Temporal Dynamics of Urinary Extracellular Vesicle Excretion and Cargo in Healthy Subjects over 24 Hours

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Abstract:

Urinary extracellular vesicles (uEVs) are promising non-invasive biomarkers of renal physiology and pathology, yet little is known about their temporal excretion patterns or optimal normalization strategies. In this basic physiology study, we used a uniquely designed collection protocol of urine collection to characterize with great detail the variation of uEV's excretion and cargo throughout a 24-hour period in healthy subjects. Thirteen healthy adults (9 F, 4 M) collected every individual void over 24 h (mean 7.2 ± 1.6 voids). An artificial 24-h sample was generated for each collection to compare individual void variation to. uEVs were enriched by low-speed centrifugation with three low-ionic-strength washes to minimize Tamm-Horsfall protein. Size and concentration were quantified by nanoparticle tracking analysis (NTA); morphology by cryo-TEM; cargo by immunoblotting (NCC, AQP2, TSG101) and single-particle flow cytometry (CD9, CD10, DPP4, Neprilysin). Dietary intake was recorded in nine participants. uEV concentrations varied up to 100-fold across the day, but differences between five predefined time blocks were not significant ($p = 0.622$). Median uEV diameter ranged from 94 nm to 220 nm without significant diurnal change ($p = 0.150$). uEV counts correlated strongly with urine creatinine ($r = 0.70$, $p < 0.001$), specific gravity ($r = 0.65$, $p < 0.001$), and TSG101 signal ($r = 0.61$, $p < 0.001$). NCC abundance showed a near-significant mid-day dip ($p = 0.06$), while AQP2 trended higher in the afternoon ($p = 0.27$); no temporal pattern was detected for CD9, CD10, DPP4, or Neprilysin. Higher urine flow rates trended toward lower uEV excretion. Food-log data suggested larger uEVs with greater fluid intake, but statistical power was limited. In healthy adults, uEV concentration, size, and most cargo markers exhibit substantial intra-day

variability without consistent circadian peaks, whereas NCC and AQP2 show reproducible diurnal trends. Creatinine and TSG101 were used as normalization factors under stable renal function. These findings offer foundational insights into uEV excretion patterns over the course of a day and emphasize key methodological considerations essential for advancing uEV biomarker research.

Introduction

Urinary extracellular vesicles (uEVs) are a heterogeneous group of membrane surrounded-vesicles which originate mostly from kidney cells but also from other parts of the urogenital tract[1]. As they carry markers of the parent cell and bioactive molecules, they are seen as novel biomarkers and cell to cell messengers in health and disease respectively[1]. However, minimal literature exists studying how uEV excretion and release into the urine changes throughout the day in healthy individuals. While circadian rhythms have been shown to control many physiologic processes in the human body, including renal function, the circadian rhythm of only a few urinary biomarkers have been well characterized[2]. The field of translational uEV investigation is also challenged by the fact that urine is the most dynamic biofluid and normalization strategies need to be established. Urine contents vary in composition between and within individuals not only from day to day but also hour to hour. These physiologic variations in uEV excretion need to be understood to explore the full diagnostic potential of uEVs as biomarkers.

In this basic physiology study, we used a uniquely designed collection protocol of urine to characterize with great detail the variation of uEV's excretion throughout a 24-hour period in healthy subjects. Thirteen healthy subjects separately collected each void throughout a 24-hour period and documented the time of their voids along with a food and drink log for the same time period. Our study separated and characterized uEVs' size, morphology, cargo, and excretion into the urine over the course of a full day which was then put in context of the subject's food intake.

Methods

Study cohort

Healthy subjects above 18 years of age were recruited through advertisement flyers and by word of mouth. All subjects had no history of diabetes mellitus, hypertension, or chronic kidney disease. Subjects were on a free diet and nine recorded their dietary intake for the 24-hour collection period using the Automated Self-Administered 24-Hour Dietary Assessment Tool[3] (ASA24). ASA24 output included kilocalories (kcal), protein, total fat, carbohydrates, and water, as well as vitamins and minerals. Study protocol was approved by the University of Virginia's Institutional Review Board (IRB#220053). All subjects provided written and verbal informed consent prior to participating in the current study.

Urine collection and storage

Healthy subjects were given an insulated bag with 5-6 ice packs and urine collection bottles. Subjects kept the ice packs in the freezer and added them to the bag once they started collecting their individual voids. These ice packs kept the insulated bags at a temperature of 4˚Celsius. Every void was collected during a 24-hour period for each healthy subject starting with the second void of the morning and ending with the first void of the following morning. Each full void was collected in a separate bottle and labeled with the time of void initiation. After the final void (the first void of the following day), the finished collection was delivered to our laboratory and processed in lab within two to six hours of the final void. Twenty percent of each void was combined to produce an artificial 24-hour collection sample. In 7 out of 13 patients we also got a serum creatinine level measured the day urine collection was finalized.

Chemical analysis of urine

After the volume of each sample was measured and an aliquot of 20% removed from each void, a urinalysis dipstick (Siemens Multistix 10 SG) analysis was performed in each raw urine sample to assess pH, glucose, bilirubin, ketone, specific gravity, blood, pH, protein, urobilinogen, nitrite, and leukocytes. All information was securely documented in a REDCap database.

Urine protein and creatinine concentrations were also measured in the raw urine aliquots from each void. Protein quantification was performed by Coomassie micro assays on a 96 well plate.

The protein in the urine binds to Coomassie Brilliant Blue G-250 which is shown with an absorption at 595 nm[4]. Standard controls were loaded on the first three lanes of the plate to make a standard curve to compare urine protein content to. Plates were scanned at 595 nm to determine absorbance strength.

Creatinine concentrations were measured utilizing a picrate alkaline Jaffe assay on a 96 well plate[5]. Creatinine and picric acid form a complex that has an absorbance at about 520 nm. Standard controls were loaded on the first three lanes of the plate to make a standard curve to compare urine creatinine content to. Plates were scanned at 520 nm to determine absorbance strength.

uEV separation

uEVs were enriched from each individual urine sample including the generated 24 hour samples by combining 20% of each individual void with differential centrifugation. A relative centrifugation force (RCF) of 4,600*g* was used to generate cell free urine from 42 ml of raw urine. The supernatant 4,600 g (SN4,600) was then centrifuged at 15,200 RPM for 30 minutes at room temperature in an Eppendorf microcentrifuge fixed angle rotor (Thermo Scientific, 75003602) in a Sorvall ST16R centrifuge (Thermo Scientific) at 4 degrees to generate an EV pellet called P21 (**Fig. 1**). The low centrifugation pellet (P21) was treated with three washes with a low ionic strength buffer (10 mM HEPES pH 7.4) supplemented with EDTA (2.5 mM pH 8.0) to remove Tamm Horsfall protein. This method allows to generate a relatively pure uEV fraction[6]. Each of the four pellets generated were equivalent to 10.5 mLs of raw urine to perform characterization and cargo analysis on. 1 mL of raw urine was frozen down for protein and creatinine assays.

uEV physical characterization

Urinary EVs were further characterized with a Nanoparticle Tracking Analysis (NTA) tool to assess size and concentration of uEVs in the Tamm Horsfall depleted uEV pellets. Cryo Electron microscopy was performed to assess uEV morphology and purity of EV prep, ruling out other protein contamination of the uEV prep.

NTA was performed using the ZetaViewPMX 120 (Particle Metrix) configured with a 488 nm laser with a long wave-pass (LWP) cut-off filter (500 nm) and a sensitive CMOS camera

640 x 480 pixels. Each P21 sample was diluted in 2 mL of 0.1 µm filtered (Minisart high flow hydrophilic 0.1 µm syringe filter Sartorious) deionized water (DI 18 MΩ/cm) to obtain a particle concentration between 1 x 10^7 and 1 x 10^8 particles/mL. The instrument was set to a constant temperature of 25°C, a sensitivity of 80, a shutter speed of 100 and a frame rate of 30 frames per second (fps). Each sample was measured at 11 different positions throughout the cell, with 5-7 cycles of readings at each position in order to have a minimum of 1000 traces. Post-acquisition parameters were set to a minimum brightness of 30, a maximum size of 200 pixels, and a minimum size of 5 pixels. Automated cell quality control was checked using high quality deionized water (DI). Camera alignment and focus optimization was performed using polystyrene 100 nm beads (Applied Microspheres). Data analysis was performed with ZetaView 8.05.12 software provided by the manufacturer. Automated reports of the particles recording across the 11 positions were manually checked and any outlier position was removed to calculate particle concentration and distribution expressed by mode, median and mean (**Supplemental Figure 4**).

uEV morphology was assessed with cryogenic transmission electron microscopy –Cryo-TEM was performed in the molecular electron microscopy core at the University of Virginia. Low speed centrifuged uEV P21 pellets were solubilized in 20 µL PBS–0.1 µm and applied to a glow-discharged, perforated carbon-coated grid (2/2-3C C-Flat; Protochips). Low-dose images were collected at a nominal magnification of 29,000 × on the Tecnai F20 Twin transmission electron microscope operating at 120 kV. Digital micrographs were recorded on a Gatan US4000 charge-coupled device camera.

uEV cargo analysis

Urinary EV cargo was assessed utilizing immunoblotting and single EV flow cytometry following the guidelines by the International Society for Extracellular Vesicles (ISEV)[7,8]. For immunoblotting P21 pellets were solubilized in an electrophoresis solubilization buffer, supplemented with 50 mM DTT (dithiothreitol) and protease and phosphatase inhibitor cocktail. Samples were run on 4–15% gradient polyacrylamide gels (Bio-Rad, 5671084) according to the manufacturer's directions. A molecular weight marker ladder was run beside the samples. Protein on the gels was transferred to PVDF membranes for subsequent staining for total protein content and then sequentially probed with antibodies to NCC, aquaporin-2 (AQP2), and

TSG101. Imaging and quantification of signal strength of blots were performed on a Li-Cor Odyssey CLx instrument using Image Studio software.

Spectral flow cytometry was performed using the Cytek Aurora 5-Laser flow cytometer equipped with the Enhanced Small Particle (ESP™) Detection tool. P21 samples were prepared from 1 mL of thawed sample using differential centrifugation (4600xg for 30 minutes, preceding 21,100xg for 30 minutes). Data acquisition was standardized by time, with events collected over a 2-minute interval. uEVs were characterized using spectral flow cytometry and analyzed with FSCExpress software to determine total particle counts based on surface marker expression. uEV concentrations were quantified using the instrument's built-in volumetric method. Specific markers were used to identify uEVs originating from distinct nephron segments based on single gene analysis, including CD26/dipeptidyl peptidase 4(DPP4)(PT S1), CD35/complement receptor 1 (podocyte), CD10/MME/neprilysin (PT S1, S2, and S3), and tetraspanin, CD9 (a canonical and tubular EV marker). Particle sizing was performed using FCMpass software, which estimates particle size using NIST traceable polystyrene particles of known diameters and refractive indices. Scatter intensity data were calibrated into standardized units of scattering cross-section (nm^2) and particle diameter (nm), enabling phenotypic analysis of medium-sized (mean size: 233.15 nm) and large (mean size: 973.3 nm) EV populations, based on software generated size distribution curves. Final size distributions were generated by extrapolating diameters from raw SSC data.

Statistical analysis

All graphs were generated using GraphPad Prism 10 (version 10.0.0, GraphPad Software). Since healthy subjects contribute several voids with each having an unique value, statistical tests must account for the same subject contributing several data points to determine statistical significance.P values for relationships between two quantitative variables were obtained using linear mixed-effects models in R (version 4.5.0), with individuals specified as a random effect. Models were implemented using the lme4 package (version 1.1-37). Linear mixed-effects models were used to evaluate associations involving uEV concentration, urine creatinine, TSG101 signal, specific gravity, and uEV excretion rate. Nonparametric one-way ANOVA (Friedman tests) were performed to assess the variance in protein signals, uEV size, and uEV concentration across different periods (Period A: second morning void. B: All voids between A and 12 pm. C:

All voids between 12 pm and 6 pm. D: All voids between 6 pm and 12 pm. E: All voids between 12 am and A) , as described in Ginsberg et al. The Friedman tests were also conducted in Prism.

Results

Study cohort

Thirteen healthy subjects were successfully recruited for this study. These 13 healthy subjects (9 F, 4M) had an average age of 44.3 +/- 11.7 years, BMI of 21.7 +/- 1.79 kg/m^2, and collected an average of 7.15 +/- 1.57 voids with an overall urine volume of 2010 +/- 734 mL over 24 hour (**Table 1**). For the subset of healthy subjects who got their serum creatinine measured at the time of collection, the estimated glomerular filtration rate was between 87 and 116 ml/min/1.73m^2 using the CKD-EPI creatinine equation[10] whereas the calculated creatinine clearance was between 77.8 and 133.8 mL/min[11]. All clinical characteristics are within normal range as expected. Clinical characteristics for each of the healthy subjects is included in **Supplemental Figure 1.**

Table 1: Overview of 13 healthy individuals' clinical characteristics and a subset of 7 individuals with serum creatinine values.

Sex (F, M)	Age	BMI	Body Surface Area	Number of Voids	Urine Flow	Urine Volume
9,4	44.3 +/- 11.7 yrs	21.7 +/- 1.79 kg/m^2	1.79 +/- 0.163 m^2	7.15 +/- 1.57 voids	0.805 +/ 0.375 mL/min	2010 +/- 734 mL over 24 hours

Subset of 7 individuals

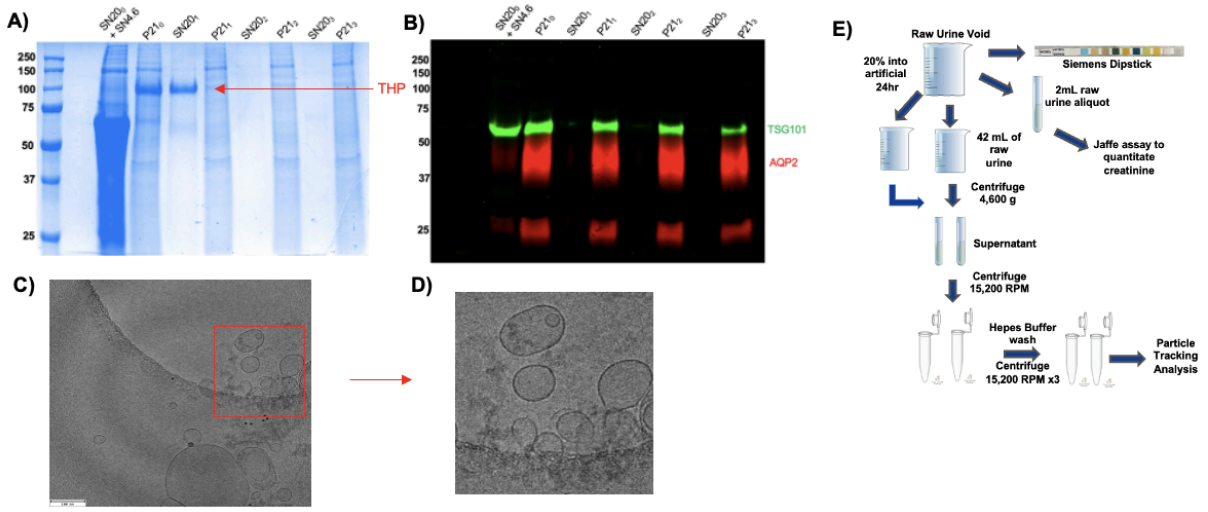
Serum Creatinine	calculated GFR	estimated GFR CKD-EPI
0.6 – 0.9 mg/dL	93-159 (ml/min/1.73m^2)	87-116 (ml/min/1.73m^2)

Reduction of Tamm Horsfall contamination

To reduce Tamm Horsfall Protein (THP) contamination, the most abundant urinary protein in healthy urine, the uEV pellets were generated with a low centrifugation spin and washed with low ionic strength buffer three times. Figure 1E shows a successful reduction of THP without

compromising the EV quantity during the process of washing. The reduction of THP filaments with the three washes of low ionic strength buffer is shown through in the Coomassie Gel as well as the preservation of TSG101 signal. The final pellet $P21_3$ (after 3 washes) is clean with the reduction of THP at 100 kd (**Fig. 1A**). The EV marker TSG101 and AQP2 signal is minimally diminished after the washing steps **(Fig. 1B**). Cryo electron microscopy shows uEV p21 pellet without protein filaments entrapping the vesicles as well as the heterogeneity of the size and shape of uEVs (**Fig. 1C**).

Figure 1: Verification of THP reduction in p20 prep. Panel A was stained with Coomassie blue to visualize protein content. After being spun in a polycarbonate tube at 15,200 RPM for 30 min, the pellet was resuspended in SN4.6 to transfer into an Eppendorf tube to then spin again at 15,200 RPM to visualize the washing steps which resulted in $SN20_0$ + SN4.6. After one wash with the low ionic strength buffer, the THP is released into the supernatant $SN20_1$. Panel B was stained with direct conjugate AQP2 680 RD, primary rabbit TSG101, anti rabbit 800 CW. Panel C is cryo electron microscopy of the p20 pellet showing a clean prep. Panel D is a zoom in of C. Panel E is a flow of the urine processing methods.

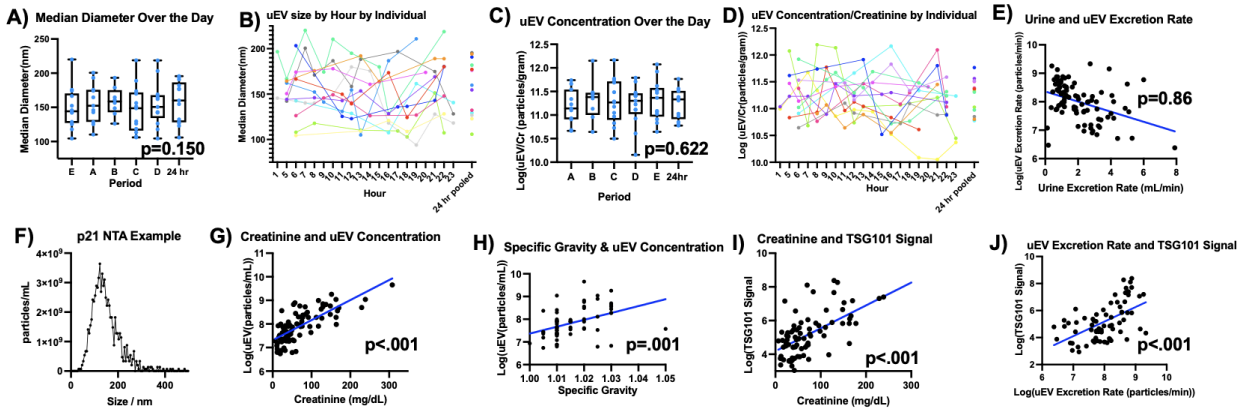


uEV sizing and counting over 24 hour period

Nanoparticle Tracking Analysis (NTA) was utilized to size and count uEVs. As described above our uEV separation protocol generates relatively clean uEV pellets without contamination with other protein complexes as visualized in our cryo electron microscopy image (**Fig. 1**). The NTA results represent mostly uEVs and not other particles. Thus, the NTA data demonstrates that the uEV concentration varies over the course of a day from $1.1 * 10^{13}$ to $1.6 * 10^{15}$ particles/gram of creatinine (**Fig. 2C/D)**. This is visualized in spaghetti plots showing individual data points as well as demonstrated by boxplots of the uEV concentration corrected to urine creatinine concentration over time periods of a 24 hour day. The changes in uEV counts are up to 100 fold,

however the changes are not statistically different (p=0.622). Median uEV size ranged between 93.9 to 220.1 nm, but the differences over the day are not significant (p=0.150, **Fig. A/B**). To be expected, the mean of uEV concentration and size of the 24 hour pooled sample (representing a 24 hour urine collection) is similar to the mean of all individual voids counts over the 24 hour period. uEVs plotted over urine excretion rate shows an indirect trend that the higher the urine flow excretion rate the lower the uEV excretion rate; however, this result was not significant (p=0.86).

Figure 2: Size, Concentration, Excretion and Normalization of uEVs. Panel A shows boxplots of the uEV median diameter over the periods (Period A: second morning void. B: All voids between A and 12 pm. C: All voids between 12 pm and 6 pm. D: All voids between 6 pm and 12 pm. E: All voids between 12 am and A) Panel B shows the uEV median diameter by hour of each void over the day by individual. Panel C shows boxplots of the uEV concentration normalized to creatinine over the periods. Panel D shows the uEV concentration normalized to creatinine by hour each void over the day by individual. Panel E shows the urine production by minute versus the uEV production by minute. Panel F shows an example of the p21 pellet size distribution on NTA. Panel G shows the correlation of urine creatinine and uEV concentration. Panel H shows the correlation of urine specific gravity and uEV concentration. Panel I shows the correlation of urine creatinine and uEV TSG101 signal via immunoblotting. Panel J shows the correlation of uEV excretion rate and uEV TSG101 signal via immunoblotting.



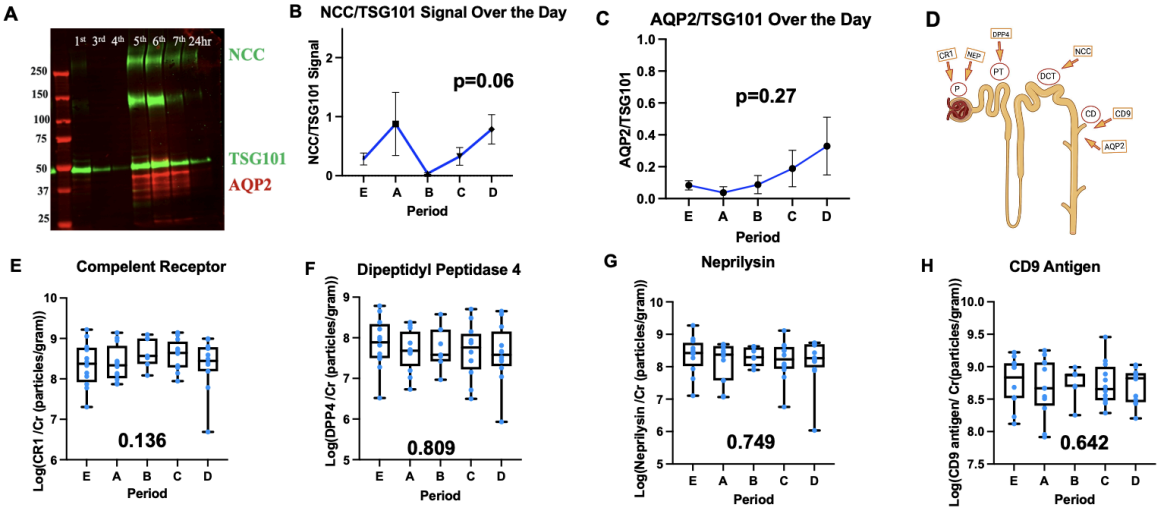
uEV count correlation to normalization markers

As urine is a very dynamic biofluid and the creatinine excretion is stable in a steady state and healthy condition we correlated uEV concentrations with urine creatinine concentrations, specific gravity and TSG101(tumor susceptibility 101), an EV marker. uEV concentrations correlated significantly with urine creatinine concentration (**Fig. 2G**) which suggests that uEV findings in spot urine can be normalized by creating an uEV/creatinine ratio in a steady and healthy state. uEV concentrations also correlated significantly with specific gravity tested by a urine dipstick (**Fig. 2H).** The TSG101 immunoblot signal also correlated significantly with urine creatinine concentrations (**Fig. 2I**). Additionally, the TSG101 signal was significantly increased as more uEVs were produced (**Fig. 2J**).

uEV cargo analysis over a 24 hour period

In order to understand temporal patterns of uEV cargo expression over a 24 hour period, EV cargo candidates representing different parts of the nephron such are glomerular (CD10, Neprilysin (MME)) and tubular markers (sodium chloride cotransporter (NCC), aquaporin 2 (AQP2), dipeptidyl peptidase 4 (DPP4), CD9) (**Fig. 3D)** were selected**.** NCC and AQP2 were tested utilizing immunoblotting. NCC varies over the day with a dip at noon with the result close to significant (**Fig. 3B**, p=0.06). AQP2 varies over the day with an increase in uEV cargo after noon but the result is not significant (**Fig. 3C,** p=0.27). Of note, the immunoblotting shows NCC protein as a dimer as we have 2 bands at 260 and 130 kd. DPP4, CD10, CD9, MME were detected with single EV flow cytometry, **see Fig. 3, Supplemental Figure 2 (gating strategy).** No significant temporal patterns were detected over the course of a day for these 4 nephron markers.

Figure 3: Cargo analysis by immunoblotting and flow cytometry. Panel A shows an example of an immunoblot stained with NCC, AQP2, and TSG101 for individual B's voids. Panel B shows the NCC normalized to TSG101 signal over the periods. Panel C shows the AQP2 normalized to TSG101 signal over the periods. Panel D shows the corresponding to their expression on the nephron For E-F, Single uEV flow cytometry the Cytek Aurora 5-Laser flow cytometer equipped with the Enhanced Small Particle (ESP™) Detection tool. Particle concentration was normalized to urine creatinine values. Panel E shows Complement Receptor 1 over the periods. Panel F shows Dipeptidyl Peptidase 4 over the periods. Panel G shows Neprilysin over the periods. Panel H shows CD9 over the periods. Friedman tests were performed on all.

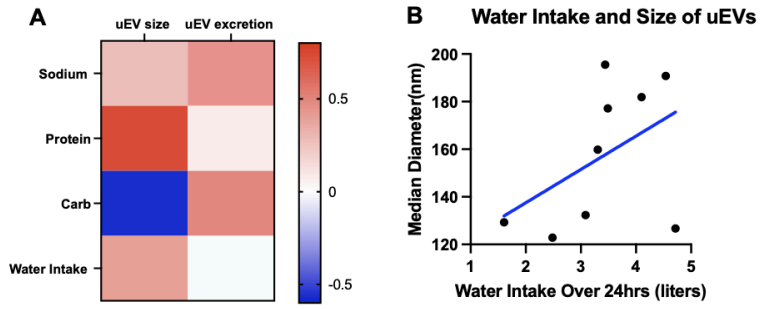


uEV counts in context of food intake over a 24 hour period

As food and fluid intake impacts the composition of urine, participants documented a food log[3]. The relationship between food intake and uEV size and excretion is summarized in a heat map (**Fig. 4A**). Protein diet had a linear trend with uEV size as did sodium and water intake. Carbohydrate intake has an inverse linear trend with uEV size. uEV excretion had a linear trend

with sodium and carbohydrates intake, but not by protein or water intake. We found a trend of increased uEV size with increased fluid intake, however our data was derived from food logs for 9 subjects out of 13 with a free diet, therefore statistical significance was not determined **(Fig. 4B**). Example of a portion of variables from the food log output is in **Supplemental Figure 4.**

Figure 4: Panel A is a heat map showing the correlation between dietary factors and uEV size and excretion. Correlation values were determined using Spearman's rank correlation coefficient, which measures the strength and direction of association between two ranked variables. Panel B is a graph showing the association between water intake and uEV size.



Discussion

Urinary EVs can reflect physiologic and pathologic processes in the kidney and have great potential as novel biomarkers. Urine is also available non-invasively and in large quantities which makes uEVs an ideal novel liquid biopsy tool. However, little is known about the overall rate of uEV excretion, which reflects the balance between their secretion and reabsorption in both health and disease. In addition, urine is the most dynamic biofluid as urine constituents change day by day and hour to hour. This dynamic process is particularly influenced by diet and fluid intake as well as medications. Normalization strategies for quantification of urine constituents and uEVs are crucially needed. Temporal patterns of biomarker excretion also need to be considered.

Since a landmark study published in 1983 in the New England Journal of Medicine (NEJM) demonstrated that the calculated protein-to-creatinine ratio of the second-morning void provided an accurate quantification of daily proteinuria, clinicians have been able to use a spot urine collection to extrapolate kidney health and function across a greater span of time than that during which the single void was produced.[9] In contrast to urinary protein, not much is currently understood about how well the characteristics of uEVs such as concentration, size, surface

markers, and internal contents/cargo within a single void reflect the range of those secreted over a longer period of time, e.g. over 24 hours. This knowledge gap surrounding the temporal variation in basic physiology represents one of the main roadblocks currently facing the field of translational uEV research, a challenge which if overcome could allow uEV markers to be utilized in clinical settings.

This study also employed an optimized uEV separation method. Washes with a low ionic strength buffer were implemented to generate clean uEV pellets to assure that true uEVs counts were measured with our Nanoparticle Tracking analysis (NTA) tool and not other non-EV particles and proteins such as Tamm Horsefall proteins. This is supported by other work which showed that adding THP to urine significantly increased particle counts by NTA[12]. Thus, our uEV separation method increased the robustness of our unique data analysis allowing correct assessment of uEV excretion.

uEV concentration, size variation and excretion over the course of a day

In a way that has not been done previously, our healthy subjects collected each void individually throughout a 24-hour period along with the exact time collected for 13 healthy subjects. Our collection approach is in line with the recommendations by the Urine Task Force of ISEV, for example performing a quality control test with a urine dipstick and cooling at proper temperature during urine collection and processing to name two examples, summarized also in the uEV collection card of the "Urine task force" of the International Society for EVs[17] .

As composition and concentrations of urinary constituents vary over the course of the day it is not surprising that uEV concentrations vary over the course of a day as shown in our study. However, we detected up to 100 fold changes, yet these variations are not statistically significant. This was visualized in individual spaghetti plots but also by combining the data into 5 collection periods over the course of the day as done in the landmark paper by Ginsberg (as discussed above)[9]. A temporal pattern of uEV excretion in timed urine collections of 4 hours over a 24 hour period was also shown in Sprague-Dawley rats, yet our data set is not directly comparable to this study by Koritzinsky et al as small uEVs utilizing a different uEV separation method were analyzed[18].

We observed a trend that a higher urine flow rate corresponded to a lower uEV excretion rate. This finding was also shown by Blijdorp et al who demonstrated that the number of uEVs

excreted in urine of 11 healthy subjects undergoing a formal water loading test varies inversely with urine excretion[12]. We can only speculate that when uEVs are excreted in context of high urine flow, there might be less time for uEV excretion which is a net effect of EV release and uptake. Several in vitro studies suggest proximal to distal tubular cell communication in which uEVs are released and uptaken by kidney cells[14]. Depending on the stimulus, here the urine flow rate, changes in uEV release and uptake could be possible. To our knowledge, the effect of urine flow rate has not been studied yet in an in vitro system. Ideally this should be studied in an in vivo system with endogenously labeled EVs.

uEV size was determined by NTA in our cohort and varied between healthy subjects from a median size for an individual void varied from 93.9 to 220.1 nm in diameter, roughly 2 fold changes, but differences were not significant over the course of a day. This is not surprising as uEVs are very heterogeneous and their size can range from 50 nm to over 1000 nm, allowing in fact 20 fold or more changes, however the majority of uEVs in general is <200nm[1,15].

uEV normalization strategies

After blood, urine is the second most studied biofluid for EV biomarker discovery. In contrast to blood samples for which homeostasis is within short margins, urine constituents change significantly over the day[36]. As done in the landmark study in the NEJM we utilized urine creatinine concentrations for normalization[9]. We observed a significant correlation between uEV concentrations and urine creatinine concentrations. This finding is in line with other uEV work[12]. Our findings were generated with samples of 13 healthy subjects providing 120 samples in total. Blijdorp et al used cell free urine from 11 subjects undergoing water loading but also random urine samples from 14 healthy subjects and 26 patients with polycystic kidney disease (PCKD)[12.] In all these samples a correlation between particle counts and creatinine concentration was observed. In order to fully understand uEV excretion rates in disease states, other chronic kidney disease processes with moderate to large proteinuria and inflammatory disease processes such as glomerulonephritis need to be studied.

We were also interested if uEV concentrations correlate with specific gravity, an indicator of more concentrated urine, and TSG101, the EV marker tumor susceptibility 101. We used SG as a surrogate for osmolality measurements and found a correlation between uEV concentration and SG[17]. In the water loading study previously mentioned, a correlation was found between

urine particle concentrations and osmolality[12]. TSG101 plays a role in the ESCRT pathway, which is involved in the sorting and release of cargo into multivesicular bodies and ultimately into EVs, therefore used as an "EV marker". We saw a correlation between urine creatinine and TSG101 and suggest that TSG101 can be used for normalization. In the above mentioned study by Korizinski et al, small uEVs/Exosomes generated from urine of rodents with ultracentrifugation from timed urine collections of 4 hours saw that TSG101 excretion had similar patterns of small uEVs excretion. The authors concluded that TSG101 could also be used for normalization[18].

As discussed earlier the average of uEV concentrations of all individual voids corresponded to the average result in this "artificial" 24 hour urine collection, supporting that 24 hour urine collection remains the gold standard for correct quantification of uEVs. We conclude that for random urine samples normalization to creatinine or TSG101 is possible if a steady state of kidney function exists. However, if the investigators wish to know the best time point to measure a biomarker, a 24 hour urine collection with individual voids should be executed as done in our study.

uEV protein cargo temporal dynamics over course of a day

Circadian patterns of extracellular vesicles have been explored previously in various disease pathways and models. Parkinson's, metabolic dysfunction, obstructive sleep apnea, PCOS, and different cancers have been studied [19,20,21]. Extracellular vesicles from a variety of sources are studied in relation to temporal patterns. Some of these sources include milk, human plasma, stem cells, mouse cell culture, rat urine, and human urine[18,22,23,24,25,]. While extracellular vesicles and their cargo have therapeutic and diagnostic potential, few comprehensive studies have been done to characterize the uEV protein cargo expression over the day in humans. We detected proteins of glomerular and tubular origin in our sample collection by immunoblotting (NCC, AQP2) and single EV flow cytometry (DPP4, Neprilysin, CD9, and CD10). Our study found a temporal/diurnal pattern of NCC expression in uEVs with a dip before the afternoon. Our NCC findings align with a study of five healthy individuals by Castagna et al., who examined the diurnal pattern of NCC and AQP2. In their study, urine was collected at five fixed time periods rather than from spontaneous voids. In addition, we also observed an afternoon increase in AQP2 similar to this study, but the differences across time points were not statistically significant for

us. Interestingly, their study used filtration to enrich uEVs, which may have captured a different uEV subpopulation. Nevertheless, both AQP2 and NCC exhibited similar circadian trends in their study and ours, supporting some consistency of these patterns[22]. In an additional animal model, NCC showed a temporal pattern[31]. In a second targeted analysis we detected glomerular and tubular markers such as DPP4, Neprilysin, CD9, and CD10 with single EV flow cytometry in individual voids of our cohort. No significant temporal patterns were detected. Mass spectrometry analysis of individual voids over a 24 hour period would be the next step to provide a more unbiased analysis of uEV protein cargo patterns over a day.

Other researchers have looked at diurnal patterns of other uEV cargo such as RNA. Circadian rhythm was found to influence adipocyte-derived exosomal miRNAs, which, in turn, regulate insulin sensitivity and metabolic function[24]. Circulating exosomal miRNAs may also serve as indicators of circadian misalignment, potentially playing a significant role in metabolic dysfunction observed in night shift workers[26]. Additionally, the circadian rhythm of adipose-derived stem cells may influence the secretion of small EVs, which modulate inflammation[27].

uEV excretion and food and fluid intake over the course of a day

Our healthy subjects had a free diet, but kept a food log. Fluid intake of our subjects showed a trend that higher fluid intake corresponding with larger uEVs but not enough healthy subjects completed the food log to determine statistical significance. This trend was also observed by the study previously mentioned which also performed a more formal water loading study demonstrating that uEV's sizes vary directly with fluid intake and osmolality[12.] The authors concluded that hypotonicity caused the increase in uEV size[12]. Protein consumption of our healthy subjects did not seem to have a difference on uEV excretion, however this needs to be verified with defined diets and also with a larger study to understand influences of diet on uEV excretion. As high protein diets lead to hyperfiltration, in particular a study needs to be designed to assess how hyperfiltration influences uEV excretion. Of note, healthy subjects did not find the food log burdensome to the 24 hour urine collection process which is recommended by the guidelines for uEV collection[13] and we encourage other investigators to include a food log into their studies.

Limitations

There are limitations in regard to design but also technology. Regarding design we studied a smaller group of healthy subjects, however our collection represents the largest cohort studied to address uEV diurnal variations and excretion, we also had over 120 individual urine samples in total. Yet our cohort was not large enough to study sex based differences. One other study found that women had a higher count of urinary EVs compared with men[28] . We added a food log to document free diets of our healthy subjects, but defined diets need to be tested to have enough power to address the effect of food intake on uEV excretion. Interestingly kidney mass can also affect uEV excretion[29] . We cannot rule out an effect of kidney size.

Each EV separation and characterization tool has its technological limitations. We focused on the low centrifugation pellet of uEVs which separates small and large uEVs[30]. As we did not study an ultracentrifugation pellet we might have missed analysis of more smaller uEVs. Detection limits of EV tools also need to be considered. NTA has a detection limit for EVs of about 60 nm and is biased towards detecting smaller EVs depending on standard operating procedure settings . The limit for our small particle flow cytometry tool was about 100-150 nm for EV size, therefore having a bias towards larger uEVs. However, immunoblotting is a bulk analysis tool and should have captured all uEVs in the uEV pellet prepared with our method. We performed targeted uEV protein cargo analysis, next step would be to utilize omic tools, such as mass spectrometry, to have an unbiased proteome analysis tool.

In summary, our study is the first physiological study to analyze uEV excretion over a period of 24 hours by collecting each individual void in healthy subjects. We observed uEV excretion variations of up to 100 fold, however this was statistically not significant. uEV protein cargo can reflect diurnal patterns of kidney specific proteins, NCC and AQP2 are showcased in this study as uEV cargo proteins. Larger cohorts in health and disease need to be studied, ideally performing unbiased uEV cargo analysis and including defined diets. Our study supports use of urine creatinine concentration and TSG101 for normalization strategies for biomarker assessment in cohorts who have a stable kidney function, which can move this field towards clinical translation.

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