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Urinary vesicles: in splendid isolation

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Urine provides an attractive, non-invasive alternative to tissue, blood or other biofluid as a potential source of biomarkers of systemic and renal disease. The urinary protein content can be divided in two parts: soluble and solid-phase components; the solid-phase components can be subdivided into sediment precipitated with low-speed centrifugation and low-density nanometer-sized particles (vesicles <100 nm in diameter) precipitated by ultracentrifugation. In normal human adult urine, 48% of the total urinary protein excreted is contained in the sediment, 49% is soluble and the remaining 3% is in urinary vesicles [1]. Pisitkun *et al.* [2] were the first to describe these vesicles in human urine and called them 'exosomes'; they went on to demonstrate the potential for exosomes as a starting material for biomarker discovery in urine. They chose the term exosomes because the vesicles originate from the membranes of internal multivesicular bodies that are released into the urine when the outer membrane of these bodies fuses with the apical plasma membrane of polarized epithelial cells [2].

Although the discovery of exosomes was intriguing, the question was why we should pay them any attention? Several recent studies have shown why exosomes are attractive for biomarker discovery. First, proteomics has shown that urinary exosomes are derived from all the epithelial cells lining the urinary tract, including glomerular podocytes and renal tubular cells from proximal and distal nephron segments [2]. The protein family commonly associated with exosomes is the tetraspanins, including CD9, CD63 and CD81. Second, other exosomal proteins directly represent the proteome of the source cells, for example, the presence of the sodium-potassium-chloride cotransporter type 2 (NKCC2), the sodium-chloride cotransporter (NCC) or the water channel aquaporin-2 (AQP2) reflects exosomes from the thick ascending limb of the loop of Henle, the distal convoluted and the collecting duct, respectively [2]. This property of exosomes is clinically useful as a biomarker not only of the nature of a renal disease, but also the site of renal injury [3]. For example, the abundance of the aldosterone-sensitive NCC was found to be higher in urinary exosomes of animal models of hyperaldosteronism and in patients with primary hyperaldosteronism [4]. Third, exosomes also contain transcription factors, messenger RNA (mRNA) and microRNA (miRNA), which have been shown to change in a disease [5–7]. Finally, the distal traffic of protein or RNA occurring after exosomes are released into the glomerular filtrate could potentially affect downstream cellular functions and be a novel mechanism of intra-renal signalling. Indeed, a recent study has demonstrated the ability of exosomes to mediate cell-to-cell communication along the nephron with functional AQP2 being transferred between cells [8].

The studies cited above provide a biological rationale for focussing more attention on urinary exosomes. However, as with many technical discoveries of this nature, there have been unresolved methodological problems. Since their initial discovery, the method of harvesting exosomes from various biological fluids, including urine, has relied on a two-step differential centrifugation process [2, 9, 10]. Although widely used, it is still uncertain whether differential centrifugation can isolate all urinary vesicles. This uncertainty was the basis of a recent and important study by Musante *et al.* [11] reported in the current issue of the journal. In their study, these investigators tried to optimize the process of vesicle isolation and yield, and, specifically, to define the properties of previously uncharacterized vesicles retained in the ultracentrifugation supernatant [11].

Using pooled second morning urines from six healthy volunteers, Musante *et al.* [11] set out to analyze whether urinary vesicles are still present in the original ultracentrifugation supernatant ('the crude fraction'). To do so, they subjected the ultracentrifugation supernatant to overnight ammonium sulfate precipitation. A 10 000 *g* centrifugation step was then used to generate supernatant and pellet. This second supernatant was subjected to two further centrifugation steps and the resulting pellet was analyzed ('the aqueous fraction'). The pellet obtained

from the 10 000 *g* centrifugation after ammonium sulfate treatment was dissolved separately in sodium hydroxide (NaOH), dialysed and centrifuged twice; the resulting pellet was then also analysed ('the NaOH fraction'). A simple protein quantification indicated that the crude fraction contained only 62% of the total protein content, while the aqueous and NaOH fractions accounted for 23 and 15%, respectively [11]. Indeed, transmission electron microscopy and dynamic light scattering identified membrane-encapsulated structures in the aqueous and NaOH fractions with a diameter consistent with exosomes (30–300 nm). Significantly, the mean size of the vesicles in the aqueous and NaOH fractions was different from that of the crude ultracentrifugation fraction, which suggested an enrichment of previously uncharacterized urinary vesicles still in solution after the initial centrifugation steps. Several exosomal markers were analysed and revealed that some protein isoforms were differentially expressed across the three different fractions. For example, an ∼55 kDa band of CD63, was only present in the aqueous and NaOH fractions, but not in the crude fraction. For the other proteins analysed (AQP2, actin, podocin and WT1), the main band was usually present in all fractions, but certain isoforms were absent in the aqueous and NaOH fractions. This was interpreted as further evidence of isolation of a specific subpopulation of urinary vesicles [11]. Thus, the authors showed unequivocally that appreciable amounts of nanovesicles can be recovered from the untreated 200 000 *g* supernatant. Although the authors also showed that some isoforms are only present in the aqueous and NaOH fractions, they did not identify proteins that were exclusive to the two treated fractions. Therefore, the question arises whether the protein isoforms identified only in the 200 000 *g* aqueous or NaOH fractions provide useful additional information that cannot be obtained from the 200 000 *g* crude pellet. According to the expression patterns of the proteins analysed in the three fractions (see Figure 5 of [11]), it seems that most isoforms were still captured in the crude ultracentrifugation pellet. The fact that isolation with ultracentrifugation alone is not complete or sufficient is only problematic if the aqueous or NaOH fractions contain biomarkers that are found exclusively in these fractions or that perform better in terms of sensitivity and specificity, which only time will tell. Furthermore, the identification of this subset of vesicles also raises the question of what determines the distribution among the crude, aqueous and NaOH fractions (62 versus 38% in this study). If this distribution changes randomly between different samples, depending, for example, on the concentration of the sample, this would require these additional isolation steps for complete urinary vesicle recovery. These questions remain for future methodological studies.

Three major steps are required for the development of urinary biomarker assays for routine application: the discovery of biomarker candidates, subsequent validation studies in larger groups of patients, and, finally, clinical testing and implementation [12]. In this respect, rigorous protocols facilitating the collection, processing and storage of urine samples are needed to ensure correct, comparable and reproducible urinary exosome analyses. Indeed, one of the conclusions of Musante *et al.* [11] is that 'Achieving a standard protocol for urinary exosomes is thus crucial to tap the full potential of urinary vesicles as new biomarkers'. While we agree that this is an important step in the wider clinical application of urinary exosomes, we also believe that the biological relevance of the subpopulations of urinary vesicles identified by Musante *et al.* needs to be clarified. In fact, if analysis of biomarkers in urinary vesicles were to reach the stage of routine clinical application, it would be more practicable if the ultracentrifugation step could be omitted. Alternatives to ultracentrifugation-based protocols, such as techniques using micro- and nano-ultrafiltration devices [13, 14], or techniques involving protein precipitation using the ExoQuick-TC reagent, have been described [15]. Recently, Alvarez *et al.* compared six different exosome isolation protocols: three were based on ultracentrifugation, one used a nanomembrane concentrator-based approach and two used a commercial exosome precipitation reagent. These authors observed that the modified technique using a commercial precipitation reagent, which has the advantage of requiring only routine laboratory equipment, is ideal for RNA profiling, because it yielded more and better quality exosomal miRNA and mRNA compared with the other methods, including those based on ultracentrifugation [15]. However, because it yielded lower quantities of exosomal proteins compared with ultracentrifugation-based methods, the authors concluded that ultracentrifugation is still a better option for exosome isolation. Therefore, particular isolation methods may be preferable, depending on whether RNA or protein is to be isolated. In addition to the optimal isolation method, other aspects of urinary vesicle preparation continue to be problematic (summarized in Table 1). The study by Musante *et al.* touches on three of these issues, including the use of protease inhibitors, the removal of Tamm–Horsfall glycoprotein (THP) and the normalization of samples.

The use of protease inhibitors is still a controversial point. Although some experimental results demonstrated the importance of using protease inhibitors, it is possible that they are not necessary for spot urine collections, which can be processed or frozen immediately (and may even interfere with some proteomic analyses) [12]. Using immunoblot analysis, protease inhibitors have been shown to preserve the integrity of NKCC2 compared with urine without protease inhibitors [16]. However, Mitchell *et al.* [17] have reported that exosomes do not degrade significantly in fresh urine, at least for the markers they tested. They concluded that exosomes can resist endogenous proteolytic activity of urine for at least 18 h at 37°C. However, at the same time, they did find lower exosome levels in their preparation, which was probably a consequence of proteolytic damage of exosomal constituents by endogenous urinary proteases [17]. Musante *et al.* [11] did not use protease inhibitors in their preparation, and despite some fragmentations in the proteins tumor suppressor gene 101 and podocin, they judged protease inhibition to be largely ineffective and unnecessary, since these proteins reside inside vesicles; moreover, they did not detect fragmentation of other exosomal markers characterized by an ectodomain (e.g. CD63, CD24 and AQP-2).

Another challenge in isolating urinary vesicles is to eliminate highly abundant urinary proteins that can form polymeric networks and entrap vesicles, mainly THP, also known as uromodulin [18, 19]. In the protocols developed by Pisitkun *et al.* [2]

Table 1. Some remaining challenges for biomarker discovery using urinary vesicles

Stage of the process	The challenges
Collection	• 24-h urine *versus* spot urine (first or second morning) • Still unclear whether protease inhibition is necessary
Processing	• Optimal isolation method still uncertain and may vary depending on the research question • Still unclear if successful isolation is possible without ultracentrifugation • Still unclear whether complete removal of Tamm–Horsfall protein (THP) is required
Storage	• Longer storage (≥1 month) likely to need −70 or −80°C, but for shorter storage 4 or −20°C may be sufficient
Analysis	• Still unclear what the best method of normalization (standardization) should be—time, urine creatinine, total protein, THP, exosomal markers or number of exosomes • Several options for protein analysis —mass spectrometry, immunoblot and enzyme-linked immunosorbent assay

and Zhou *et al.* [16], THP is still present in the ultracentrifugation pellet, although it can be reduced significantly using dithiothreitol [18, 19]. To improve the isolation of exosomes, sedimentation methods based on their buoyancy characteristics using a sucrose/D_2O gradient or cushion ultracentrifugation [19, 20] or immune-isolation using antibody-based derivatized Dynabeads, have proved effective [21, 22]. Both sucrose/D_2O gradient and cushion centrifugations provide better size-dependent separation of vesicles and are able to remove THP and other cytoplasmic filament contaminations in the exosome preparation [19, 20]. Combination of sucrose/D_2O cushion ultracentrifugation and antibody-coated bead immune-isolation also allows the collection of exosomes expressing specific proteins, such as tumour proteins [22]. In the study by Musante *et al.* [11], silver staining showed that in the NaOH fractions especially, THP was completely removed, indicating that this new isolation method gets rid of THP effectively.

The normalization between different samples prior to analysis remains something of a 'Holy Grail' in the field of urinary vesicles. Several normalization methods are available, including normalization based on the sample time (24-h versus random), urine creatinine, THP, total protein and exosomal markers considered to be stable. Recently, a nanoparticle quantization device called qNano has been introduced to analyse the actual number of exosomes in a sample, which might be another means of normalization [23]. For comparative analysis, Musante *et al.* recommend first to standardize the sample density by introducing dialysis against water, and using a molecular weight cut-off, and second to normalize protein concentration by dilution or concentration. Although this seems to make sense from an analytical standpoint, the question is whether these elaborate steps are essential for comparison across studies. A comparison between 24-h urine collections and random morning urine samples has shown, at least for glomerular proteinuria, that despite quantitative differences, the pattern of proteins excreted is similar [24]; Zhou *et al.* found no significant differences in urinary exosomes between the first (in which there would be some overnight carry-over of urine in the bladder) and second morning urine samples, but that protease inhibitors did reduce exosomal protein degradation and that sample storage should be at −80°C [16]. For THP, Uto *et al.* found no correlation in urine concentration in the same subject between a 24-h urine collection and morning urine sample, and that a fresh urine sample or one stored at −70°C is necessary for reliable estimation of THP concentration [25]. In a study by one of the co-authors of this *In Focus* article, normalization based on time (24-h urine), urine creatinine and total protein, as assessed by Coomassie staining, was similar [4]. However, it seems that more work will be needed to determine an agreed method of standardization.

In conclusion, Musante *et al.* report a new isolation method that effectively removes THP and reveals a hitherto uncharacterized population of urinary vesicles. Future studies should explore whether this subset of urinary vesicles contains biomarkers that cannot be isolated by ultracentrifugation alone. Studies such as this one are necessary to strike the right balance between reducing the complexity of urinary isolation methods and the inadvertent loss of potentially novel and valuable urinary biomarkers.

CONFLICT OF INTEREST STATEMENT

None declared.

(See related article by Musante *et al.* Recovery of urinary nanovesicles from ultracentrifugation supernatants. *Nephrol Dial Transplant* 2013; 28: 1425–1433.)

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