Supplemental Material

Blijdorp and Tutakhel *et al.*, Comparing Approaches to Normalize, Quantify, and Characterize Urinary Extracellular Vesicles

Supplemental Table of Contents

Table S1. Characteristics of healthy subjects

	Age (years)	Sex (F/M)	Weight (kg)	BMI (kg/m^2)	Creatinine excretion* (mmol/day)
Healthy subjects subjected to water deprivation followed by water loading					
Subject 1[#]	46	M	80.8	25.8	N.A.
Subject 2[#]	27	M	92.6	27.7	N.A.
Subject 3[#]	40	M	91.8	27.4	N.A.
Subject 4	27	M	96.6	25.7	21.3
Subject 5	27	M	103.5	27.2	18.5
Subject 6	30	M	85.7	25.6	17.8
Subject 7	28	M	93.0	26.9	18.7
Subject 8	27	M	65.3	21.1	17.6
Subject 9	43	M	90.4	25.0	17.8
Subject 10	34	M	75.1	22.4	14.1
Subject 11	30	M	70.9	22.4	16.1
Average	33±7	M	86±12	25±2	18±2
Healthy subjects who provided random spot urine					
Subject 12	26	M	70	23.7	N.A.
Subject 13	29	M	89	26.6	N.A.
Subject 14	42	M	74	22.6	N.A.
Subject 15	28	M	76	23.5	N.A.
Subject 16	25	M	75	22.6	N.A.
Subject 17	31	M	63	19.7	N.A.
Subject 18	55	M	90	24.9	N.A.
Subject 19	29	M	70	24.2	N.A.
Subject 20	26	F	65	23.0	N.A.
Subject 21	27	F	69	23.9	N.A.
Subject 22	35	F	55	22.9	N.A.
Subject 23	30	F	75	25.6	N.A.
Subject 24	41	F	58	21.3	N.A.
Subject 25	26	F	74	23.1	N.A.
Subject 26	53	F	85	29.8	N.A.
Average	34±10	F: 47%	73±10	24±2	N.A.

N.A., not available

* Extrapolated from excretion in 12 hours

[#] These subjects participated both in the water loading study and as time-controls on a separate day.

Table S2. Characteristics of patients

	Age (years)	Sex (F/M)	Weight (kg)	BMI (kg/m^2)	Creatinine Excretion* (mmol/day)	eGFR (ml/min /1.73m^2)	ACR (spot urine) (mg/mol)
Patient 1	41	F	54.8	19.2	11.5	64	7.2
Patient 2	22	M	82.8	24.5	N.A.	103	0.6
Patient 3	58	F	77.0	25.4	12.5	20	0.9
Patient 4	58	M	90.0	29.7	11.6	18	176.3
Patient 5	49	F	72.0	25.5	11.0	86	5.8
Patient 6	55	F	94.0	32.5	9.4	37	0.5
Patient 7	43	M	86.5	23.0	17.4	73	9.7
Patient 8	23	F	74.0	25.6	11.7	107	6.6
Patient 9	54	F	90.0	31.5	10.6	20	7.6
Patient 10	39	M	107.0	30.3	15.6	103	4.5
Patient 11	54	M	98.3	26.7	14.6	82	0.0
Patient 12	43	F	67.1	24.6	12.2	75	1.5
Patient 13	53	M	91.5	26.4	20.0	60	2.0
Patient 14	36	F	65.0	22.0	13.3	107	7.1
Patient 15	58	M	105.0	27.3	26.9	66	0.6
Patient 16	64	M	78.0	24.6	19.7	34	2.0
Patient 17	57	F	68.0	25.6	8.1	66	0.6
Patient 18	54	F	78.6	26.0	11.3	62	0.8
Patient 19	34	F	79.6	30.7	13.0	87	1.9
Patient 20	24	F	86.7	28.0	11.3	120	0.4
Patient 21	43	F	60.9	23.8	9.8	101	0.7
Patient 22	36	F	71.2	26.8	10.3	86	3.3
Patient 23	54	M	74.0	24.2	12.6	58	13.9
Patient 24	51	M	90.7	24.6	19.9	73	14.2
Patient 25	45	F	64.0	21.6	9.2	90	1.1
Patient 26	60	M	90.0	26.6	15.8	16	9.2
Average	46±12	F: 58%	81±13	26±3	14±4	70±30	2 (0.7-7)

Abbreviations: ACR, albumin to creatinine ratio; BMI, body mass index; eGFR, estimated glomerular filtration rate according to CKD-EPI equation; N.A., not available.

Table S3. Antibodies

Used with	Antibody	Type	Species	Conc.	Source	Cat#/clone	Epitope
NTA	AQP2 (intracell)	Primary	Rabbit	1:1000	Millipore	178612	254-271
	AQP2 (extracell)	Primary	Rabbit	1:1000	Fenton	Rb323	178-191
	Rabbit, alexa488	Sec.	Goat	1:300	Thermo SC	A-11008	N.A.
EVQuant	CD9 alexa-647	Primary	Mouse	1:25	Thermo SC	MA5-18154	EC2
	CD63 alexa-488	Primary	Mouse	1:80	Santa Cruz	SC5275	N.A.
	AQP2 ATTO-488	Primary	Rabbit	1:100	Stressmarq	Spc-503	253–262
CD9-TRFIA	CD9-biotin	Capture	Mouse	1:500	Thermo SC	SN4 C3-3A2	N.A.
	CD9-Europium	Primary	Mouse	25ng/mL	CellGS	CGS12A	N.A.
IP	CD9-biotin	Capture	Mouse	1:50	Thermo SC	SN4 C3-3A2	N.A.
	CD63-biotin	Capture	Mouse	1:50	Biolegend	H5C6	C-term
Immunoblot	CD9	Primary	Mouse	1:500	R&D Syst	MAB1880	1-228
	CD63	Primary	Mouse	1:500	BD Biosc	556019	N.A.
	CD81	Primary	Mouse	1:500	Novus Biol	MAB4615	N.A.
	ALIX	Primary	Mouse	1:200	Santa Cruz	SC53540	N.A.
	TSG101	Primary	Mouse	1:333	Abcam	ab83	167-374
	AQP2	Primary	Rabbit	1:1000	Stressmarq	9398	253–262
	NHE3	Primary	Rabbit	1:1000	Stressmarq	H7644	621–640
	NaPi-IIa	Primary	Rabbit	1:500	Abcam	ab151129	15-97
	NKCC2	Primary	Rabbit	1:1000	Stressmarq	Spc-401D	33-55
	NCC	Primary	Rabbit	1:2000	Millipore	AB3553	N-term
	Mouse HRP	Sec.	Goat	1:3000	Biorad	L005680	N.A.
	Rabbit HRP	Sec.	Goat	1:3000	Biorad	L005679	N.A.
IHC/IF	CD9 (1)	Primary	Mouse	1:800	R&D Syst	MAB1880	1-228
	CD63 (1)	Primary	Mouse	1:500	BD Biosc	556019	N.A.
	WT-1	Primary	Mouse	3.7mg/L	Cell Marq	348M-9	N.A.
	Villin	Primary	Rabbit	1:500	Abcam	ab133510	650-750
	NKCC2	Primary	Rabbit	1:400	Stressmarq	Spc401D	33-55
	Parvalbumin	Primary	Rabbit	1:800	Swant	PV27	N.A.
	AQP2	Primary	Rabbit	1:4000	Stressmarq	9398	253-262
	CD9 (2)	Primary	Mouse	1:250	Novusbio	5G6	N.A.
	CD63 (2)	Primary	Mouse	1:250	Novusbio	MEM-259	N.A.
	CD81	Primary	Rabbit	1:400	Genetex	Gtx101766	Center

Abbreviations: Conc., concentration; IHC/IF, immunohistochemistry/immunofluorescence; IP, immunoprecipitation; N.A., not available; Sec., secondary.

Table S4. Differential ultracentrifugation steps

Centrifuge characteristics	Step 1	Step 2	Step 3	Step 4
Force, x g	2,000	17,000	17,000	200,000
Time, min	10	20	20	120
Temperature, °C	4	4	4	4
Rotor	Standard Hettich Rotanta	45 Ti	70.1 Ti	45 Ti
Fixed angle vs swing	Swing	Fixed	Fixed	Fixed
K factor	25000	1839	965.4	156.3
Tube	Falcon 50mL	#355655	#355603	#355655
Deceleration time	90 sec	Max (6 min)	Max (6 min)	Max (6 min)

All tubings and rotors are from Beck Coulter.

Table S5. Overview of statistical analyses

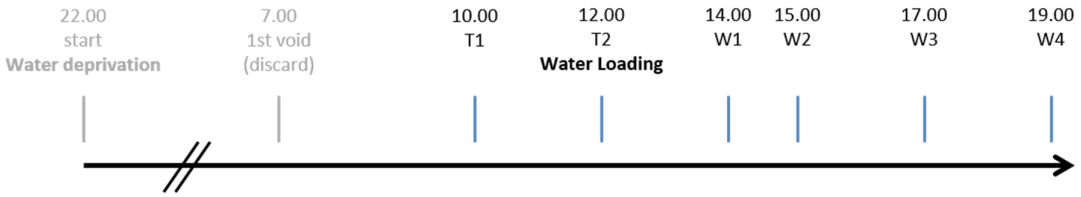
Figure	Statistical methods	Data distribution
Figure 1	Pearson correlation coefficient	Normal
Figure 2	Repeated measures ANOVA	Normal
Figure 3	Repeated measures ANOVA	Normal
Figure 4	Paired T-test (A)	Normal
	Repeated measures ANOVA (C-D)	Normal
	Mixed linear model (F-G)	Normal
Figure 5	Pearson correlation coefficient and Bland-Altman	Normal
Figure 8	ANOVA with post-hoc test (B, E, F)	Normal
	Repeated measures ANOVA (G)	Normal
	Paired T-test (H)	Normal

Table S6. Additional characteristics and urine biochemistries of the water loading experiment.

Variable	T1	T2	W1	W2	W3	W4	ANOVA
Additional characteristics							
Void deviation from schedule (min)	2±4	0±1	-2±4	-1±4	0±1	0±2	0.09
Process time (min)	62±7	55±7	58±6	57±7	52±10	65±7*	**0.001**
Time in bladder (hours)	3.06	1.97	1.96	1.02	2.02	1.99	-
Urine volume (mL)	105±24	76±27	713±188***	354±214***	248±130*	110±35	**<0.0001**
Urinary flow rate (ml/min)	0.6±0.2	0.6±0.2	6±2***	6±4***	2±0.9	1.0±0.3	**<0.0001**
Weight before time point (kg)	85±13	85±13	87±14***	86±14***	86±13*	85±13	**<0.0001**
Urine biochemistries							
Na^+ (µmol/min)	67±37	83±62	113±67	110±46	98±31	81±24	**0.03**
K^+ (µmol/min)	60±21	85±28	103±41	123±73**	78±32	56±14	**0.0005**
Cl^- (µmol/min)	88±34	113±41	113±38	115±36	96±23	82±19	**0.01**
$H_2PO_4^-$ (µmol/min)	11±5	10±3	15±4	13±6	21±8***	26±8***	**<0.0001**
Urea (µmol/min)	218±70	214±55	349±87***	335±88***	304±84**	247±56	**<0.0001**

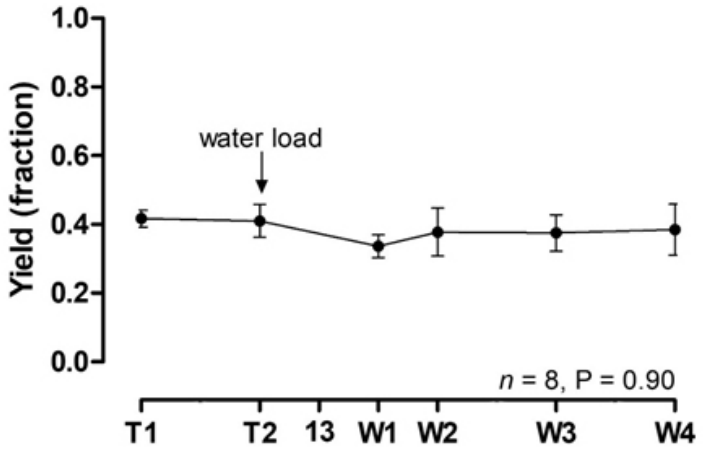
* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ *vs.* T2.

Figure S1. Schematic overview of water loading test



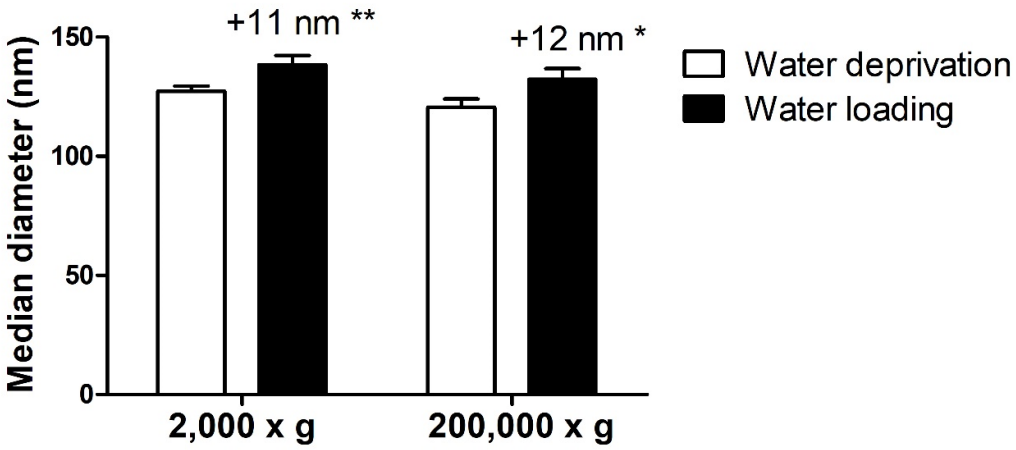
Schematic overview of the time points in the water loading test in healthy subjects. Water deprivation started at 10 p.m. the day before the test. The first urine void at 7.00 a.m. was discarded. T1-2 (urine voids at 10.00 a.m. and noon, respectively) are samples obtained during the water deprivation period, while W1-4 (urine voids at 2.00, 3.00, 5.00, and 7.00 p.m.) are samples obtained after water loading. Participants did not urinate between these time points. Water loading consisted of 20 mL/kg water within 30 minutes at noon, and was combined with a standardized meal.

Figure S2. Particle yield of differential ultracentrifugation



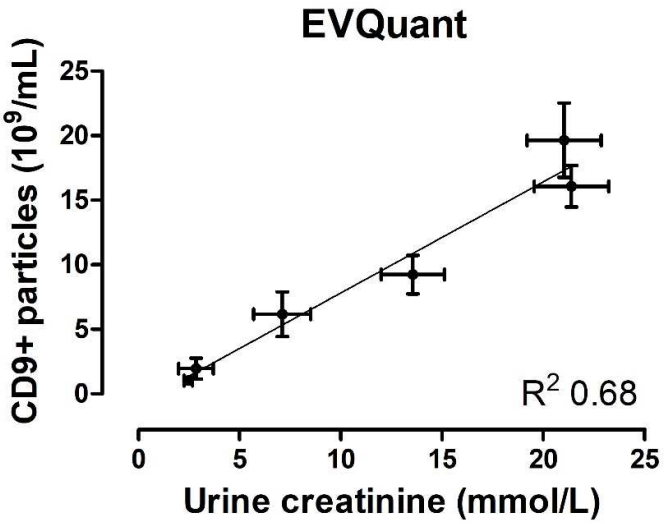
Legend: To establish the yield, the 200K pellet was re-dissolved in the original volume. EVQuant was used to count the number of particles prior to and after ultracentrifugation. The yield is expressed as the fraction of the particles present after ultracentrifugation compared to the number of particles present before ultracentrifugation.

Figure S3: Effect of centrifugation on particle size.



Legend: Particle size was measured using NTA in 6 water deprivation and 6 water loading samples in whole urine or in the 200,000 x g pellet. The difference in particle size between water loading and water deprivation was +11 nm after 2,000 x g centrifugation (** $P < 0.01$) and +12 nm after 200,000 x g centrifugation (* $P < 0.05$). After ultracentrifugation, particles were on average 6 nm smaller ($P = 0.04$).

Figure S4. Correlation CD9+ particles with urine creatinine



Legend: Analyzed in urine samples collected from 8 participants in the water loading study.

Figure S5. Urine characteristics and particle concentrations after water loading and with time-control

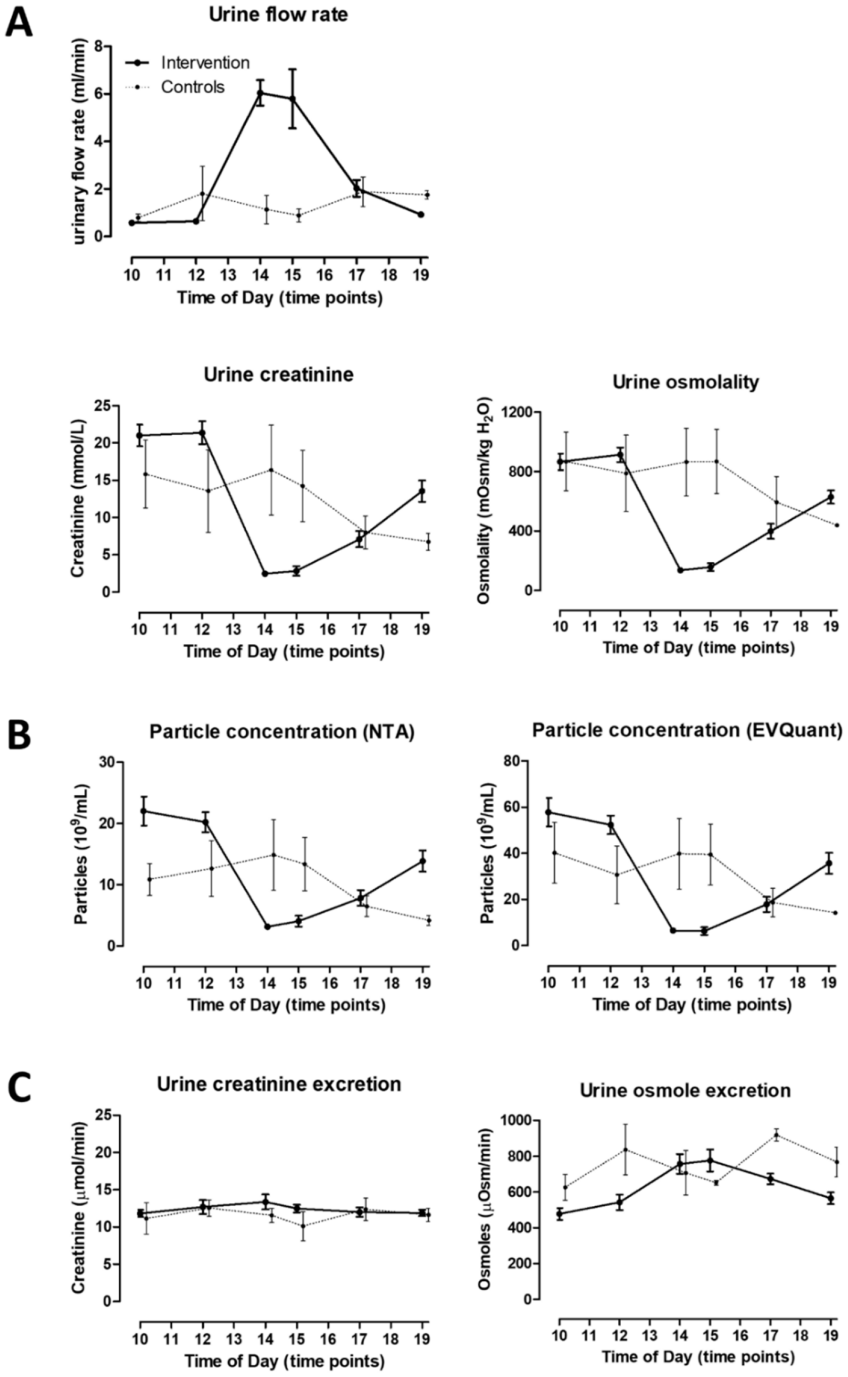
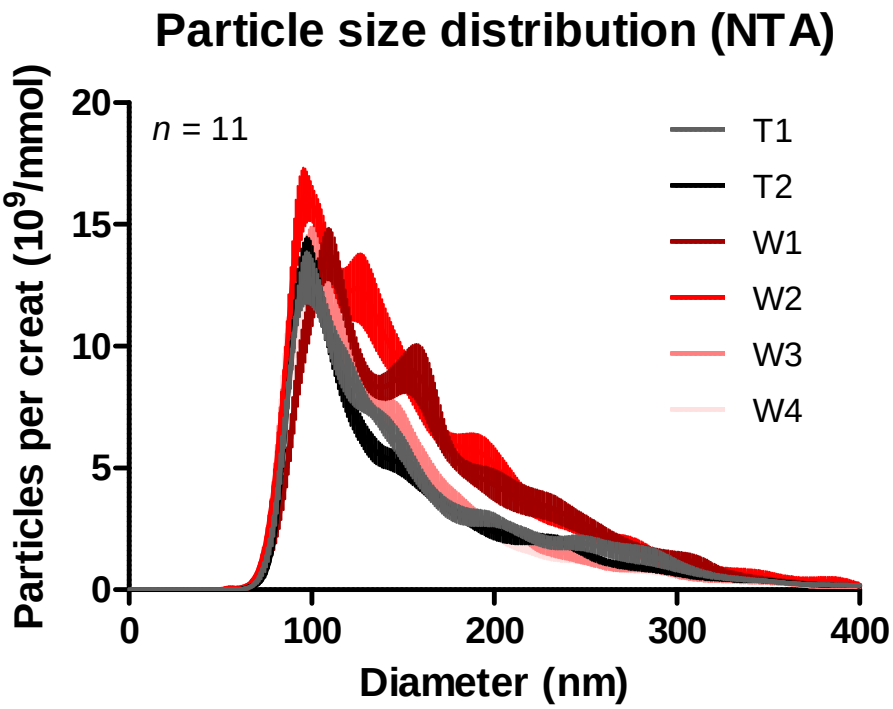


Figure S6. Particle size distribution per time point in the water loading study



Legend: Particle size distribution by NTA of each of the time points of the water loading, of combined version is shown in Figure 3B.

Figure S7. Representative raw image data of NTA (A), EVQuant (B), CD9-TR-FIA (C)

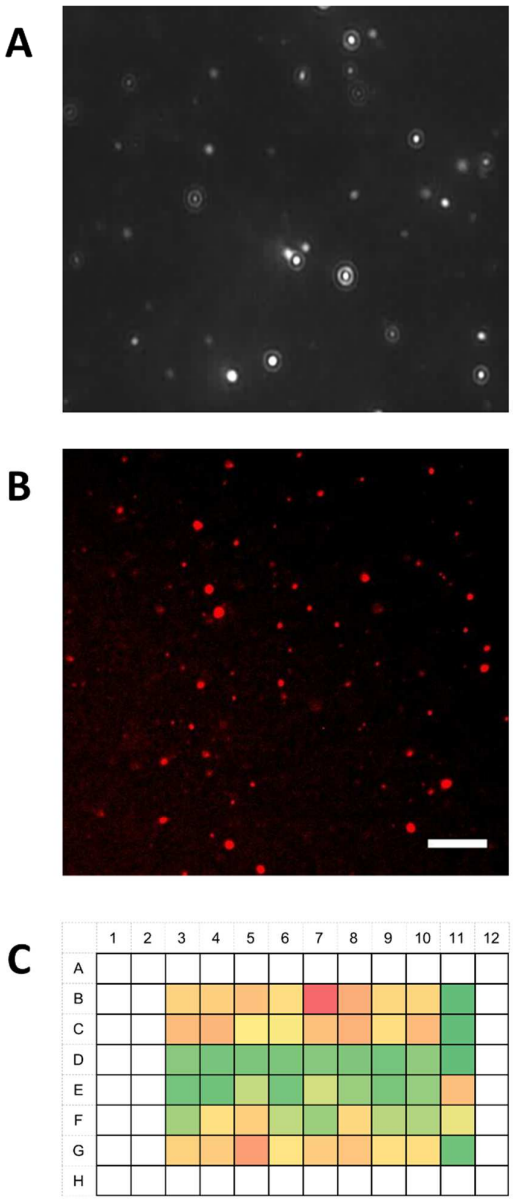
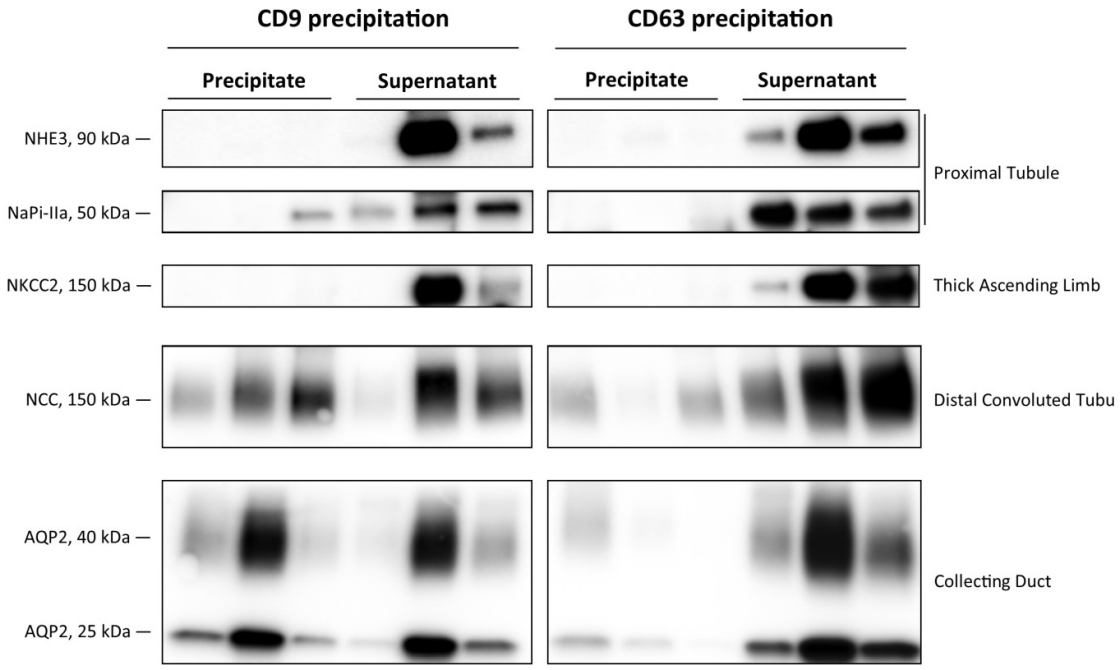


Figure S8. Additional immunoblots of CD9 and CD63 precipitations.



Legend: Characterization of $CD9^+$ and $CD63^+$ uEVs of Patients 4 – 6 (supplement to **Figure 7** which shows Patients 1 – 3). Immunoblot comparison of uEVs precipitated from 200K urine pellets by CD9- or CD63-antibody coated magnetic beads, and respective supernatant, with the nephron-segment markers NHE3, NKCC2, NCC, and AQP2.

Figure S9: Co-localization studies for a second CD9 and CD63 antibody, and for CD81.

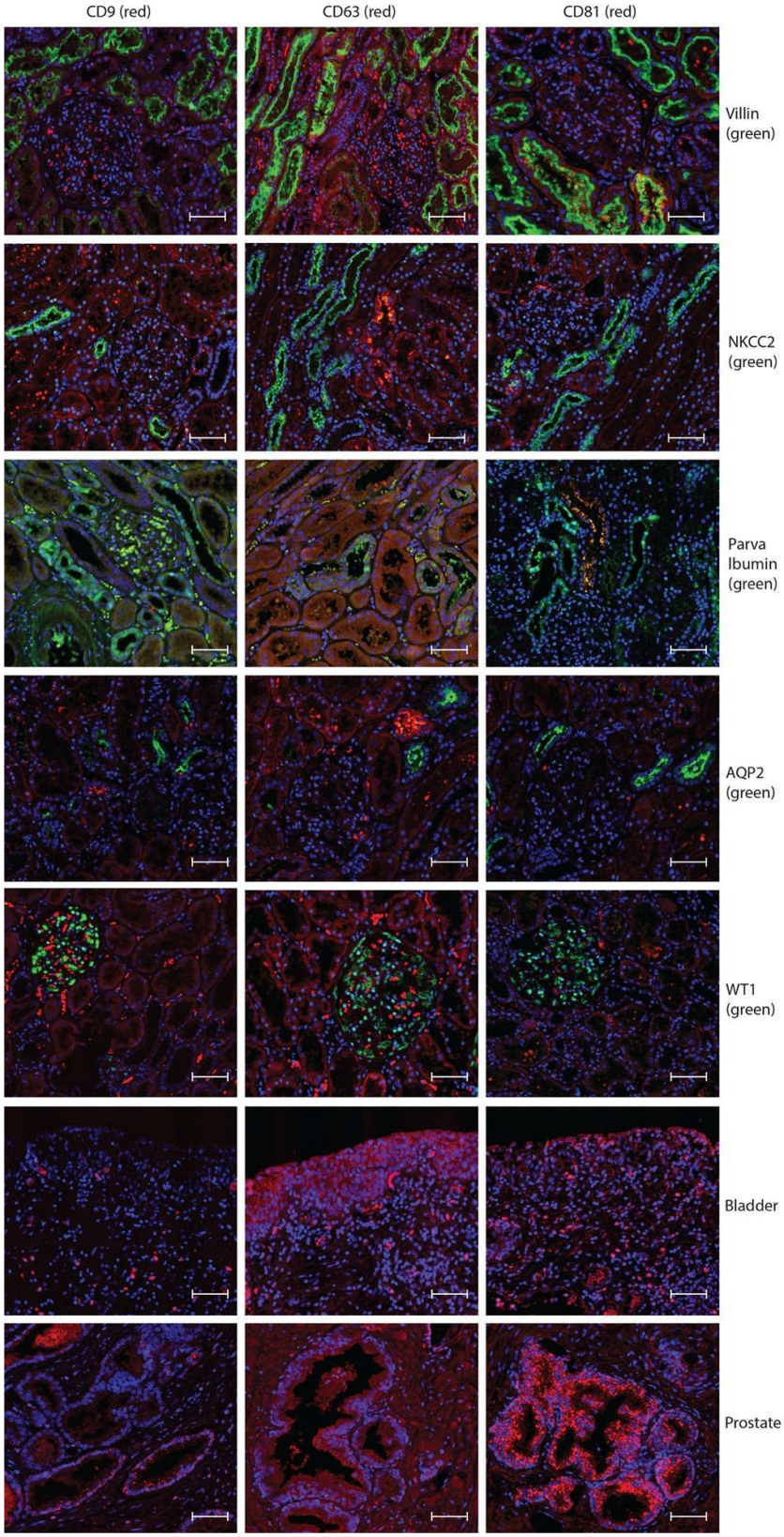
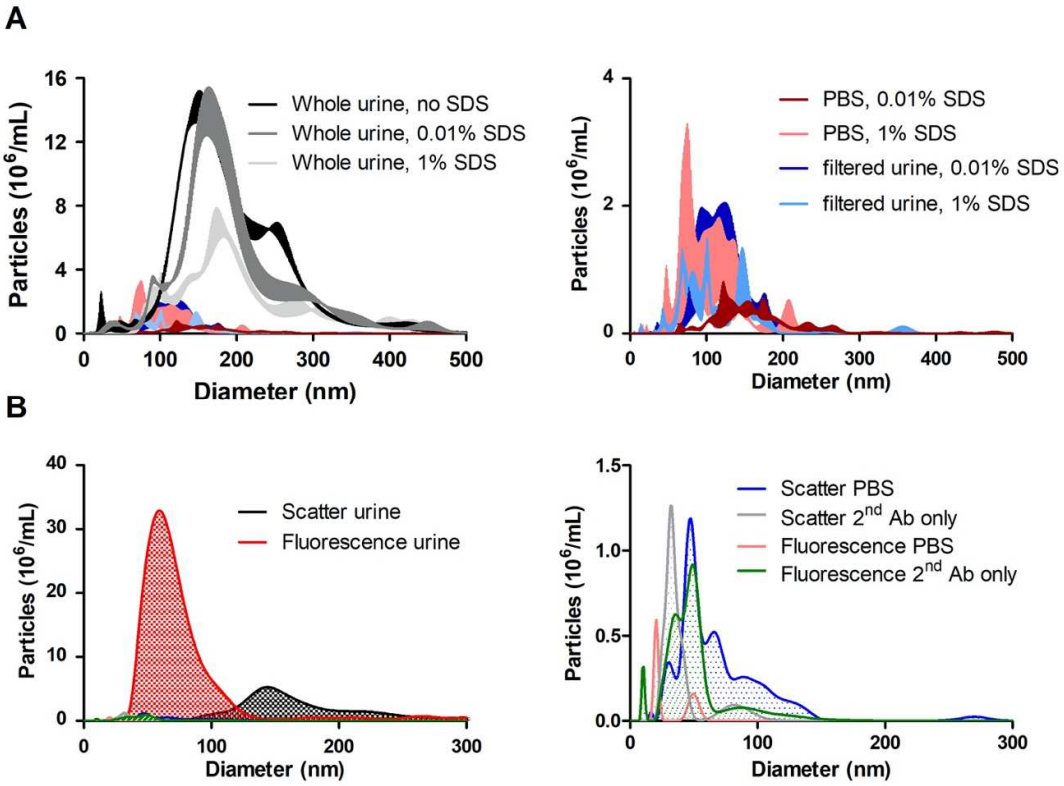


Figure S10: Additional NTA data

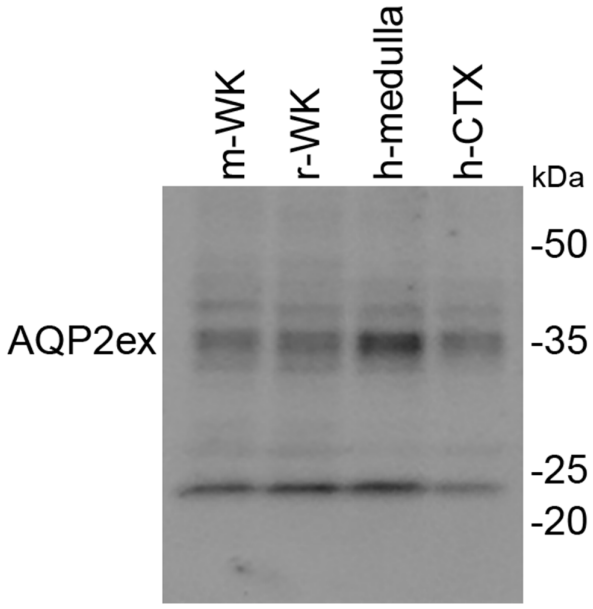


Legend: Additional controls to the data shown in Figure 8A and D.

(A) Left: Figure 8A with the addition of PBS or filtered urine (no uEVs) with 0.01% or 1% SDS. Right: close-up of the additional controls only.

(B) Left: Figure 8D with the addition of PBS only and 2nd antibody only in either scatter or fluorescence mode. Right: close-up of the additional controls only.

Figure S11: Characteristics of the extracellular-epitope AQP2 antibody



Legend: Immunoblotting of 10 µg protein homogenates from mouse whole kidney (m-WK), rat whole kidney (r-WK), human kidney medulla (h-medulla) or human cortex (h-CTX). Signals representing the glycosylated (~35 kDa) and non-glycosylated (~22 kDa) forms of AQP2 were observed in all samples.

Extracellular AQP2 antibody production: A 15-amino acid peptide, CYFTGCSMNPARSLAP (the NH2 terminal cysteine added for conjugation) corresponding to amino acids 177-191 of mouse AQP2 accession #AAB71414.1 (94% identity to human) was produced by standard solid phase techniques and conjugated to keyhole limpet hemocyanin (KLH) via covalent linkage to the NH2-terminal cysteine (Genscript USA). The antibody was affinity purified from terminal bleed serum using the immunizing peptide as described previously. The antibody titer was determined to be >1:512,000 using ELISA and AQP2 peptide conjugated plates. Antibody specificity was determined by: a) western blotting of human whole kidney, cortex or medulla tissue, showing a strong band of the characteristic molecular mass of AQP2 (Figure above); b) immunohistochemical labeling of mouse and human kidney showing characteristic labeling of tubules morphologically similar to collecting ducts (not shown).

Videos S1 and S2: Representative NTA videos before and after the addition of THP.

See separately uploaded videos.

Legend: When visually inspecting the NTA recordings for samples before and after THP addition, the increased small particle numbers appear to also include non-spherical objects. While NTA is not a platform intended to assess the shape or structural properties of particles, visual inspection is compatible with THP-vesicle or THP-protein aggregate formation, as could be expected due to THP multimer formation.