1. Collection of unprocessed urine samples

Fresh urine samples (13 mL) were transferred into sterile tubes (Ref. 188271, Greiner Bio-One, Germany) and centrifuged at 1,915 × g, room temperature, for 10 min (ROTANTA 460, Hettich, The Netherlands) to remove cells and cell debris. The supernatant (12 mL) was transferred into volumes of 1 mL aliquots into vails (catalog no. 0030122201, Eppendorf, Germany) containing 40 µl (4% v/v) of a 25X concentrated protease inhibitor cocktail (Merck, Germany) according to the manufacturer's instructions and stored at -80 ºC. Dulbecco's phosphate-buffered saline (DPBS; Thermofisher Scientific; USA) was filtered by 0.2-µm-pore syringe filters (Corning Life Sciences; The Netherlands). The filtered PBS (fDPBS) was used as a negative control or buffer.

2. Measurement of total protein, creatinine, and pH of urine

Urinary total protein concentration was measured with TPUC3/CSF Gen. 3 (Roche Diagnostics Nederland B.V., The Netherlands), and urinary creatinine was measured with CRE2U, ACN 8152 (Roche Diagnostics Nederland B.V.). The pH values of urine were measured by a calibrated pH meter (HQ440d, HACH, The Netherlands).

3. Enzyme-linked immunosorbent assay of Tamm-Horsfall protein (THP)

Enzyme-linked immunosorbent Assay (ELISA) of THP was performed according to the manufacturer's instructions (EMELCA Bioscience, The Netherlands). Optical Density (O.D.) was read out at 450 nm using an ELISA reader (Wallac 1420 Victor2 Microplate Reader, LabMakelaar Benelux B.V., The Netherlands).

4. Depletion of THP filament by dithiothreitol (DTT)

DTT was used to depolymerize THP aggregates in urine. Solid DTT (Merck) was dissolved in fDPBS at 25 mg/mL or 200 mg/mL. Urine aliquots were thawed at 37 ºC for 10 minutes, and 500 µl of urine was centrifuged at 16,000 × g, room temperature, for 20 minutes (FrescoTM 17 Microcentrifuge, Thermofisher Scientific). The supernatant was kept and transferred into a new vial, and the pellet was

resuspended with 12.5 μL DTT solution at 37 ºC degrees for 15 minutes with a vortex each 5 min. After DTT incubation, the supernatant was remixed with the DTT-treated pellet (without washing).

5. Cell culture and collection of the cell supernatant

COLO-205 cells (colon cancer; CCL-221, ATCC, France) were grown in high-glucose Dulbecco's Modified Eagle Medium (DMEM; Thermofisher Scientific) supplemented with 10% fetal bovine serum (FBS; Thermofisher Scientific) and 1% Penicillin-Streptomycin (Thermofisher Scientific) at 37 ºC, 5% CO_2. When cell density reached 70%, the culture media were exchanged for high-glucose DMEM without FBS for 48 h. After this, the cell supernatant was harvested and collected in the same procedure used for urine collection.

6. Transmission electron microscopy (TEM)

A TEM protocol with negative staining was used to observe E.V.s as described previously.[1] The large-area images of the presented close-ups in the results (Figures 2A & 7A) can be found in Supplementary Figure S2. Negative staining is for direct visualization of E.V.s and other particles, like proteins, by TEM.[2] 10 μL of thawed KTR or H.C. urine sample was loaded on a Formvar/Carbon coated 400 Mesh Cu grid (Van Loenen Instruments, The Netherlands) for 10 minutes. Then, the excess urine was removed by filter paper. Uranyless EM stain (Electron Microscopy Sciences, U.K.) was applied for the negative staining of uEVs for 1 min. The excess staining reagent was removed with filter paper and natural air drying at room temperature. The visualization of uEVs was performed using the Talos L120C TEM (ThermoFisher Scientific) equipped with a 4 × 4 K CMOS camera at an operating voltage of 100 kV.

7. Nanoparticle tracking analysis (NTA)

Size distribution and concentration of nanoparticles in 1 mL of unprocessed, healthy urine were performed using a NanoSight NS300 (NanoSight, UK) with settings as previously reported,[3] and analyzed by software NTA 3.4 (NanoSight).

8. Time-resolved fluorescence immunoassay (TR-FIA)

TR-FIA experiments were performed as previously described.[4] CD9 and CD63, belonging to the tetraspanin family, are the two most abundant uEV markers and were selected to detect the uEVs in

TR-FIA. In brief, a streptavidin-coated 96-well plate (KaiSA96, Kaivogen, Finland) was incubated with biotinylated anti-CD9 (clone 209306, MAB1880, R&D Systems, UK) or anti-CD63 (clone 460305, MAB5048, R&D Systems) in triplicate for 1 hour while shaking (750 rpm) at room temperature. After washing with buffer solution (product number: 42-01, Kaivogen), 100 µL of the sample was added per well and incubated for 1 hour, shaking at room temperature. The plate was rewashed and incubated for 1 hour, shaking at room temperature with Europium (Eu) labeled anti-CD9 and anti-CD63 (customized, Perkin-Elmer, Finland) with the same clone as the capture antibodies. Using enhancement solution (Perkin-Elmer), the Eu-signal was enhanced, and fluorescence intensity of Eu was measured by the Wallac Victor 2, 1420 multilabel counter (Perkin-Elmer) at 615 nm.

Reference

1. Larios J, Mercier V, Roux A, Gruenberg J. ALIX- And ESCRT-III-dependent sorting of tetraspanins to exosomes. *J Cell Biol*. 2020;219(3). doi:10.1083/jcb.201904113

2. Harris JR, De Carlo S. Negative staining and cryo-negative staining: Applications in biology and medicine. *Methods Mol Biol*. 2014;1117:215-258. doi:10.1007/978-1-62703-776-1_11/FIGURES/24

3. Dimuccio V, Peruzzi L, Brizzi MF, et al. Acute and chronic glomerular damage is associated with reduced CD133 expression in urinary extracellular vesicles. *Am J Physiol - Ren Physiol*. 2020;312(2):F486-F495. doi:10.1152/AJPRENAL.00404.2019

4. Duijvesz D, Versluis CYL, Fels CAM van der M Van Der, et al. Immuno-based detection of extracellular vesicles in urine as diagnostic marker for prostate cancer. *Int J Cancer*. 2015;137(12):2869-2878. doi:10.1002/IJC.29664