative to kidney size	<ul style="list-style-type: none"> <li>• Commonly used clinically (GFR)</li> <li>• May help to compare patients with different stages of kidney disease</li> </ul>	<ul style="list-style-type: none"> <li>• Non-invasive methods to estimate nephron number are unreliable</li> <li>• Requires validation in uEVs</li> </ul>
urinary PSA	Excretion relative to prostate size	<ul style="list-style-type: none"> <li>• Commonly used clinically (PSA)</li> <li>• Easy to assay</li> </ul>	<ul style="list-style-type: none"> <li>• Requires further validation in uEVs</li> </ul>

**Table 4 Summary of techniques for EV proteome analysis**

Technique	Required sample input	Strengths	Limitations	References
Mass spectrometry	High	<ul style="list-style-type: none"> <li>• Broad spectrum of analytes</li> <li>• Non-biased</li> <li>• Well established protocols</li> </ul>	<ul style="list-style-type: none"> <li>• Susceptible to “noise” from contaminants</li> <li>• Data requires trimming/cleaning</li> </ul>	(Pisitkun, Shen <i>et al.</i> 2004, Conde-Vancells, Rodriguez-Suarez <i>et al.</i> 2010, Welton, Khanna <i>et al.</i> 2010)
Aptamers	Medium	<ul style="list-style-type: none"> <li>• High sensitivity</li> <li>• High specificity</li> <li>• Can measure 1,000 s of analytes</li> <li>• Focused</li> </ul>	<ul style="list-style-type: none"> <li>• Limited coverage (analytes assessed: 1,000 s)</li> </ul>	(Welton, Brennan <i>et al.</i> 2016, Zhu, Li <i>et al.</i> 2020)
Proximity extension assays (PEA)	Medium - low	<ul style="list-style-type: none"> <li>• High sensitivity</li> <li>• High specificity</li> <li>• Focused</li> <li>• High throughput</li> </ul>	<ul style="list-style-type: none"> <li>• Severely limited coverage (analytes assessed: 100 s)</li> </ul>	(Larsen, Wik <i>et al.</i> 2017)
Proteome Profiler Arrays	Medium	<ul style="list-style-type: none"> <li>• Focused</li> <li>• No specialist equipment required</li> <li>• Relatively low cost</li> </ul>	<ul style="list-style-type: none"> <li>• Minimal coverage (analytes assessed: 10 s)</li> <li>• Low dynamic range</li> <li>• Potential interference to immuno-capture by soluble contaminants</li> </ul>	(Cha, Shin <i>et al.</i> 2018, Mata Forsberg, Bjorkander <i>et al.</i> 2019)
Immuno-affinity assays (high-resolution flow cytometry, chip/plate-based analyses)	Minimal	<ul style="list-style-type: none"> <li>• Focused</li> <li>• Relatively low cost</li> <li>• Versatility</li> </ul>	<ul style="list-style-type: none"> <li>• Minimal coverage (analytes assessed: 10 s)</li> <li>• Potential interference to immuno-capture by soluble contaminants</li> </ul>	(Musante, Tataruch-Weinert <i>et al.</i> 2017, Gori, Romanato <i>et al.</i> 2020, Rikkert, de Rond <i>et al.</i> 2020)

**Table 5 Summary of techniques for EV RNA analysis.** (Alvarez, Khosroheidari *et al.* 2012, Bryzgunova, Zaripov *et al.* 2016, Royo, Zuniga-Garcia *et al.* 2016, Khurana, Ranches *et al.* 2017, Rao, Van Vleet *et al.* 2018, Everaert, Helsmoortel *et al.* 2019, Mussack, Wittmann *et al.* 2019, Langevin, Kuhnell *et al.* 2020, Park, Lee *et al.* 2020)

Technique	Strengths	Limitations	Comments	General Recommendations	Particular Recommendations
<b>RNA-seq</b> Describes quantity and sequence of RNA using NGS	<ul style="list-style-type: none"> <li>- Detection of low and high expressed genes</li> <li>- Detection of isoforms/splice variants</li> <li>- Detection of new sequences</li> <li>- High sensitivity</li> <li>- Identifies different RNA species in one analysis (coding and non-coding)</li> <li>- Raw data can be used by different researchers to make new analysis.</li> </ul>	<ul style="list-style-type: none"> <li>- Cost</li> <li>- Training for data analysis</li> <li>- Data management and storage</li> <li>- Small amount of reference databases.</li> <li>- Lack of internal controls</li> <li>- The RNAs described by the analysis depends on the database used.</li> </ul>	<ul style="list-style-type: none"> <li>- RNA can be isolated as total RNA or small RNAs by using different RNA isolation kits, before library construction.</li> <li>- Different libraries can be created previous to NGS to enrich and/or deplete RNA populations (important in samples with low starting material): Whole transcriptome, targeted transcriptome (10 ng), targeted RNAs (500 pg- 5 ng), small RNAs.</li> <li>- Data analysis parameters, raw data, pre- and analytical conditions should be available to compare between different</li> </ul>	<p>Preanalytical:</p> <ul style="list-style-type: none"> <li>- Centrifugation of urine upon receive to remove cells, manage at 4°C to avoid cell rupture and microbial contamination.</li> <li>- Cell free urine as starting material.</li> <li>- Long term storage of cell free urine at -70°C</li> <li>- Reporting pre-analytical conditions according to MISEV2018 guidelines.</li> </ul> <p>Analytical:</p> <ul style="list-style-type: none"> <li>- Organic extraction increases RNA yield</li> <li>- RNA extraction method must be reported</li> <li>- Share raw data in public databases (EV-TRACK, Exocarta, etc.)</li> </ul>	<p>Preanalytical:</p> <ul style="list-style-type: none"> <li>- uEV isolation method: All methods available to date works well</li> </ul> <p>Analytical:</p> <ul style="list-style-type: none"> <li>- Library construction must be reported</li> </ul> <p>Data analysis:</p> <ul style="list-style-type: none"> <li>- Describe data analysis parameters</li> </ul>

<p><b>RNA array</b> Describes quantity of predefined RNA sequences</p>	<ul style="list-style-type: none"> <li>- Easier data analysis</li> <li>- Less data storage required</li> <li>- Detects expression of a set of predefined transcripts</li> <li>- High amount of reference databases</li> </ul>	<ul style="list-style-type: none"> <li>- Detection of highly expressed genes.</li> <li>- Depends on the affinity of the probes.</li> </ul>	<p>studies</p> <ul style="list-style-type: none"> <li>- RNA can be isolated as total RNA or small RNAs by using different RNA isolation kits.</li> </ul>		<ul style="list-style-type: none"> <li>- Use multiple probe sets per target</li> </ul>
<p><b>qPCR</b> Describes quantity of predefined RNA sequences</p>	<ul style="list-style-type: none"> <li>- Low cost for processing and implementation</li> <li>- Low starting material</li> </ul>	<ul style="list-style-type: none"> <li>- Lack of normalization parameters</li> <li>- Depends on the affinity of the probes.</li> </ul>	<ul style="list-style-type: none"> <li>- Targets can be obtained from RNA-seq data</li> </ul>		<p>Preanalytical:</p> <ul style="list-style-type: none"> <li>- When based in RNA-seq data, process sample under the same conditions</li> </ul> <p>Analytical:</p> <ul style="list-style-type: none"> <li>- Add synthetic RNA sequences to starting material to normalize</li> <li>- Use same volume of starting material</li> <li>- Characterize the reproducibility of the expression of internal controls</li> </ul>

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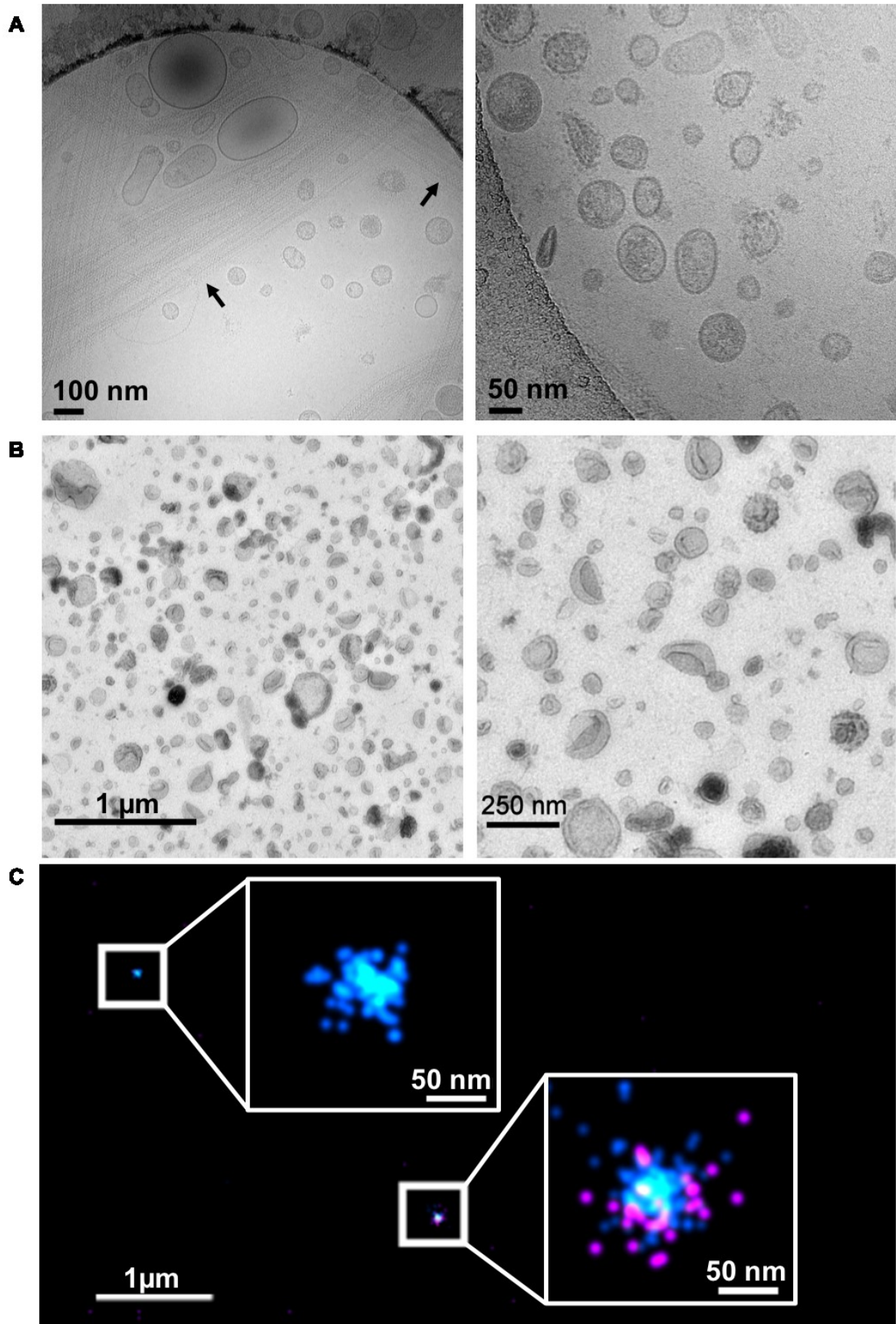


Figure 1

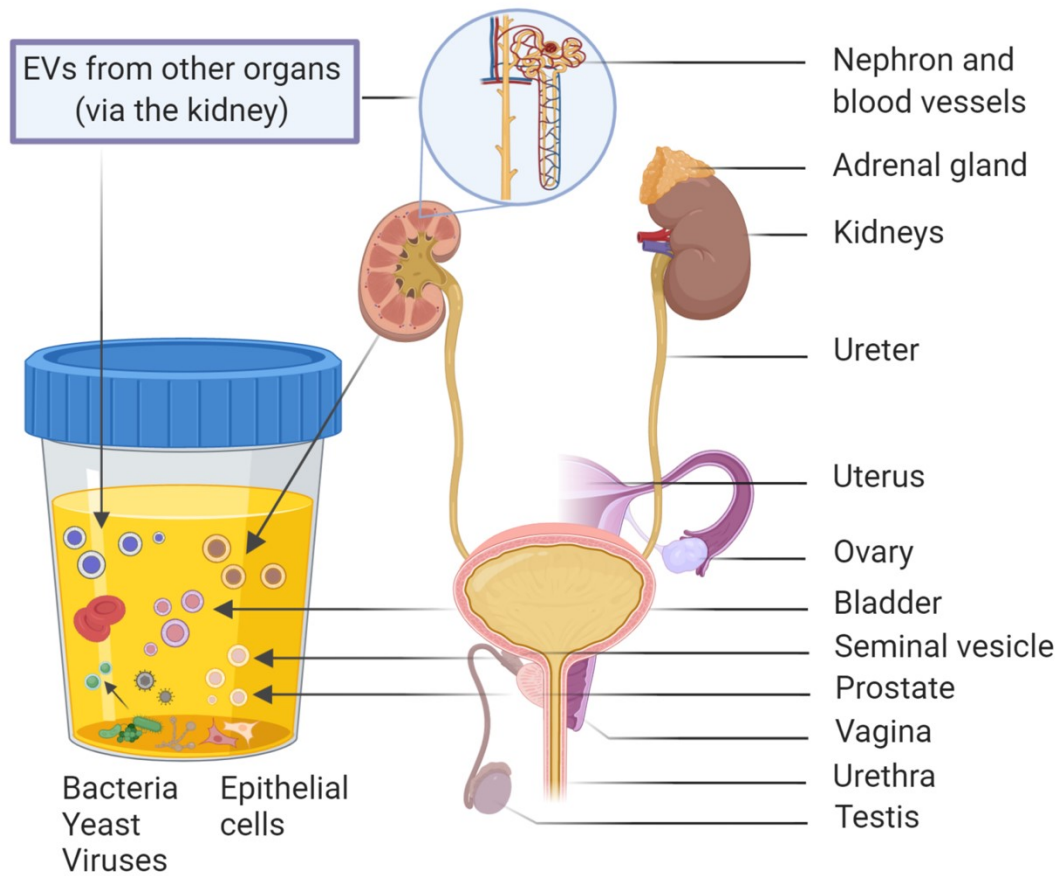


Figure 2

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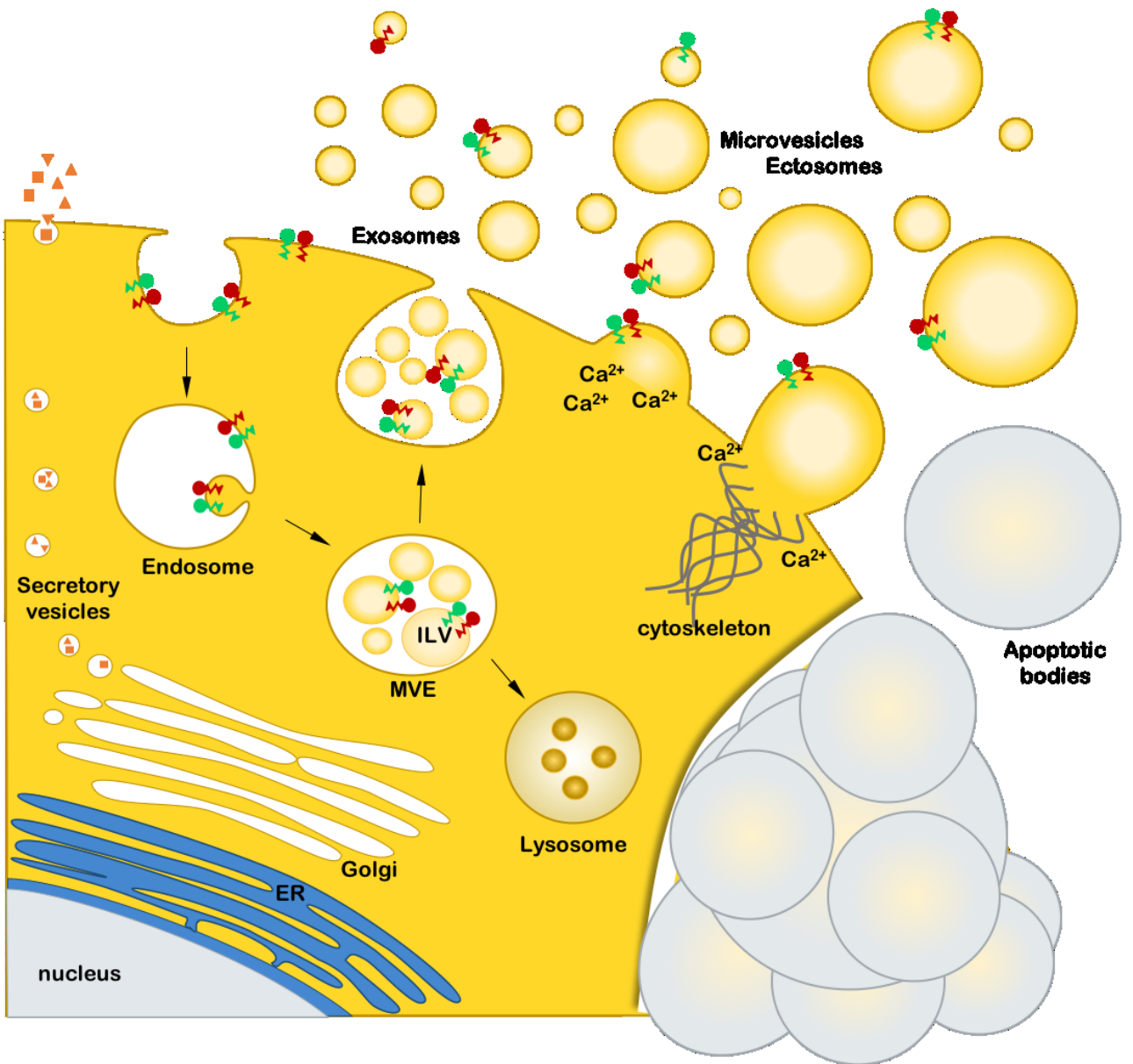


Figure 3-R1

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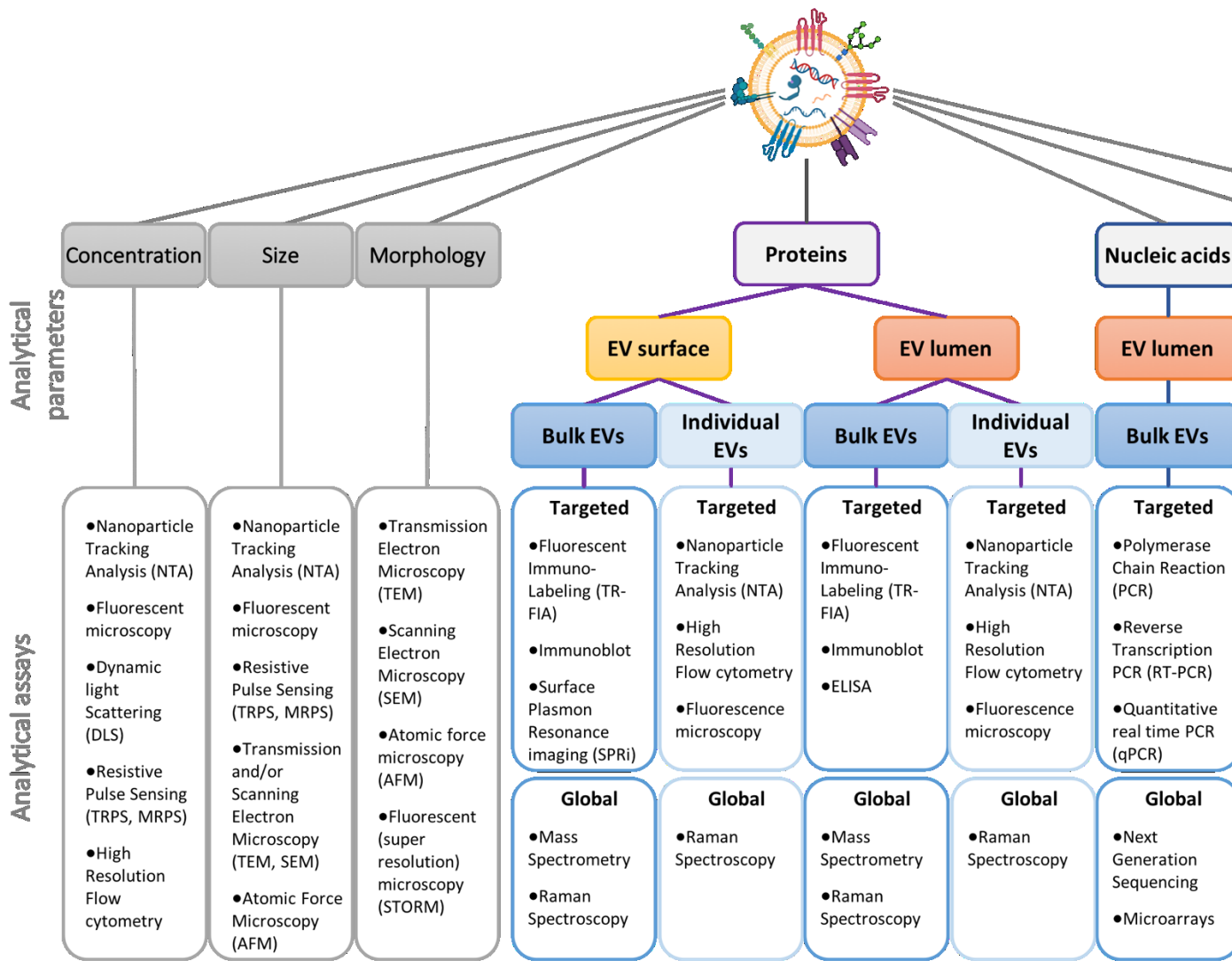


Figure 4-R1

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## References

- Abe, H., A. Sakurai, H. Ono, S. Hayashi, S. Yoshimoto, A. Ochi, S. Ueda, K. Nishimura, E. Shibata, M. Tamaki, F. Kishi, S. Kishi, T. Murakami, K. Nagai and T. Doi (2018). "Urinary Exosomal mRNA of WT1 as Diagnostic and Prognostic Biomarker for Diabetic Nephropathy." Journal of Medical Investigation **65**(3-4): 208-215.
- Adediji, A. O., T. Pourmohamad, Y. Chen, J. Burkey, C. J. Betts, S. J. Bickerton, M. Sonee and J. E. McDuffie (2019). "Investigating the Value of Urine Volume, Creatinine, and Cystatin C for Urinary Biomarkers Normalization for Drug Development Studies." Int J Toxicol **38**(1): 12-22.
- Alvarez, M. L., M. Khosroheidari, R. K. Ravi and J. K. DiStefano (2012). "Comparison of protein, microRNA, and mRNA yields using different methods of urinary exosome isolation for the discovery of kidney disease biomarkers." Kidney International **82**(9): 1024-1032.
- Ammerlaan, W., J. P. Trezzi, C. Mathay, K. Hiller and F. Betsou (2014). "Method validation for preparing urine samples for downstream proteomic and metabolomic applications." Biopreserv Biobank **12**(5): 351-357.
- Armstrong, D. A., J. A. Dessaint, C. S. Ringelberg, H. F. Hazlett, L. Howard, M. A. K. Abdalla, R. L. Barnaby, B. A. Stanton, M. A. Cervinski and A. Ashare (2018). "Pre-Analytical Handling Conditions and Small RNA Recovery from Urine for miRNA Profiling." J Mol Diagn **20**(5): 565-571.
- Avela, H. F. and H. Siren (2020). "Advances in lipidomics." Clin Chim Acta **510**: 123-141.
- Baj-Krzyworzeka, M., R. Szatanek, K. Weglarczyk, J. Baran, B. Urbanowicz, P. Branski, M. Z. Ratajczak and M. Zembala (2006). "Tumour-derived microvesicles carry several surface determinants and mRNA of tumour cells and transfer some of these determinants to monocytes." Cancer Immunol Immunother **55**(7): 808-818.
- Barreiro, K., O. P. Dwivedi, G. Leparc, M. Rolser, D. Delic, C. Forsblom, P. H. Groop, L. Groop, T. B. Huber, M. Puhka and H. Holthofer (2020). "Comparison of urinary extracellular vesicle isolation methods for transcriptomic biomarker research in diabetic kidney disease." J Extracell Vesicles **10**(2): e12038.
- Barreiro, K. and H. Holthofer (2017). "Urinary extracellular vesicles. A promising shortcut to novel biomarker discoveries." Cell Tissue Res **369**(1): 217-227.
- Barreiro, K., T. B. Huber and H. Holthofer (2020). "Isolating Urinary Extracellular Vesicles as Biomarkers for Diabetic Disease." Methods Mol Biol **2067**: 175-188.
- Barutta, F., M. Tricarico, A. Corbelli, L. Annaratone, S. Pinach, S. Grimaldi, G. Bruno, D. Cimino, D. Taverna, M. C. Deregibus, M. P. Rastaldi, P. C. Perin and G. Gruden (2013). "Urinary exosomal microRNAs in incipient diabetic nephropathy." PLoS One **8**(11): e73798.

Bazzell, B. G., W. E. Rainey, R. J. Auchus, D. Zocco, M. Bruttini, S. L. Hummel and J. B. Byrd (2018). "Human Urinary mRNA as a Biomarker of Cardiovascular Disease." Circ Genom Precis Med **11**(9): e002213.

Betsou, F., S. Lehmann, G. Ashton, M. Barnes, E. E. Benson, D. Coppola, Y. DeSouza, J. Eliason, B. Glazer, F. Guadagni, K. Harding, D. J. Horsfall, C. Kleeberger, U. Nanni, A. Prasad, K. Shea, A. Skubitz, S. Somiari, E. Gunter, B. International Society for and S. Environmental Repositories Working Group on Biospecimen (2010). "Standard preanalytical coding for biospecimens: defining the sample PReanalytical code." Cancer Epidemiol Biomarkers Prev **19**(4): 1004-1011.

Bijnsdorp, I. V., A. A. Geldof, M. Lavaei, S. R. Piersma, R. J. van Moorselaar and C. R. Jimenez (2013). "Exosomal ITGA3 interferes with non-cancerous prostate cell functions and is increased in urine exosomes of metastatic prostate cancer patients." J Extracell. Vesicles **2**.

Bijnsdorp, I. V., O. Maxouri, A. Kardar, T. Schelfhorst, S. R. Piersma, T. V. Pham, A. Vis, R. J. van Moorselaar and C. R. Jimenez (2017). "Feasibility of urinary extracellular vesicle proteome profiling using a robust and simple, clinically applicable isolation method." J Extracell Vesicles **6**(1): 1313091.

Blijdorp, C. J. and E. J. Hoorn (2019). "Urinary extracellular vesicles: the mothership connection." Am J Physiol Renal Physiol **317**(3): F648-f649.

Blijdorp, C. J., O. A. Z. Tutakhel, T. A. Hartjes, T. T. P. P. van den Bosch, M. H. van Heugten, J. P. Rigalli, R. Willemsen, U. Musterd, E. R. Barros, R. Carles-Fontana, C. A. Carvajal, O. J. Arntz, F. A. J. van de Loo, G. Jenster, M. C. Clahsen-van Groningen, C. A. C. Gallardo, D. Severs, R. A. Fenton, M. E. van Royen, J. G. J. Hoenderop, R. J. M. Bindels and E. J. Hoorn (2021). "Comparing Approaches to Normalize, Quantify, and Characterize Urinary Extracellular Vesicles." J Am Soc Nephrol.

Boulestreau, J., M. Maumus, P. Rozier, C. Jorgensen and D. Noel (2020). "Mesenchymal Stem Cell Derived Extracellular Vesicles in Aging." Front Cell Dev Biol **8**: 107.

Bourderioux, M., T. Nguyen-Khoa, C. Chhuon, L. Jeanson, D. Tondelier, M. Walczak, M. Ollero, S. Bekri, B. Knebelmann, E. Escudier, B. Escudier, A. Edelman and I. C. Guerrero (2015). "A new workflow for proteomic analysis of urinary exosomes and assessment in cystinuria patients." J Proteome Res **14**(1): 567-577.

Boyd, C., K. Wood, D. Whitaker, O. Ashorobi, L. Harvey, R. Oster, R. P. Holmes and D. G. Assimos (2018). "Accuracy in 24-hour Urine Collection at a Tertiary Center." Rev Urol **20**(3): 119-124.

Bryant, R. J., T. Pawlowski, J. W. Catto, G. Marsden, R. L. Vessella, B. Rhee, C. Kuslich, T. Visakorpi and F. C. Hamdy (2012). "Changes in circulating microRNA levels associated with prostate cancer." Br. J Cancer **106**(4): 768-774.

Bryzgunova, O. E., M. M. Zaripov, T. E. Skvortsova, E. A. Lekchnov, A. E. Grigor'eva, I. A. Zaporozhchenko, E. S. Morozkin, E. I. Ryabchikova, Y. B. Yurchenko, V. E. Voitsitskiy and P. P.

Laktionov (2016). "Comparative Study of Extracellular Vesicles from the Urine of Healthy Individuals and Prostate Cancer Patients." PLoS One **11**(6): e0157566.

Bunjevac, A., N. N. Gabaj, M. Miler and A. Horvat (2018). "Preanalytics of urine sediment examination: effect of relative centrifugal force, tube type, volume of sample and supernatant removal." Biochem Med (Zagreb) **28**(1): 010707.

Burbidge, K., V. Zwickelmaier, B. Cook, M. M. Long, B. Balva, M. Lonigro, G. Ispas, D. J. Rademacher and E. M. Campbell (2020). "Cargo and cell-specific differences in extracellular vesicle populations identified by multiplexed immunofluorescent analysis." J Extracell Vesicles **9**(1): 1789326.

Campos-Silva, C., H. Suarez, R. Jara-Acevedo, E. Linares-Espinos, L. Martinez-Pineiro, M. Yanez-Mo and M. Vales-Gomez (2019). "High sensitivity detection of extracellular vesicles immune-captured from urine by conventional flow cytometry." Sci Rep **9**(1): 2042.

Carreras-Planella, L., D. Cucchiari, L. Canas, J. Juega, M. Franquesa, J. Bonet, I. Revuelta, F. Diekmann, O. Taco, R. Lauzurica and F. E. Borrás (2020). "Urinary vitronectin identifies patients with high levels of fibrosis in kidney grafts." J Nephrol.

Carreras-Planella, L., J. Juega, O. Taco, L. Canas, M. Franquesa, R. Lauzurica and F. E. Borrás (2020). "Proteomic Characterization of Urinary Extracellular Vesicles from Kidney-Transplanted Patients Treated with Calcineurin Inhibitors." Int J Mol Sci **21**(20).

Cha, J. M., E. K. Shin, J. H. Sung, G. J. Moon, E. H. Kim, Y. H. Cho, H. D. Park, H. Bae, J. Kim and O. Y. Bang (2018). "Efficient scalable production of therapeutic microvesicles derived from human mesenchymal stem cells." Sci Rep **8**(1): 1171.

Cheah, S., S. Dee, A. Cole, L. Matzke, S. O'Donoghue and P. H. Watson (2012). "An online tool for improving biospecimen data element reporting." Biopreserv Biobank **10**(6): 501-510.

Chen, C. L., Y. F. Lai, P. Tang, K. Y. Chien, J. S. Yu, C. H. Tsai, H. W. Chen, C. C. Wu, T. Chung, C. W. Hsu, C. D. Chen, Y. S. Chang, P. L. Chang and Y. T. Chen (2012). "Comparative and targeted proteomic analyses of urinary microparticles from bladder cancer and hernia patients." J Proteome Res **11**(12): 5611-5629.

Cheng, L., X. Sun, B. J. Scicluna, B. M. Coleman and A. F. Hill (2014). "Characterization and deep sequencing analysis of exosomal and non-exosomal miRNA in human urine." Kidney Int **86**(2): 433-444.

Cheng, Y., X. Wang, J. Yang, X. Duan, Y. Yao, X. Shi, Z. Chen, Z. Fan, X. Liu, S. Qin, X. Tang and C. Zhang (2012). "A translational study of urine miRNAs in acute myocardial infarction." J Mol Cell Cardiol **53**(5): 668-676.

Cheng, Y., Q. Zeng, Q. Han and W. Xia (2019). "Effect of pH, temperature and freezing-thawing on quantity changes and cellular uptake of exosomes." Protein Cell **10**(4): 295-299.

Cheruvanky, A., H. Zhou, T. Pisitkun, J. B. Kopp, M. A. Knepper, P. S. T. Yuen and R. A. Star (2007). "Rapid isolation of urinary exosomal biomarkers using a nanomembrane ultrafiltration concentrator." American Journal of Physiology-Renal Physiology **292**(5): F1657-F1661.

Chiriaco, M. S., M. Bianco, A. Nigro, E. Primiceri, F. Ferrara, A. Romano, A. Quattrini, R. Furlan, V. Arima and G. Maruccio (2018). "Lab-on-Chip for Exosomes and Microvesicles Detection and Characterization." Sensors (Basel) **18**(10).

Chun-Yan, L., Z. Zi-Yi, Y. Tian-Lin, W. Yi-Li, L. Bao, L. Jiao and D. Wei-Jun (2018). "Liquid biopsy biomarkers of renal interstitial fibrosis based on urinary exosome." Exp Mol Pathol **105**(2): 223-228.

Ciftcioglu, N., M. Bjorklund, K. Kuorikoski, K. Bergstrom and E. O. Kajander (1999). "Nanobacteria: an infectious cause for kidney stone formation." Kidney Int **56**(5): 1893-1898.

Clayton, A., E. Boilard, E. I. Buzas, L. Cheng, J. M. Falcon-Perez, C. Gardiner, D. Gustafson, A. Gualerzi, A. Hendrix, A. Hoffman, J. Jones, C. Lasser, C. Lawson, M. Lenassi, I. Nazarenko, L. O'Driscoll, R. Pink, P. R. Siljander, C. Soekmadji, M. Wauben, J. A. Welsh, K. Witwer, L. Zheng and R. Nieuwland (2019). "Considerations towards a roadmap for collection, handling and storage of blood extracellular vesicles." J Extracell Vesicles **8**(1): 1647027.

Clos-Garcia, M., A. Loizaga-Iriarte, P. Zuniga-Garcia, P. Sanchez-Mosquera, A. Rosa Cortazar, E. Gonzalez, V. Torrano, C. Alonso, M. Perez-Cormenzana, A. Ugalde-Olano, I. Lacasa-Viscasillas, A. Castro, F. Royo, M. Unda, A. Carracedo and J. M. Falcon-Perez (2018). "Metabolic alterations in urine extracellular vesicles are associated to prostate cancer pathogenesis and progression." J Extracell Vesicles **7**(1): 1470442.

Colombo, M., C. Moita, G. van Niel, J. Kowal, J. Vigneron, P. Benaroch, N. Manel, L. F. Moita, C. Théry and G. Raposo (2013). "Analysis of ESCRT functions in exosome biogenesis, composition and secretion highlights the heterogeneity of extracellular vesicles." J Cell Sci **126**(Pt 24): 5553-5565.

Conde-Vancells, J., E. Rodriguez-Suarez, E. Gonzalez, A. Berisa, D. Gil, N. Embade, M. Valle, Z. Luka, F. Elortza, C. Wagner, S. C. Lu, J. M. Mato and M. Falcon-Perez (2010). "Candidate biomarkers in exosome-like vesicles purified from rat and mouse urine samples." Proteomics Clin Appl **4**(4): 416-425.

Consortium, E.-T., J. Van Deun, P. Mestdagh, P. Agostinis, O. Akay, S. Anand, J. Anckaert, Z. A. Martinez, T. Baetens, E. Beghein, L. Bertier, G. Berx, J. Boere, S. Boukouris, M. Bremer, D. Buschmann, J. B. Byrd, C. Casert, L. Cheng, A. Cmoch, D. Daveloose, E. De Smedt, S. Demirsoy, V. Depoorter, B. Dhondt, T. A. Driedonks, A. Dudek, A. Elsharawy, I. Floris, A. D. Foers, K. Gartner, A. D. Garg, E. Geurickx, J. Gettemans, F. Ghazavi, B. Giebel, T. G. Kormelink, G. Hancock, H. Helmoortel, A. F. Hill, V. Hyenne, H. Kalra, D. Kim, J. Kowal, S. Kraemer, P. Leidinger, C. Leonelli, Y. Liang, L. Lippens, S. Liu, A. Lo Cicero, S. Martin, S. Mathivanan, P. Mathiyalagan, T. Matusek, G. Milani, M. Monguio-Tortajada, L. M. Mus, D. C. Muth, A. Nemeth, E. N. Nolte-'t Hoen, L. O'Driscoll, R. Palmulli, M. W. Pfaffl, B. Primdal-Bengtson, E. Romano, Q. Rousseau, S. Sahoo, N. Sampaio, M. Samuel, B. Scicluna, B. Soen, A. Steels, J. V. Swinnen, M. Takatalo, S. Thaminy, C. Thery, J. Tulkens, I. Van

Audenhove, S. van der Grein, A. Van Goethem, M. J. van Herwijnen, G. Van Niel, N. Van Roy, A. R. Van Vliet, N. Vandamme, S. Vanhauwaert, G. Vergauwen, F. Verweij, A. Wallaert, M. Wauben, K. W. Witwer, M. I. Zonneveld, O. De Wever, J. Vandesompele and A. Hendrix (2017). "EV-TRACK: transparent reporting and centralizing knowledge in extracellular vesicle research." Nat Methods **14**(3): 228-232.

Corbetta, S., F. Raimondo, S. Tedeschi, M. L. Syren, P. Rebora, A. Savoia, L. Baldi, A. Bettinelli and M. Pitto (2015). "Urinary exosomes in the diagnosis of Gitelman and Bartter syndromes." Nephrol Dial Transplant **30**(4): 621-630.

Coughlan, C., K. D. Bruce, O. Burgy, T. D. Boyd, C. R. Michel, J. E. Garcia-Perez, V. Adame, P. Anton, B. M. Bettcher, H. J. Chial, M. Konigshoff, E. W. Y. Hsieh, M. Graner and H. Potter (2020). "Exosome Isolation by Ultracentrifugation and Precipitation and Techniques for Downstream Analyses." Curr Protoc Cell Biol **88**(1): e110.

Coumans, F. A., E. van der Pol, A. N. Boing, N. Hajji, G. Sturk, T. G. van Leeuwen and R. Nieuwland (2014). "Reproducible extracellular vesicle size and concentration determination with tunable resistive pulse sensing." J Extracell Vesicles **3**: 25922.

Coumans, F. A. W., A. R. Brisson, E. I. Buzas, F. Dignat-George, E. E. E. Drees, S. El-Andaloussi, C. Emanuelli, A. Gasecka, A. Hendrix, A. F. Hill, R. Lacroix, Y. Lee, T. G. van Leeuwen, N. Mackman, I. Mager, J. P. Nolan, E. van der Pol, D. M. Pegtel, S. Sahoo, P. R. M. Siljander, G. Sturk, O. de Wever and R. Nieuwland (2017). "Methodological Guidelines to Study Extracellular Vesicles." Circ Res **120**(10): 1632-1648.

Daaboul, G. G., P. Gagni, L. Benussi, P. Bettotti, M. Ciani, M. Cretich, D. S. Freedman, R. Ghidoni, A. Y. Ozkumur, C. Piotto, D. Prospero, B. Santini, M. S. Unlu and M. Chiari (2016). "Digital Detection of Exosomes by Interferometric Imaging." Sci Rep **6**: 37246.

Decramer, S., A. Gonzalez de Peredo, B. Breuil, H. Mischak, B. Monsarrat, J. L. Bascands and J. P. Schanstra (2008). "Urine in clinical proteomics." Mol Cell Proteomics **7**(10): 1850-1862.

Del Boccio, P., F. Raimondo, D. Pieragostino, L. Morosi, G. Cozzi, P. Sacchetta, F. Magni, M. Pitto and A. Urbani (2012). "A hyphenated microLC-Q-TOF-MS platform for exosomal lipidomics investigations: application to RCC urinary exosomes." Electrophoresis **33**(4): 689-696.

Delic, D., C. Eisele, R. Schmid, P. Baum, F. Wiech, M. Gerl, H. Zimdahl, S. S. Pullen and R. Urquhart (2016). "Urinary Exosomal miRNA Signature in Type II Diabetic Nephropathy Patients." PLoS One **11**(3): e0150154.

Dhondt, B., E. Geeurickx, J. Tulkens, J. Van Deun, G. Vergauwen, L. Lippens, I. Miinalainen, P. Rappu, J. Heino, P. Ost, N. Lumen, O. De Wever and A. Hendrix (2020). "Unravelling the proteomic landscape of extracellular vesicles in prostate cancer by density-based fractionation of urine." J Extracell Vesicles **9**(1): 1736935.

Dhondt, B., N. Lumen, O. De Wever and A. Hendrix (2020). "Preparation of Multi-omics Grade Extracellular Vesicles by Density-Based Fractionation of Urine." STAR Protocols **1**(2): 100073.

Dhondt, B., J. Van Deun, S. Vermaerke, A. de Marco, N. Lumen, O. De Wever and A. Hendrix (2018). "Urinary extracellular vesicle biomarkers in urological cancers: From discovery towards clinical implementation." Int J Biochem Cell Biol **99**: 236-256.

Dijkstra, S., I. L. Birker, F. P. Smit, G. H. Leyten, T. M. de Reijke, I. M. van Oort, P. F. Mulders, S. A. Jannink and J. A. Schalken (2014). "Prostate cancer biomarker profiles in urinary sediments and exosomes." J Urol **191**(4): 1132-1138.

Dimuccio, V., L. Peruzzi, M. F. Brizzi, E. Cocchi, F. Fop, A. Boido, M. Gili, S. Gallo, L. Biancone, G. Camussi and B. Bussolati (2020). "Acute and chronic glomerular damage is associated with reduced CD133 expression in urinary extracellular vesicles." Am J Physiol Renal Physiol **318**(2): F486-f495.

Dimuccio, V., A. Ranghino, L. Pratico Barbato, F. Fop, L. Biancone, G. Camussi and B. Bussolati (2014). "Urinary CD133+ extracellular vesicles are decreased in kidney transplanted patients with slow graft function and vascular damage." PLoS One **9**(8): e104490.

Donovan, M. J., M. Noerholm, S. Bentink, S. Belzer, J. Skog, V. O'Neill, J. S. Cochran and G. A. Brown (2015). "A molecular signature of PCA3 and ERG exosomal RNA from non-DRE urine is predictive of initial prostate biopsy result." Prostate Cancer Prostatic Dis **18**(4): 370-375.

Duijvesz, D., C. Y. Versluis, C. A. van der Fels, M. S. Vredendregt-van den Berg, J. Leivo, M. T. Peltola, C. H. Bangma, K. S. Pettersson and G. Jenster (2015). "Immuno-based detection of extracellular vesicles in urine as diagnostic marker for prostate cancer." Int J Cancer **137**(12): 2869-2878.

Erdbrugger, U. and T. H. Le (2016). "Extracellular Vesicles in Renal Diseases: More than Novel Biomarkers?" J Am Soc Nephrol **27**(1): 12-26.

Erozenci, L. A., F. Bottger, I. V. Bijnsdorp and C. R. Jimenez (2019). "Urinary exosomal proteins as (pan-)cancer biomarkers: insights from the proteome." FEBS Lett.

Everaert, C., H. Helsmoortel, A. Decock, E. Hulstaert, R. Van Paemel, K. Verniers, J. Nuytens, J. Anckaert, N. Nijs, J. Tulkens, B. Dhondt, A. Hendrix, P. Mestdagh and J. Vandesompele (2019). "Performance assessment of total RNA sequencing of human biofluids and extracellular vesicles." Sci Rep **9**(1): 17574.

Fan, W., G. Ankawi, J. Zhang, K. Digvijay, D. Giavarina, Y. Yin and C. Ronco (2018). "Current understanding and future directions in the application of TIMP-2 and IGFBP7 in AKI clinical practice." Clin Chem Lab Med.

Fernandez-Llama, P., S. Khositseth, P. A. Gonzales, R. A. Star, T. Pisitkun and M. A. Knepper (2010). "Tamm-Horsfall protein and urinary exosome isolation." Kidney Int **77**(8): 736-742.

Firsov, D. and O. Bonny (2018). "Circadian rhythms and the kidney." Nat Rev Nephrol **14**(10): 626-635.

Fisher, L. A., T. S. Johnson, D. Porter, H. L. Bleich and W. V. Slack (1977). "Collection of a clean voided urine specimen: a comparison among spoken, written, and computer-based instructions." Am J Public Health **67**(7): 640-644.

Forni Ogha, V., A. Ogha, P. Vuistiner, M. Pruijm, B. Ponte, D. Ackermann, L. Gabutti, N. Vakilzadeh, M. Mohaupt, P. Y. Martin, I. Guessous, A. Pechere-Bertschi, F. Paccaud, M. Bochud, M. Burnier and G. Swiss Survey on Salt (2015). "New anthropometry-based age- and sex-specific reference values for urinary 24-hour creatinine excretion based on the adult Swiss population." BMC Med **13**: 40.

Fraser, K. B., A. B. Rawlins, R. G. Clark, R. N. Alcalay, D. G. Standaert, N. J. Liu, A. B. West and P. s. D. B. Program (2016). "Ser(P)-1292 LRRK2 in urinary exosomes is elevated in idiopathic Parkinson's disease." Movement Disorders **31**(10): 1543-1550.

Freitas, D., M. Balmana, J. Pocas, D. Campos, H. Osorio, A. Konstantinidi, S. Y. Vakhrushev, A. Magalhaes and C. A. Reis (2019). "Different isolation approaches lead to diverse glycosylated extracellular vesicle populations." J Extracell Vesicles **8**(1): 1621131.

Fujita, K., H. Kume, K. Matsuzaki, A. Kawashima, T. Ujike, A. Nagahara, M. Uemura, Y. Miyagawa, T. Tomonaga and N. Nonomura (2017). "Proteomic analysis of urinary extracellular vesicles from high Gleason score prostate cancer." Sci. Rep. **7**: 42961.

Fujita, K. and N. Nonomura (2018). "Urinary biomarkers of prostate cancer." Int J Urol **25**(9): 770-779.

Gaffney, A., RAC (2014). Flip a Coin: FDA Approves Kidney Diagnostic With 50% False Positive Results. Regulatory Focus. Rockville, Maryland RAPS, Regulatory Affairs Professionals Society.

Gamez-Valero, A., S. I. Lozano-Ramos, I. Bancu, R. Lauzurica-Valdemoros and F. E. Borrás (2015). "Urinary extracellular vesicles as source of biomarkers in kidney diseases." Front Immunol **6**: 6.

Ghai, V., X. Wu, A. Bheda-Malge, C. P. Argyropoulos, J. F. Bernardo, T. Orchard, D. Galas and K. Wang (2018). "Genome-wide Profiling of Urinary Extracellular Vesicle microRNAs Associated With Diabetic Nephropathy in Type 1 Diabetes." Kidney Int Rep **3**(3): 555-572.

Ghosh, A., M. Davey, I. C. Chute, S. G. Griffiths, S. Lewis, S. Chacko, D. Barnett, N. Crapoulet, S. Fournier, A. Joy, M. C. Caissie, A. D. Ferguson, M. Daigle, M. V. Meli, S. M. Lewis and R. J. Ouellette (2014). "Rapid isolation of extracellular vesicles from cell culture and biological fluids using a synthetic peptide with specific affinity for heat shock proteins." PLoS One **9**(10): e110443.

Gildea, J. J., J. E. Seaton, K. G. Victor, C. M. Reyes, D. Bigler Wang, A. C. Pettigrew, C. E. Courtner, N. Shah, H. T. Tran, R. E. Van Sciver, J. M. Carlson and R. A. Felder (2014). "Exosomal transfer from



human renal proximal tubule cells to distal tubule and collecting duct cells." *Clin Biochem* **47**(15): 89-94.

Ginsberg, J. M., B. S. Chang, R. A. Matarese and S. Garella (1983). "Use of single voided urine samples to estimate quantitative proteinuria." *N Engl J Med* **309**(25): 1543-1546.

Gleadle, J., K. McNicholas, J. Li, M. Michael and D. Rojas-Canales (2018). "Nanoparticle Tracking Analysis of Urine to Detect Exosomes Can Be Confounded by Albuminuria." *J Am Soc Nephrol* **29**(6): 1784.

Glover, S. C., M. Z. Nouri, K. M. Tuna, L. B. Mendoza Alvarez, L. K. Ryan, J. F. Shirley, Y. Tang, N. D. Denslow and A. A. Alli (2019). "Lipidomic analysis of urinary exosomes from hereditary alpha-tryptasemia patients and healthy volunteers." *FASEB Bioadv* **1**(10): 624-638.

Gonzales, P. A., T. Pisitkun, J. D. Hoffert, D. Tchapyjnikov, R. A. Star, R. Kleta, N. S. Wang and M. A. Knepper (2009). "Large-scale proteomics and phosphoproteomics of urinary exosomes." *J Am Soc Nephrol* **20**(2): 363-379.

Gonzales, P. A., H. Zhou, T. Pisitkun, N. S. Wang, R. A. Star, M. A. Knepper and P. S. Yuen (2010). "Isolation and purification of exosomes in urine." *Methods Mol. Biol* **641**: 89-99.

Gonzalez-Calero, L., P. J. Martinez, M. Martin-Lorenzo, M. Baldan-Martin, G. Ruiz-Hurtado, F. de la Cuesta, E. Calvo, J. Segura, J. A. Lopez, J. Vazquez, M. G. Barderas, L. M. Ruilope, F. Vivanco and G. Alvarez-Llamas (2017). "Urinary exosomes reveal protein signatures in hypertensive patients with albuminuria." *Oncotarget* **8**(27): 44217-44231.

Gori, A., A. Romanato, B. Greta, A. Strada, P. Gagni, R. Frigerio, D. Brambilla, R. Vago, S. Galbiati, S. Picciolini, M. Bedoni, G. G. Daaboul, M. Chiari and M. Cretich (2020). "Membrane-binding peptides for extracellular vesicles on-chip analysis." *J Extracell Vesicles* **9**(1): 1751428.

Grange, C., E. Papadimitriou, V. Dimuccio, C. Pastorino, J. Molina, R. O'Kelly, L. J. Niedernhofer, P. D. Robbins, G. Camussi and B. Bussolati (2020). "Urinary Extracellular Vesicles Carrying Klotho Improve the Recovery of Renal Function in an Acute Tubular Injury Model." *Mol Ther* **28**(2): 490-502.

Gunasekaran, P. M., J. M. Luther and J. B. Byrd (2019). "For what factors should we normalize urinary extracellular mRNA biomarkers?" *Biomol Detect Quantif* **17**: 100090.

Guo, Z., Y. Zhang, L. Zou, D. Wang, C. Shao, Y. Wang, W. Sun and L. Zhang (2015). "A Proteomic Analysis of Individual and Gender Variations in Normal Human Urine and Cerebrospinal Fluid Using iTRAQ Quantification." *PLoS One* **10**(7): e0133270.

Harpole, M., J. Davis and V. Espina (2016). "Current state of the art for enhancing urine biomarker discovery." *Expert Rev Proteomics* **13**(6): 609-626.

Hartjes, T. A., S. Mytnyk, G. W. Jenster, V. van Steijn and M. E. van Royen (2019). "Extracellular Vesicle Quantification and Characterization: Common Methods and Emerging Approaches." Bioengineering (Basel) **6**(1).

Hartjes, T. A., J. A. Slotman, M. S. Vredenburg, N. Dits, R. Van der Meel, D. Duijvesz, J. A. Kulkarni, P. J. French, W. A. Van Cappellen, R. M. Schiffelers, A. B. Houtsmuller, G. W. Jenster and M. E. Van Royen (2020). "EVQuant; high-throughput quantification and characterization of extracellular vesicle (sub)populations." bioRxiv: 2020.2010.2021.348375.

Havanapan, P. O. and V. Thongboonkerd (2009). "Are protease inhibitors required for gel-based proteomics of kidney and urine?" J Proteome Res **8**(6): 3109-3117.

Headland, S. E., H. R. Jones, A. S. D'Sa, M. Perretti and L. V. Norling (2014). "Cutting-edge analysis of extracellular microparticles using ImageStream(X) imaging flow cytometry." Sci Rep **4**: 5237.

Hendriks, R. J., S. Dijkstra, S. A. Jannink, M. G. Steffens, I. M. van Oort, P. F. Mulders and J. A. Schalken (2016). "Comparative analysis of prostate cancer specific biomarkers PCA3 and ERG in whole urine, urinary sediments and exosomes." Clin Chem Lab Med **54**(3): 483-492.

Hiemstra, T. F., P. D. Charles, T. Gracia, S. S. Hester, L. Gatto, R. Al-Lamki, R. A. Floto, Y. Su, J. N. Skepper, K. S. Lilley and F. E. Karet Frankl (2014). "Human urinary exosomes as innate immune effectors." J Am Soc Nephrol **25**(9): 2017-2027.

Hiemstra, T. F., P. D. Charles, S. S. Hester, F. E. Karet and K. S. Lilley (2011). "Uromodulin exclusion list improves urinary exosomal protein identification." J Biomol Tech **22**(4): 136-145.

Hjelle, J. T., M. A. Miller-Hjelle, I. R. Poxton, E. O. Kajander, N. Ciftcioglu, M. L. Jones, R. C. Caughey, R. Brown, P. D. Millikin and F. S. Darras (2000). "Endotoxin and nanobacteria in polycystic kidney disease." Kidney Int **57**(6): 2360-2374.

Hogan, M. C., K. L. Johnson, R. M. Zenka, M. C. Charlesworth, B. J. Madden, D. W. Mahoney, A. L. Oberg, B. Q. Huang, A. A. Leontovich, L. L. Nesbitt, J. L. Bakeberg, D. J. McCormick, H. R. Bergen and C. J. Ward (2014). "Subfractionation, characterization, and in-depth proteomic analysis of glomerular membrane vesicles in human urine." Kidney Int **85**(5): 1225-1237.

Hogan, M. C., J. C. Lieske, C. C. Lienczewski, L. L. Nesbitt, L. T. Wickman, C. M. Heyer, P. C. Harris, C. J. Ward, J. L. Sundsbak, L. Manganelli, W. Ju, J. B. Kopp, P. J. Nelson, S. G. Adler, H. N. Reich, L. B. Holzmann, M. Kretzler and M. Bitzer (2015). "Strategy and rationale for urine collection protocols employed in the NEPTUNE study." BMC Nephrology **16**(1): 190.

Hogan, M. C., L. Manganelli, J. R. Woollard, A. I. Masyuk, T. V. Masyuk, R. Tammachote, B. Q. Huang, A. A. Leontovich, T. G. Beito, B. J. Madden, M. C. Charlesworth, V. E. Torres, N. F. LaRusso, P. C. Harris and C. J. Ward (2009). "Characterization of PKD protein-positive exosome-like vesicles." J Am Soc Nephrol **20**(2): 278-288.

Huebner, A. R., P. Somparn, T. Benjachat, A. Leelahavanichkul, Y. Avihingsanon, R. A. Fenton and T. Pisitkun (2015). "Exosomes in urine biomarker discovery." Adv. Exp. Med. Biol **845**: 43-58.

Iliescu, F. S., D. Vrtacnik, P. Neuzil and C. Iliescu (2019). "Microfluidic Technology for Clinical Applications of Exosomes." Micromachines (Basel) **10**(6).

Inman, B. A., W. Etienne, R. Rubin, R. A. Owusu, T. R. Oliveira, D. B. Rodrigues, P. F. Maccarini, P. R. Stauffer, A. Mashal and M. W. Dewhirst (2013). "The impact of temperature and urinary constituents on urine viscosity and its relevance to bladder hyperthermia treatment." International Journal of Hyperthermia **29**(3): 206-210.

Islam, M. K., P. Syed, L. Lehtinen, J. Leivo, K. Gidwani, S. Wittfooth, K. Pettersson and U. Lamminmaki (2019). "A Nanoparticle-Based Approach for the Detection of Extracellular Vesicles." Sci Rep **9**(1): 10038.

Kalani, A., A. Mohan, M. M. Godbole, E. Bhatia, A. Gupta, R. K. Sharma and S. Tiwari (2013). "Wilm's tumor-1 protein levels in urinary exosomes from diabetic patients with or without proteinuria." PLoS One **8**(3): e60177.

Kalluri, R. and V. S. LeBleu (2020). "The biology, function, and biomedical applications of exosomes." Science **367**(6478).

Kalra, H., R. J. Simpson, H. Ji, E. Aikawa, P. Altevogt, P. Askenase, V. C. Bond, F. E. Borràs, X. Breakefield, V. Budnik, E. Buzas, G. Camussi, A. Clayton, E. Cocucci, J. M. Falcon-Perez, S. Gabrielsson, Y. S. Gho, D. Gupta, H. C. Harsha, A. Hendrix, A. F. Hill, J. M. Inal, G. Jenster, E.-M. Krämer-Albers, S. K. Lim, A. Llorente, J. Lötvall, A. Marcilla, L. Mincheva-Nilsson, I. Nazarenko, R. Nieuwland, E. N. M. Nolte-'t Hoen, A. Pandey, T. Patel, M. G. Piper, S. Pluchino, T. S. K. Prasad, L. Rajendran, G. Raposo, M. Record, G. E. Reid, F. Sánchez-Madrid, R. M. Schiffelers, P. Siljander, A. Stensballe, W. Stoorvogel, D. Taylor, C. Thery, H. Valadi, B. W. M. van Balkom, J. Vázquez, M. Vidal, M. H. M. Wauben, M. Yáñez-Mó, M. Zoeller and S. Mathivanan (2012). "Vesiclepedia: A Compendium for Extracellular Vesicles with Continuous Community Annotation." PLOS Biology **10**(12): e1001450.

Kang, C. S., M. Ban, E. J. Choi, H. G. Moon, J. S. Jeon, D. K. Kim, S. K. Park, S. G. Jeon, T. Y. Roh, S. J. Myung, Y. S. Gho, J. G. Kim and Y. K. Kim (2013). "Extracellular vesicles derived from gut microbiota, especially *Akkermansia muciniphila*, protect the progression of dextran sulfate sodium-induced colitis." PLoS One **8**(10): e76520.

Karina, B., D. O. Prakash, L. German, R. Marcel, D. Denis, F. Carol, G. Per-Henrik, G. Leif, H. T. B, P. Maija and H. Harry (2020). "Comparison of urinary extracellular vesicle isolation methods for transcriptomic biomarker research in diabetic kidney disease." J Extracell Vesicles **in press**.

Karpman, D., A. L. Stahl and I. Arvidsson (2017). "Extracellular vesicles in renal disease." Nat Rev Nephrol **13**(9): 545-562.

Kashani, K., A. Al-Khafaji, T. Ardiles, A. Artigas, S. M. Bagshaw, M. Bell, A. Bihorac, R. Birkhahn, C. M. Cely, L. S. Chawla, D. L. Davison, T. Feldkamp, L. G. Forni, M. N. Gong, K. J. Gunnerson, M. Haase, J. Hackett, P. M. Honore, E. A. Hoste, O. Joannes-Boyau, M. Joannidis, P. Kim, J. L. Koyner, D. T. Laskowitz, M. E. Lissauer, G. Marx, P. A. McCullough, S. Mullaney, M. Ostermann, T. Rimmele, N. I. Shapiro, A. D. Shaw, J. Shi, A. M. Sprague, J. L. Vincent, C. Vinsonneau, L. Wagner, M. G. Walker, R. G. Wilkerson, K. Zacharowski and J. A. Kellum (2013). "Discovery and validation of cell cycle arrest biomarkers in human acute kidney injury." Crit Care **17**(1): R25.

Keller, S., C. Rupp, A. Stoeck, S. Runz, M. Fogel, S. Lugert, H. D. Hager, M. S. Abdel-Bakky, P. Gutwein and P. Altevogt (2007). "CD24 is a marker of exosomes secreted into urine and amniotic fluid." Kidney Int **72**(9): 1095-1102.

Kerjaschki, D., M. Schulze, S. Binder, R. Kain, P. P. Ojha, M. Susani, R. Horvat, P. J. Baker and W. G. Couser (1989). "Transcellular transport and membrane insertion of the C5b-9 membrane attack complex of complement by glomerular epithelial cells in experimental membranous nephropathy." J Immunol **143**(2): 546-552.

Khamis, M. M., T. Holt, H. Awad, A. El-Aneed and D. J. Adamko (2018). "Comparative analysis of creatinine and osmolality as urine normalization strategies in targeted metabolomics for the differential diagnosis of asthma and COPD." Metabolomics **14**(9): 115.

Khurana, R., G. Ranches, S. Schafferer, M. Lukasser, M. Rudnicki, G. Mayer and A. Huttenhofer (2017). "Identification of urinary exosomal noncoding RNAs as novel biomarkers in chronic kidney disease." RNA **23**(2): 142-152.

Knol, J. C., I. de Reus, T. Schelfhorst, R. Beekhof, M. de Wit, S. R. Piersma, T. V. Pham, E. F. Smit, H. M. W. Verheul and C. R. Jimenez (2016). "Peptide-mediated 'miniprep' isolation of extracellular vesicles is suitable for high-throughput proteomics." EuPA Open Proteom **11**: 11-15.

Koliha, N., Y. Wiencek, U. Heider, C. Jungst, N. Kladt, S. Krauthauser, I. C. Johnston, A. Bosio, A. Schauss and S. Wild (2016). "A novel multiplex bead-based platform highlights the diversity of extracellular vesicles." J Extracell Vesicles **5**: 29975.

Konoshenko, M. Y., E. A. Lekchnov, A. V. Vlassov and P. P. Laktionov (2018). "Isolation of Extracellular Vesicles: General Methodologies and Latest Trends." Biomed Res Int **2018**: 8545347.

Koopman, M. G., R. T. Krediet, G. C. Koomen, J. Strackee and L. Arisz (1989). "Circadian rhythm of proteinuria: consequences of the use of urinary protein:creatinine ratios." Nephrol Dial Transplant **4**(1): 9-14.

Koppers-Lalic, D., M. Hackenberg, M. R. de, B. Misovic, M. Wachalska, A. Geldof, N. Zini, R. T. de, T. Wurdinger, A. Vis, M. J. van, M. Pegtel and I. Bijnsdorp (2016). "Noninvasive prostate cancer detection by measuring miRNA variants (isomiRs) in urine extracellular vesicles." Oncotarget **7**(16): 22566-22578.

Koritzinsky, E. H., J. M. Street, R. R. Chari, D. M. Glispie, T. R. Bellomo, A. M. Aponte, R. A. Star and P. S. T. Yuen (2019). "Circadian variation in the release of small extracellular vesicles can be normalized by vesicle number or TSG101." Am J Physiol Renal Physiol **317**(5): F1098-F1110.

Kowal, J., G. Arras, M. Colombo, M. Jouve, J. P. Morath, B. Primdal-Bengtson, F. Dingli, D. Loew, M. Tkach and C. Thery (2016). "Proteomic comparison defines novel markers to characterize heterogeneous populations of extracellular vesicle subtypes." Proc Natl Acad Sci U S A **113**(8): E968-977.

Kruglik, S. G., F. Royo, J. M. Guigner, L. Palomo, O. Seksek, P. Y. Turpin, I. Tatischeff and J. M. Falcon-Perez (2019). "Raman tweezers microspectroscopy of circa 100 nm extracellular vesicles." Nanoscale **11**(4): 1661-1679.

Ku, A., H. C. Lim, M. Evander, H. Lilja, T. Laurell, S. Scheduling and Y. Ceder (2018). "Acoustic Enrichment of Extracellular Vesicles from Biological Fluids." Anal Chem **90**(13): 8011-8019.

Ku, A., N. Ravi, M. Yang, M. Evander, T. Laurell, H. Lilja and Y. Ceder (2019). "A urinary extracellular vesicle microRNA biomarker discovery pipeline; from automated extracellular vesicle enrichment by acoustic trapping to microRNA sequencing." PLoS One **14**(5): e0217507.

Kwon, S. H., J. R. Woollard, A. Saad, V. D. Garovic, L. Zand, K. L. Jordan, S. C. Textor and L. O. Lerman (2017). "Elevated urinary podocyte-derived extracellular microvesicles in renovascular hypertensive patients." Nephrol Dial Transplant **32**(5): 800-807.

La Salvia, S., P. M. Gunasekaran, J. B. Byrd and U. Erdbrugger (2020). "Extracellular Vesicles in Essential Hypertension: Hidden Messengers." Curr Hypertens Rep **22**(10): 76.

Langevin, S. M., D. Kuhnell, J. Biesiada, X. Zhang, M. Medvedovic, G. G. Talaska, K. A. Burns and S. Kasper (2020). "Comparability of the small RNA secretome across human biofluids concomitantly collected from healthy adults." PLoS One **15**(4): e0229976.

Larsen, P., L. Wik, P. Czarnewski, M. Eldh, L. Lof, K. G. Ronquist, L. Dubois, E. Freyhult, C. J. Gallant, J. Oelrich, A. Larsson, G. Ronquist, E. J. Villablanca, U. Landegren, S. Gabrielsson and M. Kamali-Moghaddam (2017). "Tracing Cellular Origin of Human Exosomes Using Multiplex Proximity Extension Assays." Mol Cell Proteomics **16**(3): 502-511.

Lee, J., K. Q. McKinney, A. J. Pavlopoulos, M. Niu, J. W. Kang, J. W. Oh, K. P. Kim and S. Hwang (2018). "Altered Proteome of Extracellular Vesicles Derived from Bladder Cancer Patients Urine." Mol Cells **41**(3): 179-187.

Lee, Y., J. Y. Park, E. H. Lee, J. Yang, B. R. Jeong, Y. K. Kim, J. Y. Seoh, S. Lee, P. L. Han and E. J. Kim (2017). "Rapid Assessment of Microbiota Changes in Individuals with Autism Spectrum Disorder Using Bacteria-derived Membrane Vesicles in Urine." Exp Neurobiol **26**(5): 307-317.

Lehmann, S., F. Guadagni, H. Moore, G. Ashton, M. Barnes, E. Benson, J. Clements, I. Koppandi, D. Coppola, S. Y. Demiroglu, Y. DeSouza, A. De Wilde, J. Duker, J. Eliason, B. Glazer, K. Harding, J. P. Jeon, J. Kessler, T. Kokkat, U. Nanni, K. Shea, A. Skubitz, S. Somiari, G. Tybring, E. Gunter, F. Betsou, B. International Society for and S. Environmental Repositories Working Group on Biospecimen (2012). "Standard preanalytical coding for biospecimens: review and implementation of the Sample PREanalytical Code (SPREC)." Biopreserv Biobank **10**(4): 366-374.

Leiblich, A. (2017). "Recent Developments in the Search for Urinary Biomarkers in Bladder Cancer." Curr Urol Rep **18**(12): 100.

Li, Y., Y. Zhang, F. Qiu and Z. Qiu (2011). "Proteomic identification of exosomal LRG1: a potential urinary biomarker for detecting NSCLC." Electrophoresis **32**(15): 1976-1983.

Liang, L. G., M. Q. Kong, S. Zhou, Y. F. Sheng, P. Wang, T. Yu, F. Inci, W. P. Kuo, L. J. Li, U. Demirci and S. Wang (2017). "An integrated double-filtration microfluidic device for isolation, enrichment and quantification of urinary extracellular vesicles for detection of bladder cancer." Sci Rep **7**: 46224.

Londono, I. and M. Bendayan (2005). "Glomerular handling of native albumin in the presence of circulating modified albumins by the normal rat kidney." Am J Physiol Renal Physiol **289**(6): F1201-1209.

Longmire, M., P. L. Choyke and H. Kobayashi (2008). "Clearance properties of nano-sized particles and molecules as imaging agents: considerations and caveats." Nanomedicine (Lond) **3**(5): 703-717.

Lotvall, J., A. F. Hill, F. Hochberg, E. I. Buzas, D. Di Vizio, C. Gardiner, Y. S. Gho, I. V. Kurochkin, S. Mathivanan, P. Quesenberry, S. Sahoo, H. Tahara, M. H. Wauben, K. W. Witwer and C. Thery (2014). "Minimal experimental requirements for definition of extracellular vesicles and their functions: a position statement from the International Society for Extracellular Vesicles." J Extracell Vesicles **3**: 26913.

Lozano-Ramos, I., J. Bancu, A. Oliveira-Tercero, M. P. Armengol, A. Menezes-Neto, H. A. Del Portillo, R. Lauzurica-Valdemoros and F. E. Borrás (2015). "Size-exclusion chromatography-based enrichment of extracellular vesicles from urine samples." J Extracell Vesicles **4**: 27369.

Lozano-Ramos, S. I., I. Bancu, L. Carreras-Planella, M. Monguio-Tortajada, L. Canas, J. Juega, J. Bonet, M. P. Armengol, R. Lauzurica and F. E. Borrás (2018). "Molecular profile of urine extracellular vesicles from normo-functional kidneys reveal minimal differences between living and deceased donors." BMC Nephrol **19**(1): 189.

Lytvyn, Y., F. Xiao, C. R. Kennedy, B. A. Perkins, H. N. Reich, J. W. Scholey, D. Z. Cherney and D. Burger (2017). "Assessment of urinary microparticles in normotensive patients with type 1 diabetes." Diabetologia **60**(3): 581-584.

Magiorkinis, E. and A. Diamantis (2015). "The fascinating story of urine examination: From uroscopy to the era of microscopy and beyond." Diagn Cytopathol **43**(12): 1020-1036.

Manoni, F., G. Gessoni, M. G. Alessio, A. Caleffi, G. Sacconi, M. G. Silvestri, D. Poz, M. Ercolin, A. Tinello, S. Valverde, C. Ottomano and G. Lippi (2011). "Mid-stream vs. first-voided urine collection by using automated analyzers for particle examination in healthy subjects: an Italian multicenter study." Clin Chem Lab Med **50**(4): 679-684.

Mata Forsberg, M., S. Bjorkander, Y. Pang, L. Lundqvist, M. Ndi, M. Ott, I. B. Escriba, M. C. Jaeger, S. Roos and E. Sverre-remark-Ekstrom (2019). "Extracellular Membrane Vesicles from Lactobacilli Dampen IFN-gamma Responses in a Monocyte-Dependent Manner." Sci Rep **9**(1): 17109.

Mateescu, B., E. J. Kowal, B. W. van Balkom, S. Bartel, S. N. Bhattacharyya, E. I. Buzas, A. H. Buck, P. de Candia, F. W. Chow, S. Das, T. A. Driedonks, L. Fernandez-Messina, F. Haderk, A. F. Hill, J. C. Jones, K. R. Van Keuren-Jensen, C. P. Lai, C. Lasser, I. D. Liegro, T. R. Lunavat, M. J. Lorenowicz, S. L. Maas, I. Mager, M. Mittelbrunn, S. Momma, K. Mukherjee, M. Nawaz, D. M. Pegtel, M. W. Pfaffl, R. M. Schiffelers, H. Tahara, C. Thery, J. P. Tosar, M. H. Wauben, K. W. Witwer and E. N. Nolte-'t Hoen (2017). "Obstacles and opportunities in the functional analysis of extracellular vesicle RNA - an ISEV position paper." J Extracell Vesicles **6**(1): 1286095.

McKiernan, J., M. J. Donovan, E. Margolis, A. Partin, B. Carter, G. Brown, P. Torkler, M. Noerholm, J. Skog, N. Shore, G. Andriole, I. Thompson and P. Carroll (2018). "A Prospective Adaptive Utility Trial to Validate Performance of a Novel Urine Exosome Gene Expression Assay to Predict High-grade Prostate Cancer in Patients with Prostate-specific Antigen 2-10ng/ml at Initial Biopsy." Eur Urol.

McKiernan, J., M. J. Donovan, V. O'Neill, S. Bentink, M. Noerholm, S. Belzer, J. Skog, M. W. Kattan, A. Partin, G. Andriole, G. Brown, J. T. Wei, I. M. Thompson, Jr. and P. Carroll (2016). "A Novel Urine Exosome Gene Expression Assay to Predict High-grade Prostate Cancer at Initial Biopsy." JAMA Oncol **2**(7): 882-889.

McNicholas, K., J. Y. Li, M. Z. Michael and J. M. Gleadle (2017). "Albuminuria is not associated with elevated urinary vesicle concentration but can confound nanoparticle tracking analysis." Nephrology (Carlton) **22**(11): 854-863.

Merchant, M. L., I. M. Rood, J. K. J. Deegens and J. B. Klein (2017). "Isolation and characterization of urinary extracellular vesicles: implications for biomarker discovery." Nat Rev Nephrol **13**(12): 731-749.

Micanovic, R., S. Khan, D. Janosevic, M. E. Lee, T. Hato, E. F. Srour, S. Winfree, J. Ghosh, Y. Tong, S. E. Rice, P. C. Dagher, X. R. Wu and T. M. El-Achkar (2018). "Tamm-Horsfall Protein Regulates Mononuclear Phagocytes in the Kidney." J Am Soc Nephrol **29**(3): 841-856.

Minciacchi, V. R., A. Zijlstra, M. A. Rubin and D. Di Vizio (2017). "Extracellular vesicles for liquid biopsy in prostate cancer: where are we and where are we headed?" Prostate Cancer Prostatic Dis **20**(3): 251-258.

Miranda, K. C., D. T. Bond, J. Z. Levin, X. Adiconis, A. Sivachenko, C. Russ, D. Brown, C. Nusbaum and L. M. Russo (2014). "Massively parallel sequencing of human urinary exosome/microvesicle RNA reveals a predominance of non-coding RNA." PLoS One **9**(5): e96094.

Miranda, K. C., D. T. Bond, M. McKee, J. Skog, T. G. Paunescu, S. N. Da, D. Brown and L. M. Russo (2010). "Nucleic acids within urinary exosomes/microvesicles are potential biomarkers for renal disease." Kidney Int **78**(2): 191-199.

Mitchell, P. J., J. Welton, J. Staffurth, J. Court, M. D. Mason, Z. Tabi and A. Clayton (2009). "Can urinary exosomes act as treatment response markers in prostate cancer?" J. Transl. Med **7**: 4.

Moatamed, N. A. (2019). "Biobanking of Urine Samples." Methods Mol Biol **1897**: 115-124.

Molina, L., N. Salvetat, R. B. Ameer, S. Peres, N. Sommerer, F. Jarraya, H. Ayadi, F. Molina and C. Granier (2011). "Analysis of the variability of human normal urine by 2D-GE reveals a "public" and a "private" proteome." J Proteomics **75**(1): 70-80.

Momen-Heravi, F., L. Balaj, S. Alian, A. Trachtenberg, F. Hochberg, J. Skog and W. Kuo (2012). "Impact of Biofluid Viscosity on Size and Sedimentation Efficiency of the Isolated Microvesicles." Frontiers in Physiology **3**(162).

Monguio-Tortajada, M., M. Moron-Font, A. Gamez-Valero, L. Carreras-Planella, F. E. Borrás and M. Franquesa (2019). "Extracellular-Vesicle Isolation from Different Biological Fluids by Size-Exclusion Chromatography." Curr Protoc Stem Cell Biol **49**(1): e82.

Moore, H. M., A. Kelly, S. D. Jewell, L. M. McShane, D. P. Clark, R. Greenspan, P. Hainaut, D. F. Hayes, P. Kim, E. Mansfield, O. Potapova, P. Riegman, Y. Rubinstein, E. Seijo, S. Somiari, P. Watson, H. U. Weier, C. Zhu and J. Vaught (2011). "Biospecimen Reporting for Improved Study Quality." Biopreserv Biobank **9**(1): 57-70.

Morikawa, Y., N. Takahashi, K. Kamiyama, K. Nishimori, Y. Nishikawa, S. Morita, M. Kobayashi, S. Fukushima, S. Yokoi, D. Mikami, H. Kimura, K. Kasuno, T. Yashiki, H. Naiki, M. Hara and M. Iwano (2018). "Elevated Levels of Urinary Extracellular Vesicle Fibroblast-Specific Protein 1 in Patients with Active Crescentic Glomerulonephritis." Nephron: 1-11.

Munkonda, M. N., S. Akbari, C. Landry, S. Sun, F. Xiao, M. Turner, C. E. Holterman, R. Nasrallah, R. L. Hebert, C. R. J. Kennedy and D. Burger (2018). "Podocyte-derived microparticles promote proximal tubule fibrotic signaling via p38 MAPK and CD36." J Extracell Vesicles **7**(1): 1432206.

Murakami, T., M. Oakes, M. Ogura, V. Tovar, C. Yamamoto and M. Mitsuhashi (2014). "Development of glomerulus-, tubule-, and collecting duct-specific mRNA assay in human urinary exosomes and microvesicles." PLoS One **9**(9): e109074.



Musante, L., S. V. Bontha, S. La Salvia, A. Fernandez-Pineros, J. Lannigan, T. H. Le, V. Mas and U. Erdbrugger (2020). "Rigorous characterization of urinary extracellular vesicles (uEVs) in the low centrifugation pellet - a neglected source for uEVs." Sci Rep **10**(1): 3701.

Musante, L., M. Saraswat, E. Duriez, B. Byrne, A. Ravida, B. Domon and H. Holthofer (2012). "Biochemical and Physical Characterisation of Urinary Nanovesicles following CHAPS Treatment." Plos One **7**(7).

Musante, L., M. Saraswat, A. Ravida, B. Byrne and H. Holthofer (2013). "Recovery of urinary nanovesicles from ultracentrifugation supernatants." Nephrology Dialysis Transplantation **28**(6): 1425-1433.

Musante, L., D. Tataruch, D. Gu, X. Liu, C. Forsblom, P. H. Groop and H. Holthofer (2015). "Proteases and protease inhibitors of urinary extracellular vesicles in diabetic nephropathy." J Diabetes Res **2015**: 289734.

Musante, L., D. Tataruch, D. F. Gu, A. Benito-Martin, G. Calzaferri, S. Aherne and H. Holthofer (2014). "A Simplified Method to Recover Urinary Vesicles for Clinical Applications, and Sample Banking." Scientific Reports **4**.

Musante, L., D. Tataruch-Weinert, D. Kerjaschki, M. Henry, P. Meleady and H. Holthofer (2017). "Residual urinary extracellular vesicles in ultracentrifugation supernatants after hydrostatic filtration dialysis enrichment." J Extracell Vesicles **6**(1): 1267896.

Mussack, V., G. Wittmann and M. W. Pfaffl (2019). "Comparing small urinary extracellular vesicle purification methods with a view to RNA sequencing-Enabling robust and non-invasive biomarker research." Biomol Detect Quantif **17**: 100089.

Nagaraj, N. and M. Mann (2011). "Quantitative analysis of the intra- and inter-individual variability of the normal urinary proteome." J Proteome Res **10**(2): 637-645.

Nanni, U., F. Betsou, S. Riondino, L. Rossetti, A. Spila, M. G. Valente, D. Della-Morte, R. Palmirotta, M. Roselli, P. Ferroni and F. Guadagni (2012). "SPRECware: software tools for Standard PREanalytical Code (SPREC) labeling - effective exchange and search of stored biospecimens." Int J Biol Markers **27**(3): e272-279.

Nazarenko, I. (2020). "Extracellular Vesicles: Recent Developments in Technology and Perspectives for Cancer Liquid Biopsy." Recent Results Cancer Res **215**: 319-344.

Ndisang, J. F. (2018). "Glomerular Endothelium and its Impact on Glomerular Filtration Barrier in Diabetes: Are the Gaps Still Illusive?" Curr Med Chem **25**(13): 1525-1529.

Nilsson, J., J. Skog, A. Nordstrand, V. Baranov, L. Mincheva-Nilsson, X. O. Breakefield and A. Widmark (2009). "Prostate cancer-derived urine exosomes: a novel approach to biomarkers for prostate cancer." Br. J. Cancer **100**(10): 1603-1607.

Nisell, H., M. Trygg and R. Bäck (2006). "Urine albumin/creatinine ratio for the assessment of albuminuria in pregnancy hypertension." Acta Obstet Gynecol Scand **85**(11): 1327-1330.

Nolte-'t Hoen, E., T. Cremer, R. C. Gallo and L. B. Margolis (2016). "Extracellular vesicles and viruses: Are they close relatives?" Proc Natl Acad Sci U S A **113**(33): 9155-9161.

Oeyen, E., K. Van Mol, G. Baggerman, H. Willems, K. Boonen, C. Rolfo, P. Pauwels, A. Jacobs, K. Schildermans, W. C. Cho and I. Mertens (2018). "Ultrafiltration and size exclusion chromatography combined with asymmetrical-flow field-flow fractionation for the isolation and characterisation of extracellular vesicles from urine." J Extracell Vesicles **7**(1): 1490143.

Oeyen, E., H. Willems, R. t Kindt, K. Sandra, K. Boonen, L. Hoekx, S. De Wachter, F. Ameye and I. Mertens (2019). "Determination of variability due to biological and technical variation in urinary extracellular vesicles as a crucial step in biomarker discovery studies." J Extracell Vesicles **8**(1): 1676035.

Olinger, E., J. Lake, S. Sheehan, G. Schiano, T. Takata, N. Tokonami, H. Debaix, F. Consolato, L. Rampoldi, R. Korstanje and O. Devuyt (2019). "Hepsin-mediated Processing of Uromodulin is Crucial for Salt-sensitivity and Thick Ascending Limb Homeostasis." Scientific Reports **9**(1): 12287.

Oosthuyzen, W., K. M. Scullion, J. R. Ivy, E. E. Morrison, R. W. Hunter, P. J. Starkey Lewis, E. O'Duibhir, J. M. Street, A. Caporali, C. D. Gregory, S. J. Forbes, D. J. Webb, M. A. Bailey and J. W. Dear (2016). "Vasopressin Regulates Extracellular Vesicle Uptake by Kidney Collecting Duct Cells." J Am Soc Nephrol.

Oosthuyzen, W., N. E. Sime, J. R. Ivy, E. J. Turtle, J. M. Street, J. Pound, L. E. Bath, D. J. Webb, C. D. Gregory, M. A. Bailey and J. W. Dear (2013). "Quantification of human urinary exosomes by nanoparticle tracking analysis." J Physiol **591**(23): 5833-5842.

Øverbye, A., T. Skotland, C. J. Koehler, B. Thiede, T. Seierstad, V. Berge, K. Sandvig and A. Llorente (2015). "Identification of prostate cancer biomarkers in urinary exosomes." Oncotarget **6**(30): 30357-30376.

Paisrisarn, P., T. Yasui and Y. Baba (2020). "A Method to Analyze Urinary Extracellular Vesicles." Anal Sci **36**(7): 791-798.

Palanisamy, V., S. Sharma, A. Deshpande, H. Zhou, J. Gimzewski and D. T. Wong (2010). "Nanostructural and transcriptomic analyses of human saliva derived exosomes." PLoS One **5**(1): e8577.

Palomo, L., E. Casal, F. Royo, D. Cabrera, S. van-Liempd and J. M. Falcon-Perez (2014). "Considerations for applying metabolomics to the analysis of extracellular vesicles." Front Immunol **5**: 651.

Palviainen, M., M. Saraswat, Z. Varga, D. Kitka, M. Neuvonen, M. Puhka, S. Joenvaara, R. Renkonen, R. Nieuwland, M. Takatalo and P. R. M. Siljander (2020). "Extracellular vesicles from human plasma and serum are carriers of extravesicular cargo-Implications for biomarker discovery." PLoS One **15**(8): e0236439.

Park, S., K. Lee, I. B. Park, N. H. Kim, S. Cho, W. J. Rhee, Y. Oh, J. Choi, S. Nam and D. H. Lee (2020). "The profiles of microRNAs from urinary extracellular vesicles (EVs) prepared by various isolation methods and their correlation with serum EV microRNAs." Diabetes Res Clin Pract **160**: 108010.

Parolini, I., C. Federici, C. Raggi, L. Lugini, S. Palleschi, M. A. De, C. Coscia, E. Iessi, M. Logozzi, A. Molinari, M. Colone, M. Tatti, M. Sargiacomo and S. Fais (2009). "Microenvironmental pH is a key factor for exosome traffic in tumor cells." J. Biol. Chem **284**(49): 34211-34222.

Pascual, M., G. Steiger, S. Sadallah, J. P. Paccaud, J. L. Carpentier, R. James and J. A. Schifferli (1994). "Identification of membrane-bound CR1 (CD35) in human urine: evidence for its release by glomerular podocytes." J Exp Med **179**(3): 889-899.

Patrakka, J., A. T. Lahdenkari, O. Koskimies, C. Holmberg, J. Wartiovaara and H. Jalanko (2002). "The number of podocyte slit diaphragms is decreased in minimal change nephrotic syndrome." Pediatr Res **52**(3): 349-355.

Peinado, H., M. Aleckovic, S. Lavotshkin, I. Matei, B. Costa-Silva, G. Moreno-Bueno, M. Hergueta-Redondo, C. Williams, G. Garcia-Santos, C. Ghajar, A. Nitadori-Hoshino, C. Hoffman, K. Badal, B. A. Garcia, M. K. Callahan, J. Yuan, V. R. Martins, J. Skog, R. N. Kaplan, M. S. Brady, J. D. Wolchok, P. B. Chapman, Y. Kang, J. Bromberg and D. Lyden (2012). "Melanoma exosomes educate bone marrow progenitor cells toward a pro-metastatic phenotype through MET." Nat Med **18**(6): 883-891.

Pisitkun, T., R. F. Shen and M. A. Knepper (2004). "Identification and proteomic profiling of exosomes in human urine." Proc Natl Acad Sci U S A **101**(36): 13368-13373.

Ploussard, G. and A. de la Taille (2010). "Urine biomarkers in prostate cancer." Nat Rev Urol **7**(2): 101-109.

Prunotto, M., A. Farina, L. Lane, A. Pernin, J. Schifferli, D. F. Hochstrasser, P. Lescuyer and S. Moll (2013). "Proteomic analysis of podocyte exosome-enriched fraction from normal human urine." J Proteomics **82**: 193-229.

Puhka, M., M. E. Nordberg, S. Valkonen, A. Rannikko, O. Kallioniemi, P. Siljander and T. M. Af Hallstrom (2017). "KeepEX, a simple dilution protocol for improving extracellular vesicle yields from urine." Eur J Pharm Sci **98**: 30-39.

Puhka, M., M. Takatalo, M. E. Nordberg, S. Valkonen, J. Nandania, M. Aatonen, M. Yliperttula, S. Laitinen, V. Velagapudi, T. Mirtti, O. Kallioniemi, A. Rannikko, P. R. Siljander and T. M. Af Hallstrom (2017). "Metabolomic Profiling of Extracellular Vesicles and Alternative Normalization Methods

Reveal Enriched Metabolites and Strategies to Study Prostate Cancer-Related Changes." *Theranostics* **7**(16): 3824-3841.

Qi, Y., X. Wang, K. L. Rose, W. H. MacDonald, B. Zhang, K. L. Schey and J. M. Luther (2016). "Activation of the Endogenous Renin-Angiotensin-Aldosterone System or Aldosterone Administration Increases Urinary Exosomal Sodium Channel Excretion." *J Am Soc Nephrol* **27**(2): 646-656.

Rabinovitch, A., MD, PhD, L. Arzoumanian, MS, MT(CLS, NCA), K. M. Curcio, MT(ASCP), B. Dougherty and A. B. Halim, PharmD, PhD (2009). *Urinalysis; Approved Guideline. GP16-A3. 950 West Valley Road, Suite 2500, Wayne, PA 19087 USA, SLCI Clinical and Laboratory Standards Institute. 29.*

Raimondo, F., D. Cerra, F. Magni and M. Pitto (2016). "Urinary proteomics for the study of genetic kidney diseases." *Expert Rev Proteomics* **13**(3): 309-324.

Raimondo, F., C. Chinello, L. Porcaro, F. Magni and M. Pitto (2020). "Urinary Extracellular Vesicles and Salt-Losing Tubulopathies: A Proteomic Approach." *Proteomes* **8**(2).

Raimondo, F., C. Chinello, M. Stella, L. Santorelli, F. Magni and M. Pitto (2018). "Effects of Hematuria on the Proteomic Profile of Urinary Extracellular Vesicles: Technical Challenges." *J Proteome Res* **17**(8): 2572-2580.

Raimondo, F., L. Morosi, S. Corbetta, C. Chinello, P. Brambilla, P. Della Mina, A. Villa, G. Albo, C. Battaglia, S. Bosari, F. Magni and M. Pitto (2013). "Differential protein profiling of renal cell carcinoma urinary exosomes." *Mol Biosyst* **9**(6): 1220-1233.

Ransohoff, D. F. and M. L. Gourlay (2010). "Sources of bias in specimens for research about molecular markers for cancer." *J Clin Oncol* **28**(4): 698-704.

Rao, M. S., T. R. Van Vleet, R. Ciurlionis, W. R. Buck, S. W. Mittelstadt, E. A. G. Blomme and M. J. Liguori (2018). "Comparison of RNA-Seq and Microarray Gene Expression Platforms for the Toxicogenomic Evaluation of Liver From Short-Term Rat Toxicity Studies." *Front Genet* **9**: 636.

Ratajczak, J., K. Miekus, M. Kucia, J. Zhang, R. Reza, P. Dvorak and M. Z. Ratajczak (2006). "Embryonic stem cell-derived microvesicles reprogram hematopoietic progenitors: evidence for horizontal transfer of mRNA and protein delivery." *Leukemia* **20**(5): 847-856.

Rayyan, M., A. Zheutlin and J. B. Byrd (2018). "Clinical research using extracellular vesicles: insights from the International Society for Extracellular Vesicles 2018 Annual Meeting." *J Extracell Vesicles* **7**(1): 1535744.

Rigau, M., M. Olivan, M. Garcia, T. Sequeiros, M. Montes, E. Colas, M. Llaurodo, J. Planas, I. Torres, J. Morote, C. Cooper, J. Reventos, J. Clark and A. Doll (2013). "The present and future of prostate cancer urine biomarkers." *Int J Mol Sci* **14**(6): 12620-12649.

Rikkert, L. G., L. de Rond, A. van Dam, T. G. van Leeuwen, F. A. W. Coumans, T. M. de Reijke, L. Terstappen and R. Nieuwland (2020). "Detection of extracellular vesicles in plasma and urine of prostate cancer patients by flow cytometry and surface plasmon resonance imaging." PLoS One **15**(6): e0233443.

Rindler, M. J., S. S. Naik, N. Li, T. C. Hoops and M. N. Peraldi (1990). "Uromodulin (Tamm-Horsfall glycoprotein/uromucoid) is a phosphatidylinositol-linked membrane protein." J Biol Chem **265**(34): 20784-20789.

Rodriguez, M., C. Bajo-Santos, N. P. Hessvik, S. Lorenz, B. Fromm, V. Berge, K. Sandvig, A. Line and A. Llorente (2017). "Identification of non-invasive miRNAs biomarkers for prostate cancer by deep sequencing analysis of urinary exosomes." Mol Cancer **16**(1): 156.

Rood, I. M., J. K. Deegens, M. L. Merchant, W. P. Tamboer, D. W. Wilkey, J. F. Wetzels and J. B. Klein (2010). "Comparison of three methods for isolation of urinary microvesicles to identify biomarkers of nephrotic syndrome." Kidney Int **78**(8): 810-816.

Royo, F., I. Diwan, M. R. Tackett, P. Zuniga, P. Sanchez-Mosquera, A. Loizaga-Iriarte, A. Ugalde-Olano, I. Lacasa, A. Perez, M. Unda, A. Carracedo and J. M. Falcon-Perez (2016). "Comparative miRNA Analysis of Urine Extracellular Vesicles Isolated through Five Different Methods." Cancers **8**(12): E112.

Royo, F., D. Gil-Carion, E. Gonzalez, J. Mleczko, L. Palomo, M. Perez-Cormenzana, R. Mayo, C. Alonso and J. M. Falcon-Perez (2019). "Differences in the metabolite composition and mechanical properties of extracellular vesicles secreted by hepatic cellular models." J Extracell Vesicles **8**(1): 1575678.

Royo, F., P. Zuniga-Garcia, P. Sanchez-Mosquera, A. Egia, A. Perez, A. Loizaga, R. Arceo, I. Lacasa, A. Rabade, E. Arrieta, R. Bilbao, M. Unda, A. Carracedo and J. M. Falcon-Perez (2016). "Different EV enrichment methods suitable for clinical settings yield different subpopulations of urinary extracellular vesicles from human samples." J Extracell Vesicles **5**: 29497.

Royo, F., P. Zuniga-Garcia, V. Torrano, A. Loizaga, P. Sanchez-Mosquera, A. Ugalde-Olano, E. Gonzalez, A. R. Cortazar, L. Palomo, S. Fernandez-Ruiz, I. Lacasa-Viscasillas, M. Berdasco, J. D. Sutherland, R. Barrio, A. Zabala-Letona, N. Martin-Martin, A. Arruabarrena-Aristorena, L. Valcarcel-Jimenez, A. Caro-Maldonado, J. Gonzalez-Tampan, G. Cachi-Fuentes, M. Esteller, A. M. Aransay, M. Unda, J. M. Falcon-Perez and A. Carracedo (2016). "Transcriptomic profiling of urine extracellular vesicles reveals alterations of CDH3 in prostate cancer." Oncotarget **7**(6): 6835-6846.

Saetun, P., T. Semangoen and V. Thongboonkerd (2009). "Characterizations of urinary sediments precipitated after freezing and their effects on urinary protein and chemical analyses." Am J Physiol Renal Physiol **296**(6): F1346-1354.

Saliba, D. G., P. F. Cespedes-Donoso, S. Balint, E. B. Compeer, K. Korobchevskaya, S. Valvo, V. Mayya, A. Kvalvaag, Y. Peng, T. Dong, M. L. Tognoli, E. O'Neill, S. Bonham, R. Fischer, B. M. Kessler and M. L.

Dustin (2019). "Composition and structure of synaptic ectosomes exporting antigen receptor linked to functional CD40 ligand from helper T cells." Elife **8**.

Salih, M., D. M. Bovee, N. van der Lubbe, A. H. J. Danser, R. Zietse, R. A. Feelders and E. J. Hoorn (2018). "Increased Urinary Extracellular Vesicle Sodium Transporters in Cushing Syndrome With Hypertension." J Clin Endocrinol Metab **103**(7): 2583-2591.

Salih, M., J. A. Demmers, K. Bezstarosti, W. N. Leonhard, M. Losekoot, C. van Kooten, R. T. Gansevoort, D. J. Peters, R. Zietse, E. J. Hoorn and D. Consortium (2016). "Proteomics of Urinary Vesicles Links Plakins and Complement to Polycystic Kidney Disease." J Am Soc Nephrol **27**(10): 3079-3092.

Salih, M., R. A. Fenton, J. Knipscheer, J. W. Janssen, M. S. Vredenburg-van den Berg, G. Jenster, R. Zietse and E. J. Hoorn (2016). "An immunoassay for urinary extracellular vesicles." Am J Physiol Renal Physiol **310**(8): F796-F801.

Samsonov, R., T. Shtam, V. Burdakov, A. Glotov, E. Tsyrlina, L. Berstein, A. Nosov, V. Evtushenko, M. Filatov and A. Malek (2016). "Lectin-induced agglutination method of urinary exosomes isolation followed by mi-RNA analysis: Application for prostate cancer diagnostic." Prostate **76**(1): 68-79.

Santiago-Rodriguez, T. M., M. Ly, N. Bonilla and D. T. Pride (2015). "The human urine virome in association with urinary tract infections." Front Microbiol **6**: 14.

Santucci, L., M. Bruschi, G. Del Zotto, F. Antonini, G. M. Ghiggeri, I. Panfoli and G. Candiano (2019). "Biological surface properties in extracellular vesicles and their effect on cargo proteins." Scientific Reports **9**(1): 13048.

Santucci, L., G. Candiano, A. Petretto, M. Bruschi, C. Lavarello, E. Inglese, P. G. Righetti and G. M. Ghiggeri (2015). "From hundreds to thousands: Widening the normal human Urinome (1)." J Proteomics **112**: 53-62.

Saraswat, M., S. Joenvaara, L. Musante, H. Peltoniemi, H. Holthofer and R. Renkonen (2015). "N-linked (N-) glycoproteomics of urinary exosomes. [Corrected]." Mol Cell Proteomics **14**(2): 263-276.

Scherberich, J. E. (1989). "Immunological and ultrastructural analysis of loss of tubular membrane-bound enzymes in patients with renal damage." Clin Chim Acta **185**(3): 271-282.

Sequeiros, T., M. Rigau, C. Chiva, M. Montes, I. Garcia-Grau, M. Garcia, S. Diaz, A. Celma, I. Bijnsdorp, A. Campos, P. Di Mauro, S. Borros, J. Reventos, A. Doll, R. Paciucci, M. Pegtel, I. de Torres, E. Sabido, J. Morote and M. Olivan (2017). "Targeted proteomics in urinary extracellular vesicles identifies biomarkers for diagnosis and prognosis of prostate cancer." Oncotarget **8**(3): 4960-4976.

Shoskes, D. A., K. D. Thomas and E. Gomez (2005). "Anti-nanobacterial therapy for men with chronic prostatitis/chronic pelvic pain syndrome and prostatic stones: preliminary experience." J Urol **173**(2): 474-477.

Silvers, C. R., Y. R. Liu, C. H. Wu, H. Miyamoto, E. M. Messing and Y. F. Lee (2016). "Identification of extracellular vesicle-borne periostin as a feature of muscle-invasive bladder cancer." Oncotarget **7**(17): 23335-23345.

Simeone, P., G. Bologna, P. Lanuti, L. Pierdomenico, M. T. Guagnano, D. Pieragostino, P. Del Boccio, D. Vergara, M. Marchisio, S. Miscia and R. Mariani-Costantini (2020). "Extracellular Vesicles as Signaling Mediators and Disease Biomarkers across Biological Barriers." Int J Mol Sci **21**(7).

Skotland, T., K. Ekroos, D. Kauhanen, H. Simolin, T. Seierstad, V. Berge, K. Sandvig and A. Llorente (2017). "Molecular lipid species in urinary exosomes as potential prostate cancer biomarkers." Eur. J. Cancer **70**: 122-132.

Skotland, T., N. P. Hessvik, K. Sandvig and A. Llorente (2019). "Exosomal lipid composition and the role of ether lipids and phosphoinositides in exosome biology." J Lipid Res **60**(1): 9-18.

Skotland, T., K. Sagini, K. Sandvig and A. Llorente (2020). "An emerging focus on lipids in extracellular vesicles." Adv Drug Deliv Rev.

Smith, J. T., B. H. Wunsch, N. Dogra, M. E. Ahsen, K. Lee, K. K. Yadav, R. Weil, M. A. Pereira, J. V. Patel, E. A. Duch, J. M. Papalia, M. F. Lofaro, M. Gupta, A. K. Tewari, C. Cordon-Cardo, G. Stolovitzky and S. M. Gifford (2018). "Integrated nanoscale deterministic lateral displacement arrays for separation of extracellular vesicles from clinically-relevant volumes of biological samples." Lab Chip **18**(24): 3913-3925.

Soekmadji, C., B. Li, Y. Huang, H. Wang, T. An, C. Liu, W. Pan, J. Chen, L. Cheung, J. M. Falcon-Perez, Y. S. Gho, H. B. Holthofer, M. T. N. Le, A. Marcilla, L. O'Driscoll, F. Shekari, T. L. Shen, A. C. Torrecilhas, X. Yan, F. Yang, H. Yin, Y. Xiao, Z. Zhao, X. Zou, Q. Wang and L. Zheng (2020). "The future of Extracellular Vesicles as Theranostics - an ISEV meeting report." J Extracell Vesicles **9**(1): 1809766.

Sole, C., T. Moline, M. Vidal, J. Ordi-Ros and J. Cortes-Hernandez (2019). "An Exosomal Urinary miRNA Signature for Early Diagnosis of Renal Fibrosis in Lupus Nephritis." Cells **8**(8).

Sonoda, H., N. Yokota-Ikeda, S. Oshikawa, Y. Kanno, K. Yoshinaga, K. Uchida, Y. Ueda, K. Kimiya, S. Uezono, A. Ueda, K. Ito and M. Ikeda (2009). "Decreased abundance of urinary exosomal aquaporin-1 in renal ischemia-reperfusion injury." Am J Physiol Renal Physiol **297**(4): F1006-1016.

Srinivasan, S., A. Yeri, P. S. Cheah, A. Chung, K. Danielson, P. De Hoff, J. Filant, C. D. Laurent, L. D. Laurent, R. Magee, C. Moeller, V. L. Murthy, P. Nejad, A. Paul, I. Rigoutsos, R. Rodosthenous, R. V. Shah, B. Simonson, C. To, D. Wong, I. K. Yan, X. Zhang, L. Balaj, X. O. Breakefield, G. Daaboul, R. Gandhi, J. Lapidus, E. Londin, T. Patel, R. L. Raffai, A. K. Sood, R. P. Alexander, S. Das and L. C. Laurent (2019). "Small RNA Sequencing across Diverse Biofluids Identifies Optimal Methods for exRNA Isolation." Cell **177**(2): 446-462 e416.

Stokman, M. F., I. V. Bijnsdorp, T. Schelfhorst, T. V. Pham, S. R. Piersma, J. C. Knol, R. H. Giles, E. Bongers, N. Knoers, M. R. Lilien, C. R. Jimenez and K. Y. Renkema (2019). "Changes in the urinary

extracellular vesicle proteome are associated with nephronophthisis-related ciliopathies." J Proteomics **192**: 27-36.

Street, J. M., W. Birkhoff, R. I. Menzies, D. J. Webb, M. A. Bailey and J. W. Dear (2011). "Exosomal transmission of functional aquaporin 2 in kidney cortical collecting duct cells." J Physiol **589**(Pt 24): 6119-6127.

Street, J. M., E. H. Koritzinsky, D. M. Glispie, R. A. Star and P. S. Yuen (2017). "Urine Exosomes: An Emerging Trove of Biomarkers." Adv Clin Chem **78**: 103-122.

Suarez, H., A. Gamez-Valero, R. Reyes, S. Lopez-Martin, M. J. Rodriguez, J. L. Carrascosa, C. Cabanas, F. E. Borrás and M. Yanez-Mo (2017). "A bead-assisted flow cytometry method for the semi-quantitative analysis of Extracellular Vesicles." Sci Rep **7**(1): 11271.

Sun, A. L., J. T. Deng, G. J. Guan, S. H. Chen, Y. T. Liu, J. Cheng, Z. W. Li, X. H. Zhuang, F. D. Sun and H. P. Deng (2012). "Dipeptidyl peptidase-IV is a potential molecular biomarker in diabetic kidney disease." Diab Vasc Dis Res **9**(4): 301-308.

Svenningsen, P., R. Sabaratnam and B. L. Jensen (2020). "Urinary extracellular vesicles: Origin, role as intercellular messengers and biomarkers; efficient sorting and potential treatment options." Acta Physiol (Oxf) **228**(1): e13346.

Tangtanatakul, P., S. Klinchanhom, P. Sodsai, T. Sutichet, C. Promjeen, Y. Avihingsanon and N. Hirankarn (2018). "Down-regulation of let-7a and miR-21 in urine exosomes from lupus nephritis patients during disease flare." Asian Pac J Allergy Immunol.

Tataruch-Weinert, D., L. Musante, O. Kretz and H. Holthofer (2016). "Urinary extracellular vesicles for RNA extraction: optimization of a protocol devoid of prokaryote contamination." J Extracell Vesicles **5**: 30281.

Thery, C., K. W. Witwer, E. Aikawa, M. J. Alcaraz, J. D. Anderson, R. Andriantsitohaina, A. Antoniou, T. Arab, F. Archer, G. K. Atkin-Smith, D. C. Ayre, J. M. Bach, D. Bachurski, H. Baharvand, L. Balaj, S. Baldacchino, N. N. Bauer, A. A. Baxter, M. Bebawy, C. Beckham, A. Bedina Zavec, A. Benmoussa, A. C. Berardi, P. Bergese, E. Bielska, C. Blenkinsop, S. Bobis-Wozowicz, E. Boilard, W. Boireau, A. Bongiovanni, F. E. Borrás, S. Bosch, C. M. Boulanger, X. Breakefield, A. M. Breglio, M. A. Brennan, D. R. Brigstock, A. Brisson, M. L. Broekman, J. F. Bromberg, P. Bryl-Gorecka, S. Buch, A. H. Buck, D. Burger, S. Busatto, D. Buschmann, B. Bussolati, E. I. Buzas, J. B. Byrd, G. Camussi, D. R. Carter, S. Caruso, L. W. Chamley, Y. T. Chang, C. Chen, S. Chen, L. Cheng, A. R. Chin, A. Clayton, S. P. Clerici, A. Cocks, E. Cocucci, R. J. Coffey, A. Cordeiro-da-Silva, Y. Couch, F. A. Coumans, B. Coyle, R. Crescitelli, M. F. Criado, C. D'Souza-Schorey, S. Das, A. Datta Chaudhuri, P. de Candia, E. F. De Santana, O. De Wever, H. A. Del Portillo, T. Demaret, S. Deville, A. Devitt, B. Dhondt, D. Di Vizio, L. C. Dieterich, V. Dolo, A. P. Dominguez Rubio, M. Dominici, M. R. Dourado, T. A. Driedonks, F. V. Duarte, H. M. Duncan, R. M. Eichenberger, K. Ekstrom, S. El Andaloussi, C. Elie-Caille, U. Erdbrugger, J. M. Falcon-Perez, F. Fatima, J. E. Fish, M. Flores-Bellver, A. Forsonits, A. Frelet-Barrand, F. Fricke, G. Fuhrmann, S. Gabrielsson, A. Gamez-Valero, C. Gardiner, K. Gartner, R. Gaudin, Y. S. Gho, B. Giebel, C. Gilbert,



M. Gimona, I. Giusti, D. C. Goberdhan, A. Gorgens, S. M. Gorski, D. W. Greening, J. C. Gross, A. Gualerzi, G. N. Gupta, D. Gustafson, A. Handberg, R. A. Haraszti, P. Harrison, H. Hegyesi, A. Hendrix, A. F. Hill, F. H. Hochberg, K. F. Hoffmann, B. Holder, H. Holthofer, B. Hosseinkhani, G. Hu, Y. Huang, V. Huber, S. Hunt, A. G. Ibrahim, T. Ikezu, J. M. Inal, M. Isin, A. Ivanova, H. K. Jackson, S. Jacobsen, S. M. Jay, M. Jayachandran, G. Jenster, L. Jiang, S. M. Johnson, J. C. Jones, A. Jong, T. Jovanovic-Talisman, S. Jung, R. Kalluri, S. I. Kano, S. Kaur, Y. Kawamura, E. T. Keller, D. Khamari, E. Khomyakova, A. Khvorova, P. Kierulf, K. P. Kim, T. Kislinger, M. Klingeborn, D. J. Klinke, 2nd, M. Kornek, M. M. Kosanovic, A. F. Kovacs, E. M. Kramer-Albers, S. Krasemann, M. Krause, I. V. Kurochkin, G. D. Kusuma, S. Kuypers, S. Laitinen, S. M. Langevin, L. R. Languino, J. Lannigan, C. Lasser, L. C. Laurent, G. Lavieu, E. Lazaro-Ibanez, S. Le Lay, M. S. Lee, Y. X. F. Lee, D. S. Lemos, M. Lenassi, A. Leszczynska, I. T. Li, K. Liao, S. F. Libregts, E. Ligeti, R. Lim, S. K. Lim, A. Line, K. Linnemannstons, A. Llorente, C. A. Lombard, M. J. Lorenowicz, A. M. Lorincz, J. Lotvall, J. Lovett, M. C. Lowry, X. Loyer, Q. Lu, B. Lukomska, T. R. Lunavat, S. L. Maas, H. Malhi, A. Marcilla, J. Mariani, J. Mariscal, E. S. Martens-Uzunova, L. Martin-Jaular, M. C. Martinez, V. R. Martins, M. Mathieu, S. Mathivanan, M. Maugeri, L. K. McGinnis, M. J. McVey, D. G. Meckes, Jr., K. L. Meehan, I. Mertens, V. R. Minciocchi, A. Moller, M. Moller Jorgensen, A. Morales-Kastresana, J. Morhayim, F. Mullier, M. Muraca, L. Musante, V. Mussack, D. C. Muth, K. H. Myburgh, T. Najrana, M. Nawaz, I. Nazarenko, P. Nejsun, C. Neri, T. Neri, R. Nieuwland, L. Nimrichter, J. P. Nolan, E. N. Nolte-'t Hoen, N. Noren Hooten, L. O'Driscoll, T. O'Grady, A. O'Loghlen, T. Ochiya, M. Olivier, A. Ortiz, L. A. Ortiz, X. Osteikoetxea, O. Ostergaard, M. Ostrowski, J. Park, D. M. Pegtel, H. Peinado, F. Perut, M. W. Pfaffl, D. G. Phinney, B. C. Pieters, R. C. Pink, D. S. Pisetsky, E. Pogge von Strandmann, I. Polakovicova, I. K. Poon, B. H. Powell, I. Prada, L. Pulliam, P. Quesenberry, A. Radeghieri, R. L. Raffai, S. Raimondo, J. Rak, M. I. Ramirez, G. Raposo, M. S. Rayyan, N. Regev-Rudski, F. L. Ricklefs, P. D. Robbins, D. D. Roberts, S. C. Rodrigues, E. Rohde, S. Rome, K. M. Rouschop, A. Rughetti, A. E. Russell, P. Saa, S. Sahoo, E. Salas-Huenuleo, C. Sanchez, J. A. Saugstad, M. J. Saul, R. M. Schiffelers, R. Schneider, T. H. Schoyen, A. Scott, E. Shahaj, S. Sharma, O. Shatnyeva, F. Shekari, G. V. Shelke, A. K. Shetty, K. Shiba, P. R. Siljander, A. M. Silva, A. Skowronek, O. L. Snyder, 2nd, R. P. Soares, B. W. Sodar, C. Soekmadji, J. Sotillo, P. D. Stahl, W. Stoorvogel, S. L. Stott, E. F. Strasser, S. Swift, H. Tahara, M. Tewari, K. Timms, S. Tiwari, R. Tixeira, M. Tkach, W. S. Toh, R. Tomasini, A. C. Torrecilhas, J. P. Tosar, V. Toxavidis, L. Urbanelli, P. Vader, B. W. van Balkom, S. G. van der Grein, J. Van Deun, M. J. van Herwijnen, K. Van Keuren-Jensen, G. van Niel, M. E. van Royen, A. J. van Wijnen, M. H. Vasconcelos, I. J. Vechetti, Jr., T. D. Veit, L. J. Vella, E. Velot, F. J. Verweij, B. Vestad, J. L. Vinas, T. Visnovitz, K. V. Vukman, J. Wahlgren, D. C. Watson, M. H. Wauben, A. Weaver, J. P. Webber, V. Weber, A. M. Wehman, D. J. Weiss, J. A. Welsh, S. Wendt, A. M. Wheelock, Z. Wiener, L. Witte, J. Wolfram, A. Xagorari, P. Xander, J. Xu, X. Yan, M. Yanez-Mo, H. Yin, Y. Yuana, V. Zappulli, J. Zarubova, V. Zekas, J. Y. Zhang, Z. Zhao, L. Zheng, A. R. Zheutlin, A. M. Zickler, P. Zimmermann, A. M. Zivkovic, D. Zocco and E. K. Zuba-Surma (2018). "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* **7**(1): 1535750.

Thomas, C. E., W. Sexton, K. Benson, R. Sutphen and J. Koomen (2010). "Urine collection and processing for protein biomarker discovery and quantification." *Cancer Epidemiol Biomarkers Prev* **19**(4): 953-959.

Thomas, M. E., C. Blaine, A. Dawnay, M. A. Devonald, S. Ftouh, C. Laing, S. Latchem, A. Lewington, D. V. Milford and M. Ostermann (2015). "The definition of acute kidney injury and its use in practice." Kidney Int **87**(1): 62-73.

Thomas, S., L. Hao, W. A. Ricke and L. Li (2016). "Biomarker discovery in mass spectrometry-based urinary proteomics." Proteomics Clin Appl **10**(4): 358-370.

Thongboonkerd, V. and P. Saetun (2007). "Bacterial overgrowth affects urinary proteome analysis: recommendation for centrifugation, temperature, duration, and the use of preservatives during sample collection." J Proteome Res **6**(11): 4173-4181.

Tian, Y., M. Gong, Y. Hu, H. Liu, W. Zhang, M. Zhang, X. Hu, D. Aubert, S. Zhu, L. Wu and X. Yan (2020). "Quality and efficiency assessment of six extracellular vesicle isolation methods by nano-flow cytometry." J Extracell Vesicles **9**(1): 1697028.

Tiphara, P. and V. Thongboonkerd (2016). "Differential human urinary lipid profiles using various lipid-extraction protocols: MALDI-TOF and LIFT-TOF/TOF analyses." Sci Rep **6**: 33756.

Trilok, G. and H. H. Draper (1989). "Effect of a high protein intake on acid-base balance in adult rats." Calcif Tissue Int **44**(5): 339-342.

Trilok, G. and H. H. Draper (1989). "Sources of protein-induced endogenous acid production and excretion by human adults." Calcif Tissue Int **44**(5): 335-338.

Turco, A. E., W. Lam, A. D. Rule, A. Denic, J. C. Lieske, V. M. Miller, J. J. Larson, W. K. Kremers and M. Jayachandran (2016). "Specific renal parenchymal-derived urinary extracellular vesicles identify age-associated structural changes in living donor kidneys." J Extracell Vesicles **5**: 29642.

Valadi, H., K. Ekstrom, A. Bossios, M. Sjostrand, J. J. Lee and J. O. Lotvall (2007). "Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells." Nat. Cell Biol **9**(6): 654-659.

van der Lubbe, N., P. M. Jansen, M. Salih, R. A. Fenton, A. H. van den Meiracker, A. H. Danser, R. Zietse and E. J. Hoorn (2012). "The phosphorylated sodium chloride cotransporter in urinary exosomes is superior to prostaticin as a marker for aldosteronism." Hypertension **60**(3): 741-748.

van Dongen, H. M., N. Masoumi, K. W. Witwer and D. M. Pegtel (2016). "Extracellular Vesicles Exploit Viral Entry Routes for Cargo Delivery." Microbiol Mol Biol Rev **80**(2): 369-386.

van Niel, G., G. D'Angelo and G. Raposo (2018). "Shedding light on the cell biology of extracellular vesicles." Nat Rev Mol Cell Biol **19**(4): 213-228.

Vergauwen, G., B. Dhondt, J. Van Deun, E. De Smedt, G. Berx, E. Timmerman, K. Gevaert, I. Miinalainen, V. Cocquyt, G. Braems, R. Van den Broecke, H. Denys, O. De Wever and A. Hendrix

(2017). "Confounding factors of ultrafiltration and protein analysis in extracellular vesicle research." Sci Rep **7**(1): 2704.

Veziroglu, E. M. and G. I. Mias (2020). "Characterizing Extracellular Vesicles and Their Diverse RNA Contents." Front Genet **11**: 700.

Wachalska, M., D. Koppers-Lalic, M. van Eijndhoven, M. Pegtel, A. A. Geldof, A. D. Lipinska, R. J. van Moorselaar and I. V. Bijnsdorp (2016). "Protein Complexes in Urine Interfere with Extracellular Vesicle Biomarker Studies." J Circ Biomark **5**: 4.

Waikar, S. S., V. S. Sabbiseti and J. V. Bonventre (2010). "Normalization of urinary biomarkers to creatinine during changes in glomerular filtration rate." Kidney Int **78**(5): 486-494.

Wang, D. and W. Sun (2014). "Urinary extracellular microvesicles: isolation methods and prospects for urinary proteome." Proteomics **14**(16): 1922-1932.

Wang, J. and M. M. Barr (2016). "Ciliary Extracellular Vesicles: Txt Msg Organelles." Cell Mol Neurobiol **36**(3): 449-457.

Wang, S., K. Kojima, J. A. Mobley and A. B. West (2019). "Proteomic analysis of urinary extracellular vesicles reveal biomarkers for neurologic disease." EBioMedicine **45**: 351-361.

Wang, Y. T., T. Shi, S. Srivastava, J. Kagan, T. Liu and K. D. Rodland (2020). "Proteomic Analysis of Exosomes for Discovery of Protein Biomarkers for Prostate and Bladder Cancer." Cancers (Basel) **12**(9).

Welsh, J. A., E. Van Der Pol, G. J. A. Arksteijn, M. Bremer, A. Brisson, F. Coumans, F. Dignat-George, E. Duggan, I. Ghiran, B. Giebel, A. Gorgens, A. Hendrix, R. Lacroix, J. Lannigan, S. Libregts, E. Lozano-Andres, A. Morales-Kastresana, S. Robert, L. De Rond, T. Tertel, J. Tigges, O. De Wever, X. Yan, R. Nieuwland, M. H. M. Wauben, J. P. Nolan and J. C. Jones (2020). "MIFlowCyt-EV: a framework for standardized reporting of extracellular vesicle flow cytometry experiments." J Extracell Vesicles **9**(1): 1713526.

Welton, J. L., P. Brennan, M. Gurney, J. P. Webber, L. K. Spary, D. G. Carton, J. M. Falcon-Perez, S. P. Walton, M. D. Mason, Z. Tabi and A. Clayton (2016). "Proteomics analysis of vesicles isolated from plasma and urine of prostate cancer patients using a multiplex, aptamer-based protein array." J Extracell Vesicles **5**: 31209.

Welton, J. L., S. Khanna, P. J. Giles, P. Brennan, I. A. Brewis, J. Staffurth, M. D. Mason and A. Clayton (2010). "Proteomic analysis of bladder cancer exosomes." Mol. Cell Proteomics **9**(6): 1324-1338.

Wiggins, R. C., A. Glatfelter, B. Kshirsagar and J. Brukman (1986). "Procoagulant activity in normal human urine associated with subcellular particles." Kidney Int **29**(2): 591-597.

Wiklander, O. P. B., R. B. Bostancioglu, J. A. Welsh, A. M. Zickler, F. Murke, G. Corso, U. Feldin, D. W. Hagey, B. Evertsson, X. M. Liang, M. O. Gustafsson, D. K. Mohammad, C. Wiek, H. Hanenberg, M. Bremer, D. Gupta, M. Bjornstedt, B. Giebel, J. Z. Nordin, J. C. Jones, S. El Andaloussi and A. Gorgens (2018). "Systematic Methodological Evaluation of a Multiplex Bead-Based Flow Cytometry Assay for Detection of Extracellular Vesicle Surface Signatures." Front Immunol **9**: 1326.

Williams, C., M. Palviainen, N. C. Reichardt, P. R. Siljander and J. M. Falcon-Perez (2019). "Metabolomics Applied to the Study of Extracellular Vesicles." Metabolites **9**(11).

Williams, C., R. Pazos, F. Royo, E. Gonzalez, M. Roura-Ferrer, A. Martinez, J. Gamiz, N. C. Reichardt and J. M. Falcon-Perez (2019). "Assessing the role of surface glycans of extracellular vesicles on cellular uptake." Sci Rep **9**(1): 11920.

Williams, C., F. Royo, O. Aizpurua-Olaizola, R. Pazos, G. J. Boons, N. C. Reichardt and J. M. Falcon-Perez (2018). "Glycosylation of extracellular vesicles: current knowledge, tools and clinical perspectives." J Extracell Vesicles **7**(1): 1442985.

Williams, T. L., C. Bastos, N. Faria and F. E. Karet Frankl (2020). "Making urinary extracellular vesicles a clinically tractable source of biomarkers for inherited tubulopathies using a small volume precipitation method: proof of concept." J Nephrol **33**(2): 383-386.

Witwer, K. W., E. I. Buzas, L. T. Bemis, A. Bora, C. Lasser, J. Lotvall, E. N. Nolte-'t Hoen, M. G. Piper, S. Sivaraman, J. Skog, C. Thery, M. H. Wauben and F. Hochberg (2013). "Standardization of sample collection, isolation and analysis methods in extracellular vesicle research." J Extracell Vesicles **2**.

Wolley, M. J., A. Wu, S. Xu, R. D. Gordon, R. A. Fenton and M. Stowasser (2017). "In Primary Aldosteronism, Mineralocorticoids Influence Exosomal Sodium-Chloride Cotransporter Abundance." J Am Soc Nephrol **28**(1): 56-63.

Woo, H. K., V. Sunkara, J. Park, T. H. Kim, J. R. Han, C. J. Kim, H. I. Choi, Y. K. Kim and Y. K. Cho (2017). "Exodisc for Rapid, Size-Selective, and Efficient Isolation and Analysis of Nanoscale Extracellular Vesicles from Biological Samples." ACS Nano **11**(2): 1360-1370.

Wu, Z., G. I. Bagarolo, S. Thoree-Boveleth and J. Jankowski (2020). ""Lipidomics": Mass spectrometric and chemometric analyses of lipids." Adv Drug Deliv Rev.

Xu, X., K. Barreiro, L. Musante, O. Kretz, H. Lin, H. Zou, T. B. Huber and H. Holthofer (2019). "Management of Tamm-Horsfall Protein for Reliable Urinary Analytics." Proteomics Clin Appl **13**(6): e1900018.

Yaghobee, S., M. Bayani, Samiel and N. Jahedmanesh (2015). "What are nanobacteria?" Journal Biotechnology & biotechnological Equipment(26): 826-833

Yamamoto, C. M., T. Murakami, M. L. Oakes, M. Mitsuhashi, C. Kelly, R. R. Henry and K. Sharma (2018). "Uromodulin mRNA from Urinary Extracellular Vesicles Correlate to Kidney Function Decline in Type 2 Diabetes Mellitus." Am J Nephrol **47**(5): 283-291.

Yasui, T., T. Yanagida, S. Ito, Y. Konakade, D. Takeshita, T. Naganawa, K. Nagashima, T. Shimada, N. Kaji, Y. Nakamura, J. A. Thiodorus, Y. He, S. Rahong, M. Kanai, H. Yukawa, T. Ochiya, T. Kawai and Y. Baba (2017). "Unveiling massive numbers of cancer-related urinary-microRNA candidates via nanowires." Sci Adv **3**(12): e1701133.

Yoo, J. Y., M. Rho, Y. A. You, E. J. Kwon, M. H. Kim, S. Kym, Y. K. Jee, Y. K. Kim and Y. J. Kim (2016). "16S rRNA gene-based metagenomic analysis reveals differences in bacteria-derived extracellular vesicles in the urine of pregnant and non-pregnant women." Exp Mol Med **48**: e208.

Yuana, Y., R. M. Bertina and S. Osanto (2011). "Pre-analytical and analytical issues in the analysis of blood microparticles." Thromb Haemost **105**(3): 396-408.

Yuana, Y., A. N. Boing, A. E. Grootemaat, E. van der Pol, C. M. Hau, P. Cizmar, E. Buhr, A. Sturk and R. Nieuwland (2015). "Handling and storage of human body fluids for analysis of extracellular vesicles." J Extracell Vesicles **4**: 29260.

Zaichick, V. (2014). "The prostatic urethra as a Venturi effect urine-jet pump to drain prostatic fluid." Med Hypotheses **83**(1): 65-68.

Zhan, Y., L. T. Du, L. S. Wang, X. M. Jiang, S. J. Zhang, J. Li, K. Q. Yan, W. L. Duan, Y. H. Zhao, L. L. Wang, Y. S. Wang and C. X. Wang (2018). "Expression signatures of exosomal long non-coding RNAs in urine serve as novel non-invasive biomarkers for diagnosis and recurrence prediction of bladder cancer." Molecular Cancer **17**.

Zhao, Y., K. Chen, H. Li and H. Wu (2017). "Effect of pH on the isolation of urinary exosome." Int Urol Nephrol **49**(1): 165-169.

Zhou, H., P. S. Yuen, T. Pisitkun, P. A. Gonzales, H. Yasuda, J. W. Dear, P. Gross, M. A. Knepper and R. A. Star (2006). "Collection, storage, preservation, and normalization of human urinary exosomes for biomarker discovery." Kidney Int **69**(8): 1471-1476.

Zhu, C., L. Li, Z. Wang, M. Irfan and F. Qu (2020). "Recent advances of aptasensors for exosomes detection." Biosens Bioelectron **160**: 112213.

Zubiri, I., M. Posada-Ayala, A. Sanz-Maroto, E. Calvo, M. Martin-Lorenzo, L. Gonzalez-Calero, F. de la Cuesta, J. A. Lopez, B. Fernandez-Fernandez, A. Ortiz, F. Vivanco and G. Alvarez-Llamas (2014). "Diabetic nephropathy induces changes in the proteome of human urinary exosomes as revealed by label-free comparative analysis." J Proteomics **96**: 92-102.

Zullig, T. and H. C. Kofeler (2020). "High Resolution Mass Spectrometry in Lipidomics." Mass Spectrom Rev.