GUT-DERIVED UREMIC RETENTION SOLUTES IN PATIENTS WITH MODERATE CHRONIC KIDNEY DISEASE AND HEALTHY ADULTS

by

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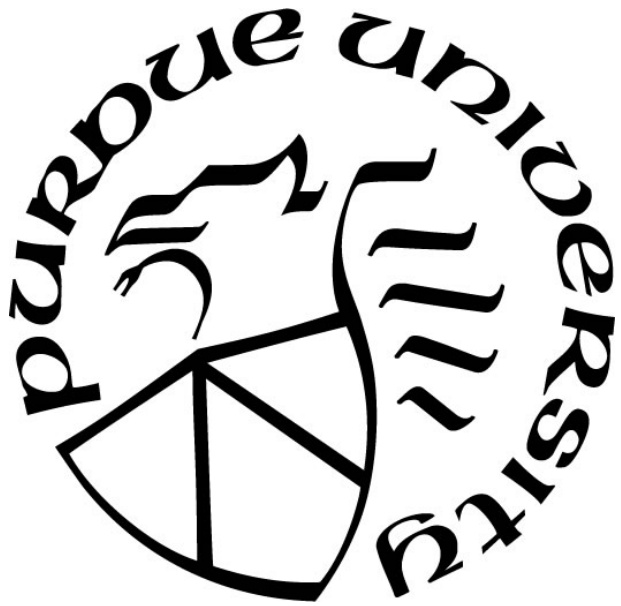

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To my many families near and far, whom without I would not have learned to smile, take a breath, and finish the work

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LIST OF ABBREVIATIONS

SYMBOL	DESCRIPTION
CKD	Chronic kidney disease
CVD	Cardiovascular disease
eGFR	Estimated Glomerular Filtration Rate
TMA	Trimethylamine
TMAO	Trimethylamine-N-Oxide
IS	Indoxyl Sulfate
P-CS	P-Cresol Sulfate
Cr	Creatinine
HD	Hemodialysis
ESKD	End stage kidney disease
LC-MS/MS	Liquid Chromatography-Tandem Mass Spectrometry
GC-MS	Gas Chromatography-Mass Spectrometry
GRE	Glycyl Radical Enzymes
FE	Fractional Excretion
OATp	Organic anion transporting polypeptide
OAT	Organic anion transporter
Mrp	Multidrug resistant protein
OCT	Organic cationic transporter

ABSTRACT

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Elevated levels of uremic retention solutes (URS), breakdown products of protein digestion, are associated with an increase in overall mortality, cardiovascular risk, and chronic kidney disease (CKD) progression. Increased levels of the URS Indoxyl sulfate (IS), p-cresol sulfate (PCS), and Trimethylamine N-oxide (TMAO) have been observed in patients with late stages of kidney disease (1-3). URS are formed via bacterial fermentation in the colon and are removed by urinary excretion, a task easily accomplished by healthy kidneys. However, in individuals with CKD, kidney function declines, resulting in decreased removal and subsequent accumulation of URS in the serum. However, few studies have evaluated URS in pre-dialysis CKD patients or have controlled for diet. Only one cross-sectional study evaluated levels of TMAO in both serum and urine of non-dialysis CKD patients compared with healthy adults (1), and no studies have examined all three of these URS while controlling dietary intake. Thus, in this secondary analysis, we aimed to determine serum and urine levels of IS, TMAO, and PCS in patients with moderate CKD compared with matched healthy adults who participated in a one-week controlled feeding study. Participants included patients with CKD (N=7) and healthy controls (N=7) matched for age, sex, and race. Participants consumed a diet controlled for macronutrients, fiber, phosphorus, calcium, potassium, and sodium for one week. Fasting serum and urine samples were collected at the end of the week. IS, PCS, and TMAO were quantified using LC-MS. Paired comparisons were used to determine differences between the groups and

associations were examined with Pearson's correlations. Results show that fasting serum URS were higher in CKD compared with controls ($p<0.05$). Urine URS tended to be higher in CKD patients, with IS reaching significance. Overall, kidney function (eGFR) was inversely related to each serum URS ($p<0.05$) and urine URS. However, when the relationship between eGFR and URS was evaluated within groups, strong inverse relationships only persisted in the CKD group. There were strong relationships among the serum and urine metabolites, and higher levels of serum URS corresponded with higher levels of the respective urine URS. When evaluated by group, these relationships remained strong in the CKD group, and slightly weakened in the control group. In conclusion, we have found that serum levels of URS are significantly elevated when compared with healthy adults even in early to moderate stages of CKD. Increased intestinal URS production via intestinal microbiome and altered liver function remain potential confounding variables in elevated serum URS levels over simply reduced excretion. Therapeutics, such as modified dietary protein intake or pre/pro/synbiotics, aimed at reducing URS production in the gut, may have the potential to reduce overall serum URS levels. Larger, longer studies evaluating diurnal serum URS and 24-hour urine URS excretion are needed to better understand URS retention and production in moderate chronic kidney disease patients.

CHAPTER 1: INTRODUCTION

CKD patients experience cardiovascular comorbidities that contribute to poor survival rates. Beyond traditional risk factors, such as diabetes mellitus and hypertension, elevated circulating metabolites, known as uremic retention solutes (URS), are associated with the acceleration of adverse cardiovascular-related outcomes. Animal models support epidemiological findings that elevated levels of URS may cause damage to renal (4-6) and cardiovascular tissue. L-carnitine and choline, tryptophan, and tyrosine and phenylalanine are dietary precursors of IS, PCS, and TMAO, respectively, that are modified by the intestinal microbiota to form intermediate metabolites that are further modified in via hepatic enzymes to form the final URS. In healthy adults, these URS are excreted via the kidneys, resulting in low circulating levels. However, elevated blood URS levels have been observed in CKD patients with kidney failure, as well as a return to levels of healthy adults following successful kidney transplant (4), indicating the role of kidney function in the elevation of serum URS levels. Dietary modifications and therapeutics modulating the gut microbiota have been suggested as a potential therapy to reduce the production of URS, potentially reducing circulating levels and the proposed adverse outcomes.

Two recent feeding studies evaluated patients with moderate stage CKD and found that a low protein diet (0.6g/kg/day) and a very low protein diet (0.3g/kg/day) can reduce blood levels of IS (5) and PCS (6). Cross-sectional studies measuring serum URS levels have found that even in early to moderate CKD patients circulating URS levels are elevated compared to healthy adults (4, 5, 7, 8). However, few studies control for dietary intake, which can profoundly alter the intestinal microbiota (9), and potentially the production of URS (10).

The purpose of this study was to assess the differences in blood and urine levels of all three URS in patients with moderate stage CKD and healthy adults who participated in a controlled feeding study consisting of adequate protein intake (RDA=0.8g/kg/day). We hypothesized that 1) blood URS levels would be elevated in CKD patients when compared to healthy adults, and 2) urine URS levels would be lower in CKD patients when compared to healthy adults.

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CHAPTER 2. LITERATURE REVIEW

Around 30 million American adults are living with chronic kidney disease (CKD) and many more are at increased risk of the disease (1). CKD is associated with myriad health problems, poor quality of life, expensive medical treatments, and increased mortality risk, making it a significant public health concern. Xie et al. (2) found that between the years 1990 and 2016 the global incidence and prevalence of CKD has increased by 89% and 87%, respectively. It is known that renal function declines with age, however factors such as hypertension, diabetes, and obesity accelerate renal function decline, making them the leading causes of kidney failure. Kidney failure results in the need for renal replacement therapy or kidney transplant. The most common is hemodialysis (HD) therapy to remove wastes and maintain fluid and electrolyte balance in the blood. HD therapy results in poor quality of life for patients and increased healthcare cost burden. The increasing aged population and increasing trends of CKD-related etiologies (diabetes, hypertension, and obesity) contributes to the increase of CKD prevalence globally (2-4). Other potential risk factors include race and ethnicity, family history, kidney injury, and prolonged consumption of over-the-counter painkillers.

Staging of Chronic Kidney Disease (CKD)

CKD is the progressive and irreversible loss of excretory, metabolic, and endocrine functions of the kidneys. Indicators used to define kidney damage include imaging, persistent albumin in the urine (albuminuria), and decreased estimated glomerular filtration rate (eGFR). The 2012 Kidney Outcomes Quality Initiative (KDIGO), classify CKD with a combination of eGFR (kidney function) and albuminuria (a kidney damage marker) **(Figure 1)** (5). CKD is classified into 5 stages that move from normal function to kidney failure as GFR declines and

albuminuria increases. Kidney function in stage 1 is normal (90-120 mL/min/1.73 m^2) or even slightly increased (~120-130 mL/min/1.72m^2). As the kidney disease progresses to stage 2, eGFR is mildly decreased to 60-89mL/min/1.73m^2. Stage 3 is split into two categories, with category "a" including individuals with an eGFR of 45-59mL/min/1.73m^2 and category "b" including individuals with eGFR 30-44mL/min/1.73m^2. The subdivision of stage 3 occurred in the 2012 KDIGO guidelines when evidence showed a difference in adverse outcomes between the groups based on eGFR. Individuals in stages 4 or 5 will have an eGFR of 15-29mL/min/1.73m^2 or <15mL/min/1.73m^2, respectively. If the cause of kidney failure is known, this is included with the classification of the CGA Staging accounting for the cause (C), eGFR (G), and albuminuria (A). Causes may include glomerular (diabetes, systemic autoimmune diseases), tubulointerstitial (systemic inflammation, drugs, environmental toxins), vascular (atherosclerosis, hypertension, ischemia), and cystic and congenital diseases (polycystic kidney syndrome). However, the most common causes of CKD are diabetes and hypertension, accounting for around 36% and 29% of all new CKD cases, respectively (6).

Prognosis of CKD by GFR and Albuminuria Categories: KDIGO 2012

GFR categories (ml/min/ 1.73 m²) Description and range				Persistent albuminuria categories Description and range		
				A1	A2	A3
				Normal to mildly increased	Moderately increased	Severely increased
				<30 mg/g <3 mg/mmol	30-300 mg/g 3-30 mg/mmol	>300 mg/g >30 mg/mmol
	G1	Normal or high	≥90			
	G2	Mildly decreased	60-89			
	G3a	Mildly to moderately decreased	45-59			
	G3b	Moderately to severely decreased	30-44			
	G4	Severely decreased	15-29			
	G5	Kidney failure	<15			

Green: low risk (if no other markers of kidney disease, no CKD); Yellow: moderately increased risk; Orange: high risk; Red, very high risk.

Figure 1 KDIGO clinical guidelines for staging of CKD. *Reprinted with permission from KDIGO (5).*

What are uremic retention solutes?

The gut microbiome generates URS that are removed from the blood via tubular secretion in kidneys with normal function. However, the decline of excretory function in CKD progression becomes increasingly problematic as URS accumulate in circulation and in tissues, leading to negative consequences. URS can be either protein-bound or nonprotein-bound. However, elevated levels of both have been associated with poor health outcomes in CKD (7-9). The most commonly studied URS include PCS, IS, and TMAO. Vanholder et al. (10) reviewed and evaluated studies for biologically relevant toxicity of many URS and indeed found that the data

confirm the role of these three URS in vascular and kidney disease progression. The following sections give an overview of studies on each of these three URS in CKD.

Indoxyl Sulfate

IS is an aryl compound (11) that originates from indole formed in the colon via bacterial modification and is sulfated in the liver **(Figure 2)**. IS has a high affinity for binding sites on albumin and is typically found to be ~90% protein-bound (12). PCS competes for these same albumin binding sites, and when present at elevated levels, there tends to be a larger fraction of free circulating IS (12).

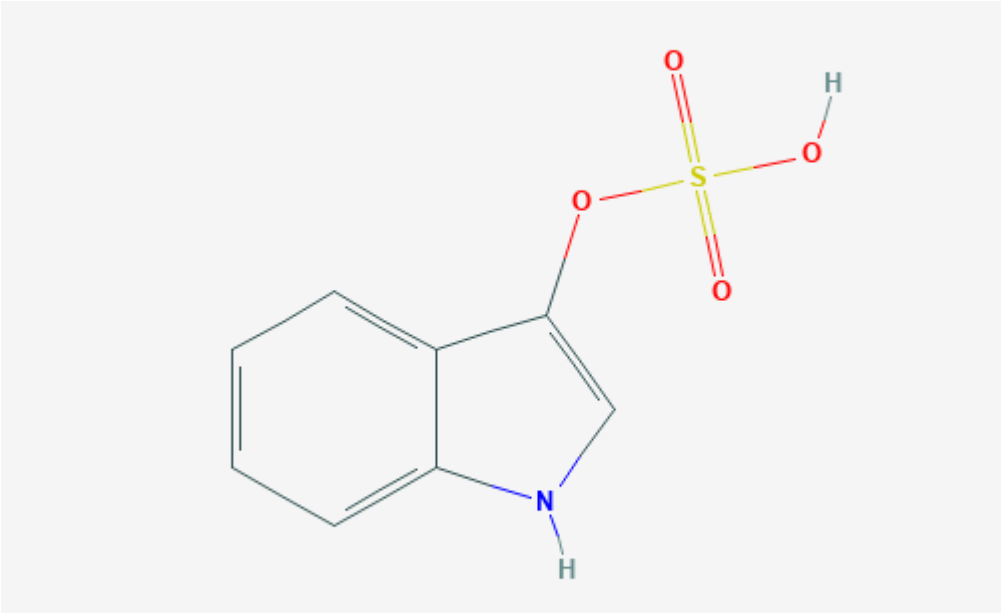

Figure 2 Chemical structure of Indoxyl Sulfate (11)

Recent studies demonstrate the relationship between serum IS levels and poor health outcomes in CKD patients. An early study by Barreto et al. (13) investigated the association between serum IS levels, vascular calcification, vascular stiffness and mortality in a cohort of patients with stages 2-5 CKD. At baseline they found that IS serum levels were inversely related

to renal function and directly related to aortic calcification. Overall, the highest tertile of serum IS was found to be a strong predictor of mortality in a follow-up, even after adjusting for age, gender, diabetes, aortic calcification and other factors. Similarly, Shimazu et al. (14) also found that patients with dilated cardiomyopathies and an eGFR equivalent to patients with stage 3 CKD had higher IS serum levels and were at increased risk for hospital admissions for heart failure and cardiovascular-related mortality. However, the risk for cardiovascular-related mortality may have been influenced by the comorbidity of dilated cardiomyopathy.

Several potential mechanisms may explain the damage inflicted by chronically elevated levels of IS. Preclinical studies have linked increased IS serum levels with various contributors of cardiovascular disease and the progression of kidney disease, including oxidative stress, inflammation, and tissue alteration in cardiac and renal tissues. In rats, IS directly stimulated vascular smooth muscle cell proliferation, a fundamental event in the progression of arteriosclerosis, in a dose-dependent matter (15). Dou et al. (16) found that biologically relevant concentrations of IS inhibited vascular endothelium proliferation and reduced wound repair, in vitro. Moreover, a recent study by Edamatsu et al. (17) showed that IS administration decreased the antioxidant, glutathione, leaving renal tubular cells vulnerable to oxidative stress. Other animal models of renal failure corroborate these findings that IS may damage renal tubular cells (17, 18).

p-Cresol Sulfate

PCS is similar to IS in that it is an aryl compound (19) (**Figure 3**) that is sulfated in the liver following bacterial modification from p-Cresol in the colon (20). As mentioned previously, PCS competes for the same binding site on albumin (12, 20), and may be found in free form when binding site capacity is saturated.

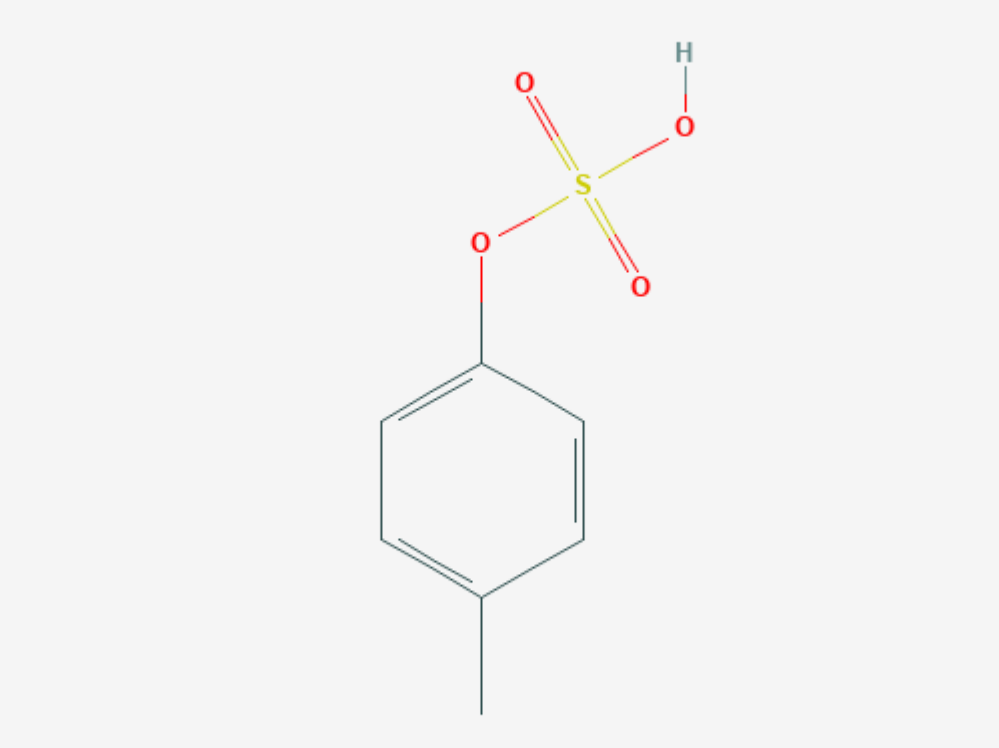

Figure 3 Chemical structure of p-Cresol Sulfate (19)

Several studies have noted the toxic effects of elevated PCS levels as CKD progresses to kidney failure. Several studies have investigated the additive contribution of elevated PCS levels to clinical outcomes in patients with ESKD undergoing hemodialysis. Among the first were Bammens et al. (21) who used a prospective observational cohort design to determine if elevated free serum levels of p-cresol are related to all-cause mortality in hemodialysis patients. Results showed that higher levels of free PCS were independently associated with higher mortality in hemodialysis patients. Lin et al. (22) corroborated these findings in a separate prospective cohort study, and in addition to higher all-cause mortality among HD patients, found that elevated PCS was also a valuable predictor of cardiovascular events. However, in a similar prospective cohort study of patients undergoing hemodialysis, Melamed et al. (23). found no significant changes in risk of cardiovascular-related and all-cause mortality in HD patients.

Trimethylamine N-Oxide

TMAO is a small, water soluble molecule (24) derived from multiple nutrients found in animal protein that is formed when trimethylamine undergoes oxidation in the liver (25) (Figure 4). In marine organisms TMAO functions as a protein stabilizing molecule that appears to counteract the effects of high urea (26).

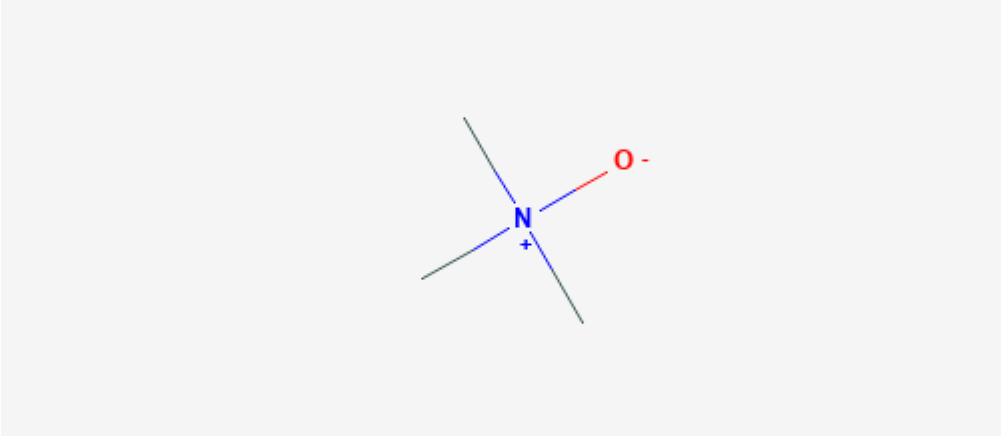

Figure 4 Chemical structure of Trimethylamine N-Oxide (24)

Inflammation and oxidative stress are implicated in risk of CKD progression and mortality. Two studies have evaluated levels of PCS and markers of inflammation and oxidative stress in CKD patients. A cross-sectional study by Rossi et al. (27) determined that elevated serum PCS levels in stage 3-4 CKD patients (N=149) were significantly associated with arterial stiffness, increased levels of (TNF-α) and interleukin-6 (IL-6), and decreased glutathione peroxidase (GPx). Borges et al. (8) found similar trends in IL-6 levels as well as increased levels of monocyte chemoattractant protein 1 (MCP-1), a chemokine that results from oxidative stress and mediates pro-atherosclerotic activity in vascular tissue. Trimethylamine N-Oxide

Recent studies have shown that elevated serum concentrations of TMAO have been linked to an increased risk of developing cardiovascular disease across all populations, including

patients with CKD (28, 29). TMAO levels are significantly elevated in CKD patients when compared to healthy controls (30-36). Bain et al. (33) was one of the first groups to recognize elevated serum levels of TMAO in hemodialysis patients compared with healthy controls. They evaluated levels of HD patients both before and after dialysis and found that serum TMAO levels were significantly elevated compared to healthy controls, but these levels fell comparable to those of healthy controls following dialysis. One limitation of this study was the use of gas-chromatography coupled with mass-spectrometry (GC-MS), which did not allow an internal standard to correctly quantify the metabolite. Despite this difference in methods, other studies have corroborated these findings. For example, after comparing HD patients with healthy controls matched for age and gender, Xu et al. (35) found that serum TMAO levels were significantly higher in the HD group ($p<0.001$). However, findings from a similar study in Missailidis et al. (32) contend that hemodialysis therapy is not effective at removing TMAO from circulation in HD patients. Investigators aimed to assess the contribution of TMAO to inflammatory biomarkers and the predictive value for all-cause mortality and the effects of various therapies undergone by patients with stage 3-5 CKD. While higher serum TMAO levels observed agree with previous studies, their results indicate that dialysis did not significantly affect TMAO levels in HD patients. However, a subset of patients in this study that underwent renal transplant surgery experienced significantly reduced serum TMAO levels, comparable to those of the healthy controls. A study by Stubbs et al. (31) also found that serum TMAO levels fell to that of healthy controls following renal transplant therapy.

Among these studies, many have shown a relationship between elevated TMAO levels and inflammatory biomarkers (36), all-cause mortality (32), cardiovascular risk (29), and all-cause mortality (30, 32). Not all findings regarding this relationship have been consistent.

Despite higher levels serum levels TMAO, low-density lipoprotein (LDL), and total cholesterol, results from Kaysen et al. (34) did not show increased risk for all-cause mortality or cardiovascular outcomes in HD patients. However, they note that by the time a patient reaches hemodialysis, the damage inflicted by elevated TMAO levels may not contribute any further risk.

URS Production and Metabolism

Colon

URS are products of protein catabolism that are further metabolized by colonic bacteria. Protein digestion occurs in the stomach via the enzymatic action of pepsin, yielding oligopeptides which are further degraded to di- and tripeptides for uptake at the brush border membrane in the intestine (37). Protein uptake and absorption in healthy adults is efficient, with approximately 95-98% of protein breakdown products taken up in the small intestine (38). The remaining 3-5% of catabolism products arrive in the colon, where they are modified by intestinal bacteria to synthesize amino acids and URS-intermediates.

Tryptophan, L-Carnitine and Choline, and L-Tyrosine or L-Phenylalanine are precursors for IS, TMAO, and PCS, respectively. Tryptophan is processed by bacterial tryptophanase to form indole. L-Tyrosine and L-phenylalanine are processed by bacterial to form p-Cresol, Choline, lecithin, and L-carnitine are endogenously cleaved to form trimethylamine (TMA) by various enzymes. Choline is a substrate for bacteria that associate with Glycyl Radical Enzymes (GRE), known as TMA-lyase, which works with GRE activase to form TMA (39). Additionally, L-Carnitine and other derivatives, specifically yBB, serve as substrates for bacteria which use Rieske-type oxygenase/reductases to form TMA (40). Aranov et al. (41) found that many uremic

toxins are either completely missing or significantly reduced in the serum CKD patients and controls with and without colons, strongly suggesting that URS originate in the colon. Most of the intermediates are absorbed via passive diffusion (25) and travel to the liver for further modification.

To quantify protein metabolism in the context of colonic microbiota it is useful to think of protein the way that microbes view it: a nitrogen source. The current assumptions are as follows: 1) lower amounts of net nitrogen are being absorbed in the large intestine due to the rapid and efficient uptake and absorption in the small intestine (42, 43), and 2) the form of nitrogen entering the large intestine (free amino acids, peptides, other metabolites, etc.,) depends upon the amount and source of protein, as well as dietary fiber (37, 44).To further complicate things, the ability of microbes in the large intestine to access the nitrogen in these precursors also depends on the favorability of the chemical environment (pH) for deamination to occur (45). Until this point, the current assumptions and discussions have been surrounding conditions in that of a healthy person. In the CKD patient, the microenvironement is inundated with factors affecting both pH and substrate availability, which are believed to alter the composition of the gut microbiota.

URS are gut-derived metabolites

Studies have found that the community composition of the gut microbiota is altered across all stages of CKD (46-48). There is a bidirectional relationship between the gut microbiota and CKD progression. This bidirectional relationship refers to the factors in CKD progression, specifically, higher circulating levels of urea, that contribute to changes in the gut microbiome which in turn, contribute to further disease progression (49). Factors that influence microbial communities include nutrient availability and diversity, pH, and interactions among

community members and the chemical and physical components in their environment (50). Vaziri et al. (50) performed the first study to explore the relationship between ESKD and the composition of the gut microbiome. They found that uremia profoundly altered the microbial community in both humans and rats with ESKD when compared to healthy controls. However, many factors are known to exert an influence on the gut microbiome in CKD patients including urea influx, phosphate binders, antibiotics, and CKD-related dietary restrictions (**Figure 5**).

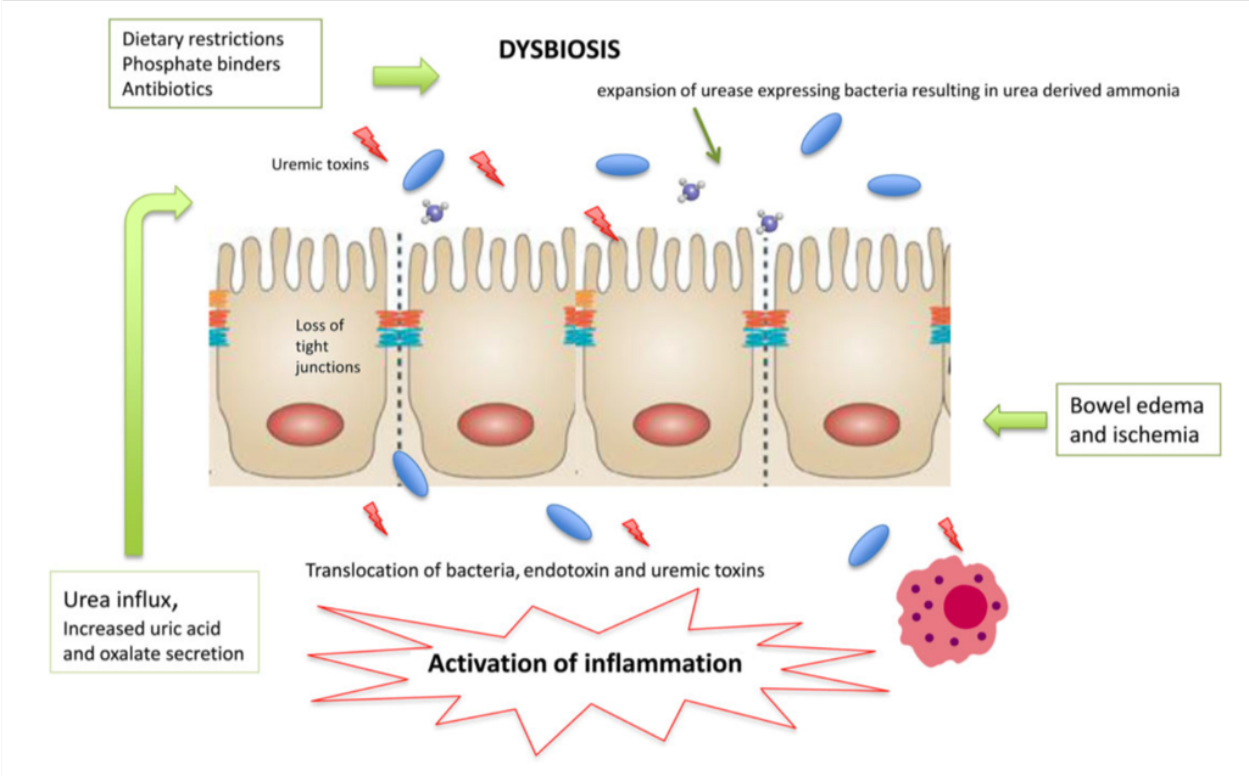

Figure 5 CKD influences gut microbiota and alters URS production. *Reprinted with permission from Oxford University Press (50).*

Among many factors, urea, another protein waste product that accumulates in CKD progression, is thought to alter microbial fermentation via lowering the pH of the lumen. In healthy adults roughly 25% of urea is secreted from the blood back into the lumen. High blood levels of urea (uremia) can be found in CKD patients and animal studies have shown a higher

amount of urea influx into the lumen of the large intestine (51). Mineral-altering medications such as phosphate binders and calcimimetics are common medications prescribed to patients with ESKD, while antibiotics and medications for the management of diabetes and hypertension are frequently used in earlier stages of CKD as well (52). Medications such as phosphate binders (53) and antibiotics (54, 55) have been found to profoundly alter the composition and interactions of the gut microbiota via changes to the chemical environment and altered nutrient availability. However, many CKD patients are on multiple medications, and this number increases upon progression to ESKD, posing a difficult task of measuring the effects of a medication cocktail on complex microbial interactions. Dietary restrictions differ in moderate-severe stages of CKD and ESKD and will be described in detail later on.

Liver

The URS intermediates indole, TMA, and p-cresol undergo enzymatic modification in the liver before entering circulation, as shown in below **(Figure 6)**. Indole is oxidized in the liver by cytochrome p450-2E1 and is then sulfated by sulfotransferase 1A1 to form IS (56). Similarly, p-Cresol is sulfated to form PCS by aryl sulfotransferases (57). TMA undergoes oxidation via hepatic Flavin monooxygenase 3 (FMO3) to form TMAO. Lin et al. (58) evaluated the role of liver function in CKD stages 1-4 and found that the liver has a role independent of kidney function in determining IS and PCS levels. Total serum IS and PCS levels were significantly lower in patients with early liver cirrhosis, but this value did not see any further increase as liver disease advanced. In the same study, findings were confirmed in a rat model of liver disease that showed significantly reduced total serum and urine IS and PCS levels with disease progression (58). These findings indicate that damage to hepatic tissue early in liver disease is associated reduced IS and PCS production, and potentially variation, in CKD patients.

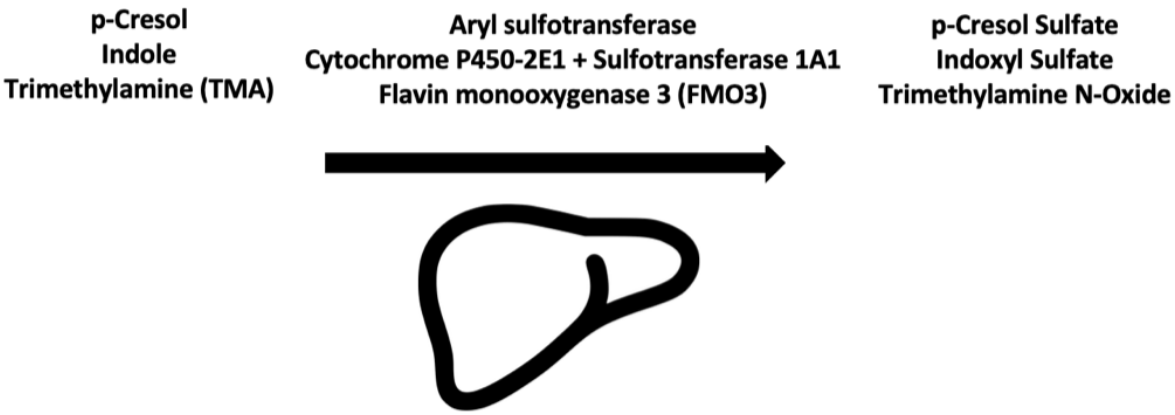

Figure 6 Enzymatic modification of URS intermediates in the liver

Kidney

IS, TMAO, and PCS are removed from circulation via proximal tubular secretion and ultimately eliminated from the body via urinary excretion. As blood flows through the afferent arteriole into the glomerulus, ultrafiltrate is formed, which is similar in composition to plasma with the exception of being virtually free of cells and large proteins. After glomerular filtration, renal blood flows through capillaries where smaller molecules not removed by filtration, such as URS and certain drugs, are secreted into the ultrafiltrate for excretion as urine (59). At the cellular level, IS, PCS, and TMAO are transported across the apical membrane of proximal tubule cells via the uptake transporters known as organic anion transporters (OAT) 1 and OAT 3 (60). Additionally, TMAO possesses both positively and negatively charged groups, allowing it to also make use of the organic cation transporter 2 (OCT2) (61). Solutes are transported across the cell by efflux transporters known as multi-drug resistance proteins (Mrp) and bidirectional transporters at the basolateral side known as organic anion transporting polypeptides (OATp) (62-64). These renal transporters required for URS transport are shown below (**Figure 2.7**).

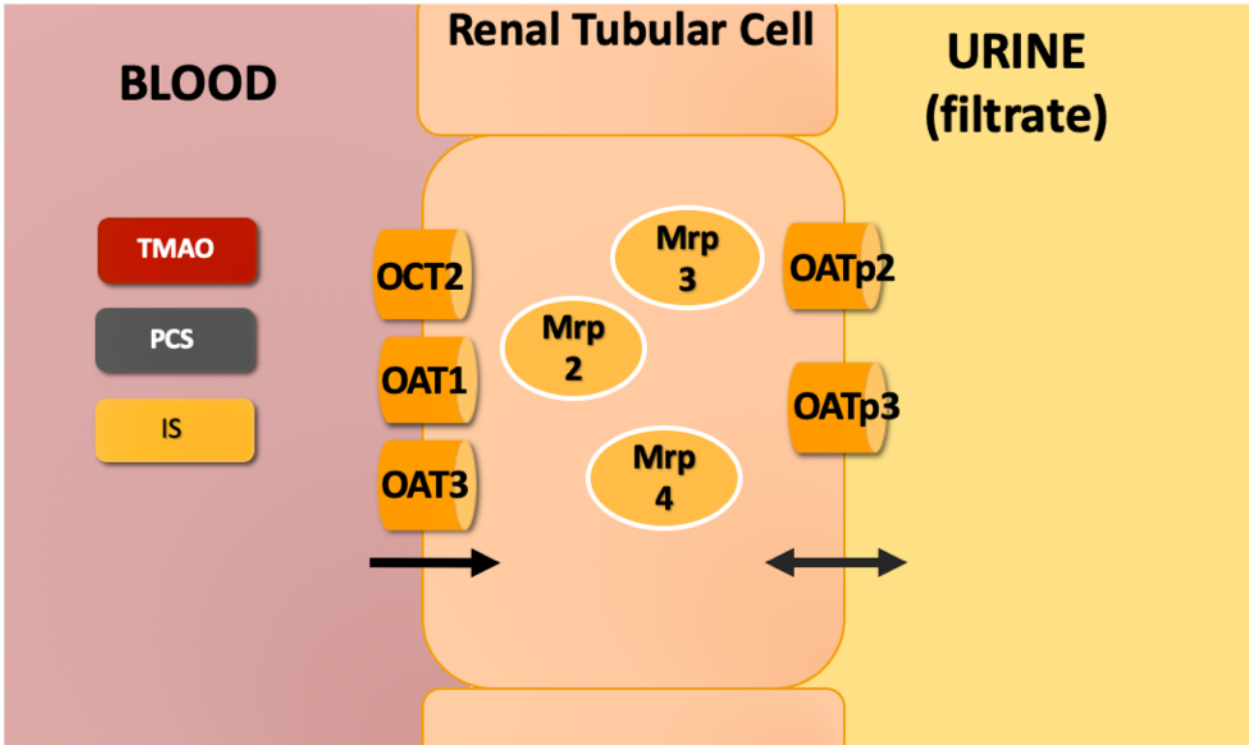

Figure 7 URS transport in the kidney

Animal studies have shown that CKD leads to reduced expression of OAT1/3 transporters, resulting in decreased proximal tubular secretion of these wastes (65, 66). Additionally, Naud et al. (65) found that chronic renal failure in male rats also led to the upregulation of efflux transporters Mrp2, Mrp3, and Mrp4 and downregulation of the bidirectional transporters OATp2 and OATp3. They suggest that this combination of changes may be a protective mechanism against the accumulation of URS and subsequent injury in the renal tissue. In addition to the changes in URS transport, commonly prescribed drugs are also known to compete for the use of these OAT transporters, all potentially contributing to further accumulation of URS in circulation. While several experimental studies corroborate these findings (60, 65-67), the measurement of urinary URS excretion in humans is lacking, particularly for 24-hour urine excretion.

Tubular URS handling in the kidney is difficult to measure directly, and future studies should employ multiple measurements to better determine renal contribution to serum URS levels. Typical measurements include the total concentration excreted in the urine (usually in mg/dL), URS urine excretion rate, measured as URS concentration normalized to urine creatinine (URS ug/mL: Cr mg/mL), and fractional excretion (FE), or the proportion of filtered URS that is excreted into the urine. While all three are useful measurements, they each have strengths and limitations in their applications. Total urine URS concentration is a valuable measurement to measure variation in renal clearance of URS; however, this measurement may be influenced by differences in the volume and the concentration of urine output. The urine URS:Cr ratio, under steady-state conditions, is assumed to be a measurement of URS excretion rate (31). Fractional URS excretion measures the percent of filtered URS that is excreted in the urine. As CKD progresses, fractional excretion of solutes has been noted to rise as the kidneys attempt to ameliorate the declining filtration in the nephron (68, 69). Measurements of urine URS levels, when used appropriately, may help elucidate the mechanisms behind URS balance in CKD.

Serum and Urine URS Levels in CKD patients and Healthy Adults

IS

Studies evaluating healthy individuals have found normal total serum IS levels to be somewhere between <1-4 uM (70, 71). Serum levels in patients with moderate CKD can range from 9-15 uM (8, 27, 46, 70), with sharp increases to >100 uM (72) in kidney failure. A study in healthy adults showed that IS levels can vary greatly over days and weeks and may require more measurements to account for biological variation (71). Currently, there is no evidence of a difference in urinary levels of IS between healthy adults and patients with CKD.

PCS

Though many studies have concluded that elevated serum PCS contribute to poor health outcomes in CKD patients, a clinical reference range has not yet been determined. Studies evaluating healthy individuals have found normal total serum levels to be around 15-35uM (73, 74). Studies evaluating PCS levels in CKD patients show 48-60 uM for moderate stage CKD (70, 8) and >100 uM in ESKD (8, 75, 76). Currently, evidence neither supports nor refutes differences in urinary PCS concentrations, which may be due to large interindividual variation. One study by Rivara et al. (71) measured PCS in 25 healthy adults over a 24-hour baseline period and in two subsequent follow up visits at 2-weeks and 14-weeks. Results showed that serum and urinary P-CS concentrations vary greatly between individuals in both the short-term and long-term.

TMAO

Consistently shown across studies, CKD patients have progressively increased serum levels of TMAO compared to healthy adults (31, 35, 36). Reported median serum TMAO levels for healthy adults range from ~1-5uM (30-32, 35). However, the intraindividual variation of TMAO levels in healthy, older adults has been shown to be even greater than variation between individuals (77). Serum TMAO levels in stage 3 CKD are consistently shown to be elevated ~8-10uM (30-32). Currently, only Stubbs et al. (31) has compared the urine concentration and fractional excretion of TMAO in CKD patients and healthy adults. Interestingly, neither the concentration nor the fractional excretion of urine TMAO levels varied between healthy controls and patients with moderate to severe CKD (excluding ESKD).

The Role of Dietary Protein Intake in Blood Levels of Uremic Retention Solutes

Nutritional management plays an important role in the preventing or slowing kidney disease progression (78, 79), but despite this fact, 90% of non-dialysis kidney disease patients never see a registered dietitian (80). In early to moderate stages (Stages 1-3) dietary modifications are aimed at controlling the etiologies contributing to renal failure, including controlled carbohydrate diets (type II diabetes mellitus) and reduced sodium intake (hypertension). In later stages of CKD (stages 4-5) where most patients are nearing the need for dialysis therapy, dietary recommendations are aimed at maintaining electrolyte and fluid balance by controlling the intake of sodium, potassium, fluid, and phosphorus, and reducing the production of nitrogenous waste through protein restriction. While current guidelines recommend that patients with pre-dialysis CKD restrict protein intake to 0.6-0.8 (g protein/kg/day), protein quantity and source (animal-based v. plant-based) are still being evaluated for long-term effects on disease progression.

Lower protein intake is associated with greater constriction of the afferent arteriole, reducing blood delivered to the functional unit of the kidney known as the glomerulus, thereby reducing intraglomerular pressure. Reduced glomerular pressure protects the glomerulus from excessive filtering of wastes (hyperfiltration), which can cause damage over time (81). Findings from the Modification of Diet in Renal Disease (MDRD) study showed that a low protein diet (0.58g protein/kg/day) minimally slows the progression of kidney disease in moderate staged patients (82). Many large trials since the MDRD, including meta-analyses, have shown a modest beneficial effect of low protein on kidney disease progression (83). Taken together, these findings guide the current recommendations for modest protein intake in patients (0.6-0.8g/kg/day) before the initiation of dialysis therapy.

Recent studies have found that dietary protein intake influences circulating levels of URS in both healthy adults and patients with varying stages of kidney disease (84, 85). The circulating levels of URS can be visualized as a bathtub **(Figure 5)** in which the faucet is always producing URS at varying levels following endogenous and exogenous protein metabolism. The drain in this case represents the extent to which kidney function contributes to elevated URS from the blood, maintaining a homeostasis with the various inputs contributing to URS blood levels. Individuals with healthy kidneys are able to clear URS from the blood via proximal tubular secretion kidneys and subsequent urinary excretion; however, this becomes increasingly problematic for patients with declining kidney function. Progressively higher levels of TMAO, IS, an PCS have been described with advancing kidney disease in several cross-sectional studies (13, 31, 36, 75). As previously described, elevated blood levels of URS may contribute to vascular and renal tissue damage, and many studies have examined therapies aimed at modulating the gut microbiota as a means to reduce URS blood levels (86). Dietary protein restriction has been suggested as a potential therapy to lower URS levels and control uremic symptoms.

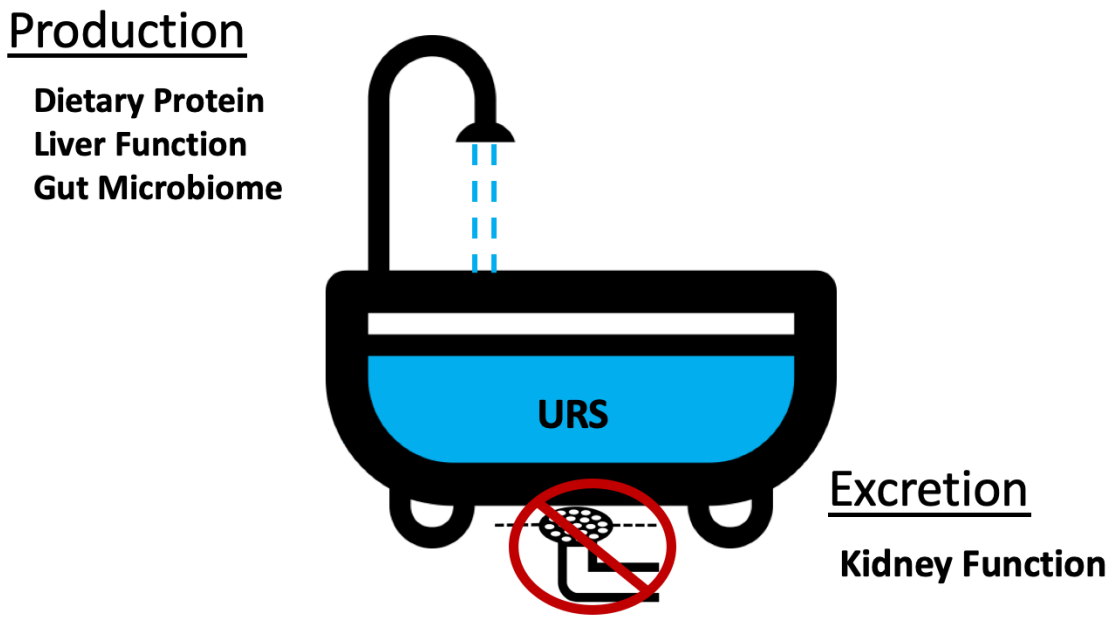

Figure 8 Factors affecting URS levels

Two studies (46, 84) have found that lower levels of dietary protein intake in moderate stage kidney disease patients result in lower blood levels of IS and PCS. Marzocco et al. (84) studied blood levels of IS in moderate stage kidney disease patients in a two-week controlled feeding study. Participants were given a low protein diet (LPD, 0.6g protein/kg/day) for one week, immediately followed by a very low protein diet (VLPD, 0.3 g protein/kg/ day) supplemented with ketoanalogues for a second week. Results showed that a very low protein diet significantly reduced serum levels of IS in moderate stage CKD patients. However, a study by Black et al. (46) found that the same population could achieve a significant reduction in blood levels of PCS, but not IS, after consuming a LPD. Blood levels of IS and PCS were evaluated at baseline and at the end of the six-month intervention. Their findings show that a LPD results in a significant reduction in the blood PCS levels, but not in IS levels.

Currently, there are no controlled feeding studies in patients with CKD evaluating levels of TMAO. A study by Pignanelli et al. (70) evaluated the relationship between levels all three URS and dietary intake of known URS-precursors in older adults in attending stroke prevention centers. TMAO levels were higher with reduced kidney function of <66 mL/min/m2 compared to >90 mL/min/m^2. A unique measurement in this study included quantification of the dietary precursors of TMAO consumed by each quartile of metabolite levels. Interestingly, the dietary precursors only predicted TMAO levels in patients with eGFR >60. The authors discuss potential reasons for these outcomes, including the idea that the gut-renal axis may contribute to these findings. While this did not specifically assess patients diagnosed with CKD, their findings indicate that even modest renal impairment is related to elevated fasting blood levels of TMAO.

Conclusions and Future Work

The accumulation of URS, even in early stages of CKD, may contribute to the progression toward kidney failure and cardiovascular outcomes. Dietary modifications in moderate stage CKD can help slow disease progression. However, reference ranges for serum and urine URS of interest have not been established. Further, it is still unclear how earlier stages of CKD affect URS blood and urine levels. Few studies have evaluated the effect of moderate stage CKD, and even fewer have controlled for dietary intake. Diet can produce rapid and significant changes in the composition of the gut microbiome (87), and some evidence suggests that diet influences the gut microbiome and subsequent metabolic products more so than changes in kidney function (88).

Measuring in blood and urine URS values could be used to assess further risk and guide therapies targeting high serum URS levels. Controlled feeding studies are needed to determine the effect of kidney function integrity on levels of blood and urine URS in patients with

moderate stage CKD. However, there are gaps in the current literature laying the foundation for developing these targeted therapies. In order to investigate the effect of kidney function on blood and urine URS levels, future studies should control protein intake quantitatively. Ideally, studies matching CKD patients with healthy controls would allow for a reduction of variation that may result from demographic differences.

This secondary analysis aims to address the gap in basic information surround URS levels in the blood and urine between CKD patients and healthy adults. By controlling for diet and confounding demographic variables with matched participants, our findings will contribute to the current knowledge of the relationship between kidney function and levels of URS in the blood and urine.

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CHAPTER 3: METHODS

Participant Selection

Seven moderate stage CKD patients and seven healthy adults matched for sex, race and age (+/- 10 years) to the enrolled CKD patients between ages 30-75 years were recruited to participate in a controlled feeding study with the primary objective of determining intestinal fractional phosphorus absorption in CKD compared with healthy controls. The present study of serum and urine URS was an ancillary aim. Subjects received verbal and written descriptions of the study and provided signed informed consent prior to the study. After giving informed consent, participants underwent screening to assess eligibility at the Indiana Clinical Research Center. The screening included vital signs, a review of medical history, and laboratory values.

If available, laboratory values from 3 months prior to the study were used to determine study eligibility according to criteria below **(Table 1).** If values from the last 3 months were unavailable, then a fasting (at least 8 hours over night) blood collection of 7 mL, by trained phlebotomist, and fasting urine for screening with measurements of eGFR and urine protein to creatinine ration (UPCR) and safety biochemistry were obtained (see screening tool, Appendix A). Healthy participants met inclusion criteria if serum phosphate, calcium, potassium, sodium, and blood urea nitrogen were within normal ranges, along with no evidence or diagnosis of kidney disease. Participants in the CKD group were considered eligible if fasting serum calcium and potassium were not elevated in the last three months, and eGFR and/or UPCR measurements according to inclusion criteria.

Table 1 Participant inclusion/exclusion criteria

Moderate CKD	Healthy Controls
Inclusion	
Men and women ages 30-75 years old, any race	Men and women matched for race and age (within +/-10 years) of CKD patients enrolled in this study
Moderate CKD, based on the last set of labs done in the past year, defined as: GFR category G3a (eGFR 45-59 mL/min) with A2 or A3 albuminuria or proteinuria by positive dipstick or UPCR; or confirmed progressive CKD by a study nephrologist. -or- G3b (eGFR 30-44 mL/min) or early G4 (eGFR 25-29 mL/min), with or without evidence of albuminuria (any A1-A3) or proteinuria. (**Figure 1**)	No diagnosis or evidence of CKD
Female subjects must be post-menopausal, surgically sterile, or confirmed not pregnant by pregnancy test and not breastfeeding.	same
Subjects must be on stable doses of medications (except those noted in exclusion criteria) for at least 4 weeks prior to the study	same
Exclusion	
Plans to initiate dialysis within 6 months	Diagnosis or evidence of CKD using GFR and albuminuria categories (**Figure 1**) G1 or G2 with A2 or A3 (or positive for proteinuria on dipstick or UPCR); G3a-G5 with or without albuminuria/proteinuria (subjects in G1A1 and G2A1 will meet inclusion, if no other evidence of CKD)
Labs based on the last set done in the past 3 months: Hypercalcemia defined as serum calcium > 10.5 mg/dL Hyperkalemic > 5.5 mg/dL	Labs based on the last set done in the past 3 months: Serum phosphate, serum calcium, potassium, or sodium outside of normal ranges; high blood urea nitrogen (>24 mg/dL)

Table 1 continued

Prescribed a phosphate binder medication	same
Small bowel resection, bariatric surgery. Medically unstable or poorly controlled hypertension, diabetes, or gastrointestinal disorders in the opinion of the physicians on the study team	same
Calcitriol, vitamin D analogs, calcimimetics, PTH analogues, and other medications that may alter phosphorus metabolism – must be off for at least 4 weeks prior to study	same
Unwilling to discontinue vitamin D supplements (ergocalciferol, cholecalciferol), calcium supplements, multivitamins/minerals, other nutritional supplements during the controlled diet study.	same

Experimental Design

Enrolled subjects participated in an 8-day controlled feeding study ending with a two-day, inpatient period with the primary aim of determining intestinal phosphorus absorption using an oral and IV administration of the radioisotope P-33. Enrolled subjects were asked to discontinue nutritional supplements at the time of enrollment and through completion of the study and complete a 4-day diet record prior to the Day 1 outpatient visit.

Day 1: After successfully completing informed consent and a screening visit determined eligibility, participants reported to the Indiana Clinical Research Center for an outpatient visit (approximately 30-60 minutes). During the inpatient period, all food intake was monitored by study staff and all excreta (urine and feces) were collected. Subjects were instructed by study staff on how to complete a diet record and were asked to complete a 4-day diet record prior to

Day 1 of the controlled feeding period, for assessment of usual dietary intake. For enrolled subjects with diabetes, the physicians on the protocol along with the dietitian, reviewed their dietary intake from the pre-study 4-day food record and their medications and made adjustments in study diet and diabetes medications as appropriate prior to the start of the study. The subjects were given the 4-day food record and instructions to take home at the screening/enrollment visit and returned the completed record to the study coordinator prior to Day 1 so that study diet and medications could be adjusted prior to beginning the study.

Day 4: Patients returned to the hospital to collect the food remaining for the last three days.

Day 7: Patients reported to the Indiana Clinical Research Center on the morning of Day 7 for fasting urine and blood collection.

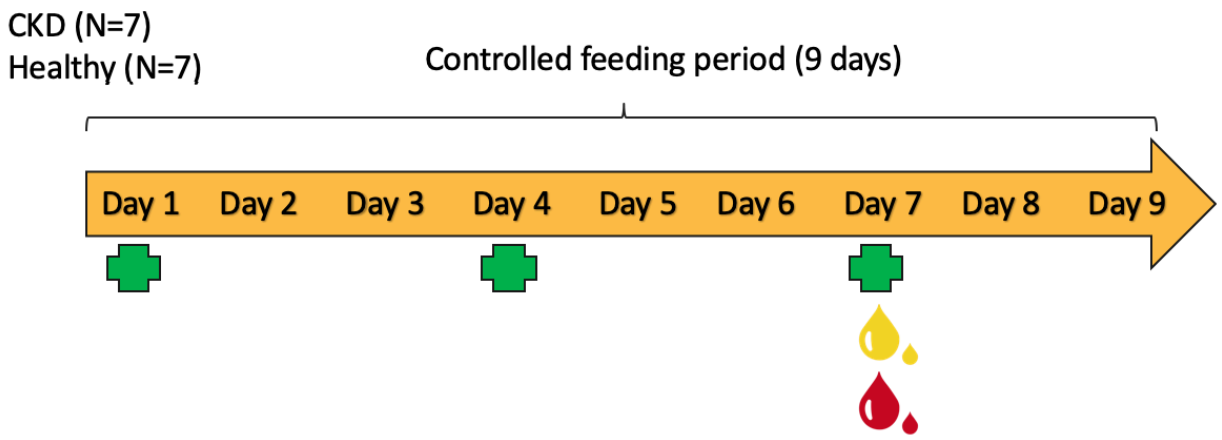

Figure 9 Study design

Study Diet

Subjects consumed a diet controlled for macronutrient (energy 2240 ± 15kcal/day, protein 108 ± 10 g/day, carbohydrate 288 ± 6 g/day, fat 76 ± 3 g/day), phosphorus (1526 ± 15mg/day), calcium (1400mg/day), sodium (2442 ± 234mg/day), and potassium (3192 ±

276mg/day) content (Appendix C). Protein content was 70% animal-based protein and 30% plant-based, and fiber content was 72% insoluble fiber and 28% soluble fiber **(Appendix A).**

Biochemical Analyses

Serum (50uL) and urine (500uL) metabolites from the fasting collections on Day 7 were measured using an Agilent 1290 Infinity II liquid chromatography (LC) system coupled to an Agilent 6470 series QQQ mass spectrometer (MS). A Water's Corporation HSS T3 2.1 mm x 150 mm, 1.8 µm column was used for LC separation (Water's Corporation, Milford, MA). The buffers were (A) water + 0.1 % formic acid and (B) acetonitrile + 0.1% formic acid. The linear LC gradient was as follows: time 0 minutes, 0 % B; time 2 minutes, 0% B; time 6 minutes, 100 % B; time 7 minutes, 100 % B; time 7.1 minutes, 0 % B; time 10 minutes, 0 % B. The flow rate was 0.3 mL/min. Multiple reaction monitoring was used for MS analysis. The data were acquired in negative and positive electrospray ionization (ESI) mode according to **(Table 1, Appendix B)**. The jet stream ESI interface had a gas temperature of 325°C, gas flow rate of 8 L/minute, nebulizer pressure of 45 psi, sheath gas temperature of 250°C, sheath gas flow rate of 7 L/minute, capillary voltage of 3500 V in positive mode and negative mode, and nozzle voltage of 1000 V. The ΔEMV voltage was 300 in both positive and negative modes. All data were analyzed with Agilent Masshunter Quantitative Analysis (Version B.08.00).

Serum and urine creatinine were measured by colorimetric assay using a RX Daytona clinical chemistry analyzer (Randox Laboratories). The serum URS values were reported as concentration (uM). The urine URS values are presented in three ways: 1) URS concentration was presented in mg/dL, 2) ratio of URS per mg of creatinine (URS:Cr), and 3) urinary fractional URS excretion (FE_{URS}) calculated by the following formula:

$$\text{Fractional Excretion} = \left[\frac{(urine\ URS\ in\ mg/ml/\ serum\ URS\ in\ mg/ml)}{(urine\ creatinine\ in\ mg/dl\ /\ serum\ creatinine\ in\ mg/dl)}\right] \times 100$$

Statistical Analyses

The data were analyzed using statistical SAS/STAT® software[1](**Appendix B).** Differences in means between CKD patients and healthy controls were determined by paired comparisons using general linear models (PROC GLM). eGFR was tested as a covariate in these models to determine its contribution to accounting for differences between the groups. Relationships among continuous variables were evaluated with Pearson's correlations. Significance was defined as $\alpha = 0.05$.

[1] *SAS and all other SAS Institute Inc. product or service names are registered trademarks or trademarks of SAS Institute Inc. in the USA and other countries. ® indicates USA registration.*

CHAPTER 4: RESULTS

Participant Characteristics

Participant characteristics are shown in **Table 2**. Approximately half of the participants were female, with approximately ½ of subjects of black race and ½ of white race with no significant differences in weight or age. Expected differences between the patients and controls were observed in eGFR ($p=0.018$), BUN ($p<0.0001$), and serum creatinine ($p<0.0001$).

Table 2 Participant Characteristics

	Patients **N=7**	**Controls** **N=7**	**P-value**
Female/Male (n)	4/7	4/7	N/A
Age (y)	60 (9.7)	56 (9.5)	0.45
Black/White (n)	3/7	3/7	N/A
BMI (kg/m^2)	33.7 (8.40)	29.2 (4.98)	0.24
eGFR (mL/min per 1.73 m^2)	41(9)*	88 (14)	0.018
BUN (mg/dL)	31 (14)**	14 (3)	<0.0001
Serum Creatinine (mg/dL)	1.62 (0.17)**	0.88 (0.13)	<0.0001

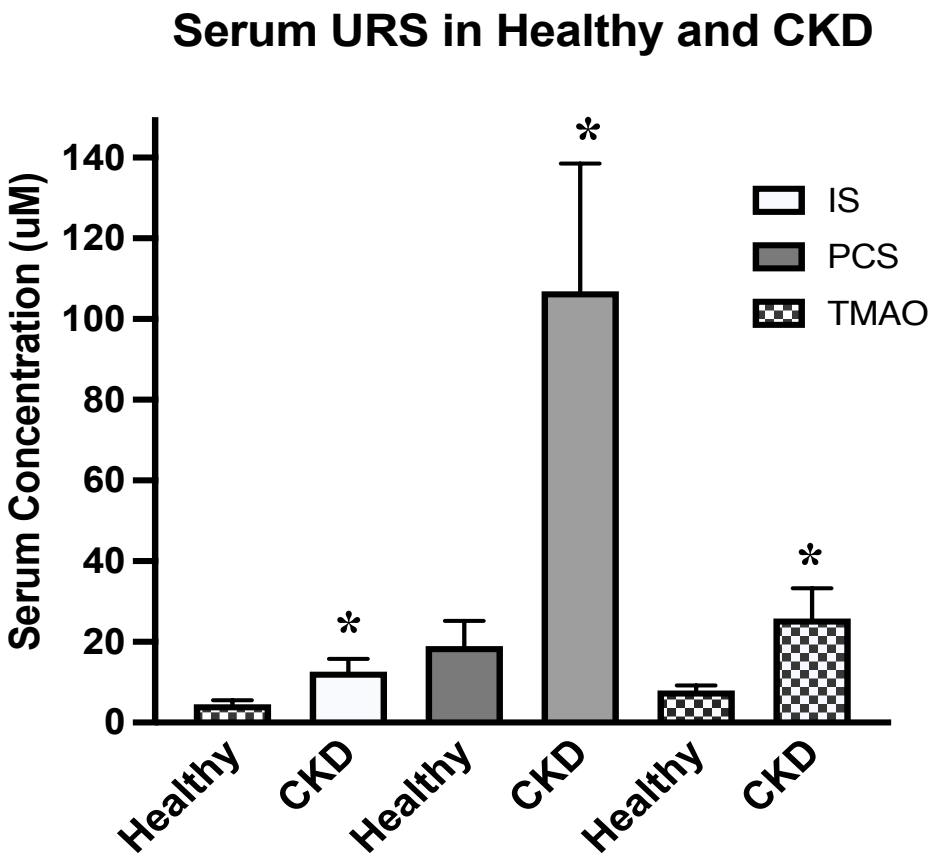

Figure 10 Fasting serum URS in CKD patients and healthy controls

Figure 10 Fasting serum URS in CKD patients and controls. Comparison of fasting serum metabolite concentrations (uM): IS (Healthy, 4.56 ± 0.94; CKD, 12.64 ± 3.16), PCS (Healthy, 18.97 ± 6.27; CKD, 106.87 ± 31.63), TMAO (Healthy, 7.92 ±1.32; CKD, 25.76 ±7.50) in healthy controls (N=7) and CKD patients (N=7). Data are presented as means ± SE. *p<0.05.

Serum and urine URS in CKD patients and healthy controls

We observed significantly higher levels of serum IS (p=0.018), PCS (p=0.019), and TMAO (p=0.035) in CKD patients compared with healthy controls **(Figure 11).** Mean urine IS, PCS, and TMAO concentrations (mg/dL) were numerically higher in CKD compared with controls,

however these differences did not reach statistical significance**.** A comparison between fasting urine URS concentration as a ratio to creatinine (URS ug: Creatinine mg) between CKD and controls showed similar results: IS (p=0.049), PCS (p=0.444), TMAO (p=0.083) tended to be higher in CKD when compared to controls, with only IS reaching significance (p<0.05).

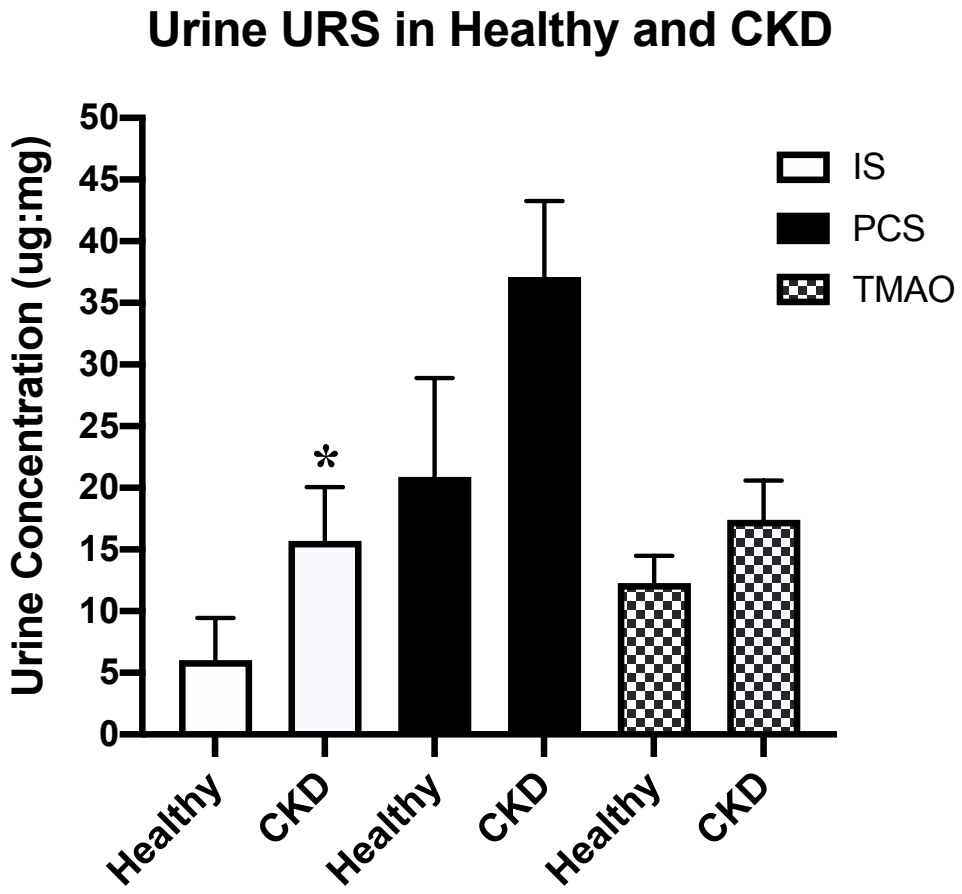

Figure 11 Fasting URS excretion in CKD patients and healthy controls

Figure 11 Comparison of fasting urine metabolite concentration (URS ug: Cr mg): IS (Healthy, 6.02 ±3.42 ; CKD, 15.69 ± 4.38), PCS (Healthy, 20.88 ± 8.02; CKD, 37.10 ± 6.17), TMAO (Healthy, 12.27 ± 2.21; CKD, 17.40 ±3.19) of healthy controls (N=6) and CKD patients (N=7). Data are presented as means ± SE. *p<0.05

There is a strong relationship between serum and urine URS levels

Pearson's correlations were used to examine relationships between all serum and urine URS and eGFR **(Appendix B)**. Overall, serum URS concentrations had a strong, positive relationship with urinary excretion rate ($r= 0.65$ to 0.91; $p<0.0001$ to 0.02). These associations were strongest within the CKD patients ($r= 0.60$ to 0.98; $p=0.0001$ to 0.16), but still apparent within controls ($r= 0.77$ to 0.98, $p=0.0008$ to 0.075). Serum PCS levels also corresponded with FE levels (PCS: $r=-0.66$, $p=0.02$), while TMAO and IS became non-significant with this measurement (TMAO: $r=-0.47$, $p=0.10$; IS: $r=0.39$, $p=0.19$). When analyzed by group, serum concentrations were strongly related to urinary excretion of IS **(Figure 12 B)** and PCS **(Figure 12 D)** in CKD patients only, while serum and urine TMAO were strongly related within both groups (Healthy: $r=0.94$, $p=0.006$; CKD: $r=0.98$, $p=0.0001$).

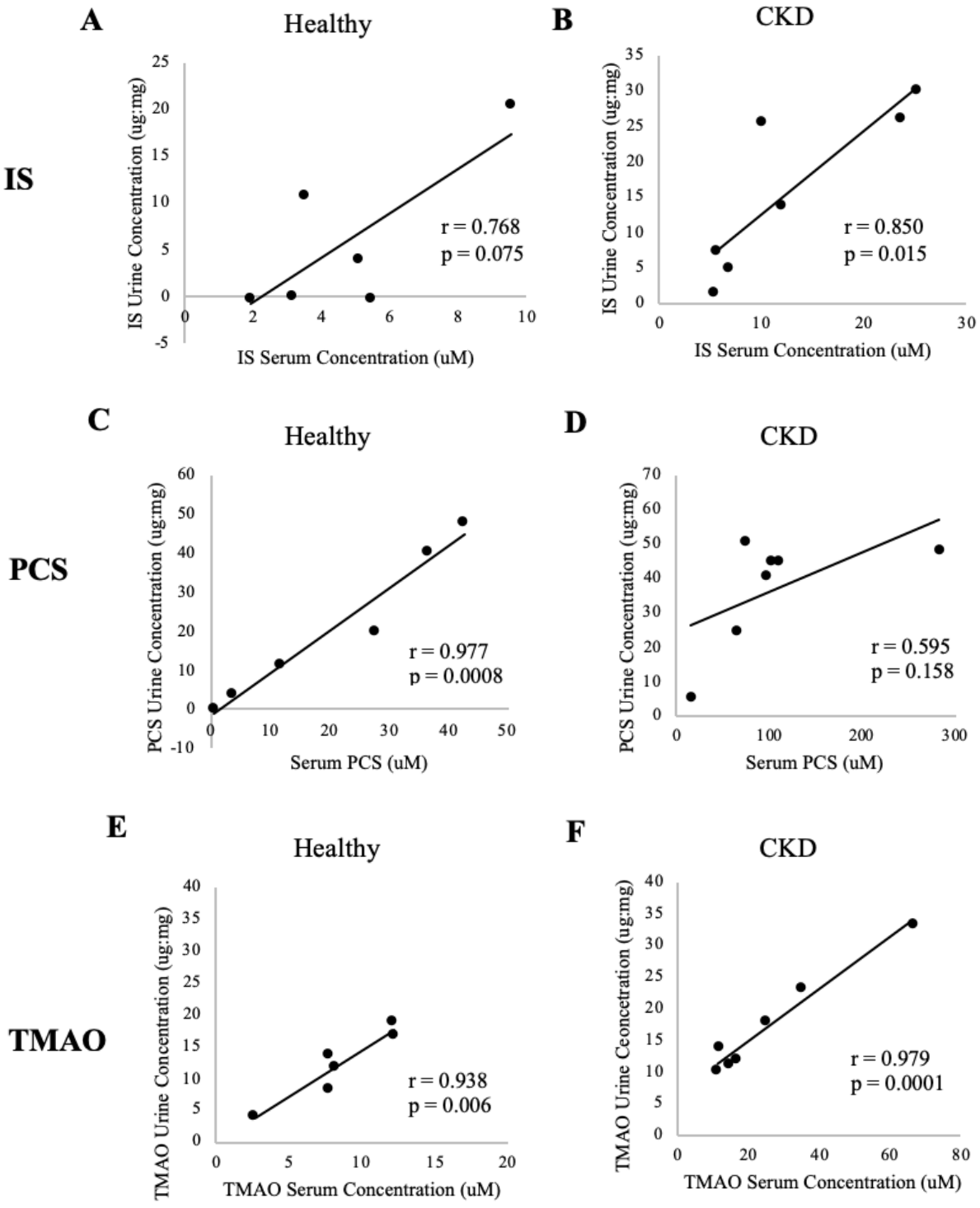

Figure 12 The relationship between serum and urine URS

There is a relationship between blood and urine URS levels and eGFR

Overall, there was a strong inverse relationship between eGFR and all serum URS (r=-0.65 to -0.70, p=0.007 to 0.02) **(Table 3)**. When CKD patients and controls were analyzed separately, these relationships between eGFR and serum URS persisted in CKD patients (r=-0.68 to -0.77, p=0.04 to 0.10), but not in the control group (r=-0.49 to 0.06, p=0.32 to 0.91). Similar trends were observed when comparing each urine URS values between CKD patients and healthy controls. URS excretion rate was inversely related to eGFR in the CKD group (r=-0.48 to -0.79, p=0.03 to 0.44) to a greater extent than the control group (r=-0.45 to 0.14, p=0.54 to 0.83). There was no relationship between eGFR and fractional excretion of any URS within the CKD group (r=-0.31 to 0.42, p=0.39 to 0.49), or controls (r=-0.12 to 0.13, p=0.85 to 0.90).

Table 3 The relationship of kidney function and URS within CKD patients and healthy controls

	All		CKD		Healthy	
Parameter	r	p	r	p	r	p
Serum		N=14		N=7		N=7
IS (uM)	-0.69	0.008*	-0.77	0.04*	-0.46	0.35
PCS (uM)	-0.7	0.007*	-0.68	0.1	-0.49	0.32
TMAO (uM)	-0.65	0.02*	-0.73	0.06	0.06	0.91
Urine						
Excretion		N=13		N=7		N=6
IS (ug:mg)	-0.54	0.07	-0.71	0.08	-0.17	0.78
PCS (ug:mg)	-0.64	0.02	-0.48	0.44	-0.45	0.54
TMAO (ug:mg)	-0.53	0.07	-0.79	0.03*	0.14	0.83
FE		N=13		N=7		N=6
IS (%)	-0.48	0.12	-0.31	0.49	-0.12	0.85
PCS (%)	0.48	0.11	0.42	0.35	0.08	0.9
TMAO (%)	-0.004	0.99	0.39	0.39	0.13	0.84

Table 3 Pearson correlation; association of eGFR with urine and serum measurements of URS within CK, Healthy, and All. The correlation matrix presenting all associations presented in **Appendix B**.

*p<0.05

Overall there was a strong inverse relationship between each serum IS, PCS, and TMAO with eGFR **(Figure 8)**.When both groups were included, the inverse relationship between serum URS values and eGFR was significant. When evaluated by group, either healthy or CKD, the relationship was weaker in both groups, but IS remained significant in the CKD group (r=-0.77, p=0.04).

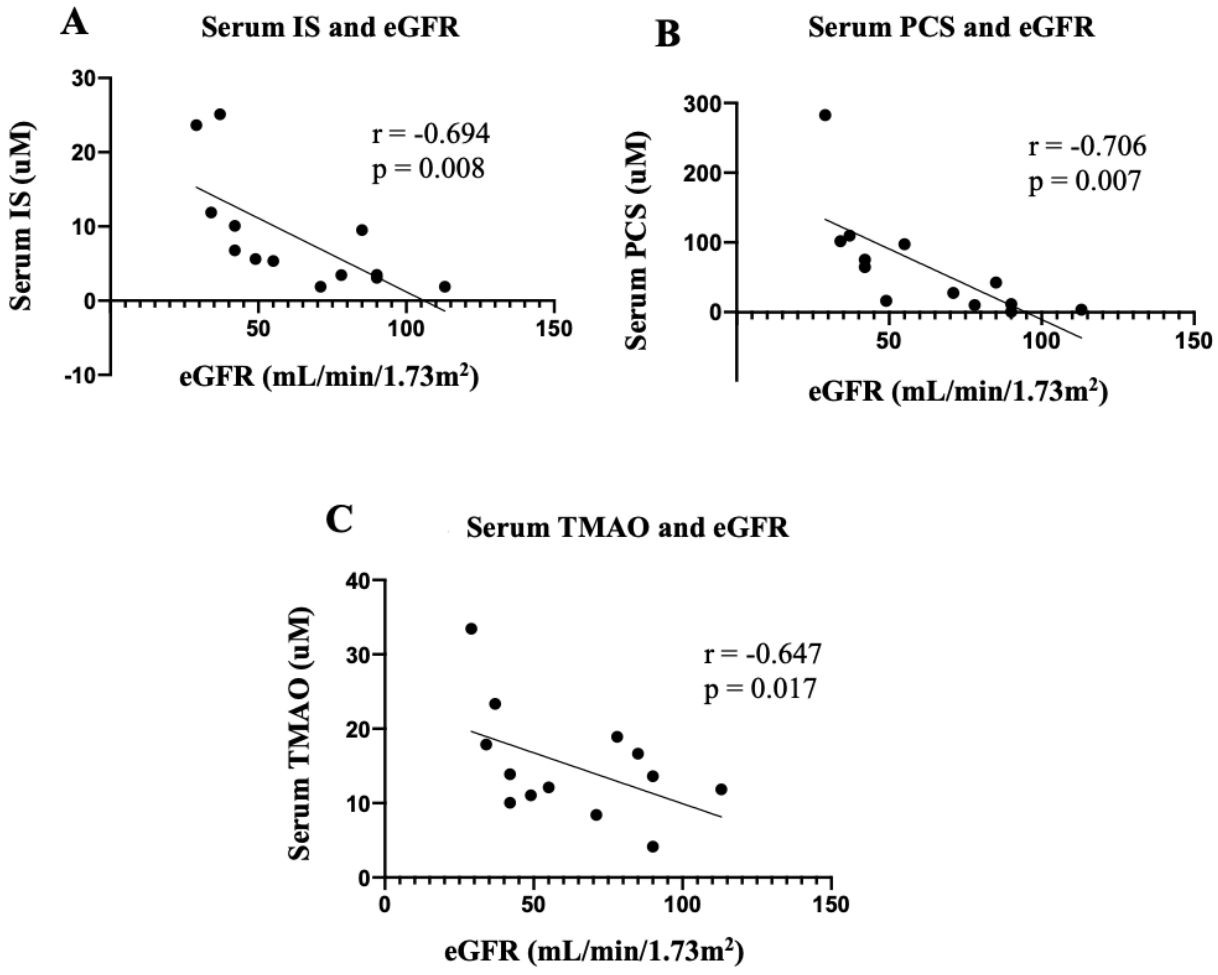

Figure 13 Serum URS and eGFR in CKD patients and healthy controls

Fractional excretion of IS tended to be numerically higher in CKD patients compared with controls **(Table 3)**, however none of the FE values for the three URS reached significance (IS: p=0.13; PCS: p=0.18; TMAO: p=0.68**) (Figure 14)**.

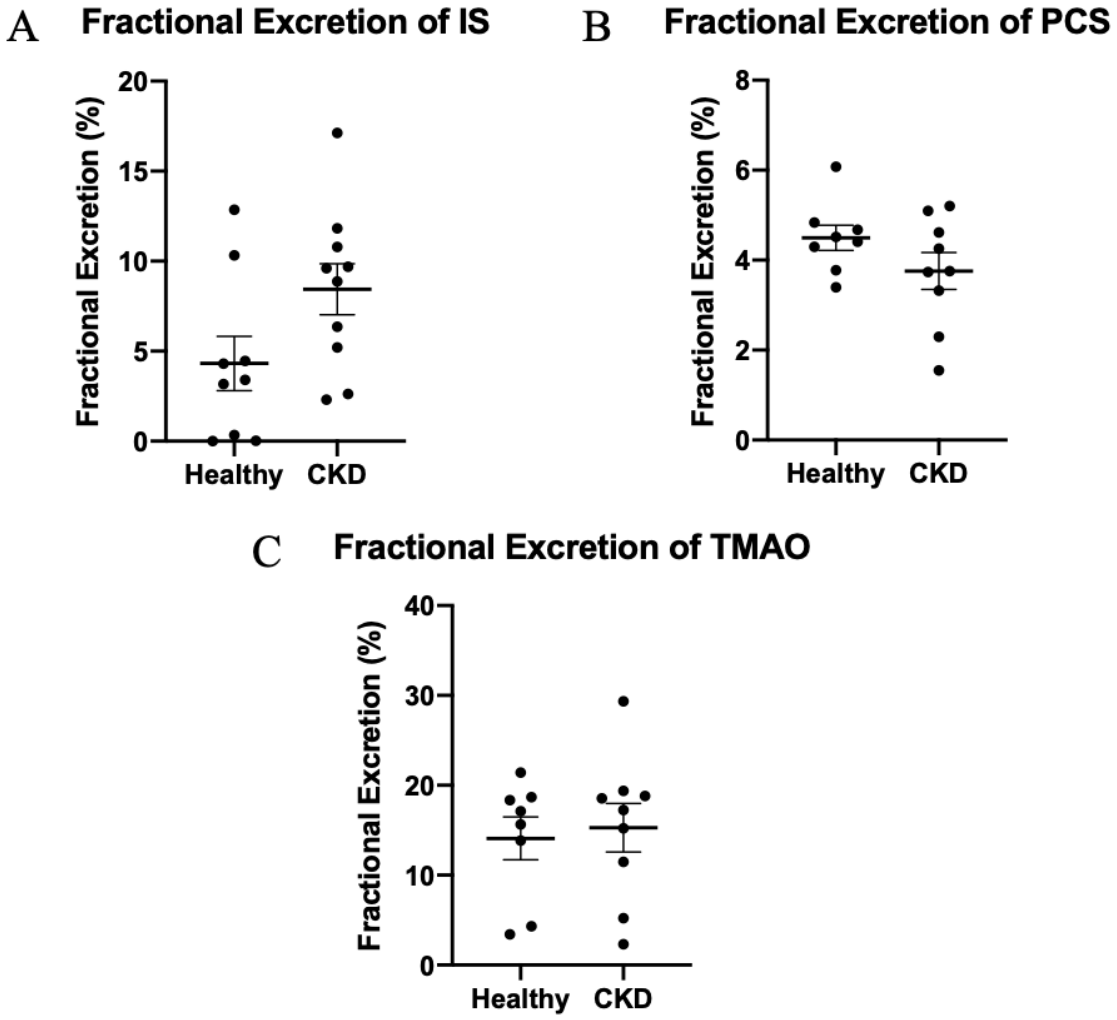

Figure 14 Fractional URS excretion in CKD patients and healthy controls

Figure 14 Fractional excretion was measured in CKD patients (N=7) and healthy controls (N=6) for (A) IS, (B) PCS, and (C) TMAO (plots depict individual data points for both groups with mean ± SE)

CHAPTER 5: DISCUSSION

The results of this study show that serum concentrations of IS, PCS, and TMAO are elevated in mild to moderate CKD when compared to matched healthy adults in a controlled feeding study and on the same, adequate dietary protein intake. Our results align with findings from recent studies evaluating the various URS (IS, PCS, or TMAO) serum levels in healthy individuals and patients with earlier stages of CKD. Elevated serum URS levels coincided with non-significantly elevated urinary levels in CKD patients.

Strong relationships between elevated serum and urine URS levels suggest that higher serum URS levels are associated with higher excretion of URS in moderate stage CKD patients. Physiologically, this makes sense for the body to prioritize elimination of wastes accumulating in circulation that have the potential to cause harm. However, this was contrary to our hypothesis that urine URS would be lower in CKD compared with control, reflecting less ability to secrete and excrete the URS, leading to their accumulation in serum. Instead, our results showed that urine concentrations of IS, PCS, and TMAO were higher (albeit non-significantly) in patients with CKD when compared with healthy adults. However, these differences sharpened when normalizing metabolite concentration to creatinine (URS:Cr), resulting in a significant difference between groups for IS excretion. These results suggest that declining kidney function may not be the only cause of elevated serum URS levels. However, a limitation in our interpretation of the urine excretion data is the single fasting collection. 24-hour urine excretion of the URS would provide additional information on the overall adequacy of URS excretion in CKD patients. Stubbs et al. (1) also found no differences in the concentration and fractional excretion of TMAO between pre-dialysis CKD patients and healthy adults and an inverse relationship between serum TMAO and eGFR. They suggest that intestinal absorption and hepatic production of TMAO

remain unchanged in CKD, and that declining kidney failure is the driving force behind elevated serum TMAO levels. While we would expect intestinal absorption and hepatic formation of URS to remain unchanged, there other factors not included in this interpretation, including increased microbial synthesis and altered intestinal barrier function in the gut.

Recent studies suggest that factors altering pH and substrate availability in CKD influence the community composition of the gut microbiota to have a higher proportion of URS-producing bacteria, resulting in higher URS production (2, 3). Significant differences in the composition of the gut microbiota have been shown as early as stage 3 (2, 3). The composition of these communities has shifted, most likely as a result of the uremic environment, to have a lower abundance of butyrate-producing families and a higher abundance of indole-, p-cresol-, and TMA-forming families (2-4). Evidence identifying specific bacterial families involved in URS production is lacking, as well as the production rate or how shifts in community composition might affect this. However, some evidence shows that bacterial families known to possess enzymes involved in URS formation are more abundant in CKD patients (5, 6). However, considering the flow of nitrogen, mentioned previously, future studies may be better suited to determine the effect microbial fermentation has on the production of URS by evaluating microbial families based on their nutrient source. A study evaluating microbes present throughout the right and left colon showed a higher abundance of sarccholytic families and a higher abundance of proteolytic families in these regions, respectively (7). Because the amount of nitrogen entering the colon depends mostly on dietary protein intake and handling in the small intestine, lowering dietary protein may be a viable way to reduce the microbes present by limiting nutrient availability. These findings suggest that shifts in the gut microbiome may also contribute to the changes in URS production, combined with declining renal clearance. It

remains a difficult task to identify the bacteria that are specifically linked to with individual URS production. Simply identifying bacterial groups present an answer to “who is there?”, but we can only infer functional changes. Many factors that fluctuate daily in the microenvironment may influence the rate at which URS are produced as well.

The major strength of this study was the one-week controlled diet. By controlling for diet, we controlled an important influencing factor on the gut microbiome to see a baseline measurement of the effect that moderate kidney function decline has on URS serum and urine levels. If elimination is relatively the same in patients with stage 3 CKD compared to healthy adults. An additional strength of this study was that the participants were matched for age, sex, and race. Matching for these demographic characteristics may reduce variation that we might see due to related biological factors. Limitations of the study include the small sample size and the use of one fasting time-point for URS measurements.

This study adds to the current knowledge of URS accumulation and elimination in CKD patients. Our study demonstrates a strong inverse relationship between circulating URS levels and urine URS levels, and that kidney function is strongly correlated with these values in moderate CKD patients. Current evidence suggests that gut-derived URS production is reliant on dietary intake of protein, and that protein restriction may be a therapeutic target in lowering URS levels in CKD patients. Future studies should determine the effects of a low protein diet in CKD compared with healthy adults on serum and urine URS, or the effects of a low protein diet versus normal protein diet on URS in CKD patients in a cross-over controlled feeding study. More thorough evaluation of urinary URS excretion, i.e. 24-hour urine excretion, is needed to further clarify the contribution of reduced excretion to the serum accumulation of URS.

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APPENDIX A. RECRUITMENT AND DATA COLLECTION MATERIALS

Purdue University Department of Nutrition Science

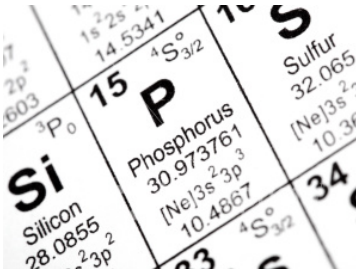
And

Indiana University School of Medicine

Chronic Kidney Disease Research Study

Participants Needed

Phosphorus is an essential mineral nutrient involved in bone, kidney, and cardiovascular health. By participating in this study, you can help us better understand how the amount of phosphorus we consume in our diets affects our ability to absorb phosphorus.



You may be eligible to participate if you are:

Male or female

Have chronic kidney disease

Not receiving dialysis

Ages 30-75 years old

Any racial or ethnic background

Research study requirements:

Participation in a short screening visit, and 8 days of controlled feeding study with 2 days of phosphorus absorption tests a the Indiana Clinical Research Center at the end of the study period. All food will be provided during the 8-day study period.

Compensation: $600 for completion of the research study

For more information, please contact Elizabeth Stremke at 317-572-5438 or estremke@purdue.edu or Dr. Kathleen Hill Gallant (principal investigator) at 765-494-0101 or hillgallant@purdue.edu.

INDIANA UNIVERSITY INFORMED CONSENT STATEMENT FOR

Phosphorus Absorption in Healthy Adults and Patients with Moderate Chronic Kidney Disease

You are invited to participate in a research study of phosphorus absorption from your food. You were selected as a possible subject because you are either a healthy adult or an adult with moderate chronic kidney disease. Please read this form and ask any questions you may have before agreeing to be in the study.

The study is being conducted by Dr. Kathleen Hill Gallant in the Department of Nutrition Science at Purdue University, in collaboration with Dr. Ranjani Moorthi and Dr. Sharon Moe in the Department of Medicine at Indiana University School of Medicine. It is funded by

departmental funds of the Purdue University Department of Nutrition Science and funding from the National Institutes of Health.

STUDY PURPOSE

The purpose of this study is to determine if patients with moderate chronic kidney disease (CKD) absorb a different amount of phosphorus from their diets than healthy adults. Phosphorus is an essential nutrient with many important functions in the body including bone health and energy metabolism. However, some people, like those who have kidney problems, may need to limit how much phosphorus they consume from the foods they eat. It is important for us to know how well people absorb phosphorus from food, and what factors influence their ability to absorb phosphorus.

NUMBER OF PEOPLE TAKING PART IN THE STUDY

If you agree to participate, you will be one of up to 30 subjects who will be participating in this research.

PROCEDURES FOR THE STUDY

If you agree to be in the study, you will do the following things:

Participate in a screening visit of 30-60 minutes to assess eligibility to participate. Screening will be conducted at the IU Clinical Research Center in Indianapolis or at your clinic at the time of recruitment, if preferred. You may need to have blood and urine collected for screening if you haven't recently had needed labs taken. If you do need blood and urine collected for screening, you will be asked to not eat anything (fast) from about midnight the morning of your visit. You will be able to drink water during the time you are fasting. For screening, you will be asked about your medical history, and blood and urine samples will be collected if needed (the amount

of blood taken will be about 1⁄2 tablespoon). You will also learn how to keep a record of the food you eat, and how to collect a stool sample at home prior to your first study visit.

If you qualify for the study based on your screening visit, and choose to enroll in the study, you will be asked to participate in an 8-day study during which time you will be asked to eat only research meals that will be provided to you, and complete two days of phosphorus absorption testing at the clinical research center at University Hospital (Indiana University School of Medicine) that will include two overnight stays. You will also be asked to stop taking any nutritional supplements as instructed by the study staff during your participation in this study. Before the 8-day study, you will be asked to keep a 4-day food record so we can determine how much phosphorus you usually eat. You will also be asked to collect one home stool collection. During the study, you will be given all of your meals, snacks, and beverages to consume for 8 days. You will be asked to pick up your meals as pack-outs at the IU Health University Hospital in Indianapolis. You will do this twice and be given food for 3 days at a time, or if this does not work for your schedule, you can ask to make a more convenient pick-up(s) arrangement(s) with the study staff. The day of the first meals pick-up you will also have an additional fasting blood draw and urine collection and will bring in your stool collection and diet record from home; this visit will take about 30-60 minutes. You will need to fast (not eat anything) from about midnight the night before. The second meal pick-up day will not involve any other study measurements. You will be expected to consume all and only the food you are given during the 8-day research study. The diets will be designed to meet your energy needs, and food preferences will be honored, when possible.

2-day Phosphorus Absorption Test: After 6 days of eating the study diet at home, you are asked to participate in a two-day phosphorus absorption test at the clinical research center. For the first day, you will be asked not to eat anything (fast) from about midnight, but you can drink plain water. You will go or be transported to the clinical research center at Indiana University School of Medicine (IUSM) in Indianapolis. Once there (around 8:15AM), you will be admitted as an inpatient on the research unit. You will have your height, weight, blood pressure, heart rate and breathing rate measured. Fasting blood and urine will be collected. If you are a person of child-bearing potential, you will have your urine tested to confirm you are not pregnant. You will have an IV placed by a trained member of the clinical research team. You will be given a radioactive form of phosphorus to drink with your breakfast. Over the next six hours, you will have your blood drawn several times using your IV, and you will also be asked to collect all urine and stool. You will continue to be provided your meals, snacks, and beverages. You will be monitored by medical and research staff during the entire testing time. You may watch TV, use a computer, read, or do other similar leisure activities while the test is being conducted. You will fast again at approximately midnight the morning of day two in the clinical research center. The second day is similar to the first day, but instead of a radioactive phosphorus drink, you will have the radioactive form of phosphorus given by IV injection. This is to see how fast your body gets rid of phosphorus in your blood. You will again have your blood drawn over 6 hours by IV and you will continue to collect all your urine and stool. You will continue to eat the study food and collect all your urine and stool through the following morning. The next morning, you will have your weight, blood pressure, heart rate, and breathing rate measured, and a final blood draw, IV removed, and then you will be sent home. This will conclude your participation in the research study.

The total amount of blood collected during the two-day phosphorus absorption test is about 11 tablespoons.

If you have diabetes, you will be asked to monitor your blood sugar by fingerstick at home during the study
and report any blood sugars lower than 100 mg/dL to the study staff.

RISKS OF TAKING PART IN THE STUDY

While on the study, the risks for side effects and/or discomforts are:

Risk of blood draw and IV: The risk involved with blood draw and IV placement in this study may include pain, discomfort, fainting, bruising, and infection. Precautions will be taken to minimize these risks by using sterile technique and applying pressure to the site after the needle or IV is withdrawn. IVs will only be placed, and blood will only be drawn by skilled blood-drawing personnel or research nursing staff.

Risk of study diet: You will be eating a fixed diet at home and in the hospital. We will give you study food to meet your needs, but you may feel hunger or fullness on the study diet compared to what you normally eat.

The amount of phosphorus in the study diet is similar to the amount that a typical American eats each day. The amount of phosphorus in the radioactive phosphorus drink is very small and not expected to cause any discomfort. All meals, snacks, and the radioactive phosphorus drink will be made up by trained study staff who will follow safe food handling methods.

If you have diabetes, there is increased risk for hypoglycemia while on the study diet because it may likely be different than your usual diet you eat. Symptoms of hypoglycemia include shakiness, sweating, tiredness, dizziness, hunger, irregular heartbeat, anxiety, irritability, tingling feeling around the mouth. Severe symptoms of hypoglycemia include confusion, blurry vision, seizures, or loss of consciousness. If you have diabetes, then we will ask you to write down everything you eat prior to the start of the study. We will then use that information to adjust your diabetic medications. If you use a sliding scale insulin at home to adjust with each meal, we will ask you to bring in your glucose monitor and self-manage while in the hospital. You will be asked to notify the study physician if you experience a hypoglycemic episode (any blood sugar lower than 100 mg/dL or if you have symptoms of hypoglycemia) while at home during the study while you are monitoring your blood sugar by fingerstick.

If you experience low blood sugar at home, you should treat it by having 15 grams of carbohydrate, waiting 15 minutes, then check your blood sugar again. If your blood sugar is still low (below 70 mg/dL), repeat these steps. Some good choices of 15 grams of carbohydrate include: a glucose tablet or gel (see instructions), 1⁄2 cup of juice or regular soda pop (not diet), 1 tablespoon of sugar, honey, or corn syrup, 15 small jelly beans or skittles, 3 pieces of hard candy such as round peppermints or jolly ranchers. **In the case of severe hypoglycemic symptoms, or when in doubt, call 911.**

Risk of radiation exposure: Your participation in this research study involves exposure to radiation in addition to what you may receive as part of your standard care. The benefit from the radiation you receive for your standard care typically outweighs the risk because it allows your doctor to provide appropriate medical care; however, the additional radiation “dose” you receive for research purposes may not benefit you personally.

Everyone is exposed to “background” radiation (e.g. radon gas in our homes, radiation from space, uranium in soil, etc.) and the radiation dose varies, depending upon where you live. Individuals who live in certain areas of the country may actually receive radiation doses that are higher than the average; however, individuals who live in those areas have not shown an increased risk of health effects (cancer and/or leukemia) above the average for the US population. The radiation dose you will receive in one year from this study is less than the average annual “background” dose received by a member of the US population. We cannot say with absolute certainty that there is no risk from the radiation dose in this study. While there is no evidence that any risk exists for humans exposed to such low levels, it is assumed that the risks rise with lifetime accumulated dose from all sources of ionizing radiation, including the doses you receive from medical procedures and the environment. The calculated effective dose resulting from your participation in this study is available upon request. If you are female of child-bearing potential, your urine will be tested again to confirm you are not pregnant before giving the radioactivity.

Risk of loss of confidentiality: Every research study carries the risk for loss of confidentiality. However, precautions will be taken to avoid this possibility.

BENEFITS OF TAKING PART IN THE STUDY

There are no direct benefits to you for participating in this study.

ALTERNATIVES TO TAKING PART IN THE STUDY

Instead of being in the study, you have these options:

- You may choose not to participate in this study or you may leave the study at any time.

WILL I RECEIVE MY RESULTS?

We may learn things about you from the study activities which could be important to your health or to your treatment. If this happens, this information will be provided to you. Examples of the type of information we may find include: blood test results showing high blood sugar, high cholesterol, or that your kidneys may not be working properly. If such information is learned, a study physician will notify you and explain the results and let you know if there is anything you should do next. You may be advised to meet with professionals with expertise to help you learn more about your research results. The study team/study will not cover the costs of any follow- up consultations or actions.

CONFIDENTIALITY

Efforts will be made to keep your personal information confidential. We cannot guarantee absolute confidentiality. Your personal information may be disclosed if required by law. Your identity will be held in confidence in reports in which the study may be published and databases in which results may be stored.

Organizations that may inspect and/or copy your research records for quality assurance and data analysis include groups such as the study investigator and his/her research associates, the Indiana University Institutional Review Board or its designees, the Indiana Clinical Research Center (ICRC), and (as allowed by law) state or federal agencies, specifically the Office for Human Research Protections (OHRP) and the National Institutes of Health (NIH) who may need to access your medical and/or research records.

A description of this clinical trial will be available on **ClinicalTrials.gov**, as required by U.S. law. This website will not include information that can identify you. At most, the website will include a summary of the results. You can search this website at any time.

For the protection of your privacy, this research is covered by a Certificate of Confidentiality from the National Institutes of Health. The researchers may not disclose or use any information, documents, or specimens that could identify you in any civil, criminal, administrative, legislative, or other legal proceeding, unless you consent to it. Information, documents, or specimens protected by this Certificate may be disclosed to someone who is not connected with the research:

(1) If there is a federal, state, or local law that requires disclosure (such as to report child abuse or communicable diseases);

(2) if you consent to the disclosure, including for your medical treatment;

(3) if it is used for other scientific research in a way that is allowed by the federal regulations that protect

research subjects

(4) for the purpose of auditing or program evaluation by the government or funding agency

You should understand that a Certificate of Confidentiality does not prevent you from voluntarily releasing information about yourself.

WILL MY INFORMATION BE USED FOR RESEARCH IN THE FUTURE?

Information or specimens collected from you for this study may be used for future research studies or shared with other researchers for future research. If this happens, information which could identify you will be removed before any information or specimens are shared. Since identifying information will be removed, we will not ask for your additional consent.

COSTS

You will not be responsible for any study-specific costs: these include meals, visits, laboratory tests, and parking.

PAYMENT

You will receive payment for taking part in this study. If you want your research information released to an insurer, medical care provider, or any other person not connected with the research, you must provide consent to allow the researchers to release it.

You will be paid for your participation in the study according to the following schedule:

Screening Visit: $25

Day 1 Visit: $25

Absorption test day 1: $200

Absorption test day 2: $350

Total for completion of the study: $600

A check for each completed visit and/or test days will be sent to the address given by you when you either complete the study or withdraw from the study. Your name, social security number and address will be provided to the business office at Purdue University for the purpose of facilitating payment for participating in this study.

COMPENSATION FOR INJURY

In the event of physical injury resulting from your participation in this research, necessary medical treatment will be provided to you and billed as part of your medical expenses. Costs not covered by your health care insurer will be your responsibility. Also, it is your responsibility to determine the extent of your health care coverage. There is no program in place for other

monetary compensation for such injuries. However, you are not giving up any legal rights or benefits to which you are otherwise entitled. If you are participating in research that is not conducted at a medical facility, you will be responsible for seeking medical care and for the expenses associated with any care received.

FINANCIAL INTEREST DISCLOSURE

The investigators involved in this research do not have any financial conflicts of interest with this study.

CONTACTS FOR QUESTIONS OR PROBLEMS

For questions about the study or a research-related injury during the study, you should contact the researcher, Kathleen Hill Gallant, PhD, at 765-494-0101. If you cannot reach the researcher during regular business hours (i.e., 8 a.m. to 5 p.m.), please call the IU Human Subjects Office at 317-278-3458 or 800-696-2949.

In the event of an emergency, call 911 or go to the emergency department and tell the study doctor as soon as possible. If you get emergency care or are hospitalized, please tell the doctor treating you that you are in a research study being conducted by Dr. Ranjani Moorthi. After hours you may call IU Health University Hospital at 317 944 5000 and ask for the Nephrology Fellow on call.

For questions about your rights as a research participant, to discuss problems, complaints, or concerns about a research study, or to obtain information or offer input, contact the IU Human Subjects Office at 317-278-3458 or 800-696-2949.

You should not sign this consent form unless you have had a chance to ask and have satisfactory answers to all of your questions.

VOLUNTARY NATURE OF THIS STUDY

Taking part in this study is voluntary. You may choose not to take part or may leave the study at any time. Leaving the study will not result in any penalty or loss of benefits to which you are entitled. Your decision whether or not to participate in this study will not affect your current or future relations with Indiana University, Purdue University, or your physician.

Your participation may be terminated by the investigator without regard to your consent in the following circumstances: if you are unable or unwilling to follow the study procedures.

You will be told about new information that may affect your health, welfare, or willingness to stay in the study.

SUBJECT'S CONSENT

In consideration of all of the above, I give my consent to participate in this research study.

I will be given a copy of this informed consent document to keep for my records. I agree to take part in this study.

Subject's Printed Name: Subject's Signature:

Date:

(must be dated by the subject)

Printed Name of Person Obtaining Consent:

Signature of Person Obtaining Consent: **Date**:

SCREENING TOOL

Phosphorus Absorption in Healthy Adults

and Patients with Moderate Chronic Kidney Disease

Department of Nutrition Science

Kathleen Hill Gallant, PhD, RD, Principal Investigator

1. Date_____/_____/_____Time _____:_____AM/PM

Name_____

Last First

Local

Address_____

Street Apt.

_____

_____City State Zip

Phone_(____)_____-_____ E-mail:_____

Home

Emergency Contact:

Name: _____Relationship:_____Phone:

(_____)_____-_____

2. Date of Birth_____ _____, _____

Month Day Year

3. Age_____years Name and address of physician:

Height _____ft_____in

_____

Weight_____(lb)

_____

_____

Tel:_____

4. Self-identified Race/Ethnicity (check all that apply):

☐African American ☐Hispanic

☐Asian ☐Non-Hispanic

☐White

☐Native American/Pacific Islander

☐Other:_____

5. Please list the year if a doctor has ever told you that you had any of the following conditions. The year should be placed in the space provided to the left of the condition.

Year	**Condition**	**Year**	**Condition**	**Year**	**Condition**
_____	Asthma	_____	Epilepsy	_____	Malaria
_____	Atherosclerosis	_____	Hay Fever	_____	Rheumatoid Arthritis
_____	Bladder Disease	_____	Heart Disease	_____	Sickle Cell Anemia
_____	Cancer	_____	HIV	_____	Stomach Ulcers
_____	Chronic Bronchitis	_____	Hypertension	_____	Thyroid Condition
_____	Colitis	_____	Kidney Disease	_____	Tuberculosis
_____	Crohn's Disease	_____	Leukemia	_____	Urinary Tract Infection
_____	Diabetes Mellitus	_____	Liver Disease (cirrhosis)	_____	Bowel obstruction/delayed bowel emptying
_____	Diverticulosis	_____	Osteoporosis	_____	Other_____

6. If "yes" for kidney disease, what type?: _____

7. Have you had any other major illness or surgeries? ☐ Yes ☐ No

If Yes, please list them and the year you had them.

Illness Year

_____ _____ _____

_____ _____ _____

8. Have you ever had bone fractures? ☐ Yes ☐ No

What bone_____? Age and date when it happened and how:

_____.

9. Do you have any known allergies (non-food)? ☐ Yes ☐ No

If Yes, please specify _____ Reaction:_____

10. Do you have any known food allergies? ☐ Yes ☐ No

If Yes, please specify _____ Reaction: _____

11. Do you have any known food intolerances? ☐ Yes ☐ No

If Yes, please specify _____ Reaction: _____

12. Are you currently pregnant or nursing?

☐ Yes (pregnant/nursing) ☐ No ☐ Unsure ☐ Male N/A

(confirm "no" or "unsure" with urine test)

13. When did your last period occur?_____ ☐ Male N/A

14. Have you had a hysterectomy? ☐ Yes ☐ No ☐ Male N/A

☐ Total or ☐ Partial

15. Do you take any medications regularly? ☐ Yes ☐ No

If yes, indicate dosages(mg), times per day, years taken:

Medication	No./Day	Years/months	Dosage
Diuretics (e.g. furosemide/Lasix, spironolactone, hydrochlorothiaszide, theophylline, theobromine, caffeine)			

ACE inhibitors _____

(e.g. ramipril, lisinopril, benazepril)

Angiotensin receptor blockers _____

(e.g. losartan/Cozaar, valsartan)

Sodium phosphates _____

(e.g. OsmoPrep, Visicol)

NSAIDS _____

(e.g. aspirin, ibuprofen, naproxen)

Vitamin D preparations or analogs _____

(e.g. ergocalciferol, cholecalciferol, calcitriol, paracalcitol)

Osteoporosis medications _____

(e.g. bisphosphonates, teriparatide, raloxifene, denosumab)

Other Medications _____

_____

_____

16. Do you take antacids regularly? ☐ Yes ☐ No

If yes, how often?_____

17. If yes, what is the name of the antacid?

☐ 1. Tums or Chooz
☐ 2. Tritralac
☐ 3. Alkamints
☐ 4. Gas-X
☐ 5. Mylanta, Maalox, Digel or Gelusil
☐ 6. Rolaids
☐ 7. Other (specify):_____

18. Do you take calcium supplements? ☐ Yes ☐ No

What is the name of the supplement?_____

How much calcium does it contain per tablet? _____mg

How many tablets do you take per day?_____

19. Do you take a multivitamin/mineral supplement? ☐ Yes ☐ No

If yes, what is the brand name?_____

How much calcium does it contain? ☐None ☐ _____mg ☐ don't know

How much vitamin D does it contain? ☐None ☐ _____mg ☐ don't know

20. Do you use any of these products? Indicate the dosage you are currently taking?

Please include any herbal supplements also.

PRODUCT		**AMOUNT**		**TIMES/DAY**		**BRAND**
Vitamin C						
Vitamin E						
Vitamin D						
Vitamin A						
Vitamin B-12						
Vitamin B-6						
Iron						
Zinc						
Magnesium						
Selenium						
Bran						
Wheat germ						
Brewer's yeast						
Cod liver oil						
Weight loss pills						
Weight loss formulas						
Prebiotics (specify)						
Probiotics (specify)						
Other (please list)						

21. Are you currently on any of the following special diets? (more than one diet may apply)

☐ No ☐ Low Sodium ☐ Low Cholesterol

☐ Weight Loss (specify): _____

☐ Weight Gain (specify): _____

☐ Vegetarian (specify): _____

☐ Other (specify):_____

Vitals

Staff initials

Height: _____ cm _____

Weight: _____ kg _____

BMI: _____ kg/m^2 _____

Pulse: _____ bpm _____

BP: _____/_____ mm Hg _____

Respirations: _____ rpm _____

Confirm Fasting State

Last ate: Date_____/_____/_____ Time _____:_____AM/PM

Fasting ☐yes _____ hours ☐ no Staff initials _____

Urine

☐ Date_____/_____/_____ Time _____:_____AM/PM

Pregnancy Test ☐ negative ☐ positive Staff initials _____

Blood Draw

Date_____/_____/_____ Time _____:_____AM/PM

☐ 7 mL in 10 mL red top Drawn by:_____

Notes:_____

_____

Results:		reference range	exclude?
Serum albumin:	_____ g/dL	3.5-5.0 g/dL	
Serum phosphorus:	_____ mg/dL	2.5-4.7 mg/dL	☐
Serum calcium:	_____ mg/dL	8.4-10.5 mg/dL	☐
Serum potassium:	_____ mmol/L	3.5-5.1 mmol/L	☐
Serum sodium:	_____ mmol/L	136-145 mmol/L	☐
Serum CO2	_____ mmol/L	18-27 mmol/L	☐
Serum glucose	_____ mg/dL	65-99 mg/dL	☐
Serum creatinine:	_____ mg/dL	0.7-1.20 mg/dL	☐
BUN:	_____ mg/dL	7-20 mg/dL	☐
eGFR: _____ mL/min/1.73m^2		>60 mL/min/1.73m^2	☐
GFR category	G1 G2 G3a G3b G4 G5		
PCR	_____ mg/g	<150 mg/g	☐
ACR	_____ mg/g	< 30 mg/g	☐
Urine protein reagent strip	_____	negative/trace	☐
Albuminuria category	A1 A2 A3		

SCREENING STATUS:

☐Accept ☐Notified ____/____/____(Date) ☐healthy group ☐CKD group

☐Deny ☐Notified ____/____/____(Date)

Reason for Denial:

_____

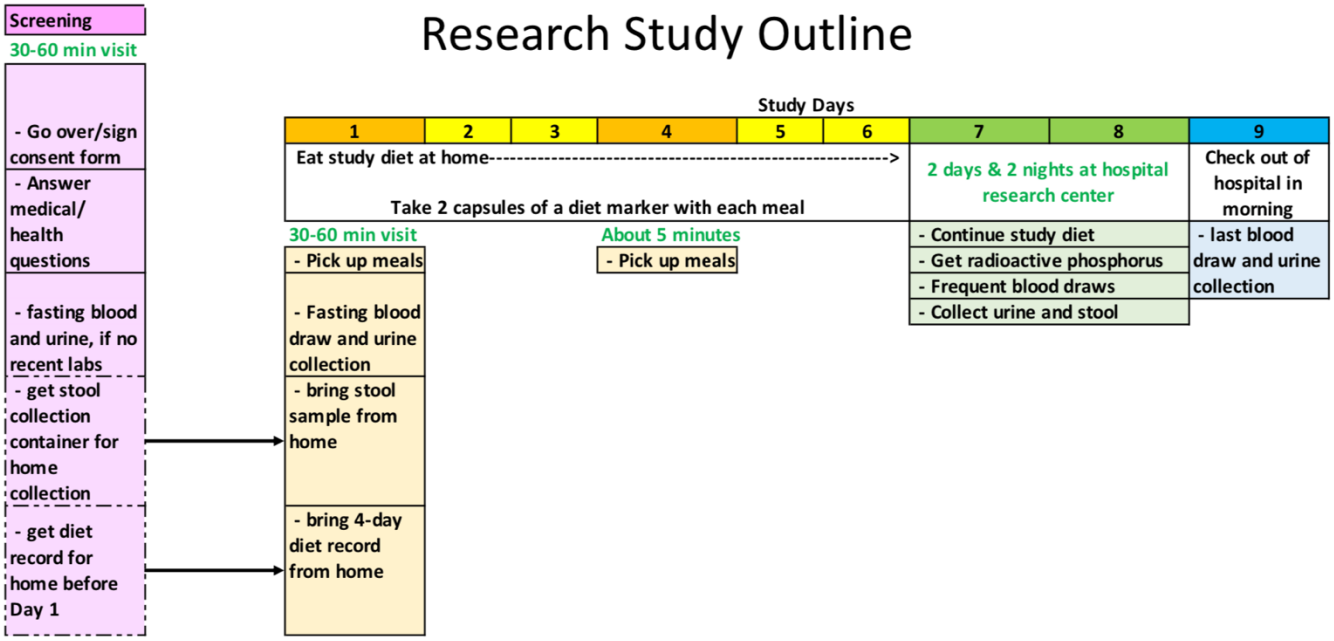
_____

Notes:_____

_____

Study Staff:_____ _____/_____/_____

Research Study Outline



3-Day Cycle Menu: Day 1

Food	*Weight (g)*	*Energy (kcal)*	*CHO (g)*	*Protein (g)*	*Fat (g)*	*Phos (mg)*	*Na (mg)*	*K (mg)*	*Total daily fiber (g)*	*Soluble fiber (g)*	*Insoluble fiber (g)*
BREAKFAST											
English muffin, whole wheat	55 g	112	22.22	4.84	1.16	155	200	116	3.69	0.52	2.98
lowfat cottage cheese	65 g	53	3.09	6.79	1.48	98	200	81	0	0	0
jelly	1 pk	39	9.64	0.05	0.01	3	4	11	0.15	0.04	0.11
butter	2 PC	136	0.01	0.16	15.34	5	1	5	0	0	0
blueberries	75 g	43	10.87	0.56	0.25	9	1	58	1.8	0.17	1.63
milk, 1%	180 g	76	8.98	6.07	1.75	171	79	270	0	0	0
apple juice, fortified	160 g	74	18.08	0.16	0.21	11	6	294	0.32	0.02	0.3
total for meal		531	72.895	18.626	20.185	451	11.901	834	5.96	0.748	5.024
LUNCH											
baked Lays	40 g	164	32.47	2.06	2.93	51	190	382	1.82	1.02	0.81
chicken breast	125 g	158	0	28.21	4.12	197	70	225	0	0	0
Kraft swiss cheese	15 g	48	0.37	3.24	3.7	62	109	11	0	0	0
Kraft ranch dressing	15 g	77	0.7	0.31	8.28	7	150	11	0.09	0.08	0.009
hamburger bun	45 g	126	22.55	4.4	1.76	45	222	55	0.81	0.23	0.59
tangerines	85 g	32	8	0.64	0.03	9	4	116	1.02	0	1.02
milk, 1 %	200 g	84	9.98	6.74	1.94	190	88	300	0	0	0
total for meal		688	74.07	45.6	22.75	561	834	1100	3.74	1.328	2.42

3-Day Cycle Menu: Day 1 continued

Food	*Wt. (g)*	*Energy (kcal)*	*CHO (g)*	*Protein (g)*	*Fat (g)*	*Phos (mg)*	*Na (mg)*	*K (mg)*	*Total daily fiber (g)*	*Soluble fiber(g)*	*Insoluble fiber(g)*
DINNER											
Minute Maid Punch	180 g	82	21.45	0.06	0.03	3	2	25	0.082	0.015	0.067
brown rice	50 g	206	42.85	4.59	1.63	173	7	144	2.68	0.35	2.33
olive oil	20 g	177	0	0	20	0	0	0	0	0	0
lentils	43 g	50	8.66	3.88	0.163	77	1	159	2.52	0.189	2.331
soy sauce	12 g	6	0.59	0.98	0.068	20	659	52	0.096	0.043	0.053
beef, tenderloin	95 g	136	0	20.4	5.99	128	27	168	0	0	0
mixed veg	200 g	126	25.4	5.55	0.29	99	68	328	8.54	4.07	4.46
cookies, choc chip	3 med	147	19.81	1.53	7.38	22	93	51	0.785	0.2	0.59
pineapple	125g	75	19.45	0.64	0.138	9	1	155	1.625	0.238	1.388
Total for meal		1005	138.2	37.62	35.68	531	859	1082	16.324	5.11	11.214
Total for menu		**2223**	**285.165**	**101.836**	**78.615**	**1543**	**2306**	**3016**	**26.026**	**7.186**	**18.658**

3-Day Cycle Menu: Day 2

Food	*Wt. (g)*	*Energy (kcal)*	*CHO (g)*	*Protein (g)*	*Fat (g)*	*Phos (mg)*	*Na (mg)*	*K (mg)*	*Total daily fiber (g)*	*Soluble fiber(g)*	*Insoluble fiber(g)*
BREAKFAST											
wheat bread	75 g	189	32.03	9.34	2.63	159	341	191	4.5	1.013	3.5
peanut butter	30 g	179	6.69	6.66	15.41	101	128	167	1.5	0.09	1.41
flax seeds	15 g	80	4.33	2.74	6.32	96	5	122	4.1	1.23	2.87
jelly	1 pkt	39	9.64	0.05	0.01	3	4	11	0.15	0.04	0.113
cantaloupe	115 g	39	9.38	0.97	0.22	17	18	307	1.04	0.173	0.863
milk, 1 %	260 g	109	12.97	8.76	2.5	247	114	390	0	0	0
total for meal		636	75.056	28.524	27.107	623	611	1188	11.284	2.547	8.744
LUNCH											
flour tortilla, whole wheat	1 ea, 8"	138	24.63	4.32	3.32	123	304	119	3.5	0.584	2.92
turkey breast	95 g	104	2.09	16.55	2.85	113	733	129	0	0	0
swiss cheese	45 g	177	0.65	12.13	13.95	258	84	32	0	0	0
mayo, Kraft light	1 packet	36	1.6	0.09	3.2	2	76	1	0	0	0
Baked Lays	35 g	143	28.4	1.81	2.56	44	167	334	1.6	0.89	0.71
Sprite	can, 12 FO	148	37.48	0.185	0.07	0	33	4	0	0	0
grapes	110 g	76	19.91	0.79	0.18	22	2	210	0.99	0	0.99
total for meal		821	114.77	35.87	26.13	562	1399	829	6.088	1.475	4.613

3-Day Cycle Menu: Day 2 continued

Food	Wt. (g)	Energy (kcal)	CHO (g)	Protein (g)	Fat (g)	Phos (mg)	Na (mg)	K (mg)	Total daily fiber (g)	Soluble fiber(g)	Insoluble fiber(g)
DINNER											
beef tenderloin	158g, raw	226	0	33.92	9.95	213	45	279	0	0	0
baked, potato	160 g	121	27.93	2.54	0.13	65	6	507	1.944	0.376	1.57
butter	1 PC	68	0.006	0.08	7.67	2	61	2	0	0	0
green beans	110 g	23	4.61	1.16	0.45	24	2	106	2.09	0.418	1.672
pears	208 g	60	16.25	0.395	0.06	15	4	110	3.33	1.75	1.58
Lorna Doone	28 g	144	19.34	1.82	6.67	20	145	24	0.57	0.27	0.3
Sprite	can, 12 FO	148	37.48	0.19	0.07	0	33	4	0	0	0
Meal Total		789	105.61	40.09	25.01	340	297	1032	7.935	2.81	5.13
Menu Total		**2246**	**295.43**	**104.48**	**78.25**	**1524**	**2307**	**3049**	**25.31**	**6.83**	**18.48**

3-Day Cycle Menu: Day 3

Food	*Wt.*	*Energy (kcal)*	*CHO (g)*	*Protein (g)*	*Fat (g)*	*Phos (g)*	*Na (g)*	*K (g)*	*Total daily fiber (g)*	*Soluble Fiber (g)*	*Insoluble Fiber (g)*
BREAKFAST											
egg beaters	95 g	48	1.27	10.1	0.16	14	185	143	0.59	0.59	0
egg whites only	65g	34	0.48	7.08	0.11	10	108	106	0	0	0
flax seeds	8 g	43	2.31	1.46	3.37	51	2	65	2.18	0.65	1.53
bacon, turkey	30g	110	1.27	8.85	7.76	125	270	200	0	0	0
green pepper	3 TS	2	0.43	0.08	0.02	2	0	16	0.16	0.06	0.1
red pepper	4 TS	4	0.75	0.123	0.04	3	0	26	0.26	0.09	0.17
hash brown pot	45 g	54	12.65	1.08	0.06	25	175	207	1.3	0.63	0.67
strawberries, fresh	125g	40	9.6	0.84	0.38	30	1	191	2.5	0.58	1.92
Greek Yogurt Vanilla, Dannon	85g	64	9.04	6.58	0.07	75	29	79	0	0	0
grape juice	140 g	84	20.68	0.52	0.18	20	7	146	0.28	0.06	0.22
total for meal		483	58.48	36.713	12.15	355	777	1179	7.27	2.66	4.61

3-Day Cycle Menu: Day 3 continued

Food	*Wt.*	*Energy (kcal)*	*CHO (g)*	*Protein (g)*	*Fat (g)*	*Phos (g)*	*Na (g)*	*K (g)*	*Total daily fiber (g)*	*Soluble Fiber (g)*	*Insoluble Fiber (g)*
LUNCH											
sub bun	85 g	237	42.6	8.31	3.32	86	420	104	1.53	0.43	1.11
beef	95 g	314	0	21.95	24.35	171	62	295	0	0	0
barbeque sauce	18 g	31	7.34	0.15	0.113	4	185	42	0.162	0.018	0.144
choc frosted mini wht	20 g	70	15.3	2.1	0.72	73	76	98	2.12	0.32	1.8
Crackin' Oat Bran cereal	20g	80	14.36	1.6	2.78	61	62	90	2.54	0.52	1.95
pretzels	25 g	100	20.06	2.61	0.87	28	397	71	0.76	0.39	0.38
craisins	10 g	31	8.28	0.02	0.11	1	1	5	0.53	0.13	0.4
raisins	10 g	22	5.74	0.14	0.02	5	1	35	0.17	0.05	0.12
Milk, 1%	195g	82	9.73	6.57	1.89	185	86	293	0	0	0
total for meal		967	123.41	43.45	34.173	614	1290	1033	7.812	1.858	5.904

3-Day Cycle Menu: Day 3 continued

Food	*Wt.*	*Energy (kcal)*	*CHO (g)*	*Protein (g)*	*Fat (g)*	*Phos (g)*	*Na (g)*	*K (g)*	*Total daily fiber (g)*	*Soluble Fiber (g)*	*Insoluble Fiber (g)*
DINNER											
spaghetti noodles	55 g, uncooked	245	47.86	9	1.44	90	203	68	2.79	0.81	1.99
tomato sauce, spaghetti no salt added	85g	43	6.85	1.2	1.26	29	26	271	1.53	0.25	1.28
ground beef, 90/10	90g, uncooked	139	0	17.25	7.19	125	40	194	0	0	0
romaine	125 g	21	4.11	1.54	0.38	38	10	309	2.63	0	2.63
chickpeas	35g	49	7.89	2.47	0.97	30	74	38	2.24	0.15	2.1
carrots, raw	15 g	6	1.44	0.14	0.04	5	10	48	0.42	0.07	0.35
Marzetti honey french	20g	100	6.51	0	8.48	0	93	4	0.01	0	0.01
Oreos	2 each	107	16.29	1.05	4.66	18	93	37	0.728	0.224	0.505
milk, 1 %	220 g	92	10.98	7.41	2.13	209	97	330	0	0	0
Meal Total		802	101.93	40.06	26.55	544	646	1299	10.348	1.504	8.865
Menu Total		**2252**	**283.82**	**120.223**	**72.873**	**1513**	**2713**	**3511**	**25.43**	**6.022**	**19.379**

APPENDIX B. STATISTICAL ANALYSIS, TABLES, AND FIGURES

Multiple reaction monitoring table for data acquisition.

Compound Name	**Precursor Ion (m/z)**	**Product Ion (m/z)**	**Collision Energy (V)**	**ESI Polarity**
3-indoxyl sulfate-d4	216	80.9	10	Negative
3-indoxyl sulfate-d4	216	79.9	20	Negative
3-indoxyl sulfate-d4	216	136	15	Negative
3-indoxyl sulfate	212	132	15	Negative
3-indoxyl sulfate	212	80.9	10	Negative
3-indoxyl sulfate	212	79.9	20	Negative
p-cresol sulfate-d7	194	114	20	Negative
p-cresol sulfate-d7	194	79.9	30	Negative
p-cresol sulfate	187	107	20	Negative
p-cresol sulfate	187	79.9	30	Negative
TMAO-d9	85.1	68.1	10	Positive
TMAO-d9	85.1	66.1	20	Positive
TMAO-d9	85.1	46.1	45	Positive
TMAO	76.1	59.1	10	Positive
TMAO	76.1	58.1	20	Positive
TMAO	76.1	42.1	45	Positive

SAS Code

```
data metabolites;
input ID$ pair$ group$ sTMAOuM eGFR
;
cards;
401   1     CKD   11.09471    42
601   1     Cont  8.17738     113
402   2     CKD   35.18482    37
502   2     Cont  12.19976    85
403   3     CKD   25.06256    34
603   3     Cont  7.77956     71
404   4     CKD   14.49827    49
504   4     Cont  7.69115     90
406   5     CKD   11.58093    42
506   5     Cont  2.56372     90
407   6     CKD   66.34726    29
507   6     Cont  12.06716    .
410   7     CKD   16.53156    55
510   7     Cont  4.95063     78
;
proc print data=metabolites;
Run;

proc glm data=metabolites;
class pair group;
model sTMAOuM =  pair group;
lsmeans group/pdiff stderr lines;
output out=stdres p=predict r=resid;
run;

proc univariate data=stdres normal;
var resid;run;

proc glm data=metabolites;
model sTMAOuM = eGFR;
run;

proc glm data=metabolites;
class group pair;
model sTMAOuM =egfr pair group;
lsmeans group / pdiff  lines;

run;

proc sort data=metabolites; by group;run;
proc glm;by group;
model sTMAOuM = eGFR;
run;

data metabolites2;
input       ID$   pair$ group$      sTMAOuM     uTMAOug_mL  TMAO_Cr_ug_mg
FE_TMAO      sISuM uISug_mL    IS_Cr_ug_mg FE_IS sPCSuM      uPCSug_mL
PCS_Cr_ug_mg      FE_PCS      sCR   eGFR
;
cards;
      401   1     CKD   11.09471    29.31560    10.05646    0.17257
10.07496      75.00690    25.73047    0.17129     75.16179    146.99951
1.20064     0.05098      1.49  42
```

```
      601   1     Cont  8.17738     22.96378    11.85900    0.15640
1.89551      0.02171     0.01121     0.00022     3.71502     7.36861
0.02571     0.04409      0.67  113
      402   2     CKD   35.18482    14.01272    23.36622    0.15208
25.10700      18.07381    30.13809    0.09684     109.69991   27.02689
0.23972     0.03755      1.49  37
      502   2     Cont  12.19976    38.62787    16.64923    0.18351
9.52275      48.13448    20.74673    0.10321     42.61745    111.92087
0.89333     0.06075      0.84  85
      403   3     CKD   25.06256    22.67958    17.88470    0.18812
11.89460      17.52887    13.82294    0.10792     101.88322   56.66353
1.71861     0.04614      1.55  34
      603   3     Cont  7.77956     11.52571    8.42646     0.13844
5.44247      0.01631     0.01192     0.00010     27.58017    27.93309
0.23207     0.03777      0.89  71
      404   4     CKD   14.49827    15.91807    11.05882    0.19397
5.62332      10.69137    7.42766     0.11833     16.33456    7.69283
0.01554     0.03321      1.55  49
      504   4     Cont  7.69115     4.68817     13.60468    0.21431
3.08438      0.08684     0.25202     0.00349     0.61024     0.19657
0.00446     0.04520      0.84  90
      406   5     CKD   11.58093    16.28460    13.87813    0.29357
6.79474      5.86664     4.99969     0.06350     64.83983    29.06915
0.25806     0.03735      1.94  42
      506   5     Cont  2.56372     10.34612    4.13547     0.18684
3.47510      27.38920    10.94780    0.12855     11.46342    29.01624
0.24163     0.04677      0.96  90
      407   6     CKD   66.34726    24.03348    33.46349    0.11483
23.64039      18.77379    26.14005    0.08868     282.76915   34.67096
0.25678     0.01551      1.77  29
      507   6     Cont  12.06716    23.75427    18.92620    0.17123
5.02707      5.20352     4.14590     0.03172     36.62092    51.02362
0.56463     0.04837      .     .
      410   7     CKD   16.53156    18.57739    12.11516    0.18538
5.34027      2.41709     1.57629     0.02630     97.51552    63.06674
0.23106     0.04258      1.58  55
      510   7     Cont  4.95063     19.82704    .     .     3.45373
30.59894     .     .     10.14432    21.85845    .     .     1.05  78
;

proc print data=metabolites2;
run;

proc glm data=metabolites2;
class pair group;
model sISuM =  pair group;
lsmeans group/pdiff stderr lines;
output out=stdres2 p=predict r=resid;
run;

proc univariate data=stdres2 normal;
var resid;run;


proc glm data=metabolites2;
model sISuM = eGFR;
run;
```

```
proc glm data=metabolites2;
class group pair;
model sISuM =egfr pair group;
lsmeans group / pdiff  lines;
run;
*;
proc sort data=metabolites2; by group;run;
proc glm;by group;
model sISuM = eGFR;
run;

proc glm data=metabolites2;
class pair group;
model sPCSuM =  pair group;
lsmeans group/pdiff stderr lines;
output out=stdres2 p=predict r=resid;
run;

proc univariate data=stdres2 normal;
var resid;run;


proc glm data=metabolites2;
model sPCSuM = eGFR;
run;

proc glm data=metabolites2;
class group pair;
model sPCSuM =egfr pair group;
lsmeans group / pdiff  lines;
run;

proc sort data=metabolites2; by group;run;
proc glm;by group;
model sPCSuM = eGFR;
run;

proc glm data=metabolites2;
class pair group;
model uTMAOug_mL =  pair group;
lsmeans group/pdiff stderr lines;
output out=stdres2 p=predict r=resid;
run;

proc univariate data=stdres2 normal;
var resid;run;


proc glm data=metabolites2;;
model uTMAOug_mL = eGFR;
run;

proc glm data=metabolites2;
class group pair;
model uTMAOug_mL =egfr pair group;
lsmeans group / pdiff  lines;
```

```
run;

proc sort data=metabolites2; by group;run;
proc glm;by group;
model uTMAOug_mL = eGFR;
run;

proc glm data=metabolites2;
class pair group;
model uISug_mL =  pair group;
lsmeans group/pdiff stderr lines;
output out=stdres2 p=predict r=resid;
run;

proc univariate data=stdres2 normal;
var resid;run;


proc glm data=metabolites2;
model uISug_mL = eGFR;
run;

proc glm data=metabolites2;
class group pair;
model uISug_mL =egfr pair group;
lsmeans group / pdiff  lines;
run;

proc sort data=metabolites2; by group;run;
proc glm;by group;
model uISug_mL = eGFR;
run;

proc glm data=metabolites2;
class pair group;
model uPCSug_mL =  pair group;
lsmeans group/pdiff stderr lines;
output out=stdres2 p=predict r=resid;
run;


proc univariate data=stdres2 normal;
var resid;run;

proc glm data=metabolites2;
model uPCSug_mL = eGFR;
run;

proc glm data=metabolites2;
class group pair;
model uPCSug_mL =egfr pair group;
lsmeans group / pdiff  lines;
run;

proc sort data=metabolites2; by group;run;

proc glm;by group;
```

```
model uPCSug_mL = eGFR;
run;

proc glm data=metabolites2;
class pair group;
model TMAO_Cr_ug_mg =  pair group;
lsmeans group/pdiff stderr lines;
output out=stdres2 p=predict r=resid;
run;
proc univariate data=stdres2 normal;
var resid;run;


proc glm data=metabolites2;
model TMAO_Cr_ug_mg = eGFR;
run;

proc glm data=metabolites2;
class group pair;
model TMAO_Cr_ug_mg =egfr pair group;
lsmeans group / pdiff  lines;
run;

proc sort data=metabolites2; by group;run;
proc glm;by group;
model TMAO_Cr_ug_mg = eGFR;
run;


proc glm data=metabolites2;
class pair group;
model IS_Cr_ug_mg =  pair group;
lsmeans group/pdiff stderr lines;
output out=stdres2 p=predict r=resid;
run;

proc univariate data=stdres2 normal;
var resid;run;


proc glm data=metabolites2;
model IS_Cr_ug_mg = eGFR;
run;

proc glm data=metabolites2;
class group pair;
model IS_Cr_ug_mg =egfr pair group;
lsmeans group / pdiff  lines;
run;

proc sort data=metabolites2; by group;run;
proc glm;by group;
model IS_Cr_ug_mg = eGFR;
run;
```

```
proc glm data=metabolites2;
class pair group;
model PCS_Cr_ug_mg =  pair group;
lsmeans group/pdiff stderr lines;
output out=stdres2 p=predict r=resid;
run;

proc univariate data=stdres2 normal;
var resid;run;

proc glm data=metabolites2;
model PCS_Cr_ug_mg = eGFR;
run;

proc glm data=metabolites2;
class group pair;
model PCS_Cr_ug_mg =egfr pair group;
lsmeans group / pdiff  lines;
run;

proc sort data=metabolites2; by group;run;
proc glm;by group;
model PCS_Cr_ug_mg = eGFR;
run;

proc glm data=metabolites2;
class pair group;
model FE_TMAO =  pair group;
lsmeans group/pdiff stderr lines;
output out=stdres2 p=predict r=resid;
run;

proc univariate data=stdres2 normal;
var resid;run;

proc glm data=metabolites2;
model FE_TMAO = eGFR;
run;

proc glm data=metabolites2;
class group pair;
model FE_TMAO =egfr pair group;
lsmeans group / pdiff  lines;
run;

proc sort data=metabolites2; by group;run;
proc glm;by group;
model FE_TMAO = eGFR;
run;

proc glm data=metabolites2;
class pair group;
model FE_IS =  pair group;
lsmeans group/pdiff stderr lines;
output out=stdres2 p=predict r=resid;
run;
```

```
proc univariate data=stdres2 normal;
var resid;run;

proc glm data=metabolites2;
model FE_IS = eGFR;
run;

proc glm data=metabolites2;
class group pair;
model FE_IS =egfr pair group;
lsmeans group / pdiff  lines;
run;

proc sort data=metabolites2; by group;run;
proc glm;by group;
model FE_IS = eGFR;
run;

proc glm data=metabolites2;
class pair group;
model FE_PCS =  pair group;
lsmeans group/pdiff stderr lines;
output out=stdres2 p=predict r=resid;
run;

proc univariate data=stdres2 normal;
var resid;run;

proc glm data=metabolites2;
model FE_PCS = eGFR;
run;

proc glm data=metabolites2;
class group pair;
model FE_PCS =egfr pair group;
lsmeans group / pdiff  lines;
run;

proc sort data=metabolites2; by group;run;
proc glm;by group;
model FE_PCS = eGFR;
run;

data metabolites2;
input       ID$   pair$ group$      sTMAOuM     uTMAOug_mL  TMAO_Cr_ug_mg
FE_TMAO      sISuM uISug_mL    IS_Cr_ug_mg FE_IS sPCSuM      uPCSug_mL
PCS_Cr_ug_mg      FE_PCS      sCR   eGFR
;
cards;
      401   1     CKD   11.09471    29.31560    10.05646    0.17257
10.07496      75.00690    25.73047    0.17129     75.16179    146.99951
1.20064     0.05098      1.49  42
      601   1     Cont  8.17738     22.96378    11.85900    0.15640
1.89551      0.02171     0.01121     0.00022     3.71502     7.36861
0.02571     0.04409      0.67  113
```

```
      402   2     CKD   35.18482    14.01272    23.36622    0.15208
25.10700      18.07381    30.13809    0.09684     109.69991   27.02689
0.23972     0.03755      1.49  37
      502   2     Cont  12.19976    38.62787    16.64923    0.18351
9.52275      48.13448    20.74673    0.10321     42.61745    111.92087
0.89333     0.06075      0.84  85
      403   3     CKD   25.06256    22.67958    17.88470    0.18812
11.89460      17.52887    13.82294    0.10792     101.88322   56.66353
1.71861     0.04614      1.55  34
      603   3     Cont  7.77956     11.52571    8.42646     0.13844
5.44247      0.01631     0.01192     0.00010     27.58017    27.93309
0.23207     0.03777      0.89  71
      404   4     CKD   14.49827    15.91807    11.05882    0.19397
5.62332      10.69137    7.42766     0.11833     16.33456    7.69283
0.01554     0.03321      1.55  49
      504   4     Cont  7.69115     4.68817     13.60468    0.21431
3.08438      0.08684     0.25202     0.00349     0.61024     0.19657
0.00446     0.04520      0.84  90
      406   5     CKD   11.58093    16.28460    13.87813    0.29357
6.79474      5.86664     4.99969     0.06350     64.83983    29.06915
0.25806     0.03735      1.94  42
      506   5     Cont  2.56372     10.34612    4.13547     0.18684
3.47510      27.38920    10.94780    0.12855     11.46342    29.01624
0.24163     0.04677      0.96  90
      407   6     CKD   66.34726    24.03348    33.46349    0.11483
23.64039      18.77379    26.14005    0.08868     282.76915   34.67096
0.25678     0.01551      1.77  29
      507   6     Cont  12.06716    23.75427    18.92620    0.17123
5.02707      5.20352     4.14590     0.03172     36.62092    51.02362
0.56463     0.04837      .     .
      410   7     CKD   16.53156    18.57739    12.11516    0.18538
5.34027      2.41709     1.57629     0.02630     97.51552    63.06674
0.23106     0.04258      1.58  55
      510   7     Cont  4.95063     19.82704    .     .     3.45373
30.59894    .      .     10.14432    21.85845    .     .     1.05  78
;

proc print data=metabolites2;
run;


proc corr data = metabolites2;
var sTMAOuM     uTMAOug_mL  TMAO_Cr_ug_mg     FE_TMAO       sISuM uISug_mL
IS_Cr_ug_mg FE_IS sPCSuM      uPCSug_mL   PCS_Cr_ug_mg      FE_PCS       sCR
eGFR;
run;
```

	sTMAOuM	TMAO_Cr_ug_mg	FE_TMAO	sISuM	IS_Cr_ug_mg	FE_IS	sPCSuM	PCS_Cr_ug_mg	FE_PCS	sCR	eGFR
sTMAOuM	1.00000 7	0.93865 0.0055 6	-0.11080 0.8345 6	0.60872 0.1469 7	0.19867 0.7059 6	-0.24489 0.6400 6	0.69066 0.0858 7	0.71512 0.1102 6	0.49251 0.3210 6	-0.55969 0.2481 6	0.05905 0.9115 6
TMAO_Cr_ug_mg	0.93865 0.0055 6	1.00000 6	0.16382 0.7565 6	0.40573 0.4248 6	0.13161 0.8037 6	-0.24451 0.6405 6	0.48329 0.3315 6	0.58063 0.2269 6	0.51395 0.2970 6	-0.53178 0.3563 5	0.13819 0.8246 5
FE_TMAO	-0.11080 0.8345 6	0.16382 0.7565 6	1.00000 6	-0.03754 0.9437 6	0.26860 0.6068 6	0.31697 0.5405 6	-0.30517 0.5565 6	-0.16097 0.7606 6	0.43111 0.3934 6	0.21959 0.7227 5	0.12701 0.8387 5
sISuM	0.60872 0.1469 7	0.40573 0.4248 6	-0.03754 0.9437 6	1.00000 7	0.76799 0.0745 6	0.43619 0.3872 6	0.88239 0.0085 7	0.87455 0.0226 6	0.70090 0.1208 6	0.08669 0.8703 6	-0.46383 0.3541 6
IS_Cr_ug_mg	0.19867 0.7059 6	0.13161 0.8037 6	0.26860 0.6068 6	0.76799 0.0745 6	1.00000 6	0.86766 0.0251 6	0.57406 0.2335 6	0.66009 0.1537 6	0.88879 0.0179 6	0.32972 0.5879 5	-0.17298 0.7809 5
FE_IS	-0.24489 0.6400 6	-0.24451 0.6405 6	0.31697 0.5405 6	0.43619 0.3872 6	0.86766 0.0251 6	1.00000 6	0.31314 0.5456 6	0.38931 0.4455 6	0.63876 0.1722 6	0.56704 0.3188 5	-0.12140 0.8458 5
sPCSuM	0.69066 0.0858 7	0.48329 0.3315 6	-0.30517 0.5565 6	0.88239 0.0085 7	0.57406 0.2335 6	0.31314 0.5456 6	1.00000 7	0.97749 0.0008 6	0.51918 0.2912 6	0.08629 0.8709 6	-0.49097 0.3227 6
PCS_Cr_ug_mg	0.71512 0.1102 6	0.58063 0.2269 6	-0.16097 0.7606 6	0.87455 0.0226 6	0.66009 0.1537 6	0.38931 0.4455 6	0.97749 0.0008 6	1.00000 6	0.66545 0.1492 6	0.21590 0.7273 5	-0.45395 0.4425 5
FE_PCS	0.49251 0.3210 6	0.51395 0.2970 6	0.43111 0.3934 6	0.70090 0.1208 6	0.88879 0.0179 6	0.63876 0.1722 6	0.51918 0.2912 6	0.66545 0.1492 6	1.00000 6	0.00155 0.9980 5	0.07743 0.9015 5
sCR	-0.55969 0.2481 6	-0.53178 0.3563 5	0.21959 0.7227 5	0.08669 0.8703 6	0.32972 0.5879 5	0.56704 0.3188 5	0.08629 0.8709 6	0.21590 0.7273 5	0.00155 0.9980 5	1.00000 6	-0.75070 0.0855 6
eGFR	0.05905 0.9115 6	0.13819 0.8246 5	0.12701 0.8387 5	-0.46383 0.3541 6	-0.17298 0.7809 5	-0.12140 0.8458 5	-0.49097 0.3227 6	-0.45395 0.4425 5	0.07743 0.9015 5	-0.75070 0.0855 6	1.00000 6

Difference between serum and urine URS in CKD and control groups

Group/Measurement	IS		PCS		TMAO	
	Mean ± SD	p	Mean ± SD	p	Mean ± SD	p
CKD serum (uM)	**12.64 (8.37)**	**0.0177**	**106.89 (83.70)**	**0.019**	**25.76 (19.85)**	**0.035**
Control serum (uM)	4.56 (0.94)		18.96 (16.58)		7.92 (3.49)	
CKD urine (ug/mL)	**21.19 (24.57)**	0.719	**52.17 (45.79)**	0.543	**20.12 (5.45)**	0.79
Control urine (ug/mL)	15.92 (19.39)		37.91 (40.45)		18.82 (11.25)	
CKD urine (ug:mg)	**15.69 (11.58)**	**0.049**	**56.01 (63.81)**	0.444	**17.40 (8.45)**	0.083
Control urine (ug:mg)	6.02 (8.37)		32.69 (34.30)		12.27 (5.41)	
CKD urine FE	**0.10 (0.05)**	0.1343	**0.05 (0.008)**	0.1792	**0.18 (0.03)**	0.6796
Control urine FE	0.04 (0.06)		0.04 (0.01)		0.16 (0.09)	

Correlations between eGFR and urine and serum URS

Measurement	**IS**		**PCS**		**TMAO**	
	p	r	p	r	p	r
Serum	**0.0084**	0.482	**0.007**	**0.500**	**0.017**	**0.418**
Urine (ug/mL)	0.595	0.027	0.40	0.065	0.73	0.011
Urine (ug:mg)	0.068	0.295	0.241	0.134	0.07	0.285
Urine FE	0.12	0.228	0.11	0.237	0.99	0.00002