Proximal Tubular Secretion, Cardiovascular Events, and Kidney Clearance of Medications

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Abstract

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Background:

Current detection and monitoring of chronic kidney disease (CKD) rely primarily on glomerular filtration rate (GFR) and albuminuria. However, the kidneys perform many important functions outside of the glomerulus. Among them, the secretion of organic anions and cations by kidney proximal tubules is a vital non-glomerular function for removing retained metabolic wastes, toxins, and drugs from the circulation. We have previously developed a novel assay to estimate proximal tubular secretory clearance based on the quantification of endogenous secretory solutes in blood and urine and detected associations of compromised tubular secretory function with more severe metabolic complications, and higher risks of CKD progression and all-cause mortality in patients with mild-to-moderate CKD. However, the association between lower proximal tubular secretory clearance and cardiovascular events, the most common cause of death among patients with CKD, is uncertain. In addition, despite the primacy of tubular secretion in kidney drug handling, current medication dosing strategies are based on estimates of GFR (or

creatinine clearance) under the assumption that secretion and filtration are tightly linked within an individual. This assumption has been challenged by multiple pharmacokinetics studies in recent years. However, few studies have empirically determined relationships between tubular secretory clearance and kidney drug elimination.

Objectives:

The overall goal of this dissertation is to further determine the clinical significance of kidney secretory clearance in the setting of CKD by determining associations with cardiovascular events and kidney drug elimination. Specifically, we aim to: 1) test associations of estimated proximal tubular secretory clearances with cardiovascular events in a national cohort study of CKD; 2) compare proximal tubular secretory clearances with measured GFR for the prediction of the kidney drug elimination in a population with a wide range of kidney function.

Methods:

For aim 1, we included 3,407 participants with CKD from the Chronic Renal Insufficiency Cohort (CRIC) study. We measured the plasma and urine concentration of eight secretory solutes using liquid chromatography tandem mass spectrometry (LC-MS/MS). We used Cox proportional hazards regression to estimate associations of secretory solute clearances at baseline with incident heart failure, myocardial infarction, and stroke events, adjusting for estimated GFR and other potential confounding characteristics. For aim 2, we evaluated 54 individuals with a wide range of estimated GFRs (21–140 ml/min/1.73m^2). We administered single doses of furosemide and famciclovir (converted to penciclovir) and calculated their kidney clearances

based on sequential plasma and timed urine measurements. Concomitantly, we quantified eight endogenous secretory solutes in plasma and urine using LC-MS/MS, and we measured GFR by iohexol disappearance (iGFR). We computed a summary secretion score as the scaled average of the secretory solute clearances. We used linear regression and leave-one-out cross-validation to calculate prediction statistics.

Results:

Secretion and cardiovascular events: In a national, representative cohort of CKD, we found kidney clearances of secretory solutes to be modestly correlated with estimated GFR. Lower 24-hour kidney clearances of secretory solutes were associated with incident heart failure and myocardial infarction, but not incident stroke, over long-term follow-up after controlling for demographics and traditional risk factors. However, these associations were removed by adjustment for estimated GFR. Secretion and kidney drug clearance: In participants with and without CKD, the kidney clearance of furosemide was correlated with iGFR ($r = 0.84$) and the summary secretion score ($r = 0.86$). The average proportionate difference between model-predicted and measured drug clearance, i.e., the mean proportionate error (MPE), between iGFR-predicted and measured furosemide clearance was 30.0%. MPEs for individual secretory solute clearances ranged from 27.3% - 48.0%, with the lowest MPE observed for the summary secretion score (24.1%). These predictive errors were statistically indistinguishable. Penciclovir kidney clearance was correlated with iGFR ($r = 0.78$) and with the summary secretion score ($r = 0.85$), with similar predictive accuracy of iGFR and secretory clearances. Combining iGFR with pyridoxic acid, indoxyl sulfate, and the summary secretion score modestly improved the prediction of furosemide clearance.

Conclusions:

In summary, we found no clinically or statistically relevant association between the kidney clearances of endogenous secretory solutes and incident heart failure, myocardial infarction, and stroke after adjustment for eGFR in a national cohort study of CKD. These findings suggest that tubular secretory clearance provides little additional information about the development of cardiovascular events beyond GFR among patients with mild-to-moderate CKD. We also found that the kidney clearance of secretory solutes and iGFR demonstrated relatively similar accuracy for predicting the clearances of furosemide and penciclovir, with some improvement from combining both kidney measures. These findings provide some reassurance that GFR is a useful surrogate for predicting secretory drug clearance in stable persons and suggest cautious optimism for future improvements in kidney drug dosing strategies by incorporating measures of secretory clearance.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF SUPPLEMENTAL MATERIALS

Chapter 1: Introduction

Approximately 30 million adults in the United States were estimated to have chronic kidney disease (CKD), which is defined by a substantial reduction in the estimated glomerular filtration rate (GFR) or the existence of albuminuria.[1] Patients with CKD are more susceptible to cardiovascular disease, infections, bone problems, kidney failure, and death.[1] CKD related deaths have doubled between 1990 and 2010.[2] CKD also poses a substantial economic burden on society. In 2010, stage 2 to stage 4 CKD cost Medicare $49 billion, while stage 5 CKD, also known as end-stage kidney disease (ESKD) cost almost $33 billion alone, mainly due to the requirement of maintenance dialysis or kidney transplantation.[3,4]

The current detection and monitoring of CKD focus primarily on measurements of glomerular functions.[5] Indeed, the development of standardized equations to estimate GFR has promoted the early detection of CKD and prediction of its complications.[5-7] However, the kidneys perform many other crucial functions outside of the glomerular compartment, including the secretion and reabsorption of organic solutes, the maintenance of acid-base and salt-water balance, and the synthesis of hormones. These functions are not typically assessed due to a lack of validated methods of measurement.

The secretion of organic anions and cations by the proximal tubules represents a vital intrinsic kidney function for removing retained solutes from the circulation.[8] Transporters on the basolateral surface of proximal tubular epithelial cells, including organic anion transporters (OAT) 1 and 3 and the organic cation transporter (OCT) 2, shuttle solutes from the post-

glomerular capillaries into the cell.[9-11] These solutes are subsequently extruded into the urine by active transporters on the luminal cell surface, primarily members of the ATP-binding cassette transporter superfamily.[12] The coordinated processes required for tubular secretion, which include mitochondrial respiration, energy-coupled cell transport against a chemical gradient, and maintenance of cell polarity, may be particularly susceptible to injury in CKD, suggesting possible contrasts between glomerular filtration and tubular secretory functions. Consequently, secretory clearances may capture novel and prognostically relevant important information about kidney health status that is not revealed by measurements of glomerular function alone. Furthermore, although the intact nephron hypothesis predicts close correlations between GFR and other essential kidney functions,[13-16] the severity of metabolic complications of kidney disease and histological features on kidney biopsy vary across people with similar GFRs, suggesting unobserved variability in tubular and glomerular functions across individuals and underlying disease processes.[17]

However, despite the biological significance of proximal tubular secretion, no established methods have been widely accepted for estimating this essential kidney function, mainly due to a lack of reliable endogenous markers and non-standardized laboratory assays. Tests based on the administration of exogenous agents that are cleared exclusively by tubular secretion are expensive and impractical to perform in large populations. In order to solve this problem, Dr. Kestenbaum and colleagues have developed a novel assay to estimate secretory clearance based on quantification of endogenous secretory solutes in blood and urine. Specifically, eight secretory solutes have shown promise for estimating proximal tubular secretory clearance, i.e., pyridoxic acid, isovalerylglycine, tiglylglycine, kynurenic acid, xanthosine, cinnamoylglycine,

indoxyl sulfate, and p-cresol sulfate. These solutes were selected based on known specificity for OAT1/3 transporters in the proximal tubules, an increase in circulating levels in rodent models following OAT1/3 knockout, a high degree of protein binding, low diurnal variation in plasma, and/or higher kidney clearances than GFR or creatinine clearance.[18-20]

CKD is associated with greatly increased risks of heart failure and atherosclerotic cardiovascular disease.[21-23] For example, in a community-based population of over one million adults, a graded association was found between lower estimated GFR and higher risk of cardiovascular events, defined as hospitalization for coronary disease, heart failure, stroke, or peripheral arterial disease.[21] The excess cardiovascular risk associated with CKD has been attributed partly to a greater burden of traditional risk factors, such as diabetes and hypertension, but also to metabolic disturbances arising from kidney disease itself.[24-27] Prolonged exposure to uremic toxins, which are primarily eliminated by tubular secretion, has been demonstrated to exert cardiovascular toxicity in experimental models. For example, indoxyl sulfate and p-cresol sulfate stimulate the production of reactive oxygen species (ROS), as well as the activity of NADH/NADPH oxidase and glutathione peroxidase, which in turn, induce oxidative stress.[28-31] Oxidative stress plays critical roles in the development of atherosclerosis, including dyslipidemia, endothelial dysfunction, initial plaque formation and progression, and plaque rupture.[30-35] In addition, our pilot study of 1,240 participants from the Chronic Renal Insufficiency Cohort (CRIC) study found that lower proximal tubular secretory solutes clearances were associated with higher levels of C-reactive protein, a marker of systemic inflammation, and higher levels of triglycerides, a cardiovascular risk factor, independent of GFR. However, the association between decreased

proximal tubular secretory function itself and cardiovascular outcomes among patients with CKD remains uncertain.

The kidneys clear administered drugs from the circulation by two distinct mechanisms: glomerular filtration and proximal tubular secretion. Of these, secretion is the primary mechanism of kidney drug elimination. Many drugs and their metabolites are fully or partially cleared by tubular secretion, including antivirals (e.g., tenofovir, famciclovir), antibiotics (e.g., penicillins, cephalosporins), diuretics (e.g., furosemide), and antidiabetic agents (e.g., metformin). Secretion is capable of eliminating protein-bound drugs that are inefficiently filtered due to the size and charge of their binding proteins and the selectivity of the glomerular basement membrane, and can achieve high rates of clearance that greatly exceed the GFR. Impaired renal function is a major risk factor for preventable medication-related hospital admission.[36] Moreover, the expression of tubular organic anion transporters is reduced in rodent models of CKD, which is likely due to tubular loss or an adaptive response to uremic solute exposure,[37-39] suggesting potentially altered kidney medication clearance by proximal tubular secretion in CKD.

Despite the primacy of tubular secretion in kidney drug handling, current medication dosing strategies are based on estimates of GFR (or creatinine clearance) under the assumption that secretion and filtration are tightly linked within an individual. The assumption that GFR represents a reliable proxy of kidney drug elimination was challenged in a study where measured GFR (^{51}Cr-EDTA clearance) was found to be poorly correlated with tubular cationic transport.[40]

Moreover, a review of published pharmacokinetic data identified differences between GFR-predicted and actual kidney elimination for 48% of evaluated drugs.[41] However, few studies have empirically determined relationships between tubular secretory clearance and kidney drug elimination.

This dissertation evaluated the consequences of compromised proximal tubular secretory clearances in a large cohort study population and a hands-on study of drug-dosing. These studies evaluated the implications of incorporating proximal tubular secretion into the assessment of kidney function with several applications. First, the presence/absence of an association between proximal tubular secretory clearance and cardiovascular events could further elucidate the role of the kidneys in the development of cardiovascular disease. Second, linking kidney medication clearance with the proven mechanism of drug elimination could promote more safe and efficacious kidney drug dosing strategies.

Chapter 2. Association Between Kidney Clearance of Secretory Solutes and Cardiovascular Events: The CRIC Study

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ABSTRACT

Background: Kidney tubular secretory clearance is an innate mechanism for eliminating protein-bound solutes that cannot be efficiently filtered through the glomerulus. Many secretory solutes that derive from dietary sources are putative uremic toxins that could exert cardiovascular toxicity in humans. However, the association between lower kidney clearances of secretory solutes and cardiovascular events among patients with chronic kidney disease (CKD) remains uncertain.

Methods: We evaluated 3,407 participants from the Chronic Renal Insufficiency Cohort (CRIC) study. We measured concentrations of secretory solutes in plasma samples and paired timed urine collections at baseline using liquid chromatography tandem mass spectrometry (LC-MS/MS). The CRIC study adjudicated heart failure, stroke, and myocardial infarction events through centralized review of medical records by at least two study physicians. We used Cox regression to evaluate associations of secretory solute clearances with cardiovascular events adjusting for estimated GFR (eGFR) and other potential confounders.

Results: Participants in this study were characterized by a mean age of 56 years; 45% women; 41% Black; and a median eGFR of 43 ml/min/1.73m^2. The kidney clearances of secretory solutes range from a median of 10 ml/min for p-cresol sulfate to a median of 456 ml/min for pyridoxic acid. Lower 24-hour kidney clearances of secretory solutes were associated with incident heart failure and myocardial infarction, but not incident stroke, over long-term follow-up after

controlling for demographics and traditional risk factors. However, these associations were attenuated and not significant after adjustment for eGFR.

Conclusions: In a national cohort study of CKD, we found no clinically or statistically relevant association between the kidney clearances of endogenous secretory solutes and incident heart failure, myocardial infarction, and stroke after adjustment for eGFR. These findings suggest that tubular secretory clearance provides little additional information about the development of cardiovascular events beyond GFR among patients with mild-to-moderate CKD.

INTRODUCTION

The presence of chronic kidney disease (CKD), as assessed by the estimated glomerular filtration rate (eGFR), is associated with greatly increased risk of heart failure and atherosclerotic cardiovascular disease.[21-23] The excess cardiovascular risk associated with CKD has been attributed partly to a greater burden of traditional risk factors, such as diabetes and hypertension, but also to metabolic disturbances arising from kidney disease itself, including the activation of pro-inflammatory and oxidative stress pathways and the promotion of vascular and soft tissue calcification.[24-27]

In addition to filtering freely circulating solutes, the kidneys directly extract retained substances from the circulation via tubular secretion. Transporters on the basolateral surface of proximal tubular epithelial cells, including organic anion transporters (OAT) 1 and 3 and the organic cation transporter (OCT) 2, shuttle solutes from the post-glomerular capillaries into the cell.[9-11] These solutes are subsequently extruded into the urine by active transporters on the luminal cell surface, primarily members of the ATP-binding cassette transporter superfamily.[12] Tubular secretory clearance is an innate mechanism for eliminating protein-bound solutes and drugs that cannot be efficiently filtered due to selectivity of the glomerular basement membrane. Many secretory solutes that derive from dietary sources are putative uremic toxins associated with wide-ranging clinical and metabolic disturbances, described by the European Uremic Toxin Work Group.[42] For example, indoxyl sulfate and p-cresol sulfate induce endothelial cell damage, stimulate vascular smooth muscle cell proliferation, and activate pro-fibrotic cardiac pathways in animal models.[43-46]. Kynurenic acid, a protein-bound metabolite of tryptophan metabolism, impairs cardiac mitochondrial coupling and ATP synthesis.[47]

We hypothesized that lower native kidney clearances of secretory solutes would be associated with greater risks of cardiovascular outcomes in non-dialyzed persons with CKD. To test this hypothesis, we measured baseline 24-hour kidney clearances of eight endogenous solutes suspected to be eliminated primarily by tubular secretion in 3,407 persons from a national cohort study of CKD. We evaluated associations of these baseline kidney clearances with incident heart failure, myocardial infarction, and stroke over follow-up after controlling for eGFR and the presence of albuminuria.

METHODS

Data source and study population

The Chronic Renal Insufficiency Cohort (CRIC) Study is a multicenter prospective cohort study that recruited 3,939 patients with chronic kidney disease (CKD) from 2003 to 2007.[48-50] The CRIC study excluded persons with an estimated glomerular filtration rate (GFR) <20 ml/min/1.73m^2 at baseline, a history of kidney transplantation, polycystic kidney disease, multiple myeloma, pregnancy, HIV infection, cirrhosis, severe heart failure, and those receiving active immunosuppression. For the current study, we further excluded 532 CRIC study participants who did not have an available 24-hour urine and plasma sample at baseline for the measurement of secretory solutes. We then restricted analyses of incident heart failure events to persons without prevalent heart failure at baseline (N=3,072); analyses of incident myocardial infarction to persons without prevalent myocardial infarction (N=3,128); and analyses of incident

stroke to persons without prevalent stroke (N=3,077). The institutional review boards at all CRIC sites approved the study protocol. All participants provided written informed consent.

Measurements of secretory solute clearance

We measured the baseline kidney clearance of eight endogenous solutes suspected to be eliminated primarily by proximal tubular secretion: pyridoxic acid, isovalerylglycine, tiglylglycine, kynurenic acid, cinnamoylglycine, indoxyl sulfate, and p-cresol sulfate. We previously selected these organic solutes based on known specificity for OAT1/3 transporters in the proximal tubules, an increase in circulating levels in rodent models following OAT1/3 knockout, a high degree of protein binding, low diurnal variation in plasma, and/or higher kidney clearances than GFR or creatinine clearance.[18-20]

We measured total (not free) plasma and urine concentration of secretory solutes using solid phase extraction and targeted liquid chromatography tandem mass spectrometry (LC-MS/MS).[51,52] Plasma samples underwent protein precipitation prior to solid phase extraction. Data were normalized to labeled purified compounds (internal standards) added to each well. A single point calibration method was used to account for potential drift by measuring five replicates of calibrators during each run (pooled human serum and urine). We determined absolute concentrations of secretory solutes in the external calibrators by quantitative nuclear magnetic resonance (NMR) and standard addition of purified compounds. Intra- and inter-assay coefficients of variation for these secretory solutes range from 3.4% to 14.7% in plasma concentrations and from 4.5% to 10.1% in urine.

We calculated the kidney clearance of each secretory solute as:

$$Clearance\ (X) = [(U_X * V) / P_X]$$

In this equation, U_X represents the concentration of the secretory solute in the 24-hour urine sample, V represents the corresponding urine volume in ml per minute, and P_X represents the concentration of the solute in plasma.

Measurement of outcomes

Individual primary outcomes of this study are incident heart failure, myocardial infarction, and stroke. The CRIC study identified hospitalizations via telephone calls alternating every six months with in-clinic visits.[53-55] Study personnel retrieved medical records with codes relevant to study outcomes for centralized adjudicated review. Two study physicians reviewed all possible heart failure, myocardial infarction, and stroke events for classification. Definite or probable heart failure events were defined using Framingham and ALLHAT (Antihypertensive and Lipid-Lowering Treatment to Prevent Heart Attack Trial) criteria based on clinical symptoms, radiographic evidence of pulmonary congestion, physical examination findings, and echocardiographic imaging, if available.[56,57] Definite or probable myocardial infarction events were determined based on characteristic symptoms, cardiac biomarkers, and electrocardiographic data.[58] Definite stroke events were defined based on the sudden onset of neurologic symptoms supported by imaging findings. Probable stroke events were determined as sudden or rapid onset of one major or two minor neurologic signs or symptoms lasting for more than 24 hours or until the patient died with no evidence of hemorrhage or infarction on computed tomography or

magnetic resonance imaging performed within 24 hours of the onset of symptoms.[53] Secondary outcomes included all heart failure events (incident and recurrent), and ischemic and hemorrhagic strokes assessed separately.

Measurements of covariates

Participant self-reported sociodemographic characteristics and medical histories at baseline. Medication use was determined using the inventory method.[48,49] GFR was estimated using an equation developed in 1,433 CRIC participants who completed ^{125}-Iothalamate GFR (iGFR) clearance studies, based on serum creatinine and cystatin C concentrations, age, sex, and Black race.[59] Serum creatinine concentrations were measured using an enzyme-based assay with values traceable to isotope dilution mass spectrometry (Hitachi Vitros 950 AT). Serum cystatin C concentrations were measured using a Siemens BNII instrument and longitudinal control materials were used to correct for drift over time when using different calibrator and reagent lots.[60] 24-hour urine albumin was measured on the Siemens Immulite.[59,61] The mean of the latter two out of three seated blood pressure measurements was used for analysis.[62] Triglyceride and high-density lipoprotein were measured by spectrophotometry, and low-density lipoprotein by β quantification after separation by ultracentrifugation.[63] Hemoglobin was measured at each CRIC clinical center.[64] The CRIC study defined diabetes mellitus by a fasting glucose concentration ≥126 mg/dL, a non-fasting glucose ≥ 200 mg/dL, or the use of antidiabetic medication. Plasma glucose was measured on the Hitachi Vitros 950 AT [65]

Statistical analyses

We evaluated the kidney clearances of each secretory solute individually and in combination using a scaled summary score. For the summary score, we first min-max normalized each clearance to a common 0-100 scale:

$$\textit{Standardized clearance} = \frac{\textit{ln(secretory clearance)} - \textit{min(ln(secretory clearance))}}{\textit{range(ln(secretory clearance))}} * 100$$

where *ln(clearance)* is the kidney clearances of secretory solutes after natural log-transformation, *min(ln(clearance))* is the minimum value of ln-clearance, and *range(ln(clearance))* is the difference between the maximum and minimum values. We then calculated the summary score as the mean of the eight standardized clearances (Supplemental Figure 2.1). For descriptive purposes, we computed univariate Pearson correlations between log-transformed secretory clearances and log-transformed eGFR, and among the individual log-transformed secretory clearances.

We used Cox proportional hazards regression to estimate associations of secretory solute clearances at baseline with each incident cardiovascular event. For analyses of incident events, follow-up time began at the baseline exam, when secretory clearances were measured, and was continued until either the first occurrence of the event of interest or the data were censored due to death, withdrawal, loss to follow-up, or the end of the follow-up period (May 2014), whichever occurred first. We built nested models to control for potential confounding characteristics. Model 1 adjusted for age, race, and sex. Model 2 additionally adjusted for clinical sites, educational attainment, smoking status, body mass index, systolic blood pressure, blood triglyceride levels,

history of diabetes, history of cardiovascular disease (except for the outcome being evaluated in the model), 24-hour urinary albumin excretion, and the use of angiotensin-converting-enzyme inhibitors or angiotensin II receptor blockers, loop diuretics, and beta blockers. Model 3 additionally adjusted for eGFR based on the CRIC study creatinine and cystatin C equation. We used analogous Poisson regression models to evaluate associations between secretory solute clearances and the rate of all heart failure events (incident and recurrent). For this analysis, we did not exclude participants with prevalent heart failure at baseline. In sensitivity analyses, we evaluated associations with ischemic stroke and hemorrhagic stroke separately. We repeated our analyses stratified by Black vs. non-Black race, baseline diabetes status, and baseline categories of eGFR. To address competing risk by death, we used the subdistribution proportional hazard model developed by Fine and Grey to evaluate associations with the subdistribution hazard ratio of cardiovascular events.[66] We then tested the association between plasma concentrations of secretory solutes and incident cardiovascular events. We tested proportional hazards assumption of Cox models using scaled Schoenfeld residuals. We detected no evidence of violation of the proportional hazards assumption. We used the Hommel method to correct for multiple comparisons (nine comparisons in Tables 2.3-2.5).[67] We used complete-cases analysis, as covariates were missing in less than 1% of study participants. Analyses were performed using Stata/IC 14.2 (StataCorp. 2015. Stata Statistical Software: Release 14. College Station, TX) and RStudio 3.6.3 (R Core Team 2017, Vienna, Austria).

RESULTS

Study population and characteristics

This ancillary study included 3,407 CRIC study participants at baseline. Their mean age was 56 years; 45% were women; 41% were Black; 12% were Hispanic; and the median $eGFR_{CRIC}$ was 43 ml/min/1.73m^2 (IQR: 32-55 ml/min/1.73m^2). Among the eight secretory solutes evaluated, pyridoxic acid exhibited the highest 24-hour kidney clearance (median: 456 ml/min, Supplemental Table 2.1) and p-cresol sulfate the lowest (median: 10 ml/min). The kidney clearances of six of the eight secretory solutes were higher than eGFR. We observed moderate correlations between eGFR and the clearances of the individual secretory solutes, ranging from 0.40 for cinnamoylglycine to 0.61 for kynurenic acid. Participants with higher summary secretion scores, defined by the scaled average of the individual solute clearances, had higher eGFRs, were relatively younger, and were more likely to be male and non-Black compared to participants with lower secretion scores (Table 2.1). Participants with higher summary secretion scores also had a lower prevalence of cardiovascular disease, lower systolic blood pressures, lower 24-hour urine albumin excretion, lower blood triglycerides, and were less likely to use beta-blockers and loop diuretics.

Associations of kidney clearances of secretory solutes with heart failure

Over a median follow-up of 8.0 years, 439 participants developed a first definite or probable heart failure event. The unadjusted incidence of heart failure was 1.2 events per 100 person-years among participants in the highest quartile of the summary secretion score and 3.2 events per 100 person-years among participants in the lowest quartile (Table 2.2). After adjustment for age, race, sex (Model 1), and other risk factors (Model 2), lower kidney clearances of most secretory solutes, and a lower summary secretion score, were associated with greater risks of incident heart failure (Table 2.3). However, these associations were substantially attenuated by further

adjustment for eGFR (Model 3), with only isovalerylglycine remaining. Similar results were observed for all heart failure events (incident and recurrent) and for the subdistribution hazard ratio of heart failure (Supplemental Tables 2.2 and 2.3). The fully adjusted association between the summary secretion score and incident heart failure was statistically similar across categories of baseline eGFR, race, and diabetes status (Figure 2.1).

Associations of kidney clearances of secretory solutes with myocardial infarction

There were 310 incident myocardial infarction events over a median follow-up of 9.2 years. Unadjusted incidence rates of myocardial infarction were higher among participants who had lower summary secretion scores (Table 2.2). The kidney clearances of three secretory solutes were associated with myocardial infarction in the demographic and health characteristics adjusted Model 2 (Table 2.4). However, again these associations largely disappeared after further adjustment for eGFR.

Associations of kidney clearances of secretory solutes with stroke

One hundred and twenty-three incident stroke events occurred over a median follow-up of 9.3 years. No appreciable associations of the individual secretory solute clearances with incident stroke were observed after adjustment for demographics and health characteristics (Model 2; Table 2.5), nor for the subdistribution hazard ratio of stroke (Supplemental Table 2.2). Null results also were observed when ischemic stroke and hemorrhagic stroke were analyzed separately (Supplemental Table 2.4).

In models evaluating plasma concentration of the secretory solutes, rather than their respective kidney clearances, none of the solutes under evaluation were associated with incident heart failure, myocardial infarction, or stroke after adjustment for eGFR and other confounding characteristics (Supplemental Table 2.5).

DISCUSSION

In a national prospective cohort study of CKD, we observed lower 24-hour kidney clearances of secretory solutes to be associated with incident heart failure and myocardial infarction, but not incident stroke, over long-term follow-up after controlling for demographic characteristics and traditional risk factors. However, these associations were largely removed by adjustment for eGFR measured contemporaneously with the secretory solute clearances. Results were similar across categories of race, diabetes status, and eGFR and in models that considered the competing risk of death. Moreover, plasma concentrations of secretory solutes were not associated to any appreciable degree with the cardiovascular outcomes of interest after adjustment. Although methodological limitations of this study preclude definitive conclusions, these findings do not provide support for a causal role of retained secretory solutes on cardiovascular outcomes in non-dialyzed persons with an eGFR >20 ml/min/1.73m^2.

Several of the solutes included in this study are uremic toxins that demonstrate cardiovascular toxicity in experimental models. Indoxyl sulfate and p-cresol sulfate stimulate the production of reactive oxygen species (ROS), as well as the activity of NADH/NADPH oxidase and

glutathione peroxidase, which in turn, induce oxidative stress.[28-31] Oxidative stress, and downstream inflammation play critical roles in the development of atherosclerosis, including dyslipidemia, endothelial dysfunction, initial plaque formation and progression, and plaque rupture.[30-35] Uremic toxins may also impair vascular endothelial cell functioning. Both indoxyl sulfate and p-cresol sulfate, when tested at uremic concentrations, were found to reduce the proliferation and repair of endothelial cells in vitro.[33] Higher concentrations of indoxyl sulfate further inhibit the migration and tube formation of endothelial cells, possibly through the depletion of nitric oxide availability.[68-70] Indoxyl sulfate and kynurenic acid also act as agonists of the transcription factor aryl hydrocarbon receptor (AhR), which mediates a prothrombotic and proatherosclerotic phenotype of endothelial cells.[71-78] In addition, animal studies have found higher blood indoxyl sulfate concentration associated with the development of diastolic dysfunction by promoting cardiac hypertrophy and fibrosis.[79] Finally, uremic toxins may also increase the sympathetic nervous activity among patients with CKD, aggravating hypertension by stimulating presympathetic neurons in the rostral ventrolateral medulla, an important brain region that regulates blood pressure.[80] These studies provide biological plausibility for an impact of the secretory solutes evaluated in this study on cardiovascular disease and suggest that diminished kidney clearances of these solutes are associated with one or more adverse cardiovascular outcomes.

Previous human studies have reported associations between plasma concentrations of uremic toxins and cardiovascular events in patients receiving maintenance hemodialysis. For example, indoxyl sulfate, p-cresol, and p-cresol sulfate are associated with incident heart failure, cardiovascular death, and a composite outcome of cardiovascular events, defined as

cardiovascular death, myocardial infarction, myocardial ischemia, ischemic stroke, and new onset of peripheral vascular disease.[81-85] Indoxyl sulfate and p-cresol were found to be associated with cardiovascular events in some studies of patients with mild-moderate CKD; however, these studies did not adjust for GFR in their analyses.[86,87] In the current study, once GFR was accounted for, neither lower kidney clearances of secretory solutes nor higher plasma concentration of these solutes themselves were associated with heart failure, myocardial infarction, or stroke in patients with CKD and an estimated GFR $\geq$20 ml/min/1.73m^2. It could well be that the burden of secretory solutes in our study population was insufficiently high to impact the selected cardiovascular outcomes above and beyond GFR.

Our findings suggest that the kidney clearances of the measured solutes and plasma concentrations of these solutes are negligibly associated with cardiovascular outcomes for a given level of GFR. It is possible that tubular secretory clearance and GFR are too closely linked to reliably distinguish their individual effects among medically stable outpatients with CKD. However, we previously observed associations of these secretory clearances with CKD progression and mortality after adjustment for GFR in this cohort, including adjustment for GFR measured by iothalamate clearance.[88,89] Another possible explanation is that only the unbound portion of these solutes, which is eliminated by glomerular filtration, exerts cardiovascular toxicity. This hypothesis is supported by previous studies in which total p-cresol and p-cresol sulfate concentrations were less strongly associated with cardiovascular events compared with their free counterparts, in spite of a high correlation between these two variables.[87] Finally, the cardiovascular outcomes under study may have heterogeneous etiologies, such that compromised

tubular clearance of secretory solutes could be more strongly associated with these outcomes due to certain etiologies, but less so with other etiologies.

We also previously reported an independent association between lower secretory clearances and all-cause mortality in the CRIC cohort.[88] This raises a question about the underlying mechanism of the association seen with death, as in the current study we found no association between lower secretory clearances and cardiovascular events, which is the leading cause of death in patients with CKD.[90-92] Taken together, this evidence may suggest potential associations between compromised proximal tubular secretion and other major causes of death in CKD, such as cancer and infections. Previous studies have linked the accumulation of uremic toxins with cancer and infection; however, the relationship between lower kidney clearances of secretory solutes and these outcomes remains unknown, warranting further investigations.[28,29,93,94]

Important strengths of this study include precise measurements of plasma and urine concentrations of secretory solutes using targeted LC-MS/MS assays and calculation of 24-hour kidney clearances of these solutes to reduce dependency on their production. Studies were conducted in a large, nationally representative prospective cohort study of CKD patients with physician adjudicated cardiovascular outcomes. Potential confounding characteristics, including eGFR, were measured using standardized procedures. Several important limitations of the study should be considered. The CRIC study excluded persons with severely reduced eGFR at baseline (<20 ml/min/1.73m^2), precluding investigation of whether impaired tubular secretory clearance contributes to cardiovascular disease in the most advanced stages of CKD, in which circulating

concentrations of secretory solutes are highest. Second, we excluded persons who reported a prior history of heart failure, myocardial infarction, and stroke at baseline to investigate associations of secretory clearance with the initial development of these outcomes. However, subclinical cardiovascular disease is highly prevalent in CKD, suggesting that the mechanistic processes contributing to the study outcomes were already underway. Third, 24-hour kidney clearances of secretory solutes are subject to measurement variability due to fluctuation in circulating concentrations of these solutes over the urine collection period. Decades of work have refined the estimations of GFR. In contrast, measurements of secretory solute clearance are less well established and prone to greater variability due to the timed urine collection procedure.

In summary, we found no clinically or statistically relevant association between the kidney clearances of endogenous secretory solutes and incident heart failure, myocardial infarction, and stroke after adjustment for eGFR in a national cohort study of CKD. These findings suggest that tubular secretory clearance provides little additional information about the development of cardiovascular events beyond GFR among patients with mild-to-moderate CKD. Further studies are needed to elucidate why cardiovascular toxicity of uremic toxins does not translate into increased risk of cardiovascular events, as well as why lower kidney clearances of secretory solutes are associated with all-cause mortality in patients with CKD despite the null associations with cardiovascular events found in the current study.

Table 2.1 Baseline participant characteristics by quartiles of the summary secretion score. [a]

Characteristics	Quartile 1 N = 851	Quartile 2 N = 852	Quartile 3 N = 853	Quartile 4 N = 851
$eGFR_{CRIC}$, ml/min/1.73m^{2} [b]	29 (24, 38)	38 (31, 47)	47 (40, 56)	59 (49, 69)
Age, years	59 ± 11	58 ± 11	58 ± 11	56 ± 10
Female	449 (53)	428 (50)	345 (40)	307 (36)
Black	406 (48)	359 (42)	323 (38)	319 (37)
Hispanic	141 (17)	109 (13)	95 (11)	60 (7)
Body mass index, kg/m^{2}	31 ± 8	32 ± 8	32 ± 7	33 ± 8
Education categories (1-4)				
Less than high school	251 (29)	193 (23)	140 (16)	95 (11)
High school graduate	185 (22)	170 (20)	149 (17)	143 (17)
Some college	244 (29)	241 (28)	253 (30)	245 (29)
College graduate or higher	171 (20)	248 (29)	311 (36)	367 (43)
Current smoker	131 (15)	108 (13)	100 (12)	96 (11)
History of Diabetes	407 (48)	438 (51)	419 (49)	368 (43)
History of cardiovascular disease	346 (41)	309 (36)	280 (33)	198 (23)
History of peripheral vascular disease	83 (10)	66 (8)	38 (4)	41 (5)
Systolic blood pressure, mmHg	134 ± 25	128 ± 22	125± 20	125 ± 20
Diastolic blood pressure, mmHg	72 ± 14	71 ± 13	71 ± 12	72 ± 12

Lab measurements				
24-hour urine albumin, mg/24h[b]	192 (27, 890)	69 (13, 586)	47 (9, 453)	24 (7, 261)
Triglyceride, mg/dL	165 ± 126	161 ± 127	160 ± 108	141 ± 102
LDL, mg/dL	102 ± 37	101 ± 35	102 ± 36	106 ± 32
HDL, mg/dL	47 ± 16	48 ± 16	47 ± 16	48 ± 15
Hemoglobin, g/dL	12.0 ± 1.7	12.4 ± 1.7	12.8 ± 1.8	13.2 ± 1.7
Medications				
Statin	450 (53)	494 (58)	485 (57)	438 (52)
ACEi / ARB	525 (62)	625 (73)	619 (73)	545 (64)
Beta Blocker	488 (58)	464 (55)	414 (49)	320 (38)
Loop diuretic	437 (52)	365 (43)	272 (32)	201 (24)
Thiazide diuretic	179 (21)	260 (31)	281 (33)	244 (29)

[a] For continuous variable: mean ± SD; for categorical variables: N (%).

[b] Median (Interquartile range)

Table 2.2 Number of cardiovascular outcomes and their incidence rate by quartiles of the summary score [a, b]

		All	Q1	Q2	Q3	Q4
Heart failure (N = 3,072) [c]	No. of events	439	154	126	89	70
	Incidence rate	2.0	3.2	2.4	1.6	1.2
Myocardial infarction (N = 3,128) [c]	No. of events	310	99	84	78	49
	Incidence rate	1.2	1.8	1.4	1.2	0.7
Stroke (N = 3,077) [c]	No. of events	123	32	33	32	26
	Incidence rate	0.5	0.6	0.5	0.5	0.4

[a] Median follow-up time of heart failure, myocardial infarction, and stroke was 8.0, 9.2 and 9.3 years, respectively.

[b] Incidence rates shown as numbers of events per 100 person-years, calculated from unadjusted Poisson regression.

[c] Participants with prevalent heart failure, myocardial infarction, or stroke at baseline were excluded from the corresponding analysis of each outcome.

Table 2.3 Associations between secretory solute clearances and incident heart failure [a, b, c]

	Model 1			Model 2			Model 3		
	HR [d]	95% CI	P-value	HR	95% CI	P-value	HR	95% CI	P-value
Pyridoxic acid	1.34	1.23-1.46	< 0.001*	1.19	1.08-1.31	< 0.001*	1.08	0.96-1.21	0.185
Isovalerylglycine	1.44	1.32-1.58	< 0.001*	1.21	1.09-1.35	< 0.001*	1.13	1.01-1.26	0.037
Tiglylglycine	1.36	1.25-1.47	< 0.001*	1.21	1.10-1.33	< 0.001*	1.11	0.99-1.23	0.066
Kynurenic acid	1.41	1.27-1.57	< 0.001*	1.20	1.06-1.35	0.004*	1.03	0.89-1.19	0.678
Xanthosine	1.25	1.15-1.35	< 0.001*	1.14	1.05-1.24	0.001*	1.08	0.99-1.17	0.081
Cinnamoylglycine	1.14	1.07-1.23	< 0.001*	1.09	1.01-1.17	0.026	1.03	0.95-1.11	0.539
Indoxyl sulfate	1.36	1.23-1.50	< 0.001*	1.18	1.05-1.32	0.005*	1.04	0.92-1.20	0.513
p-cresol sulfate	1.20	1.11-1.30	< 0.001*	1.08	0.98-1.18	0.124	0.97	0.87-1.08	0.541
Summary score	1.61	1.42-1.82	< 0.001*	1.30	1.13-1.50	< 0.001*	1.10	0.92-1.30	0.297

[a] Results from Cox proportional hazard regression. There were 439 incident heart failure events.

[b] Model 1 adjusted for age, race, and sex. Model 2 additionally adjusted for clinical sites, educational attainment, smoking status, history of cardiovascular disease, diabetes, body mass index, systolic blood pressure, triglyceride, 24-hour urinary albumin excretion, and ACEi/ARB, loop diuretics, and beta blockers. Model 3 additionally adjusted for estimated GFR.

[c] * denotes statistical significance after correction for multiple comparisons using the Hommel method.

[d] Hazard ratio expressed per 50% lower secretory solute clearance or per 10 units lower summary secretion score

Table 2.4 Associations between secretory solute clearances and incident myocardial infarction [a, b, c]

	Model 1			Model 2			Model 3		
	HR[d]	95% CI	P-value	HR	95% CI	P-value	HR	95% CI	P-value
Pyridoxic acid	1.26	1.13-1.39	< 0.001*	1.13	1.00-1.27	0.049	1.03	0.90-1.18	0.673
Isovalerylglycine	1.32	1.18-1.48	< 0.001*	1.11	0.97-1.26	0.118	1.03	0.90-1.18	0.670
Tiglylglycine	1.31	1.18-1.46	< 0.001*	1.16	1.03-1.32	0.017	1.08	0.93-1.24	0.314
Kynurenic acid	1.36	1.19-1.54	< 0.001*	1.14	0.99-1.32	0.077	1.01	0.86-1.20	0.870
Xanthosine	1.25	1.14-1.37	< 0.001*	1.15	1.05-1.26	0.003*	1.10	1.00-1.21	0.054
Cinnamoylglycine	1.06	0.97-1.16	0.185	1.01	0.93-1.11	0.790	0.96	0.87-1.06	0.407
Indoxyl sulfate	1.18	1.04-1.33	0.009*	0.99	0.86-1.14	0.891	0.85	0.73-1.00	0.049
p-cresol sulfate	1.15	1.04-1.28	0.006*	1.03	0.91-1.15	0.658	0.93	0.82-1.06	0.293
Summary score	1.48	1.27-1.72	< 0.001*	1.22	1.02-1.45	0.030	1.04	0.84-1.30	0.702

[a] Results from Cox proportional hazard regression. There were 310 incident myocardial infarction events.

[b] Model 1 adjusted for age, race, and sex. Model 2 additionally adjusted for clinical sites, educational attainment, smoking status, history of cardiovascular disease, diabetes, body mass index, systolic blood pressure, triglyceride, 24-hour urinary albumin excretion, and ACEi/ARB, loop diuretics, and beta blockers. Model 3 additionally adjusted for estimated GFR.

[c] * denotes statistical significance after correction for multiple comparisons using the Hommel method.

[d] Hazard ratio expressed per 50% lower secretory solute clearance or per 10 units lower summary secretion score

Table 2.5 Associations between secretory solute clearances and incident stroke (definite + probable) [a, b, c]

	Model 1			Model 2			Model 3		
	HR[d]	95% CI	P-value	HR	95% CI	P-value	HR	95% CI	P-value
Pyridoxic acid	1.28	1.08-1.51	0.005*	1.17	0.96-1.42	0.119	1.17	0.94-1.46	0.158
Isovalerylglycine	1.20	1.01-1.44	0.041	1.07	0.87-1.30	0.534	1.04	0.84-1.30	0.701
Tiglylglycine	1.18	1.00-1.40	0.053	1.06	0.88-1.28	0.552	1.03	0.83-1.29	0.761
Kynurenic acid	1.15	0.93-1.42	0.191	0.99	0.78-1.26	0.939	0.93	0.69-1.24	0.602
Xanthosine	1.03	0.89-1.21	0.662	1.01	0.86-1.18	0.943	0.98	0.83-1.16	0.850
Cinnamoylglycine	1.04	0.91-1.19	0.562	1.00	0.87-1.15	0.986	0.98	0.84-1.14	0.792
Indoxyl sulfate	0.99	0.81-1.20	0.897	0.84	0.67-1.05	0.134	0.79	0.62-1.02	0.067
p-cresol sulfate	1.00	0.85-1.19	0.964	0.92	0.77-1.11	0.412	0.87	0.71-1.08	0.204
Summary score	1.20	0.94-1.54	0.150	1.01	0.76-1.35	0.918	0.94	0.66-1.33	0.714

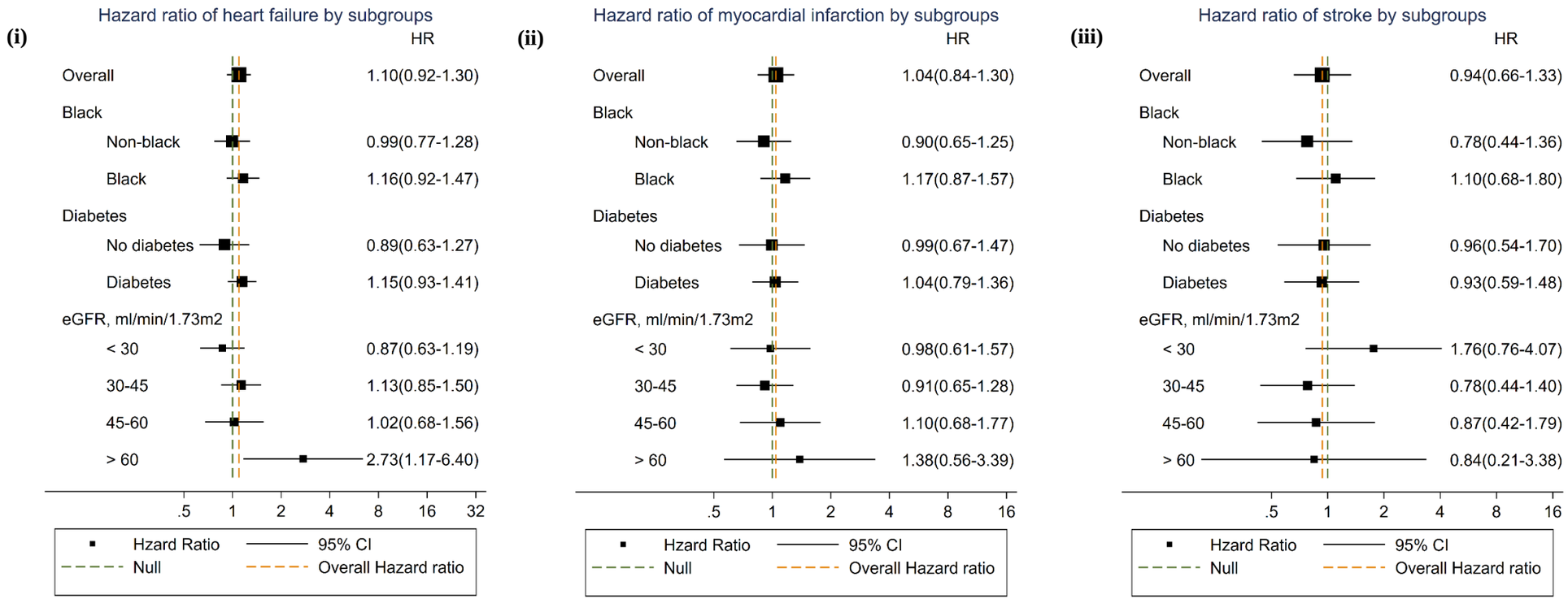
[a] Results from Cox proportional hazard regression. There were 123 incident stroke events.

[b] Model 1 adjusted for age, race, and sex. Model 2 additionally adjusted for clinical sites, educational attainment, smoking status, history of cardiovascular disease, diabetes, body mass index, systolic blood pressure, triglyceride, 24-hour urinary albumin excretion, and ACEi/ARB, loop diuretics, and beta blockers. Model 3 additionally adjusted for estimated GFR.

[c] * denotes statistical significance after correction for multiple comparisons using the Hommel method.

[d] Hazard ratio expressed per 50% lower secretory solute clearance or per 10 units lower summary secretion score

Figure 2.1 Association of summary secretion score with incident heart failure (i), myocardial infarction (ii), and stroke (iii) events by subgroups [a, b, c, d]



[a] Solid square: hazard ratio; solid line: 95% confidence interval; green dashed line: null; orange dashed line: overall hazard ratio.

[b] Results from Cox proportional hazard regression.

[c] Model adjusted for age, race, sex, clinical sites, educational attainment, smoking status, history of cardiovascular disease, diabetes, body mass index, systolic blood pressure, triglyceride, 24-hour urinary albumin excretion, ACEi/ARB, loop diuretics, beta blockers, and estimated GFR.

[d] Hazard ratio expressed per 10 units lower summary secretion score.

Supplemental Table 2.1 Kidney clearances of secretory solutes.[a]

	Median kidney clearance (ml/min, IQR)	Correlation with eGFR[b]	Molecular weight (g/mol)
Pyridoxic acid	456 (282, 715)	0.57	183
Isovalerylglycine	225 (142, 348)	0.50	159
Tiglylglycine	182 (111, 286)	0.56	157
Kynurenic acid	92 (63, 136)	0.61	189
Xanthosine	77 (46, 121)	0.44	284
Cinnamoylglycine	57 (33, 101)	0.40	205
Indoxyl sulfate	34 (22, 51)	0.56	213
p-cresol sulfate	10 (6, 15)	0.52	188

[a] IQR: inter-quartile range; eGFR: estimated GFR.

[b] Pearson correlations between log-transformed secretory solute clearances and log-transformed eGFR, each standardized to 1.73 m^2 body surface area.

Supplemental Table 2.2 Associations between secretory solute clearances and subdistribution hazard ratio of cardiovascular events[a,b]

	Heart failure			Myocardial infarction			Stroke		
	HR [c]	95% CI	P-value	HR	95% CI	P-value	HR	95% CI	P-value
Pyridoxic acid	1.06	0.94-1.19	0.361	1.00	0.88-1.14	0.949	1.14	0.91-1.42	0.264
Isovalerylglycine	1.09	0.97-1.23	0.136	1.00	0.86-1.15	0.963	1.02	0.84-1.24	0.827
Tiglylglycine	1.08	0.96-1.21	0.182	1.02	0.89-1.17	0.774	1.01	0.83-1.22	0.958
Kynurenic acid	1.01	0.87-1.17	0.893	0.99	0.83-1.17	0.881	0.90	0.68-1.18	0.437
Xanthosine	1.06	0.97-1.15	0.210	1.09	1.00-1.19	0.043[d]	0.97	0.83-1.13	0.675
Cinnamoylglycine	1.01	0.92-1.09	0.903	0.94	0.85-1.04	0.233	0.97	0.84-1.11	0.649
Indoxyl sulfate	1.04	0.91-1.19	0.529	0.86	0.73-1.01	0.071	0.77	0.58-1.01	0.058
p-cresol sulfate	0.97	0.88-1.08	0.592	0.94	0.83-1.08	0.405	0.88	0.73-1.08	0.219
Summary score	1.05	0.89-1.25	0.553	1.00	0.81-1.25	0.966	0.90	0.64-1.26	0.532

[a] Results from subdistribution proportional hazard regression with death as the competing event.

[b] Model adjusted for age, race, sex, clinical sites, educational attainment, smoking status, history of cardiovascular disease, diabetes, body mass index, systolic blood pressure, triglyceride, 24-hour urinary albumin excretion, ACEi/ARB, loop diuretics, beta blockers, and estimated GFR.

[c] Subdistribution hazard ratio expressed per 50% lower secretory solute clearance or per 10 units lower summary secretion score

[d] Not statistically significant after correction for multiple comparisons using the Hommel method.

Supplemental Table 2.3 Associations between secretory solute clearances and all heart failure events (incident and recurrent)[a, b, c, d]

	Model 1			Model 2			Model 3		
	IRR[e]	95% CI	P-value	IRR	95% CI	P-value	IRR	95% CI	P-value
Pyridoxic acid	1.36	1.26-1.47	< 0.001*	1.19	1.08-1.32	< 0.001*	1.10	0.96-1.24	0.162
Isovalerylglycine	1.32	1.19-1.46	< 0.001*	1.08	0.95-1.21	0.238	0.99	0.86-1.13	0.854
Tiglylglycine	1.34	1.23-1.46	< 0.001*	1.16	1.05-1.28	0.004*	1.07	0.95-1.20	0.253
Kynurenic acid	1.43	1.28-1.60	< 0.001*	1.18	1.04-1.35	0.012	1.04	0.89-1.21	0.618
Xanthosine	1.22	1.13-1.33	< 0.001*	1.10	1.02-1.19	0.012	1.05	0.96-1.13	0.280
Cinnamoylglycine	1.16	1.07-1.25	< 0.001*	1.09	1.01-1.18	0.025	1.04	0.95-1.14	0.408
Indoxyl sulfate	1.33	1.21-1.46	< 0.001*	1.10	0.98-1.23	0.122	0.95	0.83-1.09	0.492
p-cresol sulfate	1.23	1.14-1.34	< 0.001*	1.07	0.96-1.19	0.231	0.95	0.85-1.08	0.447
Summary score	1.60	1.43-1.79	< 0.001*	1.25	1.09-1.44	< 0.001*	1.07	0.90-1.28	0.432

[a] Results from Poisson regression.

[b] Model 1 adjusted for age, race, and sex. Model 2 additionally adjusted for clinical sites, educational attainment, smoking status, history of cardiovascular disease, diabetes, body mass index, systolic blood pressure, triglyceride, 24-hour urinary albumin excretion, ACEi/ARB, loop diuretics, and beta blockers. Model 3 additionally adjusted for estimated GFR.

[c] Overall event rate: 5.8 events per 100 person-years (95% CI: 5.3 – 6.5).

[d] * denotes statistical significance after correction for multiple comparisons using the Hommel method.

[e] Event rate ratio expressed per 50% lower secretory solute clearance or per 10 units lower summary secretion score

Supplemental Table 2.4 Associations of secretory solute clearances with ischemic stroke and hemorrhagic stroke[a, b]

	Ischemic stroke[c]			Hemorrhagic stroke[d]		
	HR [e]	95% CI	P-value	HR	95% CI	P-value
Pyridoxic acid	1.18	0.91-1.53	0.203	1.08	0.67-1.75	0.742
Isovalerylglycine	1.06	0.82-1.36	0.659	0.84	0.52-1.37	0.493
Tiglylglycine	0.94	0.73-1.22	0.650	1.15	0.72-1.84	0.566
Kynurenic acid	0.84	0.60-1.18	0.319	0.80	0.42-1.53	0.500
Xanthosine	0.93	0.77-1.14	0.499	1.08	0.75-1.56	0.685
Cinnamoylglycine	0.96	0.80-1.14	0.632	0.99	0.71-1.36	0.930
Indoxyl sulfate	0.67	0.49-0.90	0.009[f]	0.88	0.50-1.54	0.662
p-cresol sulfate	0.83	0.65-1.06	0.131	0.88	0.56-1.37	0.576
Summary score	0.84	0.56-1.28	0.427	0.95	0.43-2.09	0.899

[a] Results from Cox proportional hazard regression.

[b] Model adjusted for age, race, sex, clinical sites, educational attainment, smoking status, history of cardiovascular disease, diabetes, body mass index, systolic blood pressure, triglyceride, 24-hour urinary albumin excretion, ACEi/ARB, loop diuretics, and beta blockers, and estimated GFR.

[c] Incidence rate of ischemic stroke: 0.4 events per 100 person-years (95% CI: 0.3 – 0.5). Median follow up time: 8.2 years.

[d] Incidence rate of hemorrhagic stroke: 0.10 events per 100 person-years (95% CI: 0.07 – 0.15). Median follow up time: 9.4 years.

[e] Hazard ratio expressed per 50% lower secretory solute clearance or per 10 units lower summary secretion score

[f] Not statistically significant after correction for multiple comparisons using the Hommel method.

Supplemental Table 2.5 Associations of plasma concentrations of secretory solutes with incident heart failure, myocardial infarction, and stroke[a, b, c]

	Heart failure						Myocardial infarction						Stroke					
	Model 2			Model 3			Model 2			Model 3			Model 2			Model 3		
	HR[d]	95% CI	P-value	HR	95% CI	P-value	HR	95% CI	P-value	HR	95% CI	P-value	HR	95% CI	P-value	HR	95% CI	P-value
Pyridoxic acid	1.11	1.04-1.19	0.002*	1.07	1.00-1.15	0.059	1.07	0.99-1.16	0.102	1.04	0.96-1.13	0.374	1.03	0.90-1.18	0.645	1.02	0.88-1.17	0.791
Isovalerylglycine	1.18	1.04-1.34	0.012	1.05	0.92-1.21	0.469	1.19	1.01-1.39	0.035	1.09	0.92-1.29	0.310	0.94	0.74-1.21	0.651	0.89	0.68-1.17	0.417
Tiglylglycine	1.15	1.03-1.28	0.014*	1.03	0.92-1.16	0.609	1.17	1.03-1.34	0.019	1.09	0.95-1.26	0.221	1.07	0.88-1.30	0.517	1.04	0.84-1.29	0.715
Kynurenic acid	1.17	1.02-1.34	0.024	0.95	0.80-1.11	0.500	1.12	0.96-1.32	0.154	0.96	0.80-1.17	0.700	0.84	0.64-1.09	0.183	0.71	0.52-0.97	0.032
Xanthosine	1.10	1.03-1.18	0.006*	1.07	0.99-1.15	0.080	1.10	1.02-1.19	0.011*	1.08	1.00-1.17	0.059	0.99	0.86-1.14	0.896	0.98	0.85-1.13	0.757
Cinnamoylglycine	1.04	0.98-1.10	0.230	1.00	0.94-1.06	0.874	1.00	0.93-1.07	0.944	0.97	0.91-1.04	0.450	1.04	0.93-1.15	0.486	1.03	0.93-1.15	0.569
Indoxyl sulfate	1.04	0.93-1.16	0.525	0.91	0.82-1.02	0.109	0.98	0.86-1.12	0.795	0.89	0.78-1.02	0.085	1.05	0.86-1.28	0.638	1.02	0.83-1.26	0.843
p-cresol sulfate	1.08	1.00-1.17	0.041	1.02	0.94-1.10	0.674	1.10	1.01-1.20	0.031	1.06	0.97-1.16	0.212	1.11	0.97-1.27	0.137	1.10	0.95-1.27	0.191

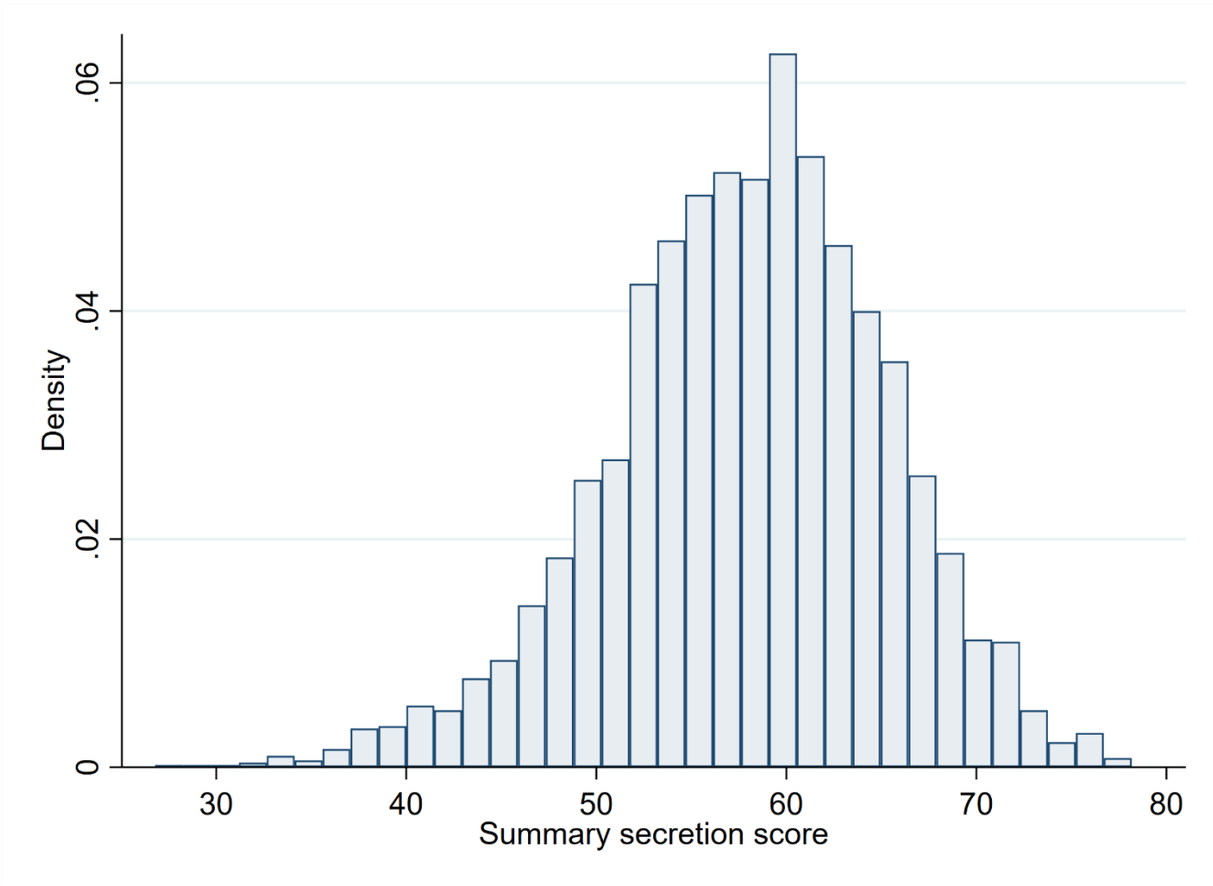
[a] Results from Cox proportional hazard regression.

[b] Model 2 adjusted for age, race, sex, clinical sites, educational attainment, smoking status, history of cardiovascular disease, diabetes, body mass index, systolic blood pressure, triglyceride, 24-hour urinary albumin excretion, ACEi/ARB, loop diuretics, and beta blockers. Model 3 additionally adjusted for estimated GFR.

[c] * denotes statistical significance after correction for multiple comparisons using the Hommel method.

[d] Hazard ratio expressed per 50% higher secretory solute plasma concentration.

Supplemental Figure 2.1 Distribution of the summary secretion score



Chapter 3: Prediction of Kidney Drug Clearance: A Comparison of Tubular Secretory Clearance and Glomerular Filtration Rate

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Key Words: kidney medication clearance, glomerular filtration rate, secretory solutes clearances, furosemide, famciclovir

ABSTRACT

Background: Current kidney drug dosing strategies are based on estimates of the glomerular filtration rate (GFR); yet, tubular secretion is the primary mechanism of kidney drug elimination. We compared GFR with tubular secretory clearance for predicting kidney drug elimination in a pharmacokinetic study.

Methods: We evaluated 54 individuals with a wide range of estimated GFRs (21–140 ml/min/1.73m^2). We administered single doses of furosemide and famciclovir (converted to penciclovir) and calculated their kidney clearances based on sequential plasma and timed urine measurements. Concomitantly, we quantified eight endogenous secretory solutes in plasma and urine using liquid chromatography-tandem mass-spectrometry, and we measured GFR by iohexol disappearance (iGFR). We computed a summary secretion score as the scaled average of the secretory solute clearances. We used linear regression and leave-one-out cross-validation to calculate prediction statistics.

Results: Participants were characterized by a median iGFR of 73 ml/min/1.73m^2 (interquartile range: 48 – 91 ml/min/1.73m^2). The kidney clearance of furosemide was correlated with iGFR (r = 0.84) and the summary secretion score (r = 0.86). The average proportionate difference between model-predicted and measured drug clearance, i.e., the mean proportionate error (MPE), between iGFR-predicted and measured furosemide clearance was 30.0%. MPEs for individual secretory solute clearances ranged from 27.3% - 48.0%, with the lowest MPE observed for the summary secretion score (24.1%). These predictive errors were statistically indistinguishable.

Penciclovir kidney clearance was correlated with iGFR (r = 0.78) and with the summary secretion score (r = 0.85), with similar predictive accuracy of iGFR and secretory clearances. Combining iGFR with pyridoxic acid, indoxyl sulfate, and the summary secretion score modestly improved the prediction of furosemide clearance.

Conclusions: These findings provide some reassurance that GFR is a useful surrogate for predicting secretory drug clearance in stable persons and suggest cautious optimism for future improvements in kidney drug dosing strategies by incorporating measures of secretory clearance.

INTRODUCTION

The kidneys play a central role in eliminating prescribed medications and their metabolites from the circulation. Accurate kidney medication dosing is necessary for reducing the risks of treatment failures and adverse drug events.[95] Impaired kidney function is a major risk factor for preventable medication-related hospital admissions.[36,96-103]

The kidneys clear administered drugs from the circulation by two distinct mechanisms: glomerular filtration and tubular secretion. Of these, secretion is the primary mechanism of kidney drug elimination. Hundreds of drugs and their metabolites are fully or partially cleared by tubular secretion, including antivirals (e.g., tenofovir, famciclovir), antibiotics (e.g., penicillins, cephalosporins), diuretics (e.g., furosemide), and anti-diabetes agents (e.g., metformin). Secretion is capable of eliminating protein-bound drugs that are inefficiently filtered due to the size and charge selectivity of the glomerular basement membrane. Moreover, tubular secretory clearance can greatly exceed the glomerular filtration rate (GFR), providing a highly efficient mechanism for eliminating retained solutes and drugs.[88]

Despite the primacy of tubular secretion in kidney drug handling, current drug dosing strategies are based on estimates of GFR (or creatinine clearance) under the assumption that secretion and filtration are tightly linked within an individual. Yet, tubular secretion is a physiologically different process from filtration that may be specifically affected by competition for secretory transporters, alterations in cellular energy generation, and disproportionate tubulointerstitial injury across disease etiologies. In animal models of chronic kidney disease (CKD), the

expression of tubular organic anion transporters is reduced, possibly as a result of tubulointerstitial fibrosis or as an adaptive response to excess uremic solutes.[37-39] The assumption that GFR represents a reliable proxy of kidney drug elimination was challenged in a review of published pharmacokinetic data, which identified differences between GFR-predicted and actual kidney elimination for 48% of evaluated drugs.[41]

Few studies have empirically determined relationships between tubular secretory clearance and kidney drug elimination. We conducted a pharmacokinetic profiling study of two avidly secreted medications in 54 individuals with a wide range of kidney function. We determined the kidney clearances of eight endogenous secretory solutes by measuring their concentrations in sequential plasma and timed urine samples, and we measured GFR by iohexol disappearance (iGFR). We then compared tubular secretory clearance and iGFR as predictors of kidney drug elimination.

METHODS

Study population

We designed the Proximal Tubular Clearance of Renal Medications (PROCLAIM) Study to investigate the role of tubular secretory solute clearance in kidney drug pharmacokinetics. Between 2017-2019, study personnel recruited 58 individuals by electronic medical record screening of outpatient primary care and Nephrology clinics at the University of Washington (UW), community advertisements, and review of the Kidney Research Institute research registry. Participants were selected based on categories of estimated glomerular filtration rates (GFRs) to include a wide range of kidney function, including normal and chronic kidney disease (CKD).

Exclusion criteria included age <18 years, receipt of any form of renal replacement therapy, current use of the study medications, allergy to the study medications, use of cimetidine, probenecid, or digoxin, and a history of nephrotic syndrome or cirrhosis (full list of exclusions in Supplemental Table 3.1). We further excluded four participants for whom reliable intravenous access could not be established for the scheduled blood collections. The institutional review board at the UW approved the study protocol. All participants provided informed consent.

Measurements of secretory solute clearance

We previously identified a set of endogenous solutes suspected to be eliminated primarily by proximal tubular secretion based on one or more of the following characteristics: affinity for proximal tubular organic anion transporters 1 and 3, elevated plasma concentrations following transporter knockout in experimental models, a high degree of protein binding, relatively low diurnal variation in plasma, and/or kidney clearances that exceed GFR or creatinine clearance.[18-20] Herein, we estimated the kidney clearances of these solutes by measuring their concentrations in sequential plasma samples and a concomitant supervised daytime urine collection. Upon arrival at the study center, participants provided a spot urine void, which marked the beginning of the timed urine collection but was excluded from the timed collection itself. Participants then voided throughout the remainder of the study visit, and the time of the last void was recorded (mean collection time 9.8 $\pm$1.2 hours). During this same period, coordinators collected and processed blood samples for secretory solute measurements at times 0, 60, 300, and 480 minutes from an indwelling intravenous catheter. Participants were provided three standard meals and ample fluids throughout the study visit.

We quantified plasma and urine concentrations of endogenous solutes using our previously published methods.[51,52] Plasma samples underwent solid phase extraction (Phree phospholipid removal plate, Phenomenex) after precipitation in organic solvent. Urine samples went through two consecutive solid phase extractions (HLB or MCX µElution plates, Waters). We reconstituted dried extracts in 80 µL of 5% acetonitrile/0.2% formic acid in H_2O followed by filtration through a large-pore filter plate (Millipore, MSBVN1210) to remove particulates before being analyzed using liquid chromatography-tandem mass spectrometry (Shimadzu and Sciex). We normalized data to internal standard peak areas based on stable isotope-labeled solutes. Calibration was achieved using a single point calibration approach. The concentrations of each solute in the single point calibrators (pooled human serum and urine) were previously quantified by standard addition of purified spiking solutions, which were characterized using quantitative nuclear magnetic resonance. Five replicates of single point calibrators were included on each plate. Intra- and inter-assay coefficients of variation were generally low (Supplemental Table 3.2).

We calculated the kidney clearance of each endogenous secretory solute as:

$$CL_R = \frac{U_x \times \dot{V}}{\frac{1}{n}\sum P_x}$$

where U_x represents the concentration of solute in the supervised daytime urine collection (ng/ml), $\dot{V}$ represents the timed urine flow rate (ml/min), n is the number of plasma measurements (49 participants provided four measurements and 5 participants provided three), and P_x represents solute concentration in plasma (ng/ml).

Measurements of drug pharmacokinetics

At the beginning of each study visit, coordinators administered a single 5 mg intravenous dose of furosemide (Hospira). Syringes were weighed pre- and post-administration to precisely calculate the administered furosemide dosage, which was then used to calculate its kidney clearance. Furosemide is highly protein bound (>95%) and eliminated primarily via secretory transporters in the proximal tubules. Coordinators simultaneously provided participants with a single 125 mg oral dose of famciclovir (Macleods Pharma). Orally administered famciclovir has high bioavailability and is rapidly metabolized by the liver to penciclovir, the active form of the drug, which is efficiently eliminated by proximal tubular secretion.[104-108] All study medications were acquired, stored, and dispensed by the Northwest Kidney Center Pharmacy.

Coordinators collected and processed blood specimens from the indwelling catheter for measurements of furosemide and penciclovir concentrations at 15, 30, 45, 60, 90, 120, 150, 180, 210, 240, 300, 480, and 600 minutes after drug administration. At the end of the study visit, participants were instructed to complete an overnight urine collection and to return to the study center the following morning to provide a final (1440-minute) blood sample.

We quantified plasma and urine concentrations of each medication using liquid chromatography tandem mass-spectrometry (LC-MS/MS) at the University of Washington Department of Pharmaceutics Pharmacokinetics Laboratory (Supplemental Methods). Briefly, plasma and urine samples were mixed with internal standards of known concentrations. The internal standards for

furosemide and penciclovir were probenecid and labeled d_4-penciclovir, respectively. Calibration samples were prepared by combining drug-free plasma or urine samples with internal standards and working standard solutions. Working standard solutions were prepared with 0.6 ng/µL of furosemide in methanol for furosemide plasma and urine samples, 1.0 ng/µL penciclovir for penciclovir plasma samples, and 160 ng/µL penciclovir for penciclovir urine samples. Samples were vortexed and centrifuged, and supernatant was then injected into the LC-MS/MS system.

The area under the concentration-time curve (AUC) for each medication was calculated using the linear up/log down trapezoidal rule, and any portion of the curve extrapolated to infinity was calculated by fitting a monoexponential, biexponential, or triexponential decay, whichever fitted best to the observed data, to the terminal portion of the curve. For the calculation of furosemide AUC, we back extrapolated the curve to time zero. This method incorporated drug exposure before the first blood sample was taken (15 minutes after administration), which usually constitutes a large portion of it. We calculated the kidney clearance of each medication as:

$$CL_{\text{renal}} = \frac{\text{mass excreted in urine}_{0\rightarrow\tau}}{\text{AUC}_{\text{plasma, }0\rightarrow\tau}}$$

where *mass excreted in urine*$_{0\rightarrow\tau}$ represents the amount of drug recovered in the timed urine sample (mg). The plasma AUC (mg/ml×min) was calculated over the same time interval. For furosemide, we used the daytime urine collection from 0 to 10 hours and blood samples over the matched time interval to calculate the urine recovery and plasma $\text{AUC}_{0\rightarrow 10\text{ hr}}$ of furosemide because the 1440-minute plasma furosemide concentration was undetectable or below the lower limit of quantification for the majority of participants. For penciclovir, by contrast, we used the

0- to 10-hour and 10- to 24-hour urine collections (supervised daytime collection plus overnight sample) and included the 1440-minute blood sample to calculate urine recovery and the plasma $AUC_{0\rightarrow\infty}$ of penciclovir.

Measurements of iohexol clearance

At the start of each study visit, coordinators administered a single 5 ml intravenous bolus of iohexol (Omnipaque, 647 mg/ml). Syringes were weighed pre- and post-administration to calculate the administered dosage. Coordinators collected blood samples at 120, 180, 240, and 600 minutes post-administration for iohexol measurements. Plasma iohexol concentrations were quantified by liquid chromatography tandem mass spectrometry at the University of Minnesota Advanced Research and Diagnostic Laboratory.[109] We calculated iohexol clearance using the slope-intercept method in conjunction with the Brochner-Mortensen equation.[110] This method provides good accuracy and reliability and does not require plasma samples collected at very short intervals immediately after iohexol administration.[111,112]

Statistical analyses

We expressed all clearances in ml/min without standardization for body surface area. We summarized correlations among the kidney clearances of each study medication, secretory solutes, and iGFR using scatter plots and Pearson's correlation. We computed a summary measure of secretory clearance by standardizing the kidney clearance of each secretory solute to a common 0 – 100 scale:

$$Standardized\ clearance_X = \frac{clearance_X - min(clearance_X)}{} * 100$$

$$range(clearance_X)$$

where $clearance_X$ represents the kidney clearance of solute X, $min(clearance_X)$ represents the minimum clearance value in the distribution of the current study, and $range(secretory\ clearance_X)$ represents the difference between the maximum and minimum values. We then calculated the summary secretion score as the average of the eight standardized clearances.

We constructed linear regression models to quantify associations between the kidney clearance of each study medication (dependent variable) and either secretory solute clearances or GFR (independent variables). We used leave-one-out cross-validation to estimate the mean absolute error (MAE) and the mean proportionate error (MPE). The MAE and MPE can be interpreted as the average absolute or proportionate difference between model-predicted and measured drug clearance. Lower MAE and MPE values indicate greater predictive accuracy. We compared the predictive accuracy of univariate iGFR and secretory clearance models by computing the difference in MAE or MPE between these models with 95% confidence intervals derived using a bootstrap approach with 500 iterations.[113] We then assessed the combined prediction of iGFR plus secretory solute clearance by comparing models that included only iGFR to models that included iGFR plus each secretory clearance measure. In sensitivity analyses, we assessed the MAE and MPE of models that included estimated GFR as a single predictor. Estimated GFR was calculated using the CKD-EPI equation using a spot creatinine concentration from blood samples collected at baseline before drug administration.[6] Analyses were performed using Stata/IC 14.2 (StataCorp. 2015. Stata Statistical Software: Release 14. College Station, TX) and RStudio 3.6.3 (R Core Team 2017, Vienna, Austria).

RESULTS

Study population and characteristics

The 54 study participants were characterized by a mean age of 55 ±15 years; 33% female; 33% Black; and a median iGFR of 73 ml/min/1.73m^2 (IQR: 48 – 91 ml/min/1.73m^2, Table 3.1). There were 23 participants (43%) with an iGFR <60 ml/min/1.73m^2 (12 with iGFR 45-60 ml/min/1.73m^2 and 11 with iGFR <45 ml/min/1.73m^2). Thirty-three percent of participants were using statins and 39% were using an angiotensin-converting-enzyme inhibitor (ACEi) or an angiotensin II receptor blocker (ARB). The kidney clearances of six of the eight secretory solutes were higher than iGFR (Supplemental table 3.2). Correlations between secretory solute clearances and iGFR ranged from 0.69 for xanthosine clearance to 0.83 for pyridoxic acid clearance. Plasma concentrations of the individual secretory solutes did not meaningfully change following the administration of the study medications (Supplemental table 3.3).

Prediction of furosemide pharmacokinetics

The median kidney clearance of furosemide was 83 ml/min (IQR: 51 to 117 ml/min). Kidney furosemide clearance was strongly correlated with iGFR and with the summary secretion score (Figure 3.1; r = 0.84 and 0.86, respectively). Using iGFR as a single predictor, the mean absolute error (MAE) between model-predicted and measured furosemide clearance was 21.5 ml/min (Table 3.2). Expressed as proportionate difference, the mean proportionate error (MPE) between iGFR-predicted and measured furosemide clearance was 30.0%. MAE and MPE values were slightly higher for the corresponding model of eGFR. Five of the eight secretory solute

clearances yielded modestly lower MAEs than the iGFR model. The lowest MAEs were observed for pyridoxic acid clearance and for the summary secretion score (2.6 and 4.5 ml/min lower than iGFR, respectively). However, differences between iGFR and secretory solute clearance-based predictions of kidney furosemide elimination were not statistically significant (all 95% confidence intervals include 0).

Prediction of penciclovir pharmacokinetics

The median kidney clearance of penciclovir was 263 ml/min (IQR: 163 to 367 ml/min), which was 3.3 times higher than iGFR on average. Correlations of penciclovir clearance with iGFR and the summary secretion score were 0.78 and 0.85, respectively (Figure 3.2). The MAE between iGFR-predicted and measured kidney penciclovir clearance was 60.3 ml/min; the corresponding MPE for this model was 26.3% (Table 3.3). Modestly higher MAE and MPE values for penciclovir clearance were observed using eGFR as a single predictor. The kidney clearances of individual secretory solutes yielded modestly higher MAEs and MPE values compared with the iGFR model. However, the summary secretion score yielded the lowest observed MAE and MPE. Neither of these differences was statistically significant.

Combining iGFR with secretory solute clearances

Adding the kidney clearances of pyridoxic acid, indoxyl sulfate, and the summary secretion score to the iGFR model each individually improved the predictive accuracy for furosemide clearance (Table 3.4). For penciclovir, the addition of the summary secretion score to iGFR yielded moderately lower errors between predicted and measured drug clearance, but this

difference was not statistically significant (Table 3.4). Further addition of age, sex, and race to the iGFR plus summary secretion score models yielded no further improvement in predictive accuracy for either drug clearance.

Discussion

In summary, we found iGFR and the kidney clearances of secretory solutes to be strongly correlated with the kidney elimination of two avidly secreted drugs among stable outpatients with and without CKD. A summary measure of kidney secretory clearance demonstrated numerically greater accuracy for predicting the clearances of furosemide and penciclovir; however, the observed differences were modest and not statistically significant. Some improvement in predicting the kidney clearance of both drugs was also achieved by combining the summary secretion score with iGFR. These findings obtained from an empiric pharmacokinetic study, provide some reassurance that GFR, even when estimated, is a useful surrogate for secretory drug clearance in healthy individuals and patients with stable CKD. The results also suggest cautious optimism for future improvements in kidney drug dosing strategies by incorporating measurements of tubular secretory clearance.

The close correlation among iGFR, secretory solute clearances, and the kidney elimination of two avidly secreted drugs, suggests tight linkage between glomerular filtration and tubular secretory clearance in stable persons with and without CKD. This result was somewhat surprising given physiological differences in the underlying mechanisms of filtration and secretion. Although both pathways are governed by hemodynamic conditions, filtration is

primarily passive and determined by size and charge selectivity of the glomerular basement membrane. In contrast, the secretion of organic solutes and drugs involves coordinated uptake by specific transporters on the basolateral cell surface, pericellular transport, and active secretion into the urine against a chemical gradient. Central to these secretory processes is efficient cellular energy generation via mitochondrial respiration, which may be differentially impacted by pathological processes. Contrasts between GFR and tubular secretory clearance may also arise from competition for cellular transporters by endogenous solutes and other drugs and from genetic variation in the transporters. For example, an intergenic polymorphism between organic anion transporter 1 and 3 (OAT1/3) modifies the effect of hydrochlorothiazide on blood pressure, and a non-synonymous polymorphism in organic cation 2 (OCT2) is associated with higher kidney metformin clearance.[114,115]

As our study included only 33 persons with CKD due to heterogenous etiologies, we cannot exclude the possibility of greater dissociation between GFR and tubular secretory clearance for specific causes of CKD or in the setting of acute kidney injury, which may preferentially impact tubular functions. It is also possible that distinctions between GFR and secretory drug clearance may be greater for cationic drugs. For example, the clearance of S-pindolol, which is avidly secreted via OCT2 transporters, was only weakly correlated with GFR measured by ^{51}Cr-EDTA clearance in a previous pharmacokinetic study.[40]

These results provide empiric evidence that tubular secretory clearance can be estimated from endogenous solutes. The solutes selected for this study are substrates of OAT1/3 transporters and

are either highly protein bound and/or exhibit kidney clearances that exceed GFR. For example, kynurenic acid is >95% bound to serum albumin, including in persons with advanced CKD, suggesting minimal glomerular filtration, is efficiently cleared by the kidneys at rates that exceed GFR, and is strongly correlated with the kidney elimination of furosemide and penciclovir in this study. Nonetheless, timed urine collections remain cumbersome to obtain in clinical practice, and plasma concentrations of endogenous secretory solutes exhibit diurnal variation, which reduces precision in estimating their clearance. Further refinement in methods for estimating tubular secretory clearance based on endogenous solutes could advance this important area.

The two medications selected for evaluation in this study are avidly secreted by the proximal tubules. Furosemide circulates bound to serum albumin (>95%), minimizing filtration,[116] and is primarily eliminated by active secretion via OAT1/3 transporters.[117] We administered furosemide intravenously to avoid individual differences in oral bioavailability, which range from 0.37 to 0.83 in healthy subjects and 0.43 to 0.76 in patients with CKD.[116] Famciclovir, an oral prodrug of penciclovir, is a nucleoside analog that inhibits herpes simplex virus DNA polymerase. Orally administered famciclovir undergoes rapid metabolism to penciclovir in the liver with a consistent oral bioavailability of approximately 60%.[118] Although the protein binding of penciclovir is low (< 20%), the drug is avidly secreted, primarily via OAT2, with a kidney clearance that greatly exceeds GFR.[105,119] We observed a median penciclovir clearance of 263 ml/min, which was 3.3 times higher than iGFR in this study.

The primary strengths of this study are the detailed procedures used to measure kidney drug pharmacokinetics, GFR, and tubular secretory clearances with high accuracy and precision. We calculated secretory clearances using time-averaged plasma concentrations of target solutes measured at multiple time points to reduce the impact of diurnal variation and urine concentrations from a supervised timed collection. We quantified the solutes of interest using targeted mass spectrometry assays with labeled internal standards and external calibrators that have been developed for this purpose. Plasma concentrations of the selected solutes were unchanged by the administration of furosemide and penciclovir, suggesting that the small doses of these drugs administered in this study were below transporter thresholds. Another strength is the inclusion of participants with a wide range of GFR and secretory solutes clearances - from healthy participants to those with CKD. Several important weaknesses deserve comment. The relatively small sample size and self-reported cause of kidney disease preclude assessment of kidney drug clearances for specific etiologies of CKD or among patients with severely reduced kidney function. The small sample size also limited study power to detecting relatively large differences between GFR and secretory clearances. We selected furosemide and penciclovir as prototypical secreted drugs; however, GFR and secretory clearance may have different relative impacts on the elimination of other drugs, particularly cationic drugs that utilize the OCT2 pathway. Some furosemide is metabolized into a glucuronide metabolite, which we were unable to measure reliably due to its instability.

In summary, we found relatively similar accuracy of iGFR and tubular secretory solute clearances for predicting the kidney elimination of two avidly secreted drugs. Some improvement in the prediction of furosemide clearance was achieved by combining secretory

solute clearance measurements with iGFR, suggesting possible future applications of secretory clearance measurements to refine kidney drug dosing. These study results also demonstrate feasibility of using endogenous secretory solutes as markers to estimate tubular secretory clearance. Future studies to evaluate other secreted drugs, particularly those that utilize different transporters, with a larger sample size and in persons with different etiologies of CKD are needed to extend these study findings.

Table 3.1 Characteristics of participants in the Proximal Tubular Clearance of Renal Medications (PROCLAIM) study [a, b]

Characteristics	
iGFR, ml/min/1.73m^2 [c]	73 (48, 91)
< 45	11 (20)
45 – 60	12 (22)
60 – 90	17 (31)
>= 90	14 (26)
Age, years	55 ± 15
Female	18 (33)
Race	
White	34 (63)
Black	18 (33)
Other	2 (4)
Body mass index, kg/m^2	29 ± 6
Education categories	
Less than high school	2 (4)
High school graduate	11 (20)
Some college	16 (30)
College graduate or higher	25 (46)
Current smoker	14 (26)
History of diabetes	6 (11)
History of cardiovascular disease	7 (13)
Systolic blood pressure, mmHg	134 ±20
Laboratory measurements	
24-hour urine albumin, mg/day [c, d]	13 (5, 68)
24-hour urine albumin > 300 mg/day [c, d]	9 (17)
Serum albumin, g/dL	4.1 ± 0.3
Serum calcium, mg/dL	9.0 ± 0.4
Serum bicarbonate, mEq/L	22.7 ± 3.4
Hemoglobin, g/dL	13.5 ± 1.7
Medications	
Insulin	3 (6)

Statin	18 (33)
ACEi / ARB	21 (39)
Thiazide diuretic	3 (6)

[a] iGFR: glomerular filtration rate measured by iohexol disappearance; ACEi: angiotensin-converting-enzyme inhibitor; ARB: angiotensin II receptor blocker.

[b] For continuous variable: mean ± SD; for categorical variables: N (%).

[c] Median (Interquartile range)

[d] Urine albumin collected during supervised daytime urine collection standardized to 24-hours.

Table 3.2 Predictive accuracy of GFR and secretory solute clearances for predicting kidney furosemide clearance. [a]

	MAE between predicted and measured drug clearance, ml/min	Difference in MAE comparing iGFR to secretory clearance, ml/min (95% CI) [b]		MPE between predicted and measured drug clearance, %	Difference in MPE comparing iGFR to secretory clearance, % (95% CI) [b]
$eGFR_{creatinine}$	22.4	-0.9 (-5.6, 3.5)		33.5	-3.5 (-12.9, 3.7)
iGFR	21.5	/		30.0	/
Clearance					
Pyridoxic acid	18.9	2.6 (-2.9, 7.0)		27.3	2.7 (-6.8, 10.1)
Isovalerylglycine	25.7	-4.2 (-11.4, 2.2)		42.5	-12.5 (-26.9, 1.1)
Tiglylglycine	27.0	-5.5 (-12.9, 1.1)		43.8	-13.8 (-32.2, 1.9)
Kynurenic acid	21.0	0.5 (-6.2, 6.3)		33.3	-3.3 (-16.8, 9.2)
Xanthosine	28.3	-6.8 (-14.3, 0.1)		48.0	-18.0 (-35.9, -1.3)
Cinnamoylglycine	20.9	0.6 (-5.1, 5.3)		29.2	0.8 (-8.2, 9.7)
Indoxyl sulfate	20.6	0.9 (-4.0, 5.6)		31.1	-1.1 (-10.4, 9.0)
p-cresol sulfate	20.0	1.5 (-3.3, 5.7)		29.5	0.5 (-5.9, 8.5)
Secretion score	17.2	4.3 (-1.1, 9.1)		24.1	5.9 (-2.1, 14.0)

[a] iGFR: glomerular filtration rate measured by iohexol disappearance; $eGFR_{creatinine}$: estimated GFR based on serum creatinine concentrations from the 2009 CKD-EPI equation; MAE: mean absolute error; MPE: mean proportionate error.

[b] Differences and 95% confidence intervals derived using leave-on-out cross validation with bootstrap with 500 iterations. Positive values indicate greater agreement for secretory solute clearances and negative values indicate greater agreement for iGFR.

Table 3.3 Predictive accuracy of GFR and secretory solute clearances for predicting kidney penciclovir clearance. [a]

	MAE between predicted and measured drug clearance, ml/min	Difference in MAE comparing iGFR to secretory clearance, ml/min (95% CI) [b]		MPE between predicted and measured drug clearance, %	Difference in MPE comparing iGFR to secretory clearance, % (95% CI) [b]
$eGFR_{creatinine}$	70.3	-10.0 (-23.9, 2.0)		32.3	-6.0 (-14.4, 0.5)
iGFR	60.3	/		26.3	/
Clearance					
Pyridoxic acid	64.2	-3.9 (-18.1, 11.7)		28.1	-1.8 (-10.7, 8.0)
Isovalerylglycine	64.4	-4.1 (-17.7, 14.6)		30.4	-4.1 (-13.1, 7.1)
Tiglylglycine	71.0	-10.7 (-25.4, 8.1)		33.1	-6.8 (-16.9, 5.1)
Kynurenic acid	66.1	-5.8 (-22.1, 16.4)		30.1	-3.8 (-13.6, 9.3)
Xanthosine	71.7	-11.4 (--29.6, 3.7)		35.9	-9.6 (-18.3 0.5)
Cinnamoylglycine	69.4	-9.1 (-23.0, 4.3)		32.1	-5.8 (-12.9, 2.0)
Indoxyl sulfate	76.5	-16.2 (-27.9, -2.6)		35.9	-9.6 (-15.2, -2.8)
p-cresol sulfate	62.2	-1.9 (-13.5, 10.8)		28.0	-1.7 (-8.0, 3.2)
Secretion score	56.2	4.1 (-9.8, 18.4)		24.2	2.1 (-5.7, 8.6)

[a] iGFR: glomerular filtration rate measured by iohexol disappearance; $eGFR_{creatinine}$: estimated GFR based on serum creatinine concentrations from the 2009 CKD-EPI equation; MAE: mean absolute error; MPE: mean proportionate error.

[b] Differences and 95% confidence intervals derived using leave-on-out cross validation with bootstrap with 500 iterations. Positive values indicate greater agreement for secretory solute clearances and negative values indicate greater agreement for iGFR.

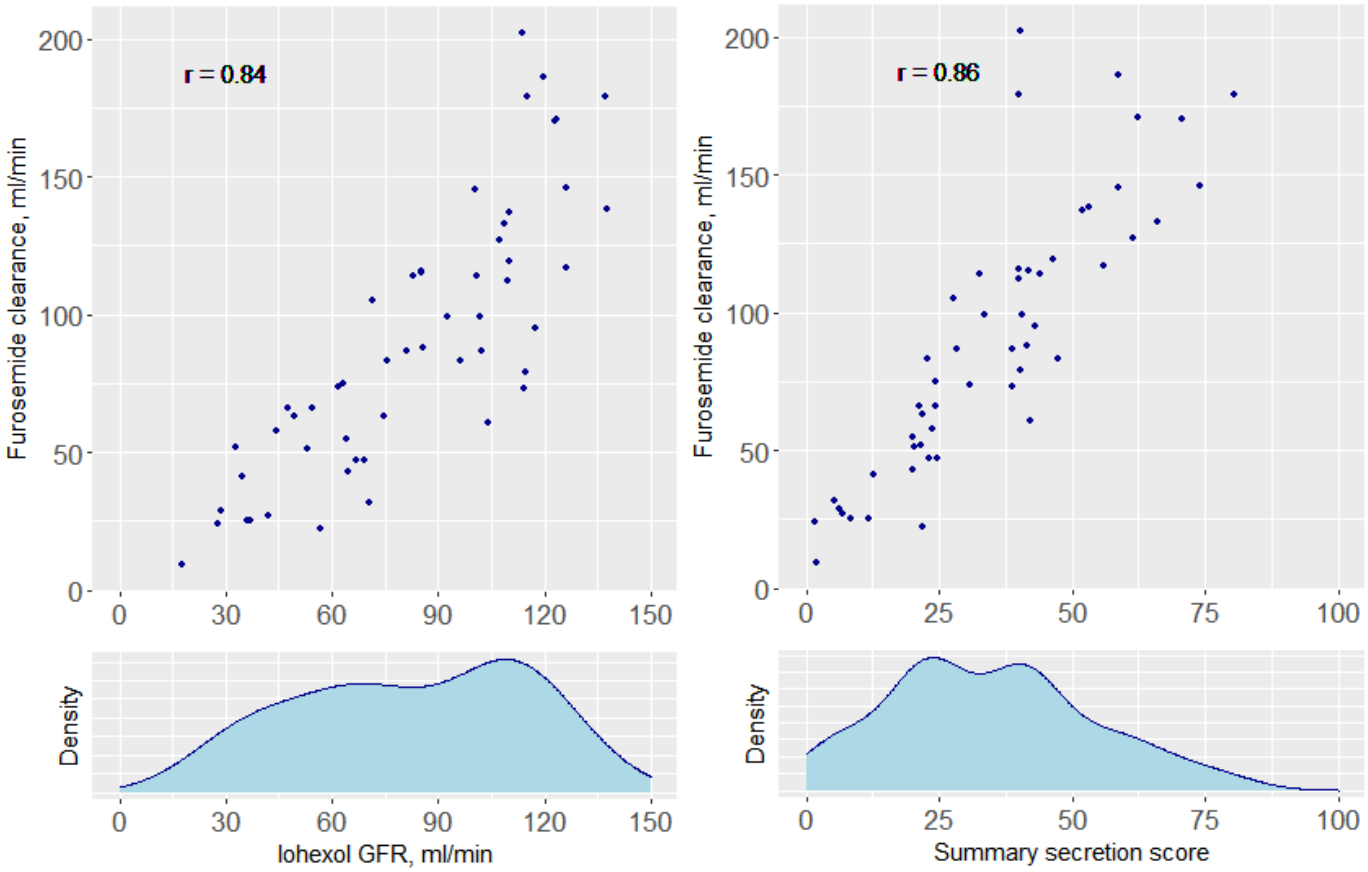
Table 3.4. Prediction of kidney drug clearances combining iGFR with secretory solute clearances. [a]

	Furosemide			Penciclovir	
	MAE between predicted and measured drug clearance, ml/min	Difference in MAE comparing iGFR alone to iGFR plus secretory clearance, ml/min (95% CI) [b]		MAE between predicted and measured drug clearance, ml/min	Difference in MAE comparing iGFR only to iGFR plus individual secretory clearance, ml/min (95% CI) [b]
iGFR	21.5	/		60.3	/
Clearance					
Pyridoxic acid	18.1	3.4 (0.2, 7.5)		58.2	2.1 (-2.8, 11.5)
Isovalerylglycine	20.7	0.8 (-0.7, 4.4)		52.7	7.6 (-1.4, 20.5)
Tiglylglycine	21.4	0.1 (-0.8, 3.3)		55.3	5.0 (-2.5, 14.5)
Kynurenic acid	18.3	3.2 (-0.4, 7.5)		56.1	4.2 (-2.6, 17.6)
Xanthosine	20.5	1.0 (-0.4, 3.7)		55.6	4.7 (-1.6, 13.5)
Cinnamoylglycine	19.7	1.8 (-0.9, 5.7)		59.4	0.9 (-3.5, 8.4)
Indoxyl sulfate	18.6	2.9 (0.2, 6.3)		61.4	-1.1 (-4.4, 4.1)
p-cresol sulfate	19.4	2.1 (-0.2, 5.3)		56.7	3.6 (-2.5, 11.3)
Secretion score	17.4	4.1 (0.1, 9.1)		53.5	6.8 (-1.6, 17.4)

[a] iGFR: glomerular filtration rate measured by iohexol disappearance; MAE: mean absolute error.

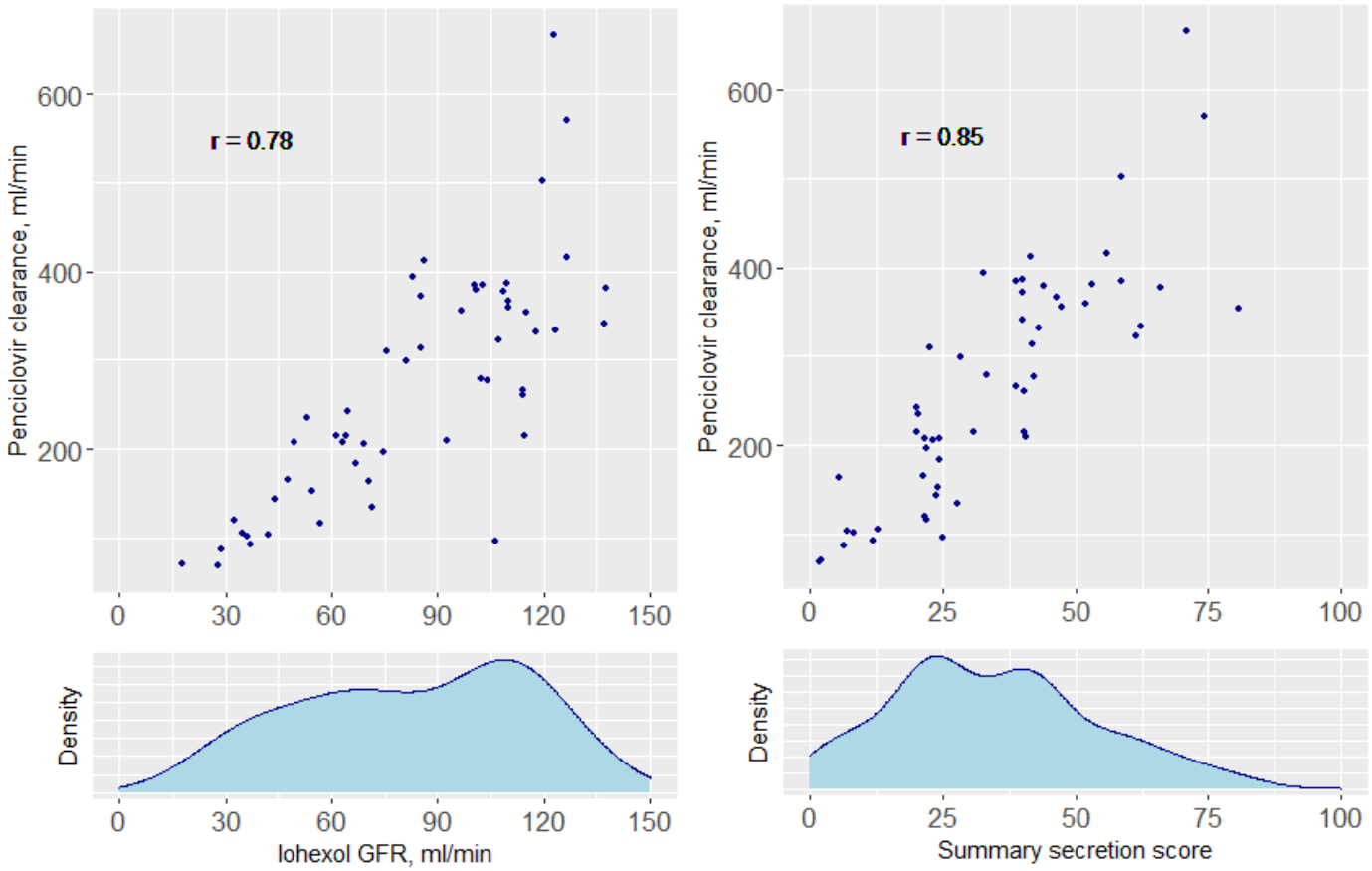
[b] Differences and 95% confidence intervals derived using leave-on-out cross validation with bootstrap with 500 iterations. Positive values indicate greater agreement for secretory solute clearances and negative values indicate greater agreement for iGFR.

Figure 3.1 Associations of iGFR and summary secretion score with kidney furosemide clearance. [a]



[a] iGFR: glomerular filtration rate measured by iohexol disappearance.

Figure 3.2 Associations of iGFR and summary secretion score with kidney penciclovir clearance. [a]



[a] iGFR: glomerular filtration rate measured by iohexol disappearance.

Supplemental Methods of the Quantification of Study Medications

Quantification of furosemide

For both plasma and urine samples, a working standard solution was prepared with 0.6 ng/µL of furosemide in methanol, and an internal standard solution contained 0.25 ng/µL of probenecid in methanol. Plasma unknown samples and quality-control samples were prepared for analysis by combining 20 µL of internal standard with 50 µL plasma or urine and 150 µL 0.1% formic acid in methanol. Calibration samples were prepared by combining 50 µL plasma free from furosemide, 20 µL internal standard, and 0 to 100 µL working standard solution plus enough of the prepared 0.1% formic acid methanol solution to make the final methanol volume 150 µL. Samples were vortexed for 30 seconds and centrifuged at 4°C for 10 minutes at 14,000 RCF and the supernatant was transferred to a 96-well plate for analysis. Urine unknown samples and quality-control samples were prepared for analysis by combining 20 µL of internal standard with 50 µL urine and 500 µL of 100 mM potassium phosphate buffer, pH 4. Calibration samples, which were made with urine free from furosemide, also contained 0 to 100 µL of the working standard solution. Samples were vortexed for 10 seconds, and then 2 ml of ethyl acetate was added. The tubes were shaken horizontally for 10 minutes. The phases were allowed to separate, and the organic phase was transferred to a glass tube and dried at 40ºC under a stream of nitrogen gas. The dried samples were reconstituted with 100 µL of 1:1 0.1% formic acid to methanol and transferred to a 96-well plate. For both plasma and urine samples, 1 µL of the prepared sample was injected onto a Shimadzu Nexera UPLC (Shimadzu Corporation, www.shimadzu.com) coupled to an AB Sciex 6500 Q-Trap tandem mass spectrometer (Sciex, sciex.com). Mobile phase A was 0.1% formic acid in water and mobile

phase B was methanol. The flow rate was 0.3 ml/min and an elution gradient started at 25% B for 0.5 minutes, increased linearly to 95% by 2.0 minutes, held at 95% until 4.0 minutes, decreased to 25% by 4.25 minutes, and equilibrated until 7.0 minutes before next injection. Chromatographic separation was achieved with an Agilent Eclipse C-18 50 mm x 2.1 mm x 1.8 μm particle-size column (Agilent). The mass spectrometer operated in electrospray mode with negative polarity. The following transitions were monitored: 328.6>204.8 m/z (furosemide) and 283.8>239.8.0 m/z (probenecid). The declustering potential was -5 V. The collision energy was -28 V for furosemide and -18 V for probenecid. The ion spray voltage was -4500 V with the source set to 550ºC.

Quantification of penciclovir

For plasma samples, a working standard solution contained 1.0 ng/μL penciclovir in methanol, and for urine samples, a working standard solution contained 160 ng/μL penciclovir in methanol. An internal standard solution contained 1.0 ng/μL of d_4-penciclovir in methanol. Unknown samples and quality-control samples were prepared for analysis by combining 20 μL of the internal standard solution with 50 μL plasma or urine and 150 μL methanol. Calibration samples were prepared by combining 50 μL of plasma or urine free from penciclovir, 20 μL of the internal standard solution, and 0 to 100 μL of working standard solution plus methanol to make the final methanol volume 150 μL. Samples were vortexed for 30 seconds and centrifuged at 4°C for 10 minutes at 14,000 RCF. The supernatant was transferred to a 96-well plate and 2 μL were injected onto an Agilent 1290 Infinity high-pressure liquid chromatography coupled to an Agilent Technologies 6410 triple-quadrupole tandem mass spectrometer. Chromatographic separation

was achieved with a Thermo Scientific Hypercarb 100 mm x 2.1 mm column with a 5 μm particle-size and 80Å pore size. Mobile phase A was 0.1% formic acid in water and mobile phase B was methanol. The flow rate was 0.4 ml/min, and an elution gradient started at 2.5% B for 4.0 minutes, increased linearly to 80% by 10.0 minutes, then decreased to 2.5% by 10.5 minutes. The mass spectrometer was operated in electrospray ionization mode with positive polarity. The following m/z transitions were monitored: 254.2>152.0 (penciclovir) and 258.2>152.0 (d_4-penciclovir). The drying gas temperature was set to 350°C at a flow rate of 10 L/minute. The nebulizer gas was nitrogen at 35 psig. The capillary voltage was 4000 V with the quadrupole temperature set to 100°C. Peak integration was performed using MassHunter Quantitation software (Agilent) and the response was measured by peak height. A second-order polynomial of analyte peak height normalized by internal standard peak height given nominal mass in the sample was fit to the data with weighting by 1/x.

Supplemental Table 3.1 Exclusion criteria of the PROCLAIM study

- Age ≤ 18
- Currently receiving maintenance hemodialysis or peritoneal dialysis
- Current or previous solid organ transplantation
- Known allergy to any of the study medications, iodine, acyclovir, or sulfa containing medications
- Current or regular use of any of the study medications (furosemide, famciclovir, tenofovir, oseltamivir)
- Current or regular use of probenecid, cimetidine, or digoxin
- Pregnancy or lactation
- Liver cirrhosis or liver failure
- Heart failure: New York Heart Association class III or greater
- Voiding problems or requirement for self-catheterization
- Nephrotic syndrome: urine albumin to creatinine ratio > 3 grams per day
- Non-English speaking
- Inability to provide written informed consent
- ALT greater than 3 times the upper limit for the test
- Hemoglobin < 9 mg/ml
- Platelet count < 100K
- Serum potassium > 5.5 or < 3.5 mEq/ml

Supplemental Table 3.2 Kidney clearances and laboratory characteristics of secretory solutes.[a]

	Median kidney clearance (ml/min, IQR)	Correlation with iGFR[b]	Molecular weight (g/mol)	Intra-assay CV plasma (%)	Inter-assay CV plasma (%)	Intra-assay CV urine (%)	Inter-assay CV urine (%)	Median Diurnal CV plasma (%, IQR)[c]
Pyridoxic acid	783 (489, 983)	0.83	183	3.4	4.7	5.7	5.8	19.5 (14.1, 26.0)
Isovalerylglycine	493 (320, 882)	0.73	159	7.1	7.3	5.4	5.9	25.2 (16.0, 32.4)
Tiglylglycine	338 (234, 565)	0.70	157	7.0	14.7	6.0	5.5	27.4 (18.1, 34.6)
Kynurenic acid	318 (222, 442)	0.74	189	4.1	5.5	5.6	8.6	17.0 (11.5, 21.2)
Xanthosine	215 (154, 313)	0.69	284	11.1	14.5	9.8	10.1	22.6 (17.7, 30.8)
Cinnamoylglycine	168 (98, 230)	0.82	205	4.5	5.4	4.9	4.5	36.0 (26.4, 54.2)
Indoxyl sulfate	60 (40, 82)	0.81	213	4.3	6.0	6.1	9.4	19.2 (10.7, 26.1)
p-cresol sulfate	24 (16, 30)	0.82	188	3.9	5.2	5.6	5.2	16.3 (11.7, 24.2)

[a] CV: coefficient of variation; IQR: inter-quartile range; iGFR: iohexol measurement of GFR.

[b] Pearson correlations between secretory solute clearances and iohexol measurement of GFR, both not standardized to 1.73 m^2 body surface area.

[c] Diurnal CV was calculated using plasma samples drawn at baseline, 1 hour, 5 hours, 8 hours, and 24 hours after baseline.

Supplemental Table 3.3 Plasma concentration of secretory solutes during study visits.

	Median plasma concentration (IQR), ng/ml				P for trend[c]
	Baseline[a]	1 hour[b]	5 hour[b]	8 hour[b]	
Pyridoxic acid	6.9 (3.9, 12.5)	5.6 (3.5, 11.0)	4.7 (3.3, 10.1)	4.9 (3.2, 11.6)	0.662
Isovalerylglycine	5.0 (3.3, 7.9)	4.9 (3.1, 7.4)	6.5 (3.9, 11.3)	6.0 (4.0, 11.3)	0.332
Tiglylglycine	6.5 (4.8, 11.9)	5.7 (4.2, 9.9)	8.0 (5.6, 13.2)	8.2 (6.1, 13.4)	0.302
Kynurenic acid	9.7 (7.3, 13.8)	8.8 (6.9, 13.2)	10.0 (7.3, 15.6)	9.6 (7.0, 14.0)	0.456
Xanthosine	6.9 (5.1, 10.5)	4.3 (3.4, 7.4)	5.5 (4.6, 7.8)	5.9 (4.8, 7.7)	0.063
Cinnamoylglycine	16.9 (5.4, 29.7)	15.5 (4.9, 24.9)	10.2 (2.6, 24.6)	9.9 (3.5, 18.7)	0.452
Indoxyl sulfate	1456.1 (961.5, 1810.0)	1280.0 (863.9, 1912.3)	1074.0 (850.8, 1695.1)	1110.2 (862.1, 1892.5)	0.110
p-cresol sulfate	4669.5 (2864.0, 6890.8)	4789.1 (2807.0, 6974.1)	4163.6 (2593.6, 7333.7)	3821.0 (2421.5, 7177.1)	0.834

[a] Before drug administration

[b] After drug administration

[c] P-values of the association between plasma concentration of secretory solutes and time was calculated from linear mixed-effects models with both random intercepts and random slopes

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