Standardization and optimization of urinary extracellular vesicle isolation by modifying hydration of a healthy cohort

Meghan R. Luellen[a]. Hayrettin Yavuz, MD, PhD[b]. Agnieszka Swiatecka-Urban, MD[c]

[a] Department of Biomedical Engineering, University of Virginia
[b] Department of Nephrology and Department of Pediatric Nephrology, University of Virginia
[c] Department of Pediatric Nephrology, University of Virginia

Abstract

Early detection of kidney injury is critical to delay chronic kidney disease (CKD) progression to the end-stage. Management for end-stage renal disease (ESRD) includes dialysis or transplantation, but neither can be considered a perfect cure. Urinary extracellular vesicles (uEvs) exhibit both diagnostic and therapeutic potential due to enclosed biomarkers indicative of kidney injury. The non-invasive, highly available nature of urine makes uEvs a suitable candidate for clinical applications, but researchers need a standardized approach to urine collection, uEv isolation, and measurement prior to clinical use. In this pilot study, we investigated the impact of water-restriction on a healthy cohort of 10 volunteers. Each volunteer consented to provide two first-morning urine samples: one after an 8-hour water-restriction period and one after no hydration limitations. The urinary creatinine concentration under both conditions validated the effectiveness of the water-restriction period. Identical methods of differential centrifugation were used to separate uEvs, followed by Nanoparticle Tracking Analysis (NTA) to determine the size and concentration of the uEvs. Results indicate that the size and concentration of uEvs are not significantly impacted by water-deprivation, and therefore, there may be no perceived benefit to abstaining from water-intake prior to first-morning urine collection for uEv analyses. Future efforts to standardize urine collection protocol may benefit from examining the impact of other pre-analytical variables on urine content and validating accessibility to biomarkers of interest in vulnerable patient samples.

Keywords: Chronic Kidney Disease (CKD), Urinary Extracellular Vesicles (uEvs)

Introduction

Chronic Kidney Disease

The renal system consists of two kidneys and the urinary tract. The kidneys conduct the removal of waste products and excess water from the blood, which are released from the body through the urinary tract. Each kidney contains approximately one million glomeruli, which are specialized bundles of capillaries that serve as primary facilitators in the filtration process (Pollak et al., 2014, p. 1461). The glomerular filtration rate (GFR) measures how effectively the kidneys are filtering waste products and excess water from the blood for clearance. Chronic kidney disease (CKD) is defined as the presence of kidney injury, clinically detected as reduced GFR, for at least three months. CKD is a progressive condition with clinical symptoms emerging slowly and silently. The severity of CKD ranges from mild (stage I) to severe (stage V) or end-stage renal disease (ESRD) when estimated GFR is less than 15 ml/min/1.73 m^2 (Vaidya & Aeddula, 2022, p. 1). Some patients develop CKD after episodes of acute kidney injury (AKI) and others have an indolent CKD course without an identifiable acute episode and may progress over time to ESRD. The risks of CKD progression are not well understood and not every episode of AKI leads to CKD. Once the disease state progresses to the end-stage, the patient will not see a return of kidney function.

Several management methods or renal replacement therapies (RRT) exist to extend the lifespan for individuals with ESRD. Approximately 71% of patients receiving treatment for ESRD undergo hemodialysis or peritoneal dialysis and 29% are recipients of kidney transplantation (Gupta, 2021, p. 72). Hemodialysis is a time-consuming demand, as treatment usually occurs three times a week for roughly three hours a session. Fatigue and discomfort are persistent symptoms leading up to and after sessions. Home hemodialysis or peritoneal dialysis allows patients to receive treatment from the comfort of their homes but requires a support system and environment capable of operating the dialysis machine safely. Kidney transplantation is accessible to those who meet extensive criteria and overcome the waitlist due to national organ shortages. Further, transplantation requires a lifelong commitment to immunosuppression management to prevent graft-rejection and subsequent failure. Despite the existence of several management methods, the lifestyle of individuals with this chronic condition is greatly altered due to the time, financial, and physical burden of CKD. Beyond physical hardship, ESRD often limits employment opportunities, worsens financial stressors, contributes to relationship strain, and impacts other important sectors of life. Thus, identification of the risks of CKD progression in patients who experienced AKI is critical to leading a longer and more comfortable life.

Cystic Fibrosis and Hypertension

It is not uncommon for chronic medical conditions to cause damage to the renal system. Cystic fibrosis (CF) is no exception. This autosomal recessive genetic disorder is caused by deleterious genetic variants in the CFTR gene, which encodes for the cystic fibrosis transmembrane conductance regulator (CFTR) protein (Dickinson & Collaco, 2021, p. 55). Lung disease is the primary manifestation in People with CF (PwCF) leading to a vicious cycle of thickened mucus secretions, chronic airway infections, inflammation, and eventually respiratory failure. In recent years, PwCF have experienced dramatic improvement in the severity of lung disease thanks to the Federal Drug Administration approved high efficiency modulator therapy (HEMT). As a result of the novel therapy, PwCF experience life expectancy approaching the general population. However, as PwCF age, they experiences extrapulmonary CF manifestations, including CF-related

diabetes mellitus (CFRD) and CKD (Dickinson & Collaco, 2021, p. 55). PwCF have at least 10-fold higher risk of ESRD than the general population and the incidence doubles every 10 years of follow up (Quon et al., 2011). Although AKI episodes resulting from frequent use of aminoglycoside antibiotics and/or CFRD have been implicated in CKD in PwCF, at least 1/3 have no identified cause (Burrows et al., 2022). As such, PwCF are a vulnerable group for CKD development and may benefit from early detection to delay disease progression.

Urinary Extracellular Vesicles

Recent research efforts demonstrate the potential that extracellular vesicles have in both clinical and diagnostic spaces due to their enclosed information. Specifically, urinary extracellular vesicles (uEvs) are membrane-bound structures that originate from the urinary tract, indicated by marker proteins such as CD9 and CD63. uEvs contain proteins and nucleic acids reflecting the physiological and possibly disease states of cells lining the urinary tract (Salih et al., 2014, p. 1). There is significant potential for uEvs for diagnostics, prognostics, and as therapeutic agents for various kidney diseases (Salih et al., 2014, p. 1). Beyond potential diagnostic and clinical applications, urine is a highly accessible, noninvasive biofluid. Urine collection can typically be self-administered, making samples easily obtainable for researchers and clinicians.

Gaps in Research

Current uEv research shows promising strides but remains inadequate. The Urine Task Force of the International Society for Extracellular Vesicles identifies shortcomings in current uEv research, emphasizing the lack of standardized approach for urine collection, uEv separation and measurement (Erdbrugger et al., 2021). The impact of pre-analytical variables on uEv quality has yet to be understood. Conditions creating variability in urine samples include, but are not limited to, the person's hydration status, diet, age, health status, time of collection and environmental factors. One possible reason that a standardized approach to urine collection and uEv isolation does not yet exist is the difficulty to control for the essentially limitless number of variables. To implement clinical use of uEvs, researchers must establish the groundwork that best positions medical professionals to access and utilize uEVs.

Proposed Solution

The first morning urine collection is a clinically accepted standardization measure limiting effects of fluid intake and the physiological effects of recumbent *versus* up-right body position on GFR. However, it is unknown how it affects uEv quantity and quality. Thus, we performed a pilot study to standardize urine collection based solely on overnight water-restricted status. Samples were provided by 10 volunteers under water-restriction (8 hours, 12AM-8AM) and non-restricted conditions. The eight-hour water-restriction period minimally impacted volunteers as restriction occurred overnight. Using identical differential centrifugation methods for uEv isolation, we seek to optimize uEv quality and quantity by size and concentration.

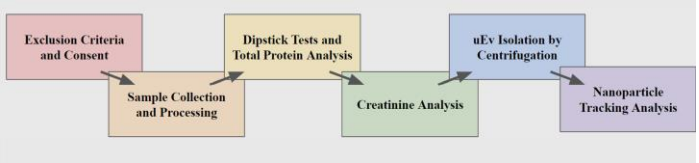


Fig. 1. Proposed methodology pipeline.

This proposed method seeks to standardize urine collection conditions and optimize uEv yield by isolating one of the many variables and establishing controls for the remaining variables. Controls in place include validating health status, age (20-22 years old), equal sex distribution (5F, 5M), consecutive collection days to minimize physiological changes, and no-alcohol period (24 hours prior to collection). The complete pipeline is outlined in **Fig. 1.**

Materials and Methods

Study Approval and Design Criteria

The approval for this study was an addition to IRB-HSR220336 (Cohort #3) for Kidney Function in PwCF. This enabled researchers to enroll volunteers to serve as a healthy reference group in the PwCF study. A study advertisement was sent via email to potential volunteers to gauge interest and go over inclusion and exclusion criteria. Of note, volunteers self-reported having no chronic conditions currently or in the past (**Appendix A**). Individuals who replied to the study advertisement and met all criteria were asked to provide basic demographics (**Appendix B**) and review consent documentation prior to signing documentation for enrollment. Once enrolled, subjects were assigned a sample ID (301-310). Samples from the water-restriction condition were labeled by the sample ID followed by -1 and samples from the no-restriction condition were labeled by the sample ID followed by -2. The 10 subjects agreed to partake in one eight-hour water-restriction period, provide two urine samples on consecutive days, and consume no alcohol within 24 hours of each collection. The water-restriction period was implemented to alter our independent variable, hydration level. Sample collection on consecutive days intended to serve as a control mechanism to minimize dietary and physiological changes that could impact results. The no-alcohol period served as another control mechanism to eliminate the influence of antidiuretics on hydration level of the volunteer samples.

Urine Collection and Processing

Each subject provided two samples: one from water-restriction conditions and one from no-restriction conditions. Sample collection occurred at individual homes upon first-morning urination. Then, samples were collected on each of the consecutive days at 8 AM and transported to the lab for processing. Initial processing involved urine dipstick analysis to measure Leukocytes, Nitrile, Urobilinogen, Protein, pH, Blood, Specific Gravity, Ketone, Bilirubin, and Glucose. These parameters are indicative of kidney health and allow us to verify that the ten volunteers are adequate to serve as part of a healthy reference group (**Appendix C**). The urine processing pipeline (**Appendix D**) separates cells from cell-free urine by centrifugation to produce the cell pellets (P1) and cell-free supernatant (S1) samples. The cell-free supernatant contains extracellular vesicles and is centrifuged once more to remove cell fragments (P2) from the cell fragments-free supernatant (S2). All pellets and supernatant samples were saved and stored in the -80 ℃ freezer.

Bradford Analysis for Total Protein

Total protein content is another useful metric to validate the kidney health. Proteinuria, or excess protein in the urine, is a common indicator of inefficient renal filtration. We prepared the protein assay with Bradford protein antibody and brought the protein assay buffer to room temperature. Then, we loaded 10 uL serial dilutions of controls (400, 200, 100, 50, 25, 12.5, 6.25, 0) in the left three columns. Next, we loaded 10 uL of raw urine to each well, followed by 200 uL of buffer to each well. Each sample was loaded in triplicates to account for variability. The microplate reader assessed the absorbance of the resultant plate at 595 nm wavelength (**Appendix E**).

Urinary Creatinine Analysis

One protein of particular interest is creatinine, as its urinary concentration provides insight regarding the subject's hydration. Creatinine is especially abundant in concentrated urine. Thus, it can be a useful tool for assessing if the water-restriction period was effective by comparing urinary creatinine concentration from water-restriction samples to no-restriction samples. First, we diluted raw urine (1:100 and 1:50) into 1.5 ml tubes and labeled them with the sample number. Next, 134 uL of standards (800, 400, 200, 100, 50, 25, 12.5, 0) and samples were loaded in triplicates into the wells followed by 34 uL of picric acid per well. Initial microplate reads at 525 nm wavelength provided baseline absorbance levels. Then, 34 uL 0.75 N was added to each well and the reaction occurred for 20 minutes. Color changes were observed and the resultant well plate was read again at 525 nm wavelength (**Appendix F**).

uEv Isolation

Centrifugation-based protocol was used to isolate uEvs **(Fig. 2)**. The cell fragments-free supernatant (S2) from initial processing served as the starting material. After bringing these samples to room temperature, roughly 25 mL of S2 samples were transferred to polycarbonate tubes. The samples were spun for 30 minutes at 16,000 rpm in 4 ℃. The new supernatants (S3) were poured out of the tubes and stored in the -80 ℃ freezer. The uEv pellets (P3) were resuspended in the polycarbonate tubes with 1 ml of 10mM HEPES + 2.5mM EDTA and transferred to the Eppendorf tubes. The P3 samples were spun down for 30 minutes at 15,200 rpm in 21 ℃. Upon completion, supernatant was carefully removed from each tube with a pipette to not disturb the pellet. The pellet was resuspended with 1 ml of the HEPES buffer. This process was repeated twice for a total of three washes and centrifugation in the Eppendorf tubes. All liquid was removed after the third round of centrifugation and P3 samples were stored in the 4 ℃ refrigerator overnight.

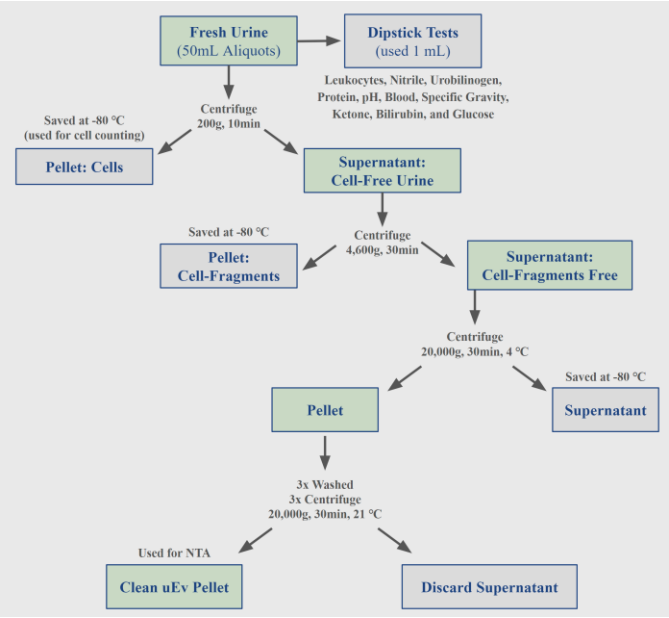


Fig. 2. Isolation pipeline adapted from protocol originally developed by Drs. Musante and Erdbrügger

Nanoparticle Tracking Analysis

The day following uEv isolation, the clean uEv pellets were quantified using the ZetaView Particle Tracking Analyzer. This enables individual tracking of nanoparticles in a physiological buffer (10mM HEPES + 2.5mM EDTA). Precise lasers track the particle movement and display video footage on the desktop application. Duplicate trial runs were performed on each of the 20 samples to account for variability. Only trials with three or fewer errors were used for analysis.

Results

Healthy Urine Sample Validation

Urine dipstick tests validated the health of this cohort. Dipstick screening for leukocytes, leukocyte esterase, nitrites, urobilinogen, protein, pH, blood, specific gravity, ketone, bilirubin, and glucose reveal that all 10 volunteers did not have medical conditions detectable by dipstick screening and were considered healthy. The conditions that can be detected by this method include most urinary tract infections, uncontrolled diabetes mellitus, liver disease, and acute or chronic kidney disease. In addition, urine specific gravity can inform about the person's hydration status.

Water-Deprivation Period Effectiveness

Our null hypothesis is that there is no difference between urinary creatinine between water-restriction conditions (day 1) and no-restriction conditions (day 2). The alternative hypothesis is that there is elevated urinary creatinine in the water-restricted samples, which we would expect to be the case if the restriction period was effective. We determined the T-Test statistic to be 3.14 and the degree of freedom to be 9. The corresponding significance level falls in the range of $0.01 < \alpha < 0.005$. We rejected the null hypothesis at an alpha level 0.01 and determined that urinary creatinine levels are elevated in water-deprivation conditions. It is important to note that all the volunteers exhibited the expected decline in urinary creatinine from day 1 to day 2, except for subjects 305 and 308 who we consider to be outliers (**Fig. 3**). One explanation for these two outliers could be that these individuals did not participate in the water-deprivation period.

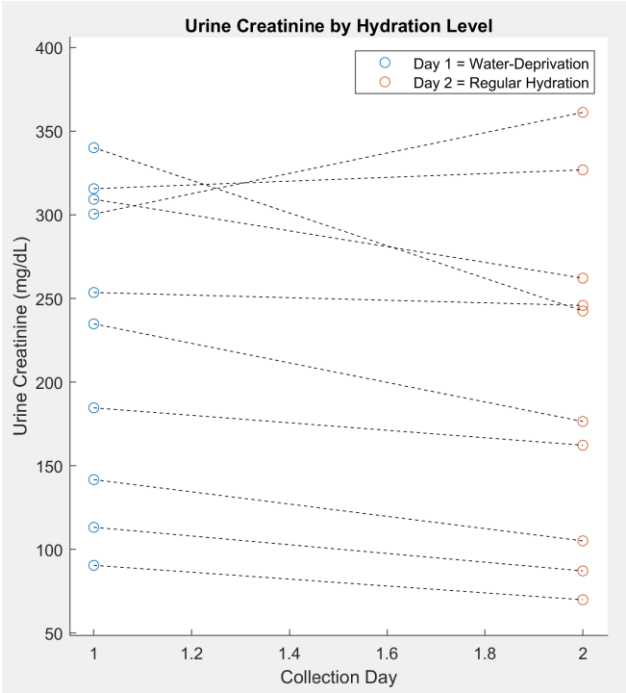


Fig. 3. Urinary creatinine shows elevated status in eight samples after water-restriction

Size and Concentration Outcomes

The clean uEv pellets provided from uEv isolation were resuspended in 10mM HEPES + 2.5mM EDTA for Nanoparticle Tracking Analysis. The ZetaView Particle Tracking Analyzer output PDFs containing critical information about each sample. Of particular interest is the average diameter and the concentration of particles in the sample. We found that there is not a significant difference between the size of uEvs or the concentration of uEvs between the water-deprivation samples and the regular hydration samples (**Figs. 4** and **5)**.

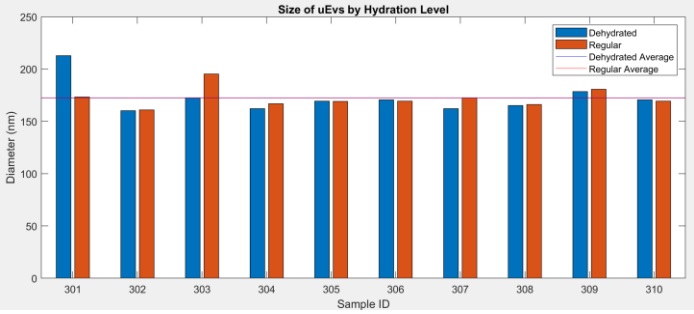


Fig. 4. Average diameter (nm) of uEVs by hydration level.

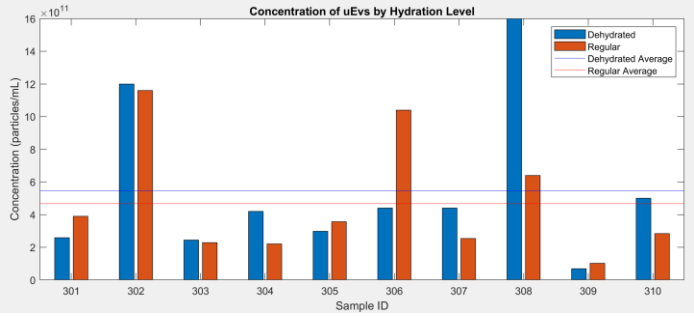


Fig. 5. Concentration (particles/mL) of uEvs by hydration level.

Discussion

Conclusion

Overall, our proposed method sought to determine if there is a difference between uEv size and concentration in water-restriction conditions compared to no-restriction conditions for a healthy volunteer cohort. These results were validated through several analyses. First, urine dipstick tests indicated that each of the volunteers demonstrated normal levels of the following metrics: Leukocytes, Nitrile, Urobilinogen, Protein, pH, Blood, Specific Gravity, Ketone, Bilirubin, and Glucose. Next, Bradford analysis for total protein ensured that no volunteers were experiencing proteinuria, which is indicative of improper kidney filtration. Last, urinary creatinine analysis verified the effectiveness of the water-restriction period as elevated creatinine content achieved statistical significance at an alpha level of 0.01. Despite the validation of adequate kidney health of the volunteers and the effectiveness of the water-restriction period, we determined that water-restriction does not have a significant impact on the size and concentration of uEvs for the methodology outlined above, including first-morning urine collection, urine processing and storage protocol, isolation via centrifugation, and Nanoparticle Tracking Analysis for measurement.

Pilot Study Limitations

The outlined work contributes to the standardization of urine collection for the purpose of uEv analyses. However, it is not without its limitations. It is worth noting that this pilot study uses a cohort of 10 subjects. Replicating this work with a greater sample size strengthens the findings. Prior to this research, we were unable to determine the sample size that enables this study to achieve statistical power. Necessary values, such as the mean and standard deviation of uEv size and concentration from a healthy urine sample, were not found in literature. Thus, this work provides the basis for power analyses to inform future sample sizes.

Another limitation in this research was in the variability of the urine samples. The establishment of controls supported the isolation of hydration as the variable of interest. However, these controls only account for health status, age, sex, consecutive collection days to minimize physiological changes, and alcohol use. There are several other factors that we did not ask about, such as food intake, time that participants slept, frequency of exercise, height, weight, and so on, that may affect urine concentration and content.

Implications for Future Research

Immediate next steps include replicating this proposed method focusing on another variable, as well as validating this proposed method using urine samples from PwCF. An interesting variable of interest is the time of day that collection occurs. Specifically, it is worth investigating how daily activities prior to collection may influence uEv content as opposed to first morning urine samples. First morning urine tends to be more concentrated and serves as another control mechanism as most study subjects had been sleeping prior to collection. However, daily activities, such as working out, may influence uEv content and could suggest an optimal time for collection. Next, it is important that this proposed methodology is applied to samples of PwCF to determine that this manner of urine collection and uEv isolation enable researchers to access the biomarkers of interest. Specifically, NGAL and Kim-1 are two transmembrane proteins that are present in uEvs upon initial injury to the kidneys. A urine sample supplied from a patient that is vulnerable to CKD may allow researchers to determine if this methodology enables identification of these markers. Additional validation should examine the presence of uEv marker proteins CD9 and CD63 to confirm that the uEvs originate from the urinary tract. Future studies may also include information on the quantity of water intake and include longer duration of water-restriction.

Downstream clinical applications include use of uEVs as diagnostic tools and therapeutic agents. Early detection of CKD is critical and uEvs can serve as a tool for doing so with their enclosed protein content. Further, uEvs are candidates for targeted therapeutic delivery due to natural targeting capacity, stability and wide biodistribution (Morrison et al., 2016).

End Matter

Acknowledgments

Agnieszka Swiatecka-Urban, MD (Lab) – Capstone Advisor

Uta Erdbrügger, MD (Lab) – Department of Nephrology

Hayrettin Yavuz, MD, PhD – Postdoctoral Research Associate

Iuliia Vitko, PhD – Clinical Research Coordinator

Soutik Goshal, PhD – Biostatician at UVA

Timothy Allen, PhD –Undergraduate Capstone Advisor

References

Burrows, N.R., Koyama, A. & Pavkov, M.E. Reported Cases of End-Stage Kidney Disease - United States, 2000-2019. MMWR Morb Mortal Wkly Rep 71, 412-415 (2022).

Dickinson, K. M., & Collaco, J. M. (2021). Cystic Fibrosis. Pediatrics in review, 42(2), 55–67. https://doi.org/10.1542/pir.2019-0212

Erdbrügger, U., Blijdorp, C. J., Bijnsdorp, I. V., Borràs, F. E., Burger, D., Bussolati, B., Byrd, J. B., Clayton, A., Dear, J. W., Falcón-Pérez, J. M., Grange, C., Hill, A. F., Holthofer, H., Hoorn, E. J., Jenster, G., Jimenez, C. R., Junker, K., Klein, J., Knepper, M. A., … Martens-Uzunova, E. S. (2021). Urinary extracellular vesicles: A position paper by the Urine Task Force of the International Society for Extracellular Vesicles. J. Extracell. Vesicles, 10, e12093. https://doi.org/10.1002/jev2.12093

Gupta, R., Woo, K., & Yi, J. A. (2021). Epidemiology of end-stage kidney disease. Seminars in vascular surgery, 34(1), 71–78. doi:10.1053/j.semvascsurg.2021.02.010

Morrison, E. E., Bailey, M. A., & Dear, J. W. (2016). Renal extracellular vesicles: from physiology to clinical application. The Journal of physiology, 594(20), 5735–5748. https://doi.org/10.1113/JP272182

National Kidney Foundation. (2023, November 15). High blood pressure and chronic kidney disease. National Kidney Foundation. https://www.kidney.org/news/newsroom/factsheets/High-Blood-Pressure-and-CKD

Pollak, M. R., Quaggin, S. E., Hoenig, M. P., & Dworkin, L. D. (2014). The glomerulus: the sphere of influence. Clinical Journal of the American Society of Nephrology : CJASN, 9(8), 1461–1469. https://doi.org/10.2215/CJN.09400913

Quon, B.S., Mayer-Hamblett, N., Aitken, M.L., Smyth, A.R. & Goss, C.H. (2011). Risk factors for chronic kidney disease in adults with cystic fibrosis. Am J Respir Crit Care Med 184, 1147-1152.

Salih, M., Zietse, R., & Hoorn, E. J. (2014). Urinary extracellular vesicles and the kidney: biomarkers and beyond. American journal of physiology. Renal physiology, 306(11), F1251–F1259. https://doi.org/10.1152/ajprenal.00128.2014

Tonelli A. R. (2013). Pulmonary hypertension survival effects and treatment options in cystic fibrosis. Current opinion in pulmonary medicine, 19(6), 652–661. https://doi.org/10.1097/MCP.0b013e3283659e9f

U.S. Department of Health and Human Services. (n.d.). Chronic kidney disease (CKD) - NIDDK. National Institute of Diabetes and Digestive and Kidney Diseases. https://www.niddk.nih.gov/health-information/kidney-disease/chronic-kidney-disease-ckd

Vaidya, S. R. & Aeddula, N. R. (2022). Chronic Renal Failure. StatPearls Publishing. 1-29. https://www.ncbi.nlm.nih.gov/books/NBK535404/

APPENDIX

Appendix A: Healthy Cohort Inclusion/Exclusion Checklist

HSR220336-Kidney Function in People with Cystic Fibrosis (PwCF)

Subject ID: _____

Healthy Cohort Inclusion/Exclusion Checklist

Must have a **YES** box checked for each of the following criteria for study inclusion:			
#	**Inclusion Criteria**	**YES**	**NO**
1	Subject called responding to the Study Advertisement		
2	Subject self-reported as Healthy		
3	Able to provide consent and verbal assent (where applicable)		
4	Age 5+		
5	Able to provide urine sample independently		
6	Subject self-reported as not having any associated chronic diseases, to the best of their knowledge, or are on chronic medication regiments		
If **NO** is answered to any of the above, the subject will not continue in the study.			

Must have a **NO** box checked for each of the following criteria for study inclusion:			
#	**Exclusion Criteria**	**YES**	**NO**
1	History of kidney disease		
2	History of cancer		
3	Any other chronic illness		
4	Current use of antibiotics		
5	Urinary symptoms or UTI (dysuria, frequency, urgency)		
6	Subject self-reported as Pregnant		
7	Currently menstruating		
If **YES** is answered to any of the above, the subject will not continue in the study.			

Study Team Signature: _____ Date: _____

Appendix B: Healthy Cohort Demographic Information

HSR220336-Kidney Function in People with Cystic Fibrosis (PwCF)

Subject ID: _____

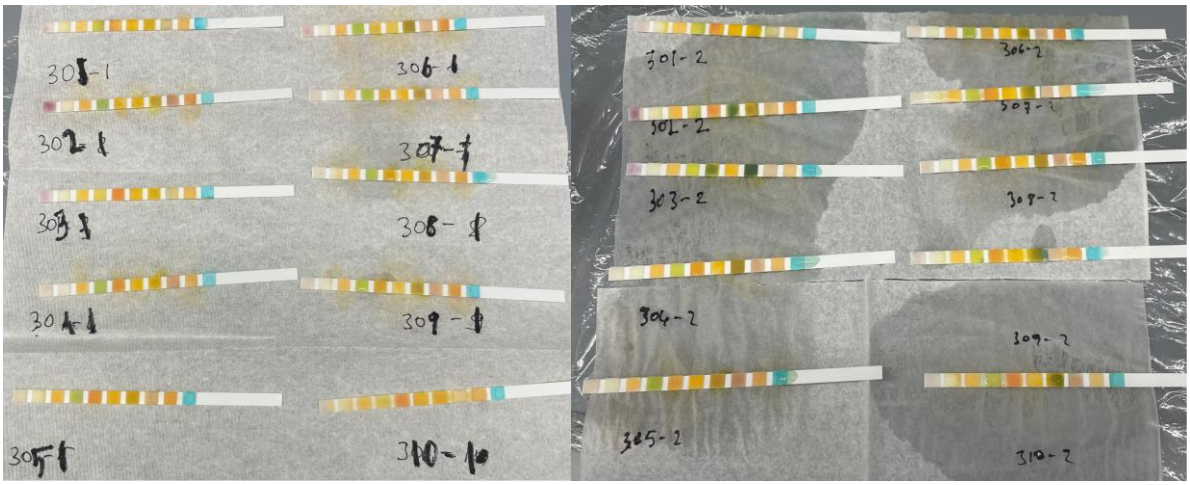
Healthy Cohort Demographic Information

#		
1	Age	
2	Gender	
3	Race	
4	Ethnicity	
5	Postal Zip Code	

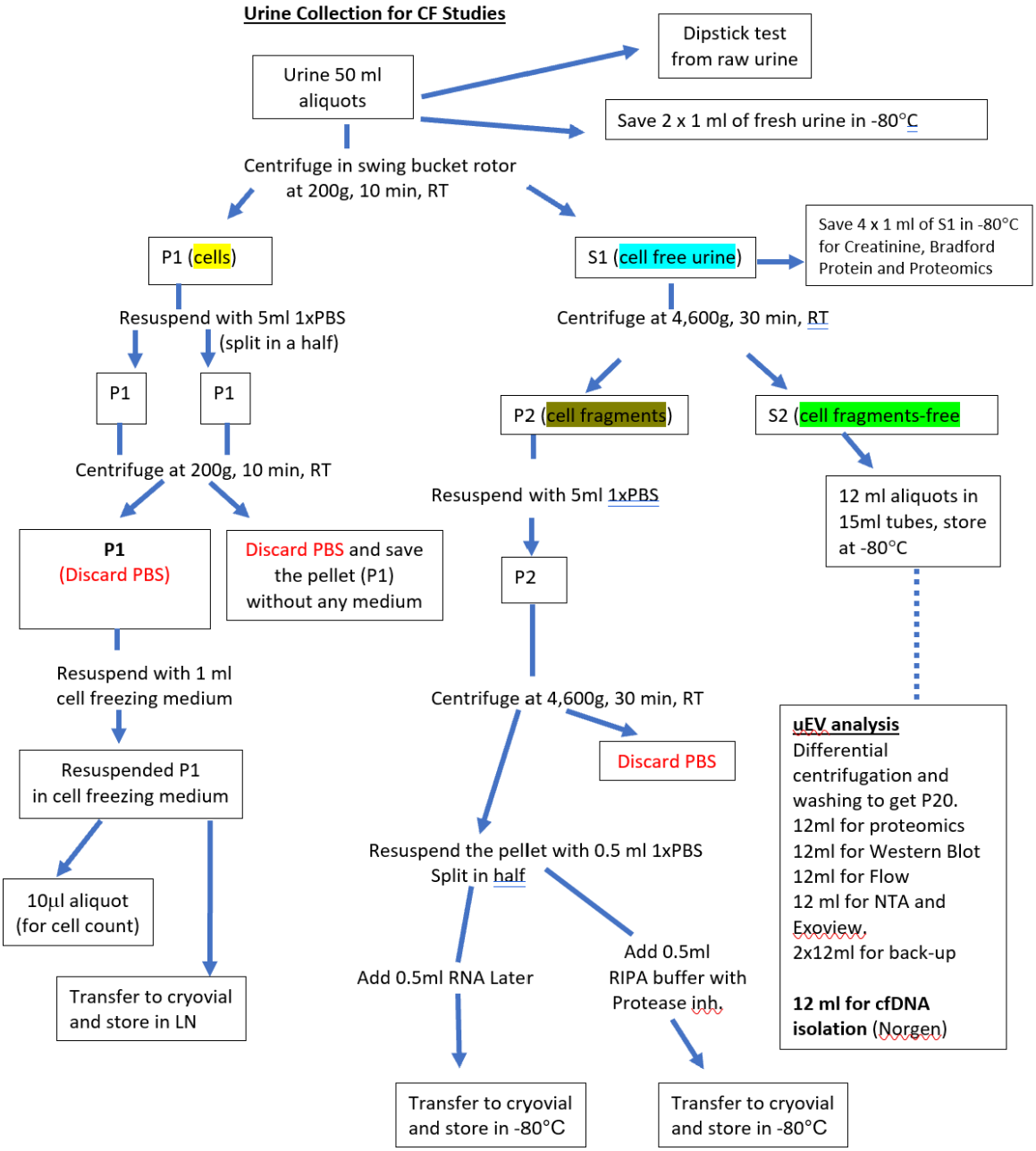
Study Team Signature: _____ Date: _____

Appendix C: Urine Dipstick Test Results

	301-1	302-1	303-1	304-1	305-1	306-1	307-1	308-1	309-1	310-1	301-2	302-2	303-2	304-2	305-2	306-2	307-2	308-2	309-2	310-2
Leu	15 +/-	125 ++	15 +/-	-	-	15 +/-	-	-	-	-	-	70+	70+	-	-	-	-	-	-	-
Nit	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Uro	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Pro	-	30+	-	-	-	15 +/-	15 +/-	15 +/-	-	-	-	15 +/-	-	-	15 +/-	-	-	-	-	-
pH	6	6	5	6	5	6	6	6	6	5	5	5	7.5	6	6	6	5	5	5	5
Blo	-	-	-	-	-	-	-	-	-	-	-	2+	-	-	-	-	-	-	-	-
SG	1.03	1.03	1.03	1.03	1.025	1.015	1.025	1.03	1.03	1.03	1.03	1.02	1.005	1.025	1.03	1.03	1.025	1.02	1.02	1.015
Ket	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Bil	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Glu	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-



Appendix D: Urine Collection Protocol Developed by Hayrettin Yavuz, MD, PhD



Legend:
P, pellet; S, supernatant; LN, liquid nitrogen

Appendix E: Total Protein Absorbance Raw Data

CONTENT	1	2	3	4	5	6	7	8	9	10	11	12
A	standard 400	standard 400	standard 400	301-1	301-1	301-1	309-1	309-1	309-1	307-2	307-2	307-2
B	standard 200	standard 200	standard 200	302-1	302-1	302-1	310-1	310-1	310-1	308-2	308-2	308-2
C	standard 100	standard 100	standard 100	303-1	303-1	303-1	301-2	301-2	301-2	309-2	309-2	309-2
D	standard 50	standard 50	standard 50	304-1	304-1	304-1	302-2	302-2	302-2	310-2	310-2	310-2
E	standard 25	standard 25	standard 25	305-1	305-1	305-1	303-2	303-2	303-2	HY	HY	HY
F	standard 12.5	standard 12.5	standard 12.5	306-1	306-1	306-1	304-2	304-2	304-2			
G	standard 6.25	standard 6.25	standard 6.25	307-1	307-1	307-1	305-2	305-2	305-2			
H	standard 0	standard 0	standard 0	308-1	308-1	308-1	306-2	306-2	306-2			

RESULTS	1	2	3	4	5	6	7	8	9	10	11	12
A	0.786	0.803	0.79	0.363	0.369	0.358	0.377	0.368	0.381	0.385	0.383	0.391
B	0.529	0.586	0.581	0.484	0.488	0.497	0.349	0.349	0.346	0.428	0.429	0.419
C	0.469	0.465	0.47	0.361	0.351	0.366	0.342	0.337	0.341	0.369	0.36	0.342
D	0.395	0.381	0.389	0.39	0.395	0.408	0.428	0.433	0.442	0.353	0.346	0.348
E	0.337	0.347	0.354	0.414	0.417	0.416	0.355	0.324	0.352	0.347	0.344	0.346
F	0.335	0.319	0.32	0.459	0.467	0.476	0.372	0.304	0.367			
G	0.314	0.309	0.307	0.418	0.421	0.422	0.435	0.436	0.445			
H	0.299	0.294	0.301	0.417	0.426	0.421	0.417	0.418	0.424			

Appendix F: Creatinine Absorbance Raw Data

CONTENT 301-305	1	2	3	4	5	6	7	8	9	10	11	12
A	standard 800	standard 800	standard 800	301-1 1:100	301-1 1:100	301-1 1:100	305-1 1:100	305-1 1:100	305-1 1:100	304-2 1:100	304-2 1:100	304-2 1:100
B	standard 400	standard 400	standard 400	301-1 1:50	301-1 1:50	301-1 1:50	305-1 1:50	305-1 1:50	305-1 1:50	304-2 1:50	304-2 1:50	304-2 1:50
C	standard 200	standard 200	standard 200	302-1 1:100	302-1 1:100	302-1 1:100	301-2 1:100	301-2 1:100	301-2 1:100	305-2 1:100	305-2 1:100	305-2 1:100
D	standard 100	standard 100	standard 100	302-1 1:50	302-1 1:50	302-1 1:50	301-2 1:50	301-2 1:50	301-2 1:50	305-2 1:50	305-2 1:50	305-2 1:50
E	standard 50	standard 50	standard 50	303-1 1:100	303-1 1:100	303-1 1:100	302-2 1:100	302-2 1:100	302-2 1:100	HY 1:100	HY 1:100	HY 1:100
F	standard 25	standard 25	standard 25	303-1 1:50	303-1 1:50	303-1 1:50	302-2 1:50	302-2 1:50	302-2 1:50	HY 1:50	HY 1:50	HY 1:50
G	standard 12.5	standard 12.5	standard 12.5	304-1 1:100	304-1 1:100	304-1 1:100	303-2 1:100	303-2 1:100	303-2 1:100			
H	standard 0	standard 0	standard 0	304-1 1:50	304-1 1:50	304-1 1:50	303-2 1:50	303-2 1:50	303-2 1:50			

BEFORE NAOH 301-305	1	2	3	4	5	6	7	8	9	10	11	12
A	0.042	0.041	0.044	0.046	0.043	0.047	0.047	0.052	0.045	0.044	0.047	0.044
B	0.042	0.042	0.043	0.043	0.047	0.058	0.046	0.047	0.046	0.044	0.045	0.044
C	0.041	0.042	0.042	0.044	0.043	0.045	0.043	0.042	0.044	0.044	0.043	0.044
D	0.042	0.041	0.041	0.045	0.045	0.045	0.043	0.043	0.043	0.044	0.045	0.044
E	0.044	0.047	0.044	0.041	0.043	0.043	0.045	0.042	0.042	0.043	0.042	0.042
F	0.041	0.04	0.042	0.044	0.043	0.044	0.044	0.047	0.045	0.043	0.042	0.044
G	0.042	0.041	0.04	0.043	0.044	0.046	0.042	0.044	0.054			
H	0.041	0.04	0.04	0.045	0.044	0.044	0.048	0.047	0.049			

RESULTS 301-305	1	2	3	4	5	6	7	8	9	10	11	12
A	1.478	1.499	1.45	0.266	0.273	0.274	0.568	0.567	0.567	0.503	0.501	0.493
B	0.804	0.812	0.818	0.431	0.43	0.446	0.958	0.98	0.974	0.868	0.88	0.859
C	0.459	0.465	0.466	0.6	0.611	0.607	0.23	0.227	0.229	0.663	0.673	0.67
D	0.265	0.267	0.268	1.122	1.123	1.122	0.35	0.356	0.352	1.118	1.129	1.121
E	0.172	0.179	0.177	0.23	0.237	0.233	0.475	0.466	0.467	0.247	0.247	0.245
F	0.134	0.135	0.134	0.365	0.364	0.359	0.833	0.797	0.792	0.383	0.379	0.387
G	0.107	0.108	0.108	0.594	0.574	0.56	0.201	0.205	0.205			
H	0.088	0.085	0.086	1.045	0.988	0.958	0.317	0.31	0.307			

CONTENT 306-310	1	2	3	4	5	6	7	8	9	10	11	12
A	standard 800	standard 800	standard 800	306-1 1:100	306-1 1:100	306-1 1:100	310-1 1:100	310-1 1:100	310-1 1:100	309-2 1:100	309-2 1:100	309-2 1:100
B	standard 400	standard 400	standard 400	306-1 1:50	306-1 1:50	306-1 1:50	310-1 1:50	310-1 1:50	310-1 1:50	309-2 1:50	309-2 1:50	309-2 1:50
C	standard 200	standard 200	standard 200	307-1 1:100	307-1 1:100	307-1 1:100	306-2 1:100	306-2 1:100	306-2 1:100	310-2 1:100	310-2 1:100	310-2 1:100
D	standard 100	standard 100	standard 100	307-1 1:50	307-1 1:50	307-1 1:50	306-2 1:50	306-2 1:50	306-2 1:50	310-2 1:50	310-2 1:50	310-2 1:50
E	standard 50	standard 50	standard 50	308-1 1:100	308-1 1:100	308-1 1:100	307-2 1:100	307-2 1:100	307-2 1:100			
F	standard 25	standard 25	standard 25	308-1 1:50	308-1 1:50	308-1 1:50	307-2 1:50	307-2 1:50	307-2 1:50			
G	standard 12.5	standard 12.5	standard 12.5	309-1 1:100	309-1 1:100	309-1 1:100	308-2 1:100	308-2 1:100	308-2 1:100			
H	standard 0	standard 0	standard 0	309-1 1:50	309-1 1:50	309-1 1:50	308-2 1:50	308-2 1:50	308-2 1:50			

BEFORE NAOH 306-310	1	2	3	4	5	6	7	8	9	10	11	12
A	0.043	0.043	0.043	0.044	0.045	0.043	0.042	0.042	0.044	0.042	0.042	0.045
B	0.042	0.043	0.044	0.045	0.044	0.044	0.044	0.044	0.043	0.044	0.043	0.044
C	0.041	0.045	0.041	0.043	0.044	0.042	0.043	0.043	0.043	0.042	0.042	0.042
D	0.042	0.043	0.042	0.043	0.043	0.045	0.046	0.055	0.051	0.048	0.042	0.042
E	0.044	0.045	0.045	0.043	0.046	0.045	0.05	0.047	0.058			
F	0.044	0.043	0.043	0.043	0.05	0.049	0.051	0.058	0.046			
G	0.046	0.041	0.042	0.042	0.043	0.043	0.044	0.045	0.047			
H	0.042	0.041	0.039	0.043	0.042	0.041	0.046	0.044	0.045			

RESULTS 306-310	1	2	3	4	5	6	7	8	9	10	11	12
A	1.424	1.319	1.365	0.471	0.468	0.475	0.296	0.302	0.303	0.323	0.328	0.324
B	0.79	0.749	0.738	0.775	0.78	0.765	0.476	0.482	0.48	0.546	0.546	0.549
C	0.439	0.436	0.433	0.439	0.447	0.444	0.456	0.465	0.461	0.239	0.245	0.246
D	0.256	0.257	0.253	0.718	0.714	0.73	0.771	0.709	0.786	0.388	0.385	0.383
E	0.167	0.168	0.167	0.584	0.576	0.545	0.356	0.357	0.362			
F	0.13	0.128	0.129	0.948	0.917	0.939	0.571	0.6	0.587			
G	0.107	0.104	0.103	0.363	0.367	0.377	0.602	0.584	0.591			
H	0.084	0.082	0.081	0.578	0.587	0.584	0.959	0.929	0.96			