interpreted as an expensive learning experience, and future trials should be aimed at specific age ranges. The gauntlet has now been thrown down for other outcomes studies in nephrology, and in particular those relevant to mineral and bone disorders in patients with chronic kidney disease. This includes studies on vitamin D metabolites. For example, is there any real benefit to analogs of 1α,25-dihydroxyvitamin D over 1α,25(OH)-vitamin D itself or the prodrug 1α(OH)-vitamin D? This has never been studied and is an open question, and we can only hope that the challenges are taken up.

In summary, DCOR was an ambitious and well performed study. It really was essentially a negative study, as younger patients showed a tendency to benefit from calcium-based P binders and, in older patients, if there was really a benefit to sevelamer, it was not shown to be due to any change in cardiovascular mortality. So intuitively it may sound correct to give a non-calcium-based P binder, but we await DCOR II for a more definitive study to know whether our intuition is based on science or skilled marketing.

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Exosomes in urine: Who would have thought…?

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Normal urine contains thousands of proteins, largely due to the presence of 'exosomes,' tiny vesicles secreted into the urine by renal epithelial cells. These exosomes, demonstrated by Keller and colleagues to be also retrievable from amniotic fluid, offer great promise for future disease biomarker discovery studies.

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Who would have thought…? Who would have thought that urine could be so complex? For hundreds of years, physicians have analyzed urine in a variety of ways to discover what is wrong with their patients.[1] Most frequently, we divide samples into two components (sediments and supernatant) and look with interest at the sediments for the telltale crystal, cast, or cell that will give us a clue to what is happening upstream in the kidney. The supernatant, or a sample of whole urine, meanwhile is examined chemically for a few properties that can provide added clues — albumin? glucose? hemoglobin? — and skilled physicians succeed with these simple measures, even today, for the benefit of their patients. We have grown comfortable with the idea that urine is a very simple fluid.

But new developments have painted a different picture of normal urine, a picture of complexity, and the findings presented by Keller and colleagues[2] (this issue) have added additional important details.[2] The first signs of complexity were early reports from proteomics studies using sensitive mass spectrometers that normal urine contains hundreds of proteins (albeit at low concentrations), including integral membrane proteins.[3,4] Another sign was the discovery that urine contains solid-phase elements that remain in the supernatant at standard centrifugation speeds for most laboratory centrifuges, but which can be pelleted at much higher centrifugation speeds, that is, via ultracentrifugation. A major component of these high-speed sediments turned out to be 'exosomes,' tiny (40–80 nm) membranous structures secreted by epithelial cells[5] (Figure 1a). These urinary exosomes were demonstrated to contain abundant aquaporin-2, providing an explanation for the earlier unexplained finding that the water channel aquaporin-2, an integral membrane protein, was plentiful in urine.[6] It turns out that many of the myriad of proteins detected in urine by mass spectrometry in those early proteomics studies are there because of the presence of exosomes in normal urine.

Exosomes are membrane-bound vesicles that originate as the internal vesicles of multivesicular bodies (MVBs; Figure 1b) in various cell types and are released to the extracellular environment by fusion of the outer membrane of the MVBs with the plasma membrane. Exosomes were previously known to be produced by many cell types, including B lymphocytes and erythrocytes, from which they are delivered into the blood. The finding of exosomes in the urine (Figure 1a) opened up new possibilities for the diagnosis of kidney diseases. Pisitkun *et al.*[5] carried out tandem mass spectrometric profiling of proteins present in urinary exosomes from normal human subjects and found 295 distinct proteins, at least 22 of which had already been implicated in various kidney and systemic abnormalities. Subsequent studies using more sensitive mass spectrometric

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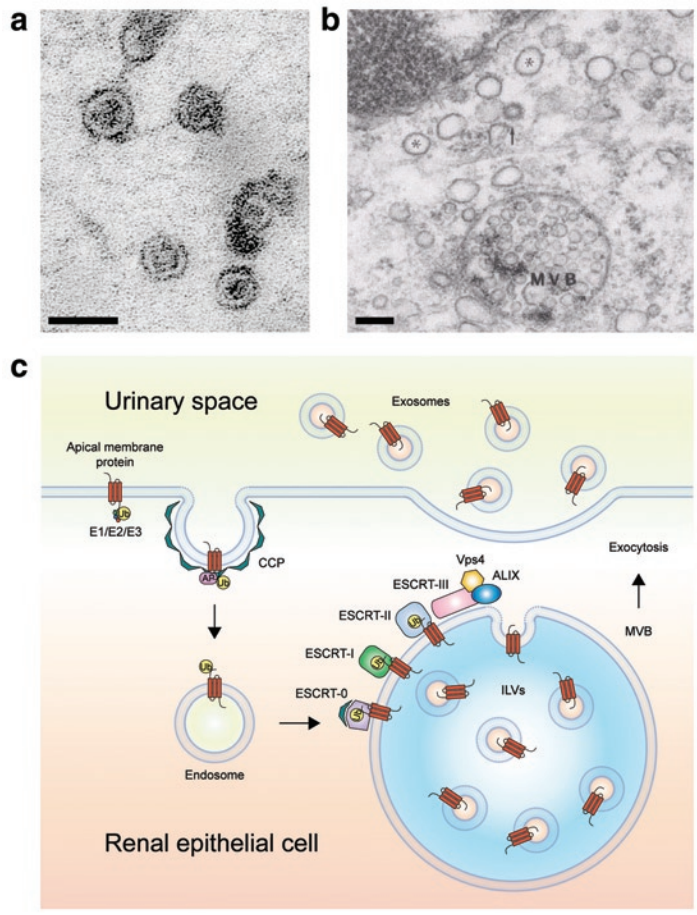

Figure 1 | Urinary exosome morphology and biogenesis. (**a**) Electron micrograph of negatively stained urinary exosomes (scale bar, 50 nm). (**b**) Electron micrograph of a renal inner medullary collecting duct cell (scale bar, 100 nm). Uncoated vesicles (asterisks) and coated vesicles (arrow) are indicated. MVB, multivesicular body. (**c**) Schematic of urinary exosome formation and release into the urine. E1, ubiquitin-activating enzyme; E2, ubiquitin-conjugating enzyme; E3, ubiquitin-protein ligase; AP, adaptor protein; Ub, ubiquitin; CCP, clathrin-coated pit (clathrin molecules are shown in green); ESCRT, endosomal sorting complex required for transport; Vps4, vacuolar protein sorting 4; ALIX, ALG-2 interacting protein X; ILVs, intraluminal vesicles.

methods have identified even more proteins (our unpublished data). The proteome of urinary exosomes includes proteins that are characteristic of every renal tubule epithelial cell type, as well as podocytes and transitional epithelia from the urinary collecting system. Not only do the exosomes contain membrane proteins, but their lumina contain cytosolic proteins trapped during the formation of MVBs. These findings open the door for the use of urinary exosomes in biomarker discovery experiments, aimed at revealing markers that could be used for low-cost screening for early detection of renal disease, for subclassification of renal diseases, and for therapeutic monitoring. With this objective in mind, we and others have been developing a technical infrastructure for efficient urinary exosome isolation, storage, and analysis.[7] Two web sites provide technical protocols (http://intramural.niddk.nih.gov/research/uroprot/) and a urinary exosome proteomics database (http://dir.nhlbi.nih.gov/papers/lkem/exosome/index.htm) for use by the general public.

The report by Keller and colleagues[2] (this issue) provides two significant advances. First, they have identified CD24 as an abundant protein present in urinary exosomes from humans and mice. CD24 is a small glycosyl phosphatidylinositol-anchored glycoprotein believed to be involved in cell–cell adhesion and signaling. Although abundant in urinary exosomes, CD24 was not discovered in earlier mass spectrometry–based profiling experiments, chiefly because of its small size and the lack of a profile of tryptic peptides of a size that would be favorable for identification by mass spectrometry. Second, Keller and colleagues[2] have demonstrated that exosomes are recoverable from amniotic fluid. It may seem self-evident that, if exosomes are present in urine, they must be present in amniotic fluid as well. However, the finding of exosomes in amniotic fluid provides a conceptual spark that may propel investigators toward studies in which amniotic fluid exosomes are used as a means of enriching markers of disease that can be used for prenatal diagnosis.

The process of exosome formation and release is illustrated in Figure 1c. Integral membrane proteins in the plasma membrane become mono- (or oligo-) ubiquitylated through the action of ubiquitin ligases such as Nedd4-2. The latter ubiquitylates the epithelial sodium channel (ENaC) in connecting tubule and collecting duct cells. The ubiquitylation marks the protein for endocytosis by creating a site for interaction with ubiquitin-binding clathrin adaptor proteins such as proteins in the epsin family.[8] The cargo in the resulting early endosomes is sorted into either recycling endosomes or late endosomes/MVBs. Those destined for the late-endosomal pathway bind to the external surfaces of MVBs by ubiquitin interaction with the ubiquitin receptor proteins HRS and Tsg101. The endosome fuses with the outer membrane of the MVB and invaginates, turning inside out as it becomes an internal vesicle of the MVB. The overall process of MVB recognition, fusion of the endosome with the MVB outer membrane, invagination, and fission of the intra-MVB vesicle is mediated by a series of protein complexes referred to as 'endosomal sorting complex required for transport,' or ESCRT. Specifically involved are newly defined ESCRT-0 (containing HRS), ESCRT-I (containing Tsg101), ESCRT-II, and ESCRT-III, as well as a protein called ALIX (also known as Bro1).[9] The intraluminal vesicles of the MVBs ('pro-exosomes') are then secreted from the cell when the outer membrane of the MVB fuses with the plasma membrane. Of note is the fact that the process of invagination from the outer membrane of the MVB causes sequestration of cytosolic proteins in the pro-exosome lumens. Thus, proteomic analysis of urinary exosomes

reveals not only an array of membrane proteins from the apical endosomal pathway of multiple cell types but a variety of entrained cytosolic proteins.[5] Not surprisingly, urinary exosomes also contain ALIX, ubiquitin, and all components of the ESCRT-I, ESCRT-II, and ESCRT-III complexes (our unpublished data). An interesting, important, and unanswered question is: What fraction of MVBs that form in renal epithelial cells are eliminated by this exocytic process versus the classical fate of late endosomes, namely, incorporation into lysosomes for degradation of their contents? Another important question is: What are the mechanisms by which MVBs translocate to the apical region of epithelial cells and fuse with the plasma membrane? Finally, are there undiscovered or unstudied disorders of exosome secretion that may shed light on their physiological roles, and are these abnormalities related to so-called disorders of lysosomal secretion such as Hermansky-Pudlak syndrome, Griscelli syndrome, and Chediak-Higashi syndrome?

Regarding the presence of exosomes in urine, it is natural to ask the 'why' question: Why would evolution select for a process that generates and excretes exosomes, as opposed to disposing of cellular waste materials through degradative processes? One answer is the '1950s-theory-of-waste-management' approach, which consisted of getting rid of wastes by dumping them into the nearest flowing stream. That is, it may be energetically efficient to dispose of waste molecules by saving them in MVBs and translocating the contents periodically to the urinary space. Although this theory lends itself to graphic metaphors, it may not be the right answer. Exosomes are very small structures, each with a very small radius of curvature (Figure 1a), which probably requires a significant amount of energy for formation. Therefore, it is not self-evident that excretion via exosomes is energy efficient. Consequently, it seems useful to look for other roles of exosomes beyond that of excretion of waste molecules. An intriguing idea was recently offered in a paper by Valadi *et al.*[10] These authors demonstrated that, in addition to proteins, exosomes isolated from a number of cell types contain a variety of mRNA and microRNA molecules. It was proposed that exosomes provide a means by which neighboring cells can influence one another's functions via transfer of these RNAs. This kind of model is of obvious interest in the area of developmental biology, where cell–cell interactions are critical for the orchestration of the differentiation of many tissues and cells in the appropriate sequence. Beyond this, if we assume that urinary exosomes also contain specific mRNAs and microRNAs, it is conceivable that day-to-day regulation of nephron function could be mediated in part through a regulated process of exosome secretion and reuptake downstream in other cell types, where their component mRNA and microRNA molecules would alter the overall structure or function of the recipient cells. Such a possibility deserves examination.

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Measuring the burden of illness for end-stage renal disease: some heavy lifting required

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Estimates for the burden of illness (BOI) attributable to end-stage renal disease (ESRD) in Canada are presented in the article by Zelmer. This Commentary describes the methodology of BOI analysis, its role in formulating public policy, and the potential application to improving care for ESRD.

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People value good health, as does society overall. For the individual, illness and disability diminish the ability to function while increasing dependence on others. Pain and distress impede the enjoyment of life. For society, impaired population health reduces productivity and decreases gross domestic product. Treatment of disease and its broader impact necessitates diversion of communal resources and increases tax burden. Individual suffering takes a toll on the national psyche.

Burden-of-illness (BOI) analysis involves methods for quantifying the societal impact of disease, disability, injury, and other forms of impaired health. Analysis of

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