Effect of Kidney Disease and Gut-Derived Uremic Toxins on Flavin Monooxygenases: Clinical and Translational Research Studies

by

Alexander John Prokopienko

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This dissertation was presented

by

Alexander John Prokopienko

It was defended on

July 24, 2019

and approved by

Samuel M. Poloyac, PharmD, PhD, Professor, School of Pharmacy

Raman Venkataramanan, PhD, Professor, School of Pharmacy

Paul A. Johnston, PhD, Associate Professor, School of Pharmacy

Lee A. McDermott, PhD, MBA, Assistant Professor, School of Pharmacy

Jason R. Stubbs, MD, MSc, Associate Professor, University of Kansas, School of Medicine

Dissertation Director: Thomas D. Nolin, PharmD, PhD, Associate Professor, School of Pharmacy

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Cardiovascular disease (CVD) is the leading cause of death in patients with kidney disease. Unfortunately, therapeutic strategies targeting traditional risk factors have limited impact on reducing CVD outcomes, suggesting that non-traditional CVD risk factors may play an important role in kidney disease. In fact, the altered kidney disease gut microbiome is a source of several non-traditional CVD risk factors. In particular, trimethylamine-*N*-oxide (TMAO) is formed from gut microbiota production of trimethylamine and hepatic flavin monooxygenase (FMO) metabolism. The overarching objective of this dissertation was to investigate the effect of kidney disease and gut-derived toxins on flavin monooxygenase activity. To achieve these goals, an analytical method was developed and validated to measure gut-derived toxins including kynurenic acid, hippuric acid, indoxyl sulfate and p-cresol sulfate.

In vitro studies utilizing rat microsomes demonstrated significant increases in the formation of TMAO in the kidney disease group versus the control, suggesting increased FMO activity. Although gene and protein expression of FMO were not changed, metabolic activation elicited by octylamine and presence of human uremic serum increased FMO-mediated TMAO formation. *Ex vivo* translational studies using primary cultures of human hepatocytes demonstrated that exposure to indoxyl sulfate, a gut-derived toxin, increases *FMO3* expression and FMO-mediated TMAO formation. The findings suggest that metabolic activation and indoxyl sulfate exposure increase FMO activity and represent novel mechanisms that contribute

to increased TMAO formation in kidney disease and may be a therapeutic target to reduce TMAO exposure and CVD.

In a pilot clinical study, a novel strategy to reduce formation of TMAO with diindolylmethane was investigated in Stage 3-4 kidney disease patients. Gut-derived uremic toxins and corresponding intestinal microbiome profiles were also assessed. The findings demonstrated that diindolylmethane reduced serum TMAO concentrations in males compared to females. The microbiota composition was also significantly altered in the kidney disease patients compared to control. Furthermore, the microbiota was a strong predictor of gut-derived toxin exposure.

Collectively, this work advances our understanding of potential increases in FMO activity and microbiota alterations in kidney disease that will facilitate future work to develop therapeutic strategies to reduce CVD.

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PREFACE

This dissertation is dedicated to my wife, Sasha and our son, Theodore; I love you both. The last 5 years in this program have been an unforgettable journey both professionally and personally, including getting married during my first year and Theodore arriving my fourth year.

I also dedicate this dissertation to my parents, John and Mary Prokopienko. You have sacrificed and dedicated your lives to provide opportunities for your six children. This dissertation is one example of the doors you have opened for us, thank you.

The work presented in this dissertation would not be possible without the support of my family, dear friends, great mentors, and supportive colleagues. Thank you all for the support, guidance and encouragement!

Sasha and I started our family and have made lifelong friends in Pittsburgh. From the labs of Salk Hall to Mellon Park, we have shared laughter and friendship. These friends, along with my faith and family, carried me through the challenging times and I am eternally grateful.

My family continues to enrich my life. My parents, John and Mary, are intelligent, kind, hardworking, and strong, with these strengths they raised 6 children. My family is full of tough love, character and masterminds. My siblings joked about my scientific theories growing up and now those theories are slightly more credible. Thank you to JD, Steve, Esther, Dave and Mark for your love and encouragement. I would also like to thank Hugh, Natalie, and Katerina for accepting me into your family and for my beautiful wife, Sasha. Thank you also to my nieces Emma, Phillipa, Bionca Anna, Audrey, Maria Francesca and my nephews Aidan, Alexander, and James for the joy you bring into our lives. Finally, thank you to all of the Prokopienko, Steenburgh, Pishenin, and Reilly families for your love and support.

I am also grateful for the support and guidance of my primary mentor, Tom Nolin, who has encouraged me to overcome challenges and have the persistence to succeed with the motto "nothing ventured, nothing gained". Tom is uncompromising, intelligent, articulate and expects excellence; these qualities make him a great scientist and mentor. His direct guidance is effective as evidenced by how fortunate I have been with grants and fellowships. I want to extend a heartfelt, thank you, for the time, effort, care, patience and confidence you have invested into my success.

My overall mentorship team has also been incredibly supportive to my career development and research. I would like to especially acknowledge Dr. Amy Pai who saw a spark of potential in 2008 when she hired me as a research assistant, thank you for your mentorship and always believing in me. In addition, I am grateful to and thank my Dissertation Committee members, Drs. Sam Poloyac, Jason Stubbs, Raman Venkataramanan, Paul Johnston and Lee McDermott for providing encouragement, guidance and support over the past 4 years. Additional contributors to my mentorship team include Drs. Randy Smith and Kevin Kraemer who have contributed to my professional and scientific development, thank you. These individuals and so many others have given me guidance, education, support and have encouraged me to rise to my true potential.

The research process is challenging, and many individuals have directly or indirectly supported the studies outlined in this dissertation. First and foremost, thank you to all of the patients that contributed to this work, it would not be possible without your time and efforts. Thank you also to the co-authors on the manuscripts within this dissertation. Your individual contributions are greatly appreciated. I would also like to acknowledge some of my fellow colleagues for their guidance and support including Raymond West III, Yoko Franchetti, Stanton Kochanek, Fanuel Hagos, Solomon Adams, Amina Shehu, Jessica Kosanovich, Katie Eichinger, Ashley Fancher, Chenxiao Tang, Osama Alshogran, Dustin Walsh, Kacey Anderson, Rujuta Joshi, Hari Kalluri, Brian Kiesel, Lingjue Li, Kunal Jhunjhunwala, and Yang Xie. All of your support and friendship has helped me learn new scientific methods and encouraged me to complete this dissertation.

ABBREVIATIONS

AhR: Arylhydrocarbon receptor

AUC: Area under the curve

BCRP: Breast cancer resistance protein

BMI: Body mass index

CKD: Chronic kidney disease

CMPF: 3- carboxy-4-methyl-5-propy-2-furanpropanoic acid

CVD: Cardiovascular disease

CYP: Cytochrome P-450

DIM: diindolylmethane

eGFR: Estimated glomerular filtration rate

ESKD: End-stage kidney disease

FDA: Food and Drug Administration

FMO: Flavin-containing monooxygenase

GDUT: Gut-derived uremic toxin

LLOQ: Lower limit of quantification

KD: Kidney disease

K_m: Michaelis-Menten constant

MS: Mass spectrometry

OAT: Organic anion transporter

OATP: Organic anion transporter polypeptide

OCT: Organic cation transporters

P-gp: P-glycoprotein

QC: Quality Control

RT-qPCR: Real time quantitative polymerase reaction

SLC: solute carrier transporters

TMAO: Trimethylamine-*N*-oxide

UGT: Uridine diphosphate (UDP)-glucuronosyltransferase

UHPLC-MS/MS: Ultra high-performance liquid chromatography- tandem mass spectrometry

V_{max}: Maximum velocity of enzymes

CHAPTER 1: INTRODUCTION AND BACKGROUND

[Prokopienko AJ and Nolin TD. *Expert Review of Clinical Pharmacology* 2018][1]

An estimated 13% of the global population or approximately 1 billion people worldwide suffer with kidney disease (KD) [2]. Cardiovascular disease (CVD) remains the leading cause of death in KD patients, despite aggressive treatment of traditional CVD risk factors such as hyperlipidemia [3]. This suggests that non-traditional CVD risk factors may play a greater role in KD. Although the mechanism is unclear, gut-derived uremic toxins (GDUTs) accumulate to supraphysiologic concentrations in KD and are implicated in the acceleration of CVD [4]. We hypothesized that the altered gut microbiota and/or altered hepatic drug metabolism enzymes increase the formation of GDUTs and contribute, in part, to the high systemic exposures [5, 6].

A major objective of this dissertation was to investigate potential changes in the formation of the non-traditional CVD risk factor trimethylamine-*N*-oxide (TMAO) in kidney disease. TMAO is associated with major adverse CVD events in the general population [7, 8]. TMAO is mechanistically linked to promoting atherosclerosis and thrombosis in preclinical mouse models and associates with CVD in humans [9]. In patients with advanced KD, serum TMAO concentrations increase disproportionately compared to earlier stages of KD and also associate with atherosclerosis burden [10]. The drug metabolism enzyme, flavin-containing monooxygenase (FMO), oxygenates trimethylamine to form TMAO [11]. To date, limited knowledge exists about FMO enzyme activity in KD patients and its potential contributions to disproportionate increases in TMAO exposure in advanced KD [10]. Therefore, clinical and translational research studies are needed to evaluate the effect of KD and GDUTs on FMO-mediated TMAO formation.

Over 100 uremic retention solutes circulate at high concentrations in KD [5] and amongst these solutes are well-known GDUTs that disrupt physiological systems such as indoxyl sulfate. GDUT disruption of “remote sensing and signaling” systems has been reviewed from a

physiological perspective by Nigham and colleagues [6]. This hypothesis broadly describes the interactions of GDUTs on biological systems in patients with KD. In particular, clinically significant interactions occur with drug metabolizing enzymes and drug transporters. For example, indoxyl sulfate upregulates p-glycoprotein (PgP) efflux transporters, and thereby, increases cyclosporine elimination in transplant patients with KD [12]. The patients who had high serum concentrations of indoxyl sulfate required higher doses of cyclosporine to maintain therapeutic concentrations and prevent transplant rejection. The cyclosporine dose adjustments demonstrate that GDUTs perpetrate interactions that impact the clinical care of KD patients, which suggests that other interactions are likely to have clinical implications. Overall, the disrupted "remote sensing and signaling" hypothesis provides a key perspective to explain how gut-derived toxins can alter drug metabolism and drug transport in KD. This chapter provides a comprehensive review to support the concept that GDUTs and/or mediators in kidney disease potentially interact with FMO enzymes, as evidenced by GDUTs interactions with several drug metabolizing enzymes and drug transporters.

KD patients have altered hepatic drug metabolism and drug transport, also known as nonrenal clearance, which affects the pharmacokinetics and pharmacodynamics of medications [13]. Briefly, the function and/or expression of some drug metabolizing enzymes and drug transporters are altered. For example, the clearance of fexofenadine, a nonspecific probe of drug transport, was reduced by 63% in end-stage kidney disease (ESKD) patients compared to healthy controls [14]. Organic anion transporting protein (OATP1B) has also been estimated to be reduced by up to 60% in KD patients [15]. The underlying mechanism(s) to explain these functional changes remains unclear; however, solutes within the uremic milieu such as GDUTs may act as perpetrators of drug interactions [1].

GDUTs originate from the gut microbiota which is a dynamic community of metabolically active microorganisms that interact bi-directionally with human physiological systems. The average human gut microbiota contains over 38 trillion microorganisms that make important contributions to overall human health [16-18]. Microbial composition is impacted by several factors, including diet, medications, host genetics, environmental interactions, disease specific changes and transient bacterial species. Gut-derived toxins, byproducts of the metabolism of dietary nutrients by gut microbiota, are implicated in multiple diseases including kidney disease [19], cardiovascular disease [10, 19, 20], cancer [21], neurological diseases [22], and inflammatory states [23]. In fact, there is high systemic exposure to several gut-derived toxins such as TMAO, indoxyl sulfate, p-cresol sulfate, hippuric acid, 3-carboxy-4-methyl-5-propyl-2-furanpropanoic acid (CMPF) and indole-3-acetic acid in kidney disease patients, likely due to increased toxin producing bacterial species, alterations to hepatic drug metabolizing enzymes and simultaneously decreased renal clearance (Figure 1) [24]. However, they may represent only the tip of the iceberg as many more gut-derived toxins likely circulate in humans.

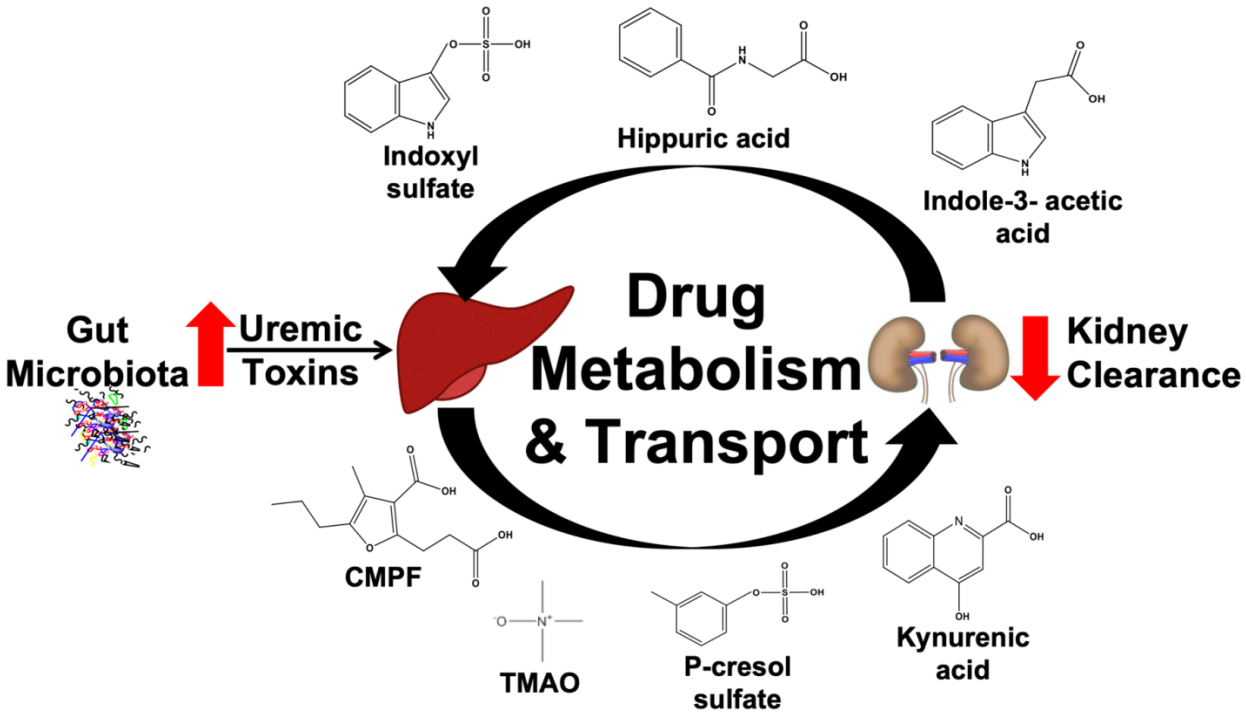


Figure 1. Microbial Toxins - Drug Interactions

The human gut microbiota produces several gut-derived toxins that accumulate in patients with kidney disease. These microbial metabolites (e.g., TMAO, p-cresol sulfate, indoxyl sulfate, indole-3-acetic acid, CMPF, kynurenic acid, and hippuric acid) interact with critical phase I/II metabolic enzymes and drug transporters. These interactions occur in the liver and kidneys at the transcriptional and/or post-transcriptional levels. Patients with kidney disease are at increased risk for adverse drug events because of the effects of these microbial toxin as well as other uremic toxins on drug disposition. Furthermore, there are increases in bacteria that produce these gut-derived toxins in kidney disease patients. Therefore, evaluating the microbiota influences on drug disposition is imperative in this population.

Microbial imbalance (i.e., disturbed microbial composition) or maladaptation of microbial communities to the diseased environment, also known as dysbiosis, is partially mediated by gut-derived toxins and influences gut microbiota-host interactions [25, 26]. Gut-derived toxins interact with several nonrenal clearance pathways, thereby potentially modifying drug dosing-exposure-response relationships in patients with kidney disease [14, 27-29]. In fact, alterations in the functional expression of drug metabolizing enzymes and transporters in kidney disease are well documented [13, 30-32], and several microbial toxins are potential mediators [33, 34]. The uremic milieu is complicated, containing thousands of compounds including numerous endogenous solutes (e.g., urate, parathyroid hormone, inflammatory cytokines) that are not derived from the microbiota, yet may elicit independent pathophysiological effects. Therefore, this chapter highlights the effects of gut-derived uremic toxins on drug metabolizing enzymes and transporters, focusing on individual toxins shown in Table 1, rather than uremia in general.

In this chapter, a comprehensive review of the effect of gut-derived uremic toxins on hepatic Phase I and Phase II drug metabolizing enzymes and drug transporters is presented. The MEDLINE bibliographic database was searched through PubMed using relevant search terms, including uremic toxins, uremic retention solutes, uremia, drug metabolism, drug transport, kidney disease, renal disease, nonrenal clearance, and human microbiome. Other articles were identified from reference lists within the retrieved manuscripts. A total of 36 articles that explicitly tested individual microbial toxin effects on drug metabolizing enzymes or drug transporters were identified. All other citations are included as supporting evidence that the microbiome and gut-derived toxins impact nonrenal clearance.

Table 1. Clinically Observed Gut-Derived Toxin Concentrations

Microbial Toxin [total]	Normal Total Concentration (SD) [μM]	Mean Uremic Free Concentration (SD) [μM]	Mean Uremic Total Concentration (SD) [μM]	Highest Uremic Total Concentration (SD or Range) [μM]
CMPF	15 (1)	-	25 (10)	135 (46) [35]
Hippuric Acid	17 (11)	231 (80)	398 (77)	487 (345)
Indole-3-acetic acid	3 (1.7)	2.1 (0.6)	12 (2)	14 (13)
Indoxyl sulfate	2.5 (1.4)	15 (5.7)	109 (61)	210 (72)
Kynurenic acid	.03 (.007)	-	-	0.8 (0.4)
P-cresol sulfate	10 (7)	9.3 (6.4)	111 (65)	218 (71)

CMPF, 3-Carboxy-4-methyl-5-propyl-2-furan-propanoic acid.

Adapted from Vanholder RC, J Am Soc Nephrol. 2012 Jul;23(7): 1258-1270 [5]

1.1 GUT MICROBIOTA – HOST LIVER INTERACTIONS

The Human Microbiome Project and the Metagenomics of the Human Intestinal Tract initiative revealed important information about the vast diversity and complexity of the human microbiome [36-38]. The diversity and function of the intestinal microbiota directly influences bacterial metabolism, which in turn impacts the systemic exposure of orally administered nutrients and xenobiotics and the formation of microbial toxins that elicit pathophysiological effects [39]. Some of the nutrients and xenobiotics transformed by gut microbiota reach the liver via portal circulation where they interact with drug metabolizing enzymes and transporters.

Hepatic drug metabolizing enzymes interact with the microbiota through biotransformation of select microbial toxins. For example, dietary tryptophan is metabolized by microbial tryptophanase to indole, which is subsequently hydroxylated by CYP2E1 to indoxyl and finally sulfonated by sulfotransferases (SULT1A1) to indoxyl sulfate. The biotransformation of indole to indoxyl sulfate requires hepatic CYP2E1 and SULT1A1 enzymes and demonstrates the importance of these pathways in microbiota-derived uremic toxin formation (Figure 2). Formation of p-cresol sulfate and indoxyl sulfate is decreased in chronic kidney disease patients and animal models with advanced liver cirrhosis (and thus impaired hepatic metabolism) [40]. Cirrhotic patients also exhibit higher serum concentrations of metabolic precursors like phenol and indole, suggesting decreased metabolic conjugation [41, 42]. The role of hepatic metabolism in biotransformation of some gut-derived toxins indicates a potential for competitive interactions with both Phase I and II metabolic pathways.

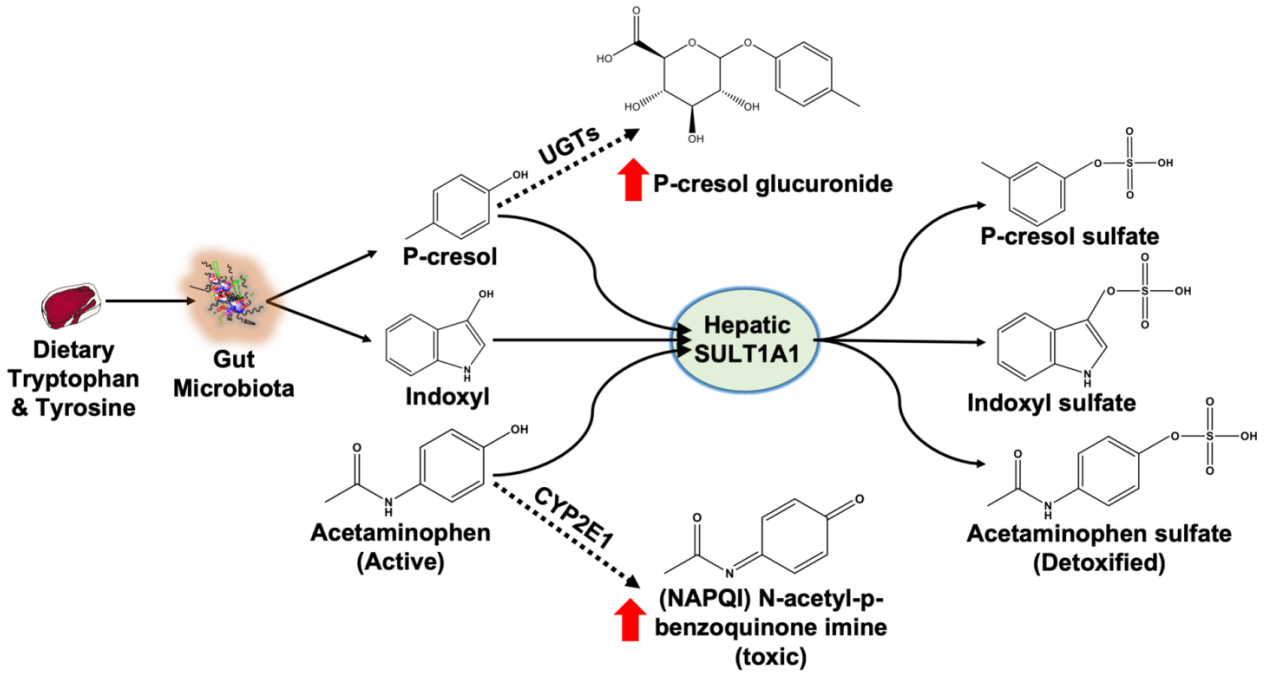


Figure 2. Microbial Toxins-Phase II SULT1A1 Interactions

P-cresol and indoxyl are both produced by bacterial protein fermentation in the human large intestine. These microbial metabolites compete with drugs for hepatic Phase II metabolic enzymes. P-cresol, indoxyl and acetaminophen are all substrates for the same enzyme, hepatic sulfotransferase SULT1A1. Therefore, this competitive inhibition is exacerbated in kidney disease by high concentrations of p-cresol and indoxyl that inhibit detoxification of acetaminophen. This competition shifts the metabolism of these substrates to alternative metabolic pathways, including the toxic NAPQI acetaminophen metabolite and p-cresol glucuronide.

The gut microbiota regulates hepatic phase I and II metabolizing enzyme and transporter expression [43, 44], therefore alterations of gut microbiota constituents might also affect these processes [43, 45]. Experimental models investigating the effects of the gut microbiota on drug metabolism and transport include un-colonized or germ-free animals. Germ-free and normally colonized mice differentially express important drug metabolism and transporter genes [45-48]. For example, germ-free mice exhibit increased expression of Cyp1a2 (+51%) and Cyp4a14 (+202%) mRNA, but decreased expression of Cyp3a11 (-87%) and Cyp2b10 (-57%) mRNA. Germ-free mice more efficiently metabolize intravenous pentobarbital, measured by total anesthesia time, suggesting enzymatic activity increases with gene expression [45]. One potential mechanism may be the continuous passing of microbial metabolites from the intestine to the liver via the portal circulation. Since these metabolites may be ligands for nuclear receptors, this could lead to altered hepatic gene expression [49]. Collectively, the evidence suggests that the gut microbiota interacts with hepatic enzymes, which might link dysbiosis in kidney disease to altered nonrenal clearance.

1.2 GUT MICROBIOTA DYSBIOSIS IN KIDNEY DISEASE

Dysbiosis in kidney disease influences microbial toxin production and absorption. Alterations to the gut microbiota in patients with kidney disease are due to a combination of factors, including concomitant use of numerous medications, frequent antibiotic use, changes in intestinal pH, and intestinal barrier dysfunction [26, 39, 50]. These factors may lead to an increase in intestinal bacteria that produce uremic toxins [25, 51]. For example, bacteria that

produce precursors to indoxyl sulfate and p-cresol sulfate are increased in patients with advanced kidney disease [51] and in animal models of kidney disease [52]. Uremic toxins in the gastrointestinal tract may engender inflammation and promote a “leaky gut” [53]. Dysbiosis also contributes to impairment of the gut epithelial barrier, which facilitates greater absorption of microbial toxins, increased serum endotoxin concentrations and bacterial translocation [24, 54]. In fact, increased microbial toxin production and absorption is associated with nearly half of the most highly accumulated uremic toxins [5]. Constipation may also worsen uremia and increase the risk of adverse renal outcomes [55]. Overall, the altered gut microbiota observed in kidney disease produces more microbial toxins that accumulate, become constituents of the uremic milieu, and are readily available at high concentrations to interact with drug metabolizing enzymes and transporters.

1.3 CONSIDERATIONS FOR INTERPRETING GUT-DERIVED UREMIC TOXIN STUDIES

Caution is warranted when interpreting studies involving gut-derived toxins due to frequent discrepancies between concentrations and metabolites of toxins observed clinically and those used experimentally. The highest clinically observed total concentrations of microbial toxins in kidney disease patients range from 3.7-fold (indole-3-acetic acid) to 83-fold (indoxyl sulfate) higher than in patients with normal kidney function (Table 1). Moreover, many microbial toxins are extensively protein bound, and since only unbound toxins elicit biological activity and/or undergo transport into cells, interpretation of data based on unbound versus total

(free and bound) concentrations may lead to different conclusions. For example, some studies acknowledge protein binding [56], while others use total concentrations [57] to interpret clinical relevance of results. The unbound concentrations at the site of interactions are likely the most relevant but are difficult to estimate [58]. Only considering plasma concentrations of microbial toxins may not accurately reflect tissue distribution. For instance, some microbial toxins accumulate to higher concentrations in liver and kidney tissues than in plasma [59].

Another important consideration when interpreting studies involving gut-derived toxins is the moiety studied. For instance, p-cresol is frequently employed in experimental models, but the sulfate and glucuronide conjugates are the only measurable forms in human plasma [60]. This raises concern over the clinical relevance of results derived in experiments using unconjugated p-cresol. Nevertheless, p-cresol is likely present in portal circulation, and must also be present at some concentrations in hepatocytes and kidney tissue [56]. Clinically observed total microbial toxin concentrations are included in Table 1 and used as a benchmark to evaluate clinical relevance of individual studies [5].

Lastly, it is important to consider limitations of experimental methods employed in individual studies. For example, experiments assessing the effect of individual microbial toxins on metabolism or transport pathways often use in vitro techniques (e.g. microsomes, isolated hepatocytes, functional assays) that may not completely translate to in vivo human processes. The strengths and weaknesses inherent in each experimental technique used should be considered in the interpretation of results.

1.4 **PHASE I METABOLIC PATHWAY – GUT-DERIVED TOXIN INTERACTIONS**

Phase I oxidative enzymes, including cytochrome P450 (CYPs) and flavin-containing monooxygenases (FMOs), metabolize approximately 80% of commonly used medications [61]. Patients with kidney disease are often prescribed 10-12 different medications [62] and many are substrates for Phase I drug metabolizing enzymes. Therefore, disruption in the expression and/or function of these pathways by uremic toxins may alter drug exposure and thereby, lead to suboptimal response or increased drug-related adverse events. Indeed, several gut-derived toxins accumulate in patients with kidney disease and interfere with Phase I hepatic metabolism (Table 2). For example, the combined effects of hippuric acid, indoxyl sulfate and p-cresol result in a >50% decrease in CYP3A4, CYP1A2, CYP2C9, and CYP2E1 human microsomal activity [56]. The combination of microbial toxins may be either additive or synergistic. Determination of individual and combined effects of microbiota-derived toxins on hepatic enzymes responsible for xenobiotic and endobiotic metabolism is therefore critical.

Table 2. Effect of Gut-Derived Toxins on Phase I Drug Metabolizing Enzymes

Enzyme	Indoxyl Sulfate	P-cresol OR P-cresol Sulfate	CMPF	Hippuric Acid	Indole-3-Acetic Acid	Combined Microbial Toxins
CYP3A	▪ Non-competitive inhibitor OR inhibitor [56, 63-65]*, [57, 66, 67]# ▪ No effect on activity [57, 66]* ▪ No significant mRNA induction [63, 66]*	▪ Competitive inhibitor [56]* ▪ Inhibitor [57]* ▪ Non-competitive inhibitor [65]*	▪ No effect on activity [56, 65, 66]* ▪ Inhibitor [57]*, [64]# ▪ No significant mRNA induction [66]*	▪ No effect on activity [56] [65, 66, 68]* ▪ Inhibitor [57]* ▪ No significant mRNA induction [66]*	▪ No effect on activity [56, 57, 63] [65, 66, 68]* ▪ No significant mRNA induction [63, 66, 67]*	▪ Inhibit activity (PC & IS) [65]* and (HA, CMPF, IS, I3A) [66]* ▪ Decreased mRNA expression (HA, CMPF, IS, I3A) [66]*
CYP1A1	▪ mRNA induction and increase in activity [69]*					
CYP1A2	▪ Weak Inhibitor or no effect on activity [56, 70]* ▪ Inhibitor [67]# ▪ mRNA induction and increase in activity [63, 69]*	▪ Weak inhibitor [56]*	▪ No effect on activity [70]*	▪ No effect on activity [56, 70]*	▪ Moderate increase in mRNA and activity [63]* ▪ Inhibitor [67, 70]#	▪ No effect on activity (HA, IS, I3A, CMPF) [70]*
CYP2E1	▪ Weak inhibitor [56]*	▪ Strong competitive inhibitor [56]*		▪ No effect on activity ([56])*		
CYP2C9	▪ No effect on activity [56]* ▪ Non-competitive inhibitor [65, 68]*	▪ Weak Inhibitor [56]* ▪ Non-competitive Inhibitor [65]*	▪ No effect on activity [56, 65]*	▪ Inhibitor [56]* ▪ No effect on activity [65]*	▪ No effect on activity [56, 65, 68]*	▪ Inhibit activity (PC & IS)[65]*
CYP2B6	▪ No effect on activity [57, 63]* ▪ No induction of mRNA [63]*	▪ No effect on activity [57]*		▪ No effect on activity [57]*	▪ No effect on activity [57] [63]* ▪ No induction of mRNA [63]*	
CYP2D6	▪ No effect on activity [70]*		▪ No effect on activity [70]*	▪ No effect on activity [70]*	▪ No effect on activity [70]*	▪ No effect on activity (HA, IS, I3A, CMPF) [70]*

*Denotes within clinically relevant concentrations. # Denotes concentration greater than clinically relevant (Table 3).
CMPF, 3-Carboxy-4-methyl-5-propyl-2-furan-propanoic acid. HA, hippuric acid. IS, Indoxyl sulfate. I3A, Indole-3-acetic acid. PC, P-cresol.
Weak inhibitor <29%, Inhibitor 30-69%, Strong inhibitor >70%

1.4.1 **CYP3A4/5**

Gut-derived toxins may affect the activity and expression of CYP3A4, a critically important isozyme that contributes to the metabolism of approximately 40% of marketed drugs [61]. P-cresol is a potent competitive inhibitor of CYP3A4 and perpetrates a >60% decrease in human microsomal activity [56]. Hippuric acid, indoxyl sulfate, CMPF and p-cresol all individually inhibit CYP3A4 mediated metabolism of testosterone in human microsomes [57]. Additionally, a combination of hippuric acid, CMPF, indoxyl sulfate and indole-3-acetic acid also perpetrate a significant decrease of CYP3A4 mRNA expression in human hepatocytes [66]. Indoxyl sulfate also inhibits CYP3A4 activity in human hepatocytes and microsomes, animal models and potentially in kidney disease patients [64, 67, 68]. Indoxyl sulfate plasma concentrations are inversely related to CYP3A4 activity measured by endogenous 4β-hydroxycholesterol formation in stable renal transplant patients [71]. Conversely, some reports suggest that indoxyl sulfate or other microbial toxins have no interactions with CYP3A4 activity or expression [63, 66]. The reason for discordant findings between studies is unclear but may be due to differences in experimental procedures. Nevertheless, the evidence to date suggests that gut-derived toxins and especially indoxyl sulfate may affect CYP3A4 activity and potentially expression.

1.4.2 **CYP1A1/2**

Gut-derived uremic toxins also affect CYP1A activity and expression [56, 63, 67, 69, 70], with idole-3-acetic acid and indoxyl sulfate being the most potent. Indole-3-acetic acid decreases CYP1A2 activity by 50% in human microsomes [70]. A combination of hippuric acid, indoxyl sulfate and p-cresol also inhibit CYP1A2 activity in human microsomes [56]. However, contradictory findings have been reported, as indole-3-acetic acid and indoxyl sulfate individually have been shown to increase mRNA expression and enzyme activity of CYP1A2 [63, 69]. Although reasons for the disparate findings are unclear, it likely involves the AhR nuclear receptor — a master regulator that activates CYP1A gene expression. Indoxyl sulfate is both a potent activating ligand of AhR that leads to increased expression of CYP1A2, and a direct inhibitor of CYP1A2 activity [63, 69]. Because of this, investigators should consider incubations that are long enough in duration to induce expression when assessing the effect of indoxyl sulfate on CYP1A2. The sum of data suggests that microbiota-derived toxins inhibit CYP1A2 enzymatic activity and that indoxyl sulfate induces CYP1A2 expression.

1.4.3 **CYP2C9**

CYP2C9 metabolism also may be inhibited by uremic toxins. Incubation of human microsomes with a combination of p-cresol and indoxyl sulfate resulted in a 46% decrease in metabolism of the dual CYP2C9 and CYP3A4 substrate losartan [65]. CMPF, indole-3-acetic acid and hippuric acid had no inhibitory effect on losartan metabolism [65]. Since losartan is a dual substrate, any effect elicited by uremic toxins may be partially attributed to inhibition of

CYP3A4 activity. Hippuric acid may inhibit CYP2C9 activity to a greater extent than p-cresol and indoxyl sulfate. Human microsomes were incubated with p-cresol, indoxyl sulfate and hippuric acid alone, and only hippuric acid decreased (by 39%) metabolism of the CYP2C9 substrate phenytoin [56].

1.4.4 **CYP2E1**

Gut-derived uremic toxins might also interact with CYP2E1. A combination of hippuric acid, indoxyl sulfate and p-cresol decreases CYP2E1 human microsomal activity by >50% [56]. At clinically relevant concentrations, p-cresol individually inhibits CYP2E1 activity by >60% in human microsomes, whereas hippuric acid and indoxyl sulfate had no individual effect [56]. This finding suggests that the microbial toxin precursor p-cresol inhibits CYP2E1 activity.

In summary, current evidence suggests that gut-derived toxins interact most extensively with CYP3A4 and CYP1A2. Interactions might also occur with CYP2C9 and CYP2E1 enzymes. These metabolic interactions and the wide variation of uremic toxin concentrations (Table 1) may partially explain the interindividual variability of alterations in Phase I drug metabolism in kidney disease patients.

1.5 **PHASE II METABOLIC PATHWAY – GUT-DERIVED TOXIN INTERACTIONS**

Gut-derived uremic toxins also appear to interact with Phase II drug metabolism pathways (Table 3). Phase II enzymes catalyze conjugation reactions and include

sulfotransferases, glutathione S-transferases, uridine diphosphate-glucuronosyltransferases, N-acetyltransferases, and methyltransferases. Like Phase I enzymes, these pathways are essential for both xenobiotic and endobiotic disposition and any disruption in their expression and/or function by uremic toxins may alter drug exposure and response.

Table 3. Effect of Gut-Derived Toxins on Phase II Drug Metabolizing Enzymes

Enzyme	Indoxyl Sulfate	P-cresol OR P-cresol Sulfate	CMPF	Indole-3-Acetic Acid
UGT1A1	• Inhibitor [56]* • mRNA induction [69]*	• Strong competitive inhibitor [56]*		• Weak inhibitor [56]*
UGT1A4	• Inhibitor [56]*	• Inhibitor [56]*		• No effect on activity [56]*
UGT1A6	• No effect on activity [56]* • mRNA induction [69]*	• Weak inhibitor [56]*		• No effect on activity [56]*
UGT1A9	• Weak inhibitor [56]*	• Non-competitive inhibitor [56]*		• Weak inhibitor [56]*
UGT2B4	• Weak inhibitor [56]*	• No effect on activity [56]*		• No effect on activity [56]*
UGT2B7	• Weak inhibitor [56]*	• Inhibitor [56]*		• Inhibitor [56]*
GST	• Inhibits activity [72]#		• Inhibits activity [72]#	• No effect on activity [72]*
SULT1A1		• Inhibits activity [73]*		

*Denotes within clinically relevant concentrations. # Denotes concentration greater than clinically relevant (Table 1). CMPF, 3-Carboxy-4-methyl-5-propyl-2-furan-propanoic acid. IS, Indoxyl sulfate. I3A, Indole-3-acetic acid. PC, P-cresol. Weak inhibitor <29%, Inhibitor 30-69%, Strong inhibitor >70%

1.5.1 **Sulfotransferases (SULT)**

Increased production and systemic exposure of p-cresol, indoxyl and perhaps other gut-derived metabolites in patients with kidney disease can perpetrate metabolic drug interactions, including competitive inhibition of hepatic sulfotransferases, and drug toxicity. The metabolic fate of acetaminophen (paracetamol) provides an important illustration of this phenomenon [73]. P-cresol and indoxyl are conjugated by SULT1A1 to the two well-known uremic toxins p-cresol sulfate and indoxyl sulfate, respectively [74]. Acetaminophen is normally metabolized by SULT1A1 conjugation as a major metabolic detoxification pathway. Interestingly, patients with high systemic concentrations of p-cresol sulfate (and thus likely increased concentrations of the unconjugated precursor) exhibit metabolic shunting of acetaminophen to CYP2E1 (Figure 2). CYP2E1 then generates hepatotoxic N-acetyl-p-benzoquinone imine (NAPQI) metabolites. Therefore, competitive SULT metabolic interactions between p-cresol and acetaminophen likely facilitate acetaminophen shunting towards NAPQI formation. Alterations to SULT function may also have cardiovascular implications, since shifts from p-cresol sulfate to glucuronide conjugation are associated with cardiovascular disease and mortality [75].

1.5.2 **Glutathione-S-transferases (GST) & Uridine Diphosphate-Glucuronosyltransferases (UGT)**

The activity and expression of GST and UGT enzymes also appear to be affected by microbiota-derived toxins. CMPF (by 37% in bovine and 21% in rat enzymes) and indoxyl sulfate (by 27% in bovine enzymes) decrease GST enzyme activity, which suggests that CMPF may be the most potent inhibitor of GST enzymes [72]. The activities of UGT1A1, UGT1A9 and UGT2B7 are decreased by 50% in human microsomes incubated with a combination of hippuric acid, indoxyl sulfate and p-cresol toxins as well as p-cresol alone at clinically observed concentrations, suggesting that p-cresol is mediating the effect [56]. P-cresol, p-cresol sulfate, kynurenic acid, indole-3-acetic acid, indoxyl sulfate and hippuric acid also can significantly decrease UGT activity in human renal proximal tubule cells [76], with p-cresol being the most potent competitive inhibitor. Similar to its effects on CYP1A2, indoxyl sulfate induces mRNA expression of UGT1A1 and UGT1A6 in primary human hepatocytes [69].

In summary, gut-derived toxins alter SULT, GST and UGT activity, which might contribute to altered drug exposure and response, and high rates of adverse drug events in kidney disease patients. Microbial toxin interactions with phase II enzymes may necessitate careful dosing of drugs that undergo biotransformation through these pathways. The effects of these direct interactions may also extend beyond drug interactions to endogenous substrates such as bilirubin, steroid hormones and bile acids.

1.6 DRUG TRANSPORTER – GUT-DERIVED TOXIN INTERACTIONS

Transporters are essential transmembrane proteins for hepatic uptake and excretion, as well as renal tubular secretion of drugs. Transporters are also located in several other organs such as the gut and blood brain barrier. Gut-derived toxins have been shown to interact with several transporters located in the kidneys and liver (Table 4).

1.6.1 Hepatic Organic Anion Transporting Polypeptides (OATP)

Hepatic organic anion transporting polypeptides (OATPs) are transporters for large hydrophobic anions (e.g., statins) and other commonly prescribed medications. OATPs transport drugs into hepatocytes, where they may undergo metabolism and biliary excretion. Gut-derived toxins affect hepatic OATP1B1, OATP1B3, and OATP2B1 activity and expression [64, 77-81]. For example, OATP1B1 and OATP1B3 transport of methotrexate is inhibited by kynurenic acid, indole-3-acetic acid, indoxyl sulfate and p-cresol in HEK293 cells [78]. Kynurenic acid has the strongest inhibitory effect. Interestingly, indoxyl sulfate and kynurenic acid are substrates of OATP1B1 and OATP1B3, and therefore might be competitive inhibitors. Uptake of the OATP1B3 substrate digoxin in rat and human hepatocytes is most notably inhibited by CMPF and p-cresol (400μM) [80]. P-cresol (300μM), indoxyl sulfate (400μM), and CMPF (400μM) significantly inhibit OATP1B1 and OATP1B3 uptake of estrone sulfate in transfected HEK293 cells [81]. The uptake of erythromycin by OATPs in rat hepatocytes is significantly inhibited by CMPF (50 μM) [64]. Inhibition of hepatic OATP transporters by gut-derived toxins likely alters intracellular drug transport and subsequent hepatic metabolism. CMPF most consistently

decreases OATP transporter function, but kynurenic acid and p-cresol also elicit inhibitory effects (Table 4). In patients with end-stage renal disease, decreased OATP transport was implicated in the 63% decrease in oral clearance of fexofenadine [14]. Increased demethylation of C^{14}-erythromycin immediately post-hemodialysis also suggests that inhibitory uremic toxins are cleared by dialysis, thereby improving OATP uptake and intracellular exposure to drug substrates [82]. In fact, OATP-mediated clearance of FDA approved medications is estimated to be decreased by up to 60% in kidney disease [15]. Therefore, Gut-derived uremic toxins likely perpetrate clinically relevant interactions with OATP transporters.

1.6.2 **Kidney Organic Anion Transporting Polypeptides (OAT)**

Organic anion transporters (OAT) secrete solutes from the basolateral membrane (blood) into the proximal tubules to facilitate secretion into the urine. These transporters, especially OAT1 and OAT3, contribute to the tubular secretion of indoxyl sulfate, p-cresol sulfate, kynurenic acid, CMPF, hippuric acid and indole-3-acetic acid [83-85]. In fact, OAT1 and OAT3 are high-capacity transporters with high affinity for kynurenic acid and p-cresol sulfate. Microbial toxins might act as competitive inhibitors of OAT tubular secretion of both endogenous solutes and drugs, and vice versa, thereby increasing systemic exposure. For example, OAT1 and OAT3 mediated tubular secretion of indoxyl sulfate is competitively inhibited by non-steroidal anti-inflammatory drugs (NSAIDs), quinapril and probenecid [86, 87], which may increase systemic exposure of drugs and/or indoxyl sulfate. Indoxyl sulfate, CMPF and hippuric acid also have inhibitory effects on OAT1 and OAT3 activity [88-90]. In rat kidney slices, hippuric acid and indoxyl sulfate in combination and independently decrease OAT1 and

OAT3 uptake of morinidazole-metabolites [91]. Also, CMPF, indoxyl sulfate, hippuric acid and indole-3-acetic acid each independently decrease OAT1 and OAT3 uptake of morinidazole-metabolites in HEK293 cells suggesting these gut-derived toxins inhibit OAT1 and OAT3 [91].

Table 4. Effect of Gut-Derived Toxins on Drug Transporters

Drug Transporter	Indoxyl Sulfate	p-Cresol OR p-Cresol Sulfate	Kynurenic Acid	CMPF	Hippuric Acid	Indole-3-Acetic Acid	Microbial Toxin Combination
OATP1B1	No effect on transport [79]* Inhibitor [78, 92]#	Inhibitor [78] [81]#	Inhibitor [78]#	Inhibitor [79]* Decreased hepatic mRNA [77]* [92]#	No effect on transport [78, 79]* [92]#	No effect on transport [79]* Inhibitor [78, 92]# [81]*	Inhibit uptake (HA, IS, I3A, CMPF) [79]* (HA, IS, I3A, CMPF) [92]*
OATP1B3	No effect on transport [79]* Inhibitor [78, 81]#	Inhibitor [78] [81]#	Inhibitor [78]#	Inhibitor [79]* [81]#	No effect on transport [79]* Inhibitor [78]#	No effect on transport [79]* Inhibitor [78]#	Inhibit uptake (HA, IS, I3A, CMPF) [79]*
OATP2	No effect on transport [64]*			Inhibitor [64]*	No effect on transport [64]*	No effect on transport [64]*	
OATP2B1	No to minimal effect on intestinal or hepatic mRNA expression [77]* Inhibitor [81]#			No to minimal effect on intestinal mRNA expression [77]* Decreased hepatic mRNA [77]* Inhibitor [81]#	No to minimal effect on intestinal mRNA expression [77]* Decreased hepatic mRNA [77]*	No to minimal effect on intestinal or hepatic mRNA expression [77]*	Decrease in intestinal mRNA expression (HA, CMPF, I3A, IS) [77]*

OAT1	Inhibitor [83, 89, 90] [91]*	Inhibitor [84]*		Inhibitor [83]#, [91]*	Inhibitor [83] [91]*	Inhibitor [83]* [91]#	
OAT3	Inhibitor [83, 89-91]*	Inhibitor [83]*		Inhibitor [83, 91]*, [88]#	Inhibitor [83, 91]*, [88]#	Inhibitor [83, 88, 91]#	
MRP2	No to minimal effect on intestinal or hepatic mRNA expression [77]*			No to minimal effect on intestinal or hepatic mRNA expression [77]*	No to minimal effect on intestinal or hepatic mRNA expression [77]*	No to minimal effect on intestinal or hepatic mRNA expression [77]*	Decrease in intestinal mRNA expression (HA, CMPF, I3A, IS) [77]*
MRP4	Non-competitive inhibitor[93]*	No effect on transport [93]*	Inhibitor [93]*		Non-competitive inhibitor [93]*	Non-competitive inhibitor [93]*	
BCRP	Non-competitive inhibitor [93]*	No effect on transport [93]*	Mixed inhibitor [93]*		Non-competitive inhibitor [93]*	No effect on transport [93]*	
* Denotes within clinically relevant concentrations, # Higher concentrations than observed in patients. CMPF, 3-Carboxy-4-methyl-5-propyl-2-furan-propanoic acid. HA, hippuric acid. IS, Indoxyl sulfate. I3A, Indole-3-acetic acid. PC, P-cresol.							

1.6.3 **Kidney Breast Cancer Resistant Protein (BCRP), Multidrug Resistance Protein 4 (MRP4) and Organic Anion Polypeptide Transporter 4 (OATP4C1)**

BCRP and MRP4 are important kidney efflux transporters located on the apical membrane of the proximal tubule epithelial cell. They are responsible for movement of solutes from within the cell into the tubular lumen. These proteins are dysregulated by microbial toxins [93]. Alterations of these transporters have significant implications for tubular secretion of several endogenous substrates and uremic toxins (e.g. urate) [93]. Hippuric acid, indoxyl sulfate and kynurenic acid inhibit these efflux transporters [93]. OATP4C1, the only OATP in human kidneys, eliminates several drugs (e.g., digoxin) and uremic toxins [94]. Indoxyl sulfate directly inhibits the function and decreases mRNA and protein expression of OATP4C1 [95].

In summary, gut-derived toxins interact with the function and expression of drug transporters in the kidneys and liver. Several microbial toxins inhibit the function and expression of renal OAT1, OAT3, BCRP, MRP4 and OATP4C1 at concentrations observed in kidney disease. Microbial toxins also interact with expression and function of hepatic OATP1B1, OATP1B3 and OATP2B1. These drug transporter-microbial toxin interactions likely contribute to accumulation of uremic toxins and/or drugs in patients with impaired kidney function.

1.7 BEYOND INTERACTIONS WITH DRUG METABOLIZING ENZYMES AND DRUG TRANSPORTERS

Effects of microbial toxins extend beyond interactions with hepatic drug metabolizing enzymes and transporters. The microbiota also impacts drug exposure and toxicity in patients with kidney disease by altering serum protein binding and enterohepatic recirculation (Figure 3). Changes in the disposition of the chemotherapy drug irinotecan illustrate each of these points. For example, irinotecan undergoes biotransformation by carboxylesterase to the active metabolite SN-38, which is a substrate of hepatic OATP1B1 and is detoxified by glucuronidation. CMPF, indoxyl sulfate, hippuric acid and indole-3-acetic acid competitively inhibit OATP-mediated transport of SN-38 in human hepatocytes and HEK 293 cells [79, 92]. Decreased hepatic uptake leads to increased systemic exposure to SN-38 and toxicity. In addition, CMPF is capable of competitively binding to plasma proteins (e.g., albumin) and displacing SN-38. This increases the unbound fraction and leads to systemic toxicity. In fact, exposure to the unbound fraction of SN-38 is increased by 2.6-fold in patients with advanced kidney disease [96, 97]. Impaired kidney function appears to be a risk factor for irinotecan-induced neutropenia, indicating increased toxicity [98], even though SN-38 is primarily eliminated by the liver.

The increased unbound fraction and decreased OATP-mediated uptake of SN-38 into hepatocytes are further complicated by potential increases in the abundance of intestinal β-glucuronidase producing bacteria *Enterobacteriaceae* [25, 99]. SN-38 undergoes hepatic glucuronidation and biliary excretion into the intestines [100], but increased β-glucuronidase activity by the microbiota can lead to increased intestinal mucosal toxicity from deconjugated

SN-38. Moreover, the deconjugated SN-38 may undergo enterohepatic recirculation, thereby increasing SN-38 systemic exposure and toxicity [97]. Collectively, these microbial toxin interactions in kidney disease lead to increased systemic exposure of a drug that undergoes predominantly nonrenal clearance. Microbial toxin interactions with drug metabolizing enzymes, transporters, plasma proteins or microbial de-conjugating activities can significantly influence drug exposure and response. Such interactions can occur independently or in concert to alter normal pharmacokinetics and pharmacodynamics of drugs in patients with kidney disease.

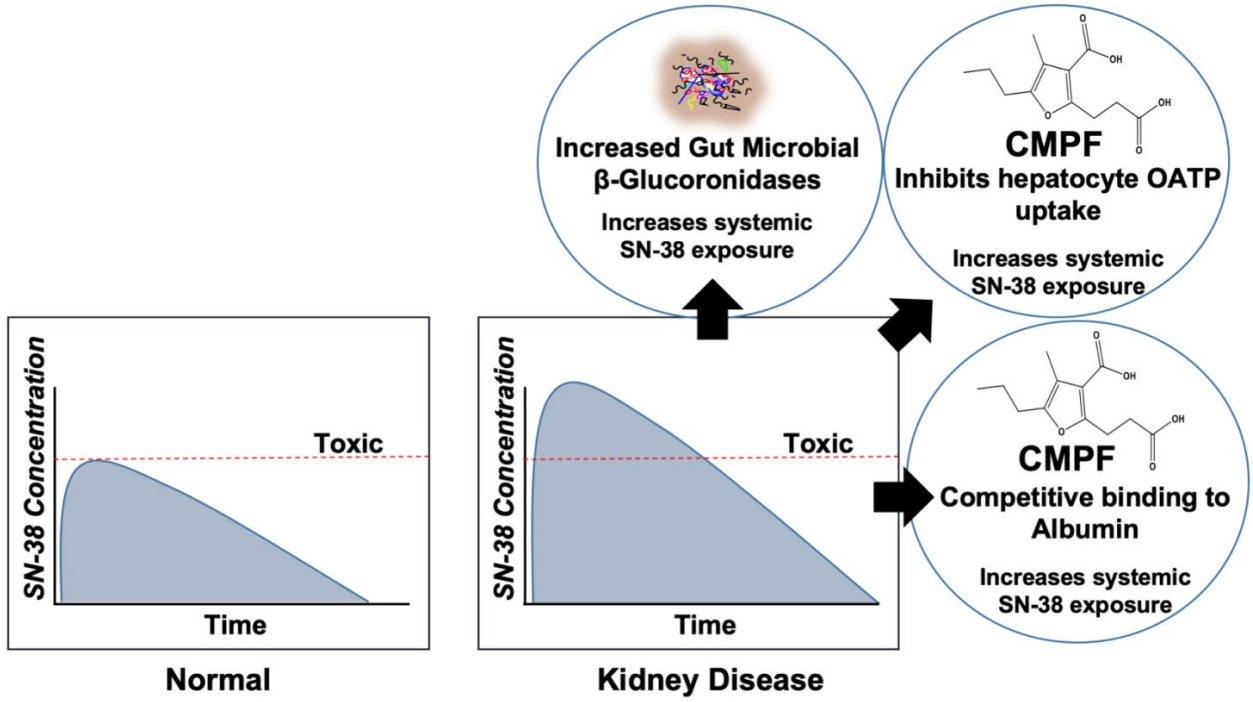


Figure 3. Gut Microbiota Interactions with Chemotherapeutic SN-38 Exposure

Increased microbial β-glucoronidases are suggested to increase de-conjugation of SN-38G and lead to enterohepatic circulation of SN-38. There is also evidence of CMPF interacting with hepatic uptake of SN-38 and could lead to decreased excretions. Finally, CMPF interacts with SN-38 plasma protein binding to albumin leading to increased exposure to the active moiety.

1.8 THERAPEUTIC STRATEGIES TO TARGET GUT-DERIVED UREMIC TOXINS

Several therapeutic strategies specifically targeting microbial toxins are possible and deserve exploration. These include antibiotics, pre- and probiotics, adsorbents, drug metabolism or transporter modulators, and enhanced dialytic elimination.

1.8.1 Targeted Antibiotics

Selective use of targeted antibiotics might beneficially alter intestinal microbiota communities [101-103]. Lethal or non-lethal antibiotic approaches can decrease microbial production of specific metabolites. For example, a non-lethal approach is targeting the TMA-lyase enzymes with a small molecule inhibitor that results in decreased microbial production of trimethylamine [104]. Trimethylamine is a gut-derived uremic toxin that is oxygenated into trimethylamine-N-oxide (TMAO). TMAO is associated with cardiovascular disease by potentially promoting atherosclerosis formation [10]. Therefore, this non-lethal approach may potentially improve cardiovascular outcomes in kidney disease patients by decreasing systemic TMAO concentrations. Genetic alteration of bacterial tryptophanase, which represents another non-lethal approach, may result in lower overall formation of indoxyl sulfate [105]. On the other hand, rifaximin is a lethal antibiotic that provides at least an acute decrease of TMAO concentrations in patients [106]. Both lethal and non-lethal antibiotics are appealing options that might reduce gut-derived uremic toxins.

1.8.2 **Pre- & Probiotics**

Prebiotics (i.e., non-digestible fiber) and probiotics (i.e., beneficial live bacteria) used alone or in combination (i.e., synbiotics) may decrease microbial toxin concentrations. A combination of pre- and probiotics has been evaluated for use in decreasing systemic exposure to p-cresol sulfate and indoxyl sulfate in kidney disease patients [107]. After controlling for oral antibiotics, significant decreases in p-cresol sulfate and indoxyl sulfate were observed. However, in a separate study prebiotic indigestible starches did not generate sustained decreases of gut-derived uremic toxins [108]. Lastly, fecal transplants from healthy individuals may be performed to re-populate the diseased microbiome (i.e., dysbiosis) with healthy microorganisms [109].

1.8.3 **Adsorbents**

AST-120 (spherical carbon adsorbent), sevelamer (phosphate binder) and other adsorbents might be therapeutic options to bind microbial toxins in the intestinal lumen and limit absorption [110]. Mixed results have been reported from prospective clinical studies evaluating the effects of AST-120 and sevelamer on systemic microbial toxin concentrations and clinical outcomes [111, 112]. Other binding resins are used in kidney disease, but further research is required to determine their efficacy in binding microbial toxins. Potential drug interactions with adsorbents are also an important consideration.

1.8.4 **Drug Metabolism and Transporter Modulators**

Drug metabolism and transport modulators might directly mitigate gut-derived uremic toxin effects. For example, hepatic SULT inhibitors (e.g., resveratrol, quercetin and meclofenamate) may protect the kidneys from indoxyl sulfate induced oxidative damage stemming from downregulation OAT1 and OAT3 renal transporter expression [89, 90]. In fact, SULT inhibitors decreased hepatic formation of indoxyl sulfate in an animal model of acute kidney injury, thereby lowering oxidative stress and restoring OAT1 and OAT3 expression [89]. Also, statins may counteract microbial toxin induced downregulation of OATP4C1 in the kidney and thereby enhance subsequent secretion of uremic toxins into the urine [94]. Increasing the tubular secretion of gut-derived toxins might be an effective strategy to decrease systemic concentrations in kidney disease patients.

1.8.5 **Dialytic Clearance**

Increased elimination by various dialytic modalities is an important therapeutic option. Although some microbial toxins are highly protein bound and are poorly cleared with hemodialysis, many other small, water soluble metabolites such as TMAO are highly cleared by dialysis. The dialysis modality, filter type, and treatment time should be evaluated to optimize elimination of these microbial toxin solutes [113, 114].

1.9 **SUMMARY AND CONCLUSIONS**

The gut microbiota is of increased importance in kidney disease and dysbiosis is linked to altered nonrenal clearance. Gut-derived uremic toxins interact with and lead to changes in the function of important phase I and II drug metabolizing enzymes and transporters, including inhibition of CYP3A4, SULT, OATP, OAT, MRP4, and BCRP activities. Alterations to these pathways can affect the pharmacokinetics and pharmacodynamics of commonly prescribed drug substrates. Dysbiosis potentially leads to an increase in the abundance of organisms expressing β-glucuronidase, which may facilitate deconjugation and enterohepatic recirculation of drugs. Limitations of uremic toxin studies, such as use of p-cresol and not the final p-cresol sulfate metabolite and inconsistent use of clinically relevant toxin concentrations, should be considered in the design and interpretation of related work. The gut microbiota or its metabolites may prove to be therapeutic targets for future research.

1.10 **OVERALL COMMENTARY**

Systematic research to investigate the functional effects of GDUTs on pharmacokinetics and pharmacodynamics is needed. Indoxyl sulfate, p-cresol sulfate, hippuric acid, indole-3-acetic acid and CMPF are important microbial toxins that warrant increased attention in clinical and translational research [115]. In addition, future systematic studies of the impact of individual and combined GDUTs on drug metabolism and transport are needed because large interindividual variability in clinically observed microbial toxin concentrations may explain some differences in

drug-related adverse events and efficacy. Important considerations include use of clinically relevant microbial toxin concentrations (Table 1), as well as use of microbial toxin precursors (e.g., p-cresol) and the most accumulated metabolites (e.g., p-cresol sulfate). In our opinion, hepatic-gut-kidney crosstalk will be an area of significant scientific research and discovery. Crosstalk individually impacts both hepatic diseases (e.g., steatohepatitis [116]) and potentially the progression of kidney disease [117]. Moreover, the implications extend to metabolic diseases, inflammatory diseases, and cardiovascular disease.

GDUT interactions with drug metabolizing enzymes and drug transporters may lead to altered drug exposure, response, and drug-related adverse events. High interindividual variability of microbial toxin concentrations may explain some variability in responses to treatment. At this point, clinical monitoring of GDUT concentrations is not available to guide drug dosing. It is possible that current drug dosing recommendations have inherently accounted for interindividual differences in toxin concentrations. Future clinical research is needed to determine effects of gut-derived toxins on drug disposition and response to facilitate personalized drug dosing.

The human microbiome presents unique challenges due to complex diversity, constant evolution and metabolically active microorganisms. It currently lacks informed clinical interventions, which could have several important therapeutic benefits. For example, microbiota-targeted interventions may decrease rates of cardiovascular morbidity and mortality observed in kidney disease patients. In addition, lowering microbial toxin concentrations is important because alterations to hepatic drug metabolism and transport pathways may impact therapeutic effectiveness and increase the risk for drug-related adverse events. Although several potential therapeutic targets are under investigation, to date no individual intervention has proved to be a viable therapeutic option for sustained decreases in microbial toxin production and/or systemic

exposure and it remains an important research opportunity. In summary, GDUTs influence drug metabolism and drug transport and may be therapeutic targets in patients with kidney disease.

1.11 **RESEARCH HYPOTHESIS AND OBJECTIVES**

Gut-derived toxins disrupt several physiological systems such as the human microbiome, hepatic drug metabolism and drug transport and/or renal function. In particular, KD and GDUTs alter the functional expression of drug metabolism and drug transport pathways, as described earlier. However, to date, limited information exists about the effect of KD and/or GDUTs on FMO activity and formation of the non-traditional CVD risk factor TMAO. A major goal of this work was to mechanistically evaluate the effect of KD and GDUTs on FMO activity. To assess FMO activity, trimethylamine was used as a probe substrate of FMO enzymes, and formation rate of TMAO was used as a surrogate measurement. This allowed for mechanistic insight into the function of hepatic FMOs in CKD. This body of work will advance our knowledge about formation of TMAO in KD and inform future therapeutic strategies to potentially reduce TMAO exposure and CVD events.

To test the overarching research hypothesis, four clinical and translational research projects were conducted. First, a robust analytical method was developed and validated to measure the GDUTs (i.e., kynurenic acid, hippuric acid, indoxyl sulfate and p-cresol sulfate). The second objective was to assess the effect of kidney disease on FMO functional expression. FMO activity was assessed *in vitro* with hepatic microsomes isolated from a 5/6th-nephrectomized experimental kidney disease animal model and control microsomes. In addition,

we evaluated FMO metabolic activation of TMAO formation (i.e., a novel type of atypical kinetics). Next, changes to FMO functional activity after exposure to the GDUT indoxyl sulfate was evaluated in primary plated human hepatocytes. Furthermore, the broader effect of indoxyl sulfate on expression of drug metabolism enzymes and drug transporters was evaluated with innovative Nanostring gene expression technologies. Finally, the effect of a novel approach to inhibit FMO-mediated TMAO formation and potential alterations to the microbiome that impact GDUT exposure were evaluated in Stage 3-4 KD patients. Investigating the altered gut microbiome and the systemic exposure to gut-derived toxins improves our understanding of the role of the microbiota in producing these non-traditional CVD risk factors in patients with KD. Lastly, the overall limitations, conclusions and future directions of this body of work are outlined in chapter 6.

CHAPTER 2: DEVELOPMENT AND VALIDATION OF A UHPLC-MS/MS METHOD FOR MEASUREMENT OF A GUT-DERIVED UREMIC TOXIN PANEL IN HUMAN SERUM: AN APPLICATION IN PATIENTS WITH KIDNEY DISEASE

[Prokopienko AJ, West III RE, Stubbs JR, and Nolin TD. *Journal of Pharmaceutical and Biomedical Analysis* 2019, [118]]

2.1 **INTRODUCTION**

Cardiovascular disease is the leading cause of death in patients with underlying chronic kidney disease (CKD) [119, 120]. Although the mechanism is unclear, gut-derived uremic toxins (GDUTs) accumulate in CKD and have been implicated in the acceleration of cardiovascular disease [5, 121]. Metabolism of tryptophan and tyrosine by gut microbes (i.e., the microbiota) leads to the generation of numerous uremic toxins, including kynurenic acid, hippuric acid, indoxyl sulfate and p-cresol sulfate [26]. Although more than 100 uremic toxins have been identified, this panel of four toxins was selected because they originate from gut metabolism, are representative of the uremic milieu, and remain persistently elevated despite treatment with hemodialysis [26, 122, 123]. Most importantly, each of these GDUTs is present in supraphysiologic concentrations in patients with kidney disease and has been independently associated with cardiovascular disease in humans [121, 124-126]. Thus, these four GDUTs are potential therapeutic targets to improve cardiovascular disease outcomes [127]. Measurement of GDUTs could be used to identify patients at risk for cardiovascular events, and to evaluate the efficacy of potential interventions to lower GDUT concentrations. Hence, simple, rapid, robust, accurate and precise analytical methods are needed to facilitate GDUT measurement and clinical-translational research in this area.

Several ultra-high performance liquid chromatography–tandem mass spectrometry (UHPLC-MS/MS) methods for measuring kynurenic acid, hippuric acid, indoxyl sulfate or p-cresol sulfate in various combinations and/or with other analytes have been reported [128-137]. Each method has limitations in terms of total run-time, sample processing, overall complexity, calibration range, validation parameters assessed, and/or the choice of analytes simultaneously

measured. For example, one of these methods has a 15 minute run-time and fairly labor-intensive sample processing [128], while another incorporates a complex LC-MS/MS detection approach with five segments requiring alternating polarity modes, co-elution of several analytes, an 8 minute total run-time, and expensive sample preparation [129]. Another multiple analyte method requires a total run-time of over 23 minutes using both positive and negative polarities, and the calibration range for indoxyl sulfate and p-cresol sulfate is not ideal for use in CKD patients [133].

The goal of this work was to develop and validate a simple, rapid, robust, accurate, and precise UPLC-MS/MS method to simultaneously measure kynurenic acid, hippuric acid, indoxyl sulfate, and p-cresol sulfate in human serum. The method was applied to a pilot study evaluating the efficacy of a novel therapeutic intervention to lower GDUTs in patients with Stage 3-4 CKD described in Chapter 5. Baseline data are presented that corroborate previously published data showing that these uremic toxins are markedly increased compared to healthy volunteers [128].

2.2 **METHODS**

2.2.1 **Chemicals and reagents**

Kynurenic acid, *d*5-kynurenic acid, hippuric acid, and indoxyl sulfate were purchased from Sigma (St. Louis, MO, USA). P-cresol sulfate, *d*7-p-cresol sulfate, *d*5-hippuric acid, and *d*4-indoxyl sulfate were purchased from Toronto Research Chemicals (North York, ON, Canada). Optima LC-MS grade methanol, acetonitrile, and water were purchased from Fisher

Scientific (Pittsburgh, PA, USA). Ultra-pure argon gas (>99.9%) was obtained from Matheson (Basking Ridge, NJ, USA). Ultra-pure nitrogen gas (>99.9%) was supplied from a nitrogen generator (Parker Balston, Haverhill, MA, USA). Double charcoal stripped, delipidized human serum was purchased from Golden West Diagnostics (Temecula, CA, USA). Oasis HLB cartridges (Waters, Milford, MA, USA) were used for the removal of residual kynurenic acid from stripped serum.

2.2.2 **UPLC-MS/MS conditions and equipment**

An Acquity UPLC system consisting of a binary solvent manager and a sample manager (Waters, Milford, MA, USA) was utilized. Chromatographic separation of analytes was achieved with an Acquity BEH C18 (2.1 × 100 mm, 1.7 µm) column fitted with an Acquity BEH C18 VanGuard pre-column (2.1 x 5 mm, 1.7 µm). The isocratic elution consisted of 85% solvent A (10 mM ammonium formate; pH 4.3) and 15% solvent B (acetonitrile). Formic acid was used to acidify solvent A to the target pH. The flow rate was 0.3 mL/min. The isocratic elution was selected to overcome limitations of gradient elution, like time for column equilibration to initial conditions. The column was held at 35˚C, and the autosampler was kept at 10˚C. Separation of analytes was achieved with a total run-time of 4 minutes.

Tandem mass spectrometric (MS/MS) detection was conducted with a TSQ Quantum Access MAX triple quadrupole mass spectrometer (Thermo Scientific, San Jose, CA, USA) fitted with a heated electrospray ionization (HESI) source. Detection of analytes was performed in positive and negative ionization modes by selected reaction monitoring (SRM). The polarity was switched from positive to negative mode at 1.4 minutes during the run. Spray voltages were

set at 3000 V in positive polarity and 2000 V in negative polarity to prevent arcing. The vaporizer temperature and the ion transfer tube were set to 300˚C. The collision gas pressure, auxiliary gas, and sheath gas and were set at 1.5 mTorr, 50 and 60 (arbitrary units), respectively. Other settings included a scan time of 0.05 seconds, scan width of 0.05 *m/z*, and full width at half maximum of 0.7 *m/z* for first and third quadrupole. Monitored ion transitions were *m/z* 190.1 → 144.0 for kynurenic acid (collision energy = 20V), *m/z* 195.1 → 149.1 for *d5*-kynurenic acid (collision energy = 18V), *m/z* 178.1 → 134.6 for hippuric acid (collision energy = 13V), *m/z* 182.9 → 139.6 for *d*5-hippuric acid (collision energy = 14V), *m/z* 212.0 → 80.4 for indoxyl sulfate (collision energy = 27V), *m/z* 215.9 → 80.4 for *d*4-indoxyl sulfate (collision energy = 31V), *m/z* 186.9→ 107.5 for p-cresol sulfate (collision energy = 26V), and *m/z* 193.9 → 114.6 for *d7*-p-cresol sulfate (collision energy = 24V). Data acquisition and processing was performed with Xcalibur software v4.0 (Thermo Scientific, San Jose, CA, USA).

2.2.3 **Preparation of stock solutions, quality control (QC) samples, and calibration standards**

Stock solutions containing *d*5-kynurenic acid, kynurenic acid, *d*5-hippuric acid, hippuric acid, *d*4-indoxyl sulfate, indoxyl sulfate, *d*7-p-cresol sulfate, and p-cresol sulfate were made in methanol. The analyte stocks were further diluted with methanol to make intermediate stocks, these were used to make serum calibration standards and QCs. Acetonitrile mixed with 0.075 µg/mL of *d*5-kynurenic acid, and 5 µg/mL of *d*4-indoxyl sulfate, *d*7-p-cresol sulfate, and *d*5-hippuric acid was used as a working internal standard solution. Double charcoal stripped human serum served as the matrix for calibrator and QC samples. Intermediate solutions were added

into stripped serum to make calibration standards at concentrations of 0.010, 0.025, 0.050, 0.075, 0.10, and 0.50 µg/mL for kynurenic acid, 0.25, 1.0, 10, 20, 40, and 80 µg/mL for p-cresol sulfate, and 0.20, 0.50, 1.0, 10, 40, and 80 µg/mL for hippuric acid and indoxyl sulfate. These ranges were selected based on concentrations reported in the literature for all stages of CKD [5]. Four quality control samples (LLOQ, LQC, MQC, and HQC) were made by spiking stripped serum at concentrations of 0.010, 0.030, 0.080, and 0.400 µg/mL for kynurenic acid, 0.250, 0.750, 15, and 60 µg/mL for p-cresol sulfate, and 0.200, 0.600, 15, and 60 µg/mL for hippuric acid and indoxyl sulfate. All calibration standards and QC samples were stored at -80˚C.

2.2.4 **Sample processing**

To begin sample preparation, 100 µL of internal standard solution was added to 50 µL of serum and briefly vortexed for 30 seconds. Following centrifugation (5 min at 10,000x*g*), the supernatant was transferred to a new micro-centrifuge tube, evaporated under nitrogen at 40˚C and then reconstituted with 150 µL of water:acetonitrile (80:20, v/v) and vortexed for 30 seconds. A 20 µL aliquot was then injected onto the UPLC-MS/MS system. The dry-down and reconstitution steps were necessary to ensure sample compatibility with the mobile phase.

2.2.5 **Validation of the assay**

2.2.5.1 **Calibration and linearity**

A six-point standard curve was constructed for each analyte. Lower limit of quantification (LLOQ) samples were processed in triplicate, and all other standards were

processed in duplicate for three days. Analyte concentrations were quantified using their respective deuterated internal standards. Standard curves were constructed by graphing absolute peak-area ratios of analytes to internal standards versus the nominal analyte concentrations.

2.2.5.2 **Accuracy and precision**

Accuracy and precision were evaluated with twelve replicate LLOQ, LQC, MQC, and HQC samples on the first day, followed by six replicate LLOQ, LQC, MQC, and HQC samples on two separate days, totaling to n=24 samples at each QC level. All 24 QC samples were used to determine inter-day accuracy and precision, and the twelve replicates from the first day were used to determine intra-day accuracy and precision (Table 5). Accuracy (% bias) was based on the calculated mean concentration relative to the nominal analyte concentration. Precision was estimated by calculating relative standard deviation (% RSD) of the QC values.

2.2.5.3 **Dilution integrity**

Dilution integrity was also assessed. Samples were prepared by initially spiking stripped serum with each analyte to twice the concentration of the highest standards. Samples were then diluted 1:1, 1:2 and 1:4 with stripped serum prior to analysis. Triplicate dilution samples were processed. Accuracy and precision relative to the nominal values was determined for each dilution factor.

2.2.5.4 **Stability**

Analyte stability was determined with LQC and HQC samples subjected to various experimental conditions compared to freshly processed LQC and HQC samples. Stability (bias

and RSD) was defined as being within 15% of the nominal freshly prepared QC sample concentrations. Stability samples were assessed in triplicate. Long-term stability was evaluated by analyzing samples that were stored for 2 months at -80°C. In order to assess the stability throughout sample processing, bench-top stability was investigated by leaving samples on the bench-top at room temperature for six hours before processing. Autosampler stability of processed samples was evaluated by injecting processed samples stored at 10˚C for 24 hours and 72 hours. Finally, stability after freeze-thaw cycles was determined by subjecting samples to three 24-hour freeze/thaw cycles.

2.2.5.5 **Matrix effect, recovery and carryover**

Matrix effect of serum on kynurenic acid, hippuric acid, indoxyl sulfate, and p-cresol sulfate was assessed by comparing stripped serum spiked at the LQC, MQC and HQC concentrations listed in Table 6 to aqueous samples spiked at the same concentrations that were not extracted. Three replicate aqueous samples and three spiked samples of each QC level were analyzed using standard curves generated from serum-based standards. Matrix effect was calculated by comparing non-extracted aqueous QC samples to extracted serum-based QC samples and was considered to be negligible if the measured concentration in serum deviated from aqueous samples by <15%.

Recovery was assessed by comparing three replicates of extracted serum-based QC samples to non-extracted blank serum samples spiked after the extraction procedure. Measured concentrations of non-extracted aqueous, and the non-extracted spiked blank serum QC samples were defined as 100%. Means, standard deviations, and coefficients of variation were determined and are listed in Table 7. Carryover was also assessed by randomly injecting at least six blank

injections of 80:20 water:acetonitrile (v/v) (mobile phase composition) during the analytical sequence.

2.2.6 **Application of the method**

The method was applied to a clinical study evaluating the efficacy of a novel therapeutic intervention to lower GDUTs in patients with CKD. The University of Pittsburgh Institutional Review Board approved the protocol, and all participants provided written informed consent. Serum samples were collected and stored at -80˚C until analysis.

2.3 **RESULTS AND DISCUSSION**

We aimed to develop and validate a simple, rapid, and robust UPLC-MS/MS method for simultaneously measuring kynurenic acid, hippuric acid, indoxyl sulfate, and p-cresol sulfate in human serum to support studies evaluating the efficacy of novel therapeutic interventions to lower GDUTs in patients with CKD. The method was comprehensively validated according to the 2018 U.S. FDA guidance for bioanalytical method validation [138].

2.3.1 **Chromatographic separation**

The MS/MS spectra of the product ion scans and compound structures are presented in Figure 4. The most intense fragment ion for each analyte and the corresponding fragment in

internal standards was selected for SRM. This allowed for selective and sensitive measurement of the gut-derived toxin analytes.

Representative chromatograms showing separation of kynurenic acid, hippuric acid, indoxyl sulfate, and p-cresol sulfate obtained from the analysis of serum samples obtained from a CKD patient and a healthy volunteer are shown in Figure 5. Analyte retention times were 1.32, 1.52, 2.11, and 3.30 minutes for kynurenic acid, hippuric acid, indoxyl sulfate, and p-cresol sulfate, respectively. All peaks were narrow and well-separated with baseline resolution. The human serum samples had comparable chromatography to all other tested samples, demonstrating no observable interference.

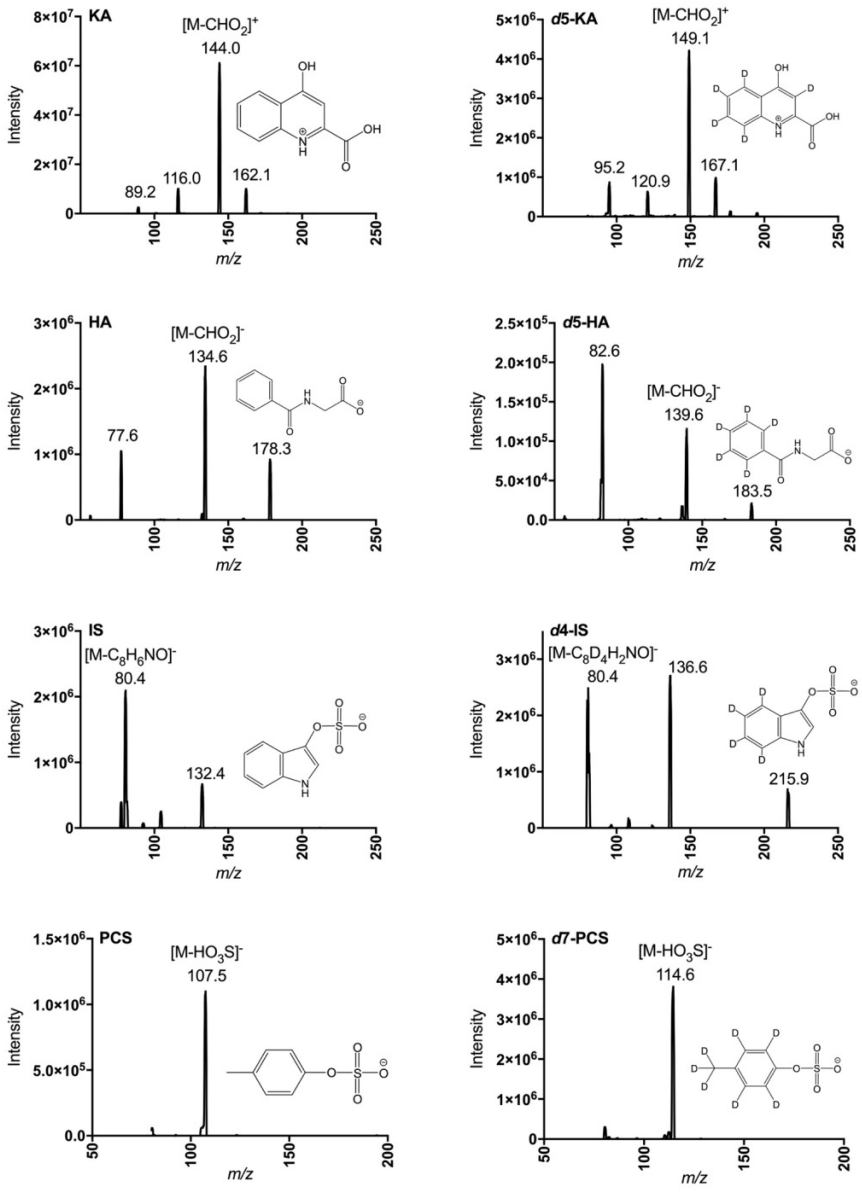


Figure 4. MS/MS Spectra

MS-MS Spectra acquired on the TSQ Quantum Access MAX triple quadrupole mass spectrometer in positive and negative mode. All respective structures of parent compounds are embedded in the mass spectras. Abbreviations: KA, kynurenic acid; *d*5-KA, *d*5-kynurenic acid; HA, hippuric acid; *d*5-HA, d5-hippuric acid; IS, indoxyl sulfate; *d*4-IS, *d*4-indoxyl sulfate; PCS, p-cresol sulfate; *d*7-PCS, *d*7-p-cresol sulfate.

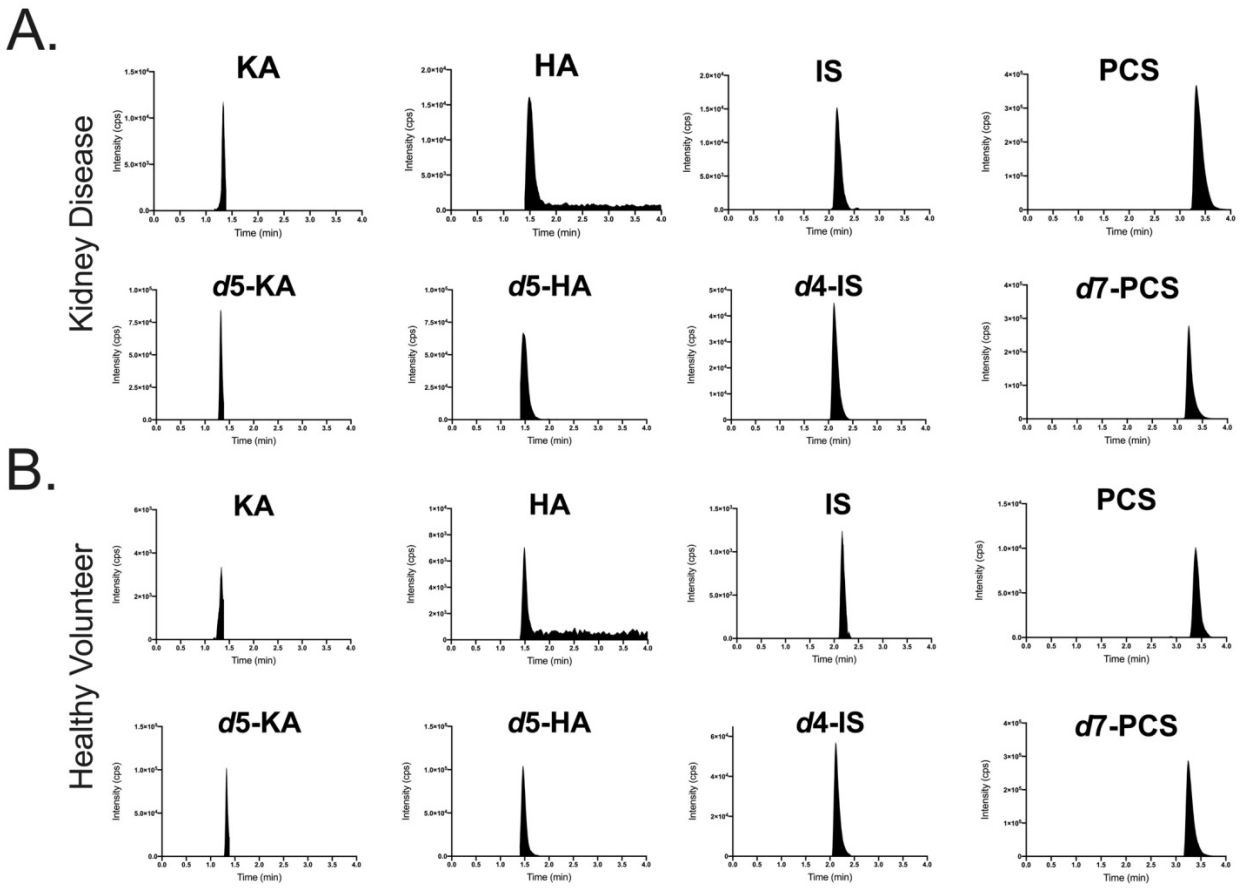


Figure 5. Representative Chromatograms from a Kidney Disease Patient and a Healthy Volunteer

Representative chromatograms obtained from the analysis of a kidney disease patient at the baseline study visit and a healthy volunteer. (A) Concentrations observed in the kidney disease patient were kynurenic acid 0.19 µg/mL, hippuric acid 1.89 µg/mL, indoxyl sulfate 4.73 µg/mL, and p-cresol sulfate 22.2 µg/mL. (B) Concentrations observed in the healthy volunteer were kynurenic acid 0.05 µg/mL, hippuric acid 0.38 µg/mL, indoxyl sulfate 0.25 µg/mL, and p-cresol sulfate 0.29 µg/mL. Abbreviations: KA, kynurenic acid; *d*5-KA, *d*5-kynurenic acid; HA, hippuric acid; *d*5-HA, *d*5-hippuric acid; IS, indoxyl sulfate; *d*4-IS, *d*4-indoxyl sulfate; PCS, p-cresol sulfate; *d*7-PCS, *d*7-p-cresol sulfate.

2.3.2 **Validation of the assay**

2.3.2.1 **Calibration and linearity**

Linear standard curves were achieved at 0.01-0.5 µg/mL for kynurenic acid, 0.25-80 µg/mL for p-cresol sulfate, and 0.2-80 µg/mL for hippuric acid and indoxyl sulfate. The correlation coefficient (r) was 0.997 or greater for all analytes. Standard curves were calculated for each analyte using weighted (1/x) linear regression analysis. LLOQ standards for each calibration curve exhibited excellent intra- and inter-day accuracy and precision (bias and RSD were within 18.9% and 12.6%, respectively), and signal-to-noise ratios were above 10:1 for all analytes. Intra- and inter-day accuracy and precision were within 12.3% for other standard levels.

2.3.2.2 **Accuracy and precision**

Intra-day and inter-day accuracy (% bias) and precision (% RSD) were determined at four levels according to the most recent FDA guidelines: the three quality controls (LQC, MQC, and HQC) and the LLOQ [138]. Assay bias for LLOQ samples was within 19.3%, while the RSD was within 13.8% (Table 5). Assay biases for all QC samples were within 10.9%, while the RSD was within 9.06% (Table 5). These values met the FDA recommendations for acceptable accuracy and precision being within 20% and 15% for LLOQs and other QCs, respectively [138].

Table 5. Intra- and inter-day accuracy (%bias) and precision (RSD) for LLOQ, LQC, MQC and HQC.

Analyte	Level	Nominal conc. (µg/mL)	Intra-day		Inter-day	
			% Bias	RSD	% Bias	RSD
Kynurenic acid	LLOQ	0.01	-5.29	13.3	-5.47	10.8
	LQC	0.03	1.44	6.13	-0.93	7.90
	MQC	0.08	6.94	4.07	2.05	9.06
	HQC	0.40	2.74	5.39	-2.07	7.27
Hippuric acid	LLOQ	0.20	-17.8	6.54	-9.40	13.8
	LQC	0.60	1.61	6.59	0.70	6.55
	MQC	15.0	-7.04	3.44	-6.15	4.48
	HQC	60.0	3.12	2.81	1.20	4.16
Indoxyl sulfate	LLOQ	0.20	-8.73	9.27	-13.35	10.9
	LQC	0.60	10.9	2.11	9.50	5.29
	MQC	15.0	3.08	5.46	1.10	5.29
	HQC	60.0	2.11	6.05	3.30	5.51
p-Cresol sulfate	LLOQ	0.25	-19.3	7.54	-17.4	6.71
	LQC	0.75	-0.50	3.10	0.40	3.46
	MQC	15.0	3.30	1.57	2.80	1.77
	HQC	60.0	-3.70	1.99	-5.30	2.74

2.3.2.3 **Dilution integrity**

Dilution integrity was assessed to determine if samples that are above the standard curve ranges can be diluted and accurately quantified. Dilution analysis exhibited excellent accuracy and precision, with bias and RSD within 11.04% and 6.19%, respectively.

2.3.2.4 **Matrix effect, recovery and carryover**

Matrix effect from serum was negligible (<15%) for all analytes over three different concentrations tested (Table 6). Recovery was acceptable ranging from 81.3% to 106% for the

LQC, MQC and HQC concentrations (Table 7). Carryover was negligible using at least six blank injections of mobile phase randomly throughout the sequence. No additional washes or column flushing was necessary, which is one advantage of the current assay over similar methods [128].

Table 6. Matrix effect human serum on kynurenic acid, hippuric acid, indoxyl sulfate and p-cresol sulfate.

	Concentration (µg/mL)			
	Nominal value	Aqueous Sample Mean ± SD	Human Serum Sample Mean ± SD	Matrix Effect (%)
Kynurenic acid	0.030	0.033 ± 0.001	0.028 ± 0.001	14.7
	0.080	0.086 ± 0.000	0.080 ± 0.003	6.8
	0.400	0.411 ± 0.010	0.410 ± 0.014	0.3
Hippuric acid	0.600	0.645 ± 0.057	0.609 ± 0.041	4.7
	15.00	15.36 ± 1.138	14.89 ± 1.503	3.1
	60.00	62.18 ± 0.796	60.21 ± 2.092	3.2
Indoxyl sulfate	0.600	0.622 ± 0.021	0.639 ± 0.022	3.0
	15.00	15.38 ± 0.450	14.55 ± 0.542	5.3
	60.00	60.39 ± 3.222	59.44 ± 3.573	1.6
p-Cresol sulfate	0.750	0.782 ± 0.026	0.752 ± 0.014	3.8
	15.00	15.87 ± 0.693	15.87 ± 0.087	0.0
	60.00	61.43 ± 4.204	57.06 ± 0.622	6.9

2.3.2.5 **Stability**

Long-term, bench-top, autosampler, and freeze-thaw stability were evaluated for analytes at LQC and HQC levels as shown in Table 8. Samples were stable for at least 6 hours on the bench-top, and at least 72-hours in the autosampler. Three 24-hour freeze-thaw cycles also did not impact analyte stability. All stability samples were within 15% of the freshly processed samples, demonstrating suitable stability at all conditions tested.

Table 7. Recovery of kynurenic acid, hippuric acid, indoxyl sulfate and p-cresol sulfate in human serum.

	Concentration (μg/mL)			
	Nominal value	Non-extracted Mean ± SD	Extracted Mean ± SD	Recovery (%)
Kynurenic acid	0.030	0.035 ± 0.001	0.031 ± 0.002	90.0
	0.080	0.100 ± 0.000	0.081 ± 0.004	81.3
	0.400	0.451 ± 0.005	0.414± 0.006	91.8
Hippuric acid	0.600	0.631 ± 0.037	0.623 ± 0.058	98.8
	15.00	14.80 ± 0.895	13.24 ± 0.174	89.6
	60.00	53.93 ± 1.598	57.03 ± 1.806	106
Indoxyl sulfate	0.600	0.696 ± 0.012	0.835 ± 0.086	106
	15.00	16.82 ± 0.450	16.70 ± 0.706	99.3
	60.00	60.38 ± 0.786	58.82 ± 6.336	97.4
p-Cresol sulfate	0.750	0.895 ± 0.012	0.831 ± 0.021	92.8
	15.00	17.56 ± 0.077	15.40 ± 0.178	87.7
	60.00	67.06 ± 0.250	59.76 ± 0.558	89.1

Table 8. Stability of kynurenic acid, hippuric acid, indoxyl sulfate and p-cresol sulfate QCs (LQC and HQC).

Analyte	Target (μg/mL)	Stability (% remaining) [#]							
		Benchtop (6 h)		Autosampler (72 h)		Freeze/Thaw (3 cycles)		Long-term Storage (60 days)	
		% of Target	% RSD	% of Target	% RSD	% of Target	% RSD	% of Target	% RSD
Kynurenic Acid	0.030	91.1	1.9	86.6	0.43	100.1	6.6	105.4	6.9
	0.400	92.1	1.6	89.9	4.2	95.2	12.6	103.7	13.7
Hippuric Acid	0.600	107.1	8.1	102.4	7.8	92.6	2.2	88.6	2.1
	60.00	99.9	5.5	104.6	5.3	99.8	7.2	103.3	7.5
Indoxyl Sulfate	0.600	96.5	4.5	98.4	2.9	98.8	3.6	98.8	3.6
	60.00	98.2	4.1	108.9	3.3	106.3	5.4	99.9	1.7
P-Cresol Sulfate	0.750	102.3	2.9	104.6	4.1	97.3	1.7	97.5	1.7
	60.00	97.6	0.9	100.9	1.0	103.2	5.8	101.1	2.0

n = 3 for LQC and HQC samples

2.3.3 **Method application**

Kynurenic acid, hippuric acid, indoxyl sulfate, and p-cresol sulfate concentrations were measured in healthy volunteers and Stage 3-4 CKD patients. Estimated glomerular filtration rate (eGFR) was used to define healthy volunteers, Stages 3a, 3b, and 4, as eGFR values of >120, 59-45, 44-30, and 29-15 mL/min/1.73 m², respectively. Representative chromatograms obtained from analysis of a Stage 3b CKD patient with an eGFR of 32 mL/min/1.73 m² and a healthy volunteer are presented in Fig. 5A. and 5B., respectively. Stepwise increases were observed in all GDUTs as CKD advanced compared to healthy volunteers. These increases were the most notable for indoxyl sulfate and p-cresol sulfate. For example, as shown in Fig. 5, the baseline serum indoxyl sulfate concentration was 18-fold higher in the CKD patient, at 4.73 μg/mL compared to 0.25 μg/mL in the healthy volunteer. Similarly, the baseline p-cresol sulfate concentration in the CKD patient was 75-fold higher, at 22.2 μg/mL compared to 0.29 μg/mL. In the cohort of study patients, the serum concentrations of indoxyl sulfate and p-cresol sulfate were 12 and 23-fold higher than healthy volunteers, respectively (Figure 6). These data suggest that GDUTs begin accumulating to high concentrations long before complete loss of kidney function (i.e., diagnosis of end-stage kidney disease). The extent of GDUT accumulation is alarming because each of these toxins is associated with and may accelerate cardiovascular disease [121, 124-126]. As such, therapeutic strategies to reduce GDUTs might decrease cardiovascular disease outcomes, especially in these earlier stages of CKD. Measurement of the GDUT panel described here may facilitate clinical investigations of the impact of lowering GDUTs on cardiovascular disease progression.

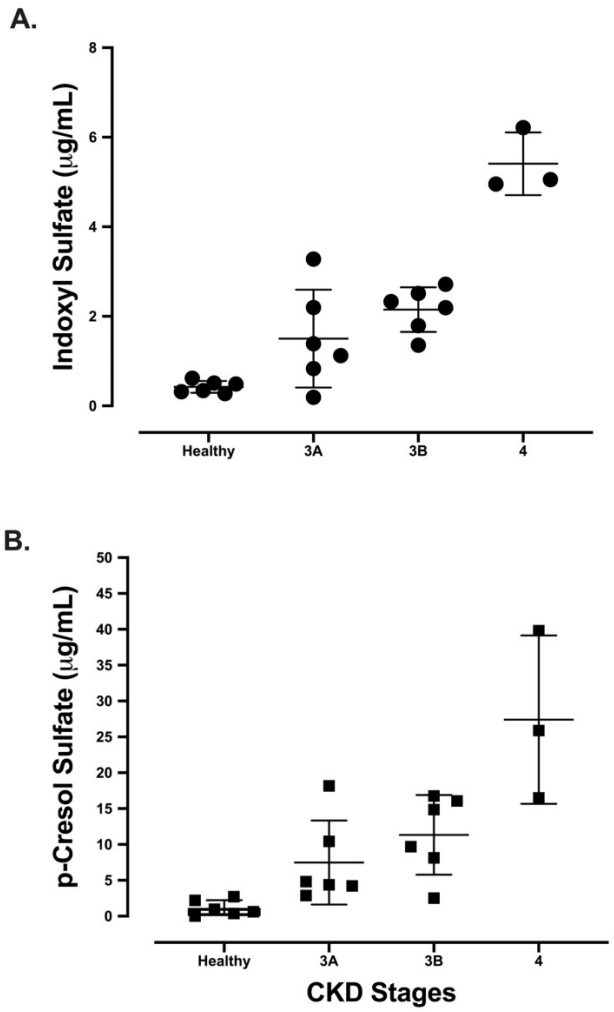


Figure 6. Serum Indoxyl Sulfate and p-Cresol Sulfate Concentrations

Serum concentrations of indoxyl sulfate and p-cresol sulfate in the total clinical study population (described fully in Chapter 5). (A.) Serum concentrations of indoxyl sulfate increased in a stepwise manner from healthy volunteers to CKD Stages 3A, 3B and 4. (B.) Serum concentrations of p-cresol sulfate increased in a stepwise manner from healthy volunteers to CKD Stages 3A, 3B and 4. The individual data points are presented, and the bars represent the mean with standard deviation. Descriptive statistics for these toxins are presented in Table 13.

2.4 **CONCLUSION**

A simple, rapid and robust UPLC-MS/MS method for the simultaneous determination of kynurenic acid, hippuric acid, indoxyl sulfate, and p-cresol sulfate in human serum was developed and validated. The assay has excellent accuracy, precision, and recovery. Additional strengths include a simple/inexpensive sample processing, short runtime (4 min), and meeting contemporary FDA validation requirements. The method was successfully applied to a clinical study evaluating the efficacy of a novel therapeutic intervention to lower GDUTs in patients with CKD.

CHAPTER 3: METABOLIC ACTIVATION OF FLAVIN-CONTAINING MONOOXYGENASE-MEDIATED TRIMETHYLAMINE-N-OXIDE FORMATION

[Prokopienko AJ, West III RE, Schrum DP, Stubbs JR, Leblond F, Pichette V and Nolin TD. 2019, In Preparation]

3.1 **INTRODUCTION**

CVD events are the leading cause of death in chronic kidney disease (CKD) patients despite aggressive treatment of traditional risk factors [139], suggesting that non-traditional CVD risk factors may play an important role [26, 140]. In Chapter 1, the effect of GDUTs on drug metabolizing enzymes were reviewed and this lead us to hypothesize that FMO enzyme activity could also be altered in KD. Specifically, higher FMO activity will enhance FMO-mediated TMAO formation and may have implications for CVD in KD patients. Trimethylamine-*N*-oxide (TMAO) promotes atherosclerosis in preclinical models [19], is elevated and is associated with CVD in CKD patients [141-143], and may be a novel non-traditional CVD risk factor [7]. For instance, we have shown that circulating TMAO concentrations are 30-fold higher in patients with end-stage kidney disease (ESKD) than in the general population and correlate with coronary atherosclerosis burden [10]. TMAO concentrations increase disproportionately from Stage 4 to 5 CKD (i.e., 20 μM to 94 μM) compared to Stage 1 to 3 CKD (i.e., 3.3 μM to 10 μM),[10, 143] strongly implicating increased FMO-mediated TMAO formation as a contributor to elevated serum TMAO concentrations in advanced CKD.

We previously demonstrated that enhanced hepatic flavin monooxygenase (FMO)-mediated TMAO formation along with decreased renal clearance contributes to increased TMAO exposure model of experimental kidney disease in mice (Figure 7) [144]. The mouse model demonstrated clear gender differences and significant increases in FMO-mediated TMAO formation for both males and female mice with CKD. In humans, FMO3 (the primary isoform responsible for TMAO formation in humans) gene and protein expression are also lower in males compared to females [145, 146]. However, the underlying mechanism driving increased TMAO

formation in CKD remained unclear. For example, FMO3 expression was unchanged in CKD female mice suggesting that increased FMO activity may not be explained by changes in expression. In fact, other investigators have also observed that increased FMO3 gene and protein expression do not always correlate with increased plasma TMAO concentrations, indicating alternative mechanisms and/or posttranscriptional events may be involved [145]. Collectively, the previous research findings demonstrate that other factors, beyond increased FMO3 expression can lead to increased FMO activity in CKD. Therefore, we aimed to mechanistically evaluate increased FMO-mediated TMAO formation in an experimental kidney disease model in rats.

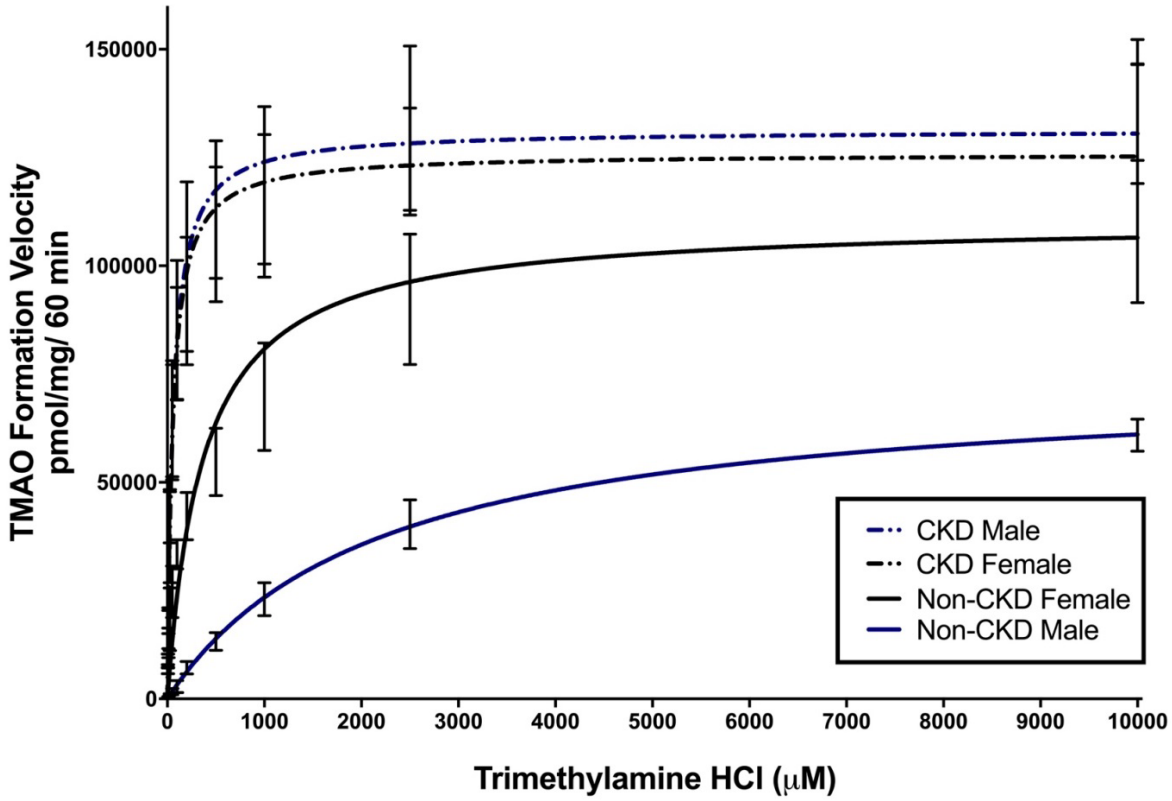


Figure 7. Increased FMO Activity in Mice with CKD.

FMO activity was deteremined and compared in microsomes isolated from male non-CKD (n=3), male CKD (n=3), female non-CKD (n=3) and female CKD (n=3) mice. Michaelis-Menten nonlinear regression kinetic models were used to fit the formation of TMAO to estimate V_{max} and K_m. The corresponding V_{max} values were 74,629 ± 1,558, 131,286 ± 2,776, 110,299 ± 4,196, and 125,920 ± 2,181 pmol/ mg (microsomes)/ 60 min., for non-CKD males, CKD males, non-CKD females and CKD females, respectively. The corresponding K_m values were 2,167 ± 120.2, 58.8 ± 6.0, 364.5 ± 51.1, and 55.6 ± 4.7 μM, for non-CKD males, CKD males, non-CKD females and CKD females, respectively.

Mechanistic evaluation of increased TMAO formation could shed light on novel therapeutic strategies for the prevention of CVD in CKD. In fact, recent studies provide proof of principle that FMOs are a potential therapeutic target [147]. In mice, antisense oligonucleotide-mediated knockdown of *FMO3* leads to decreases in serum TMAO concentrations and atherosclerosis formation [148, 149]. Therapeutically targeting FMOs may be particularly effective in the setting of increased FMO-mediated TMAO formation as seen in diabetes and CKD [144, 148, 150].

The objective of this study was to elucidate the mechanism of increased hepatic FMO-mediated TMAO formation in CKD. We accomplished this by conducting FMO activity experiments with microsomal fractions from CKD and control rats. We also investigated changes in mRNA and protein expression of FMOs.

3.2 **METHODS**

3.2.1 **Chemical Reagents**

Trimethylamine hydrochloride, TMAO, NADPH, magnesium chloride, tris (hydroxymethyl) aminomethane (Trizma® base), Trizma® hydrochloride, n-octylamine, methimazole, l-arginine and formic acid (≥95%) were purchased from Sigma-Aldrich (St. Louis, MO). Deuterated internal standard (*d*9-trimethylamine N-oxide) was purchased from Cambridge Isotopes (Cambridge, MA, USA). Optima LC–MS grade water, acetonitrile, and methanol was purchased from Fisher Scientific (Pittsburgh, PA). Taqman® primers used for mRNA

quantification were purchased from Applied Biosystems (Foster City, CA). All fluorescent antibodies were purchased from Abcam (Cambridge, MA) (Codes: ab2769, ab8226, ab22717, ab126790, ab195627, ab186693 and ab175774).

3.2.2 **Experimental Model**

Male Sprague-Dawley rats (Charles River, Saint-Charles, PQ, Canada) that weighed 200 to 300 g were fed standard rat chow and water *ad libitum* on a 12-hour light/dark cycle. Control rats were pair-fed matching amounts of standard rat chow consumed by CKD rats. The Canadian Council on Animal Care guidelines were observed for care and use of laboratory animals. Experimental CKD was surgically induced by first performing a 2/3rd nephrectomy of the left kidney followed 7 days later by a complete right nephrectomy, as previously described [151]. Control rats underwent to two sham laparotomies. Rats were sacrificed 42 days after the initial surgery and livers were immediately harvested and stored at -80ºC.

3.2.3 **Determination of FMO Activity**

Metabolic activity of hepatic FMOs was assessed with microsomes isolated from control (n=6) and CKD (n=6) rat livers. Specifically, trimethylamine was used as a probe substrate for FMO enzymes, and the formation rate of TMAO was used as a surrogate measurement of FMO activity. Hepatic microsomes (i.e., the liver fraction containing FMOs) were isolated by differential ultra-centrifugation as previously described.[152] Incubation times and microsomal

protein concentrations were optimized to achieve linear formation of TMAO in the experiments. Microsomal incubations using 0.5 mg/mL of microsomal protein in 0.02 M Tris-HCl buffer (pH 7.4) containing 1 mM NADPH and 5 mM $MgCl_2$ were conducted. The microsomal incubations were pre-warmed in the presence of NADPH for 3 minutes at 37°C. To start the reaction, 3 µL of increasing concentrations of trimethylamine (2.5, 5, 10, 25, 50, 100, 200, 500, 1000 µM) was added, and microsomes were incubated for 60 min at 37°C. Final reaction volumes were 300 µL. Each trimethylamine incubation experiment (i.e., each concentration) was conducted in duplicate. Negative controls omitting NADPH and trimethylamine were assessed for all incubations. Reactions were stopped by adding 300 µL of ice-cold methanol. TMAO was quantified by ultra-performance liquid chromatography tandem mass spectrometry (LC-MS/MS) as reported previously [40].

3.2.4 **mRNA Analysis**

RNA isolation and real-time quantitative polymerase chain reaction (RT-qPCR) were conducted as recommended by MIQE guidelines [153]. Total RNA was extracted from homogenized liver tissue from n=12 CKD and n=12 control rats, using QiaShredder and RNeasy Mini Kit (Qiagen, Valencia, CA). RNA purity (260/280 ratios ranging from 1.8 to 2.0) and concentrations were determined by measuring the optical density at 260nm and 280nm using NanoDrop (ThermoFisher Scientific, Waltham, MA). cDNA was then prepared using SuperScript III reverse transcriptase (Invitrogen, San Diego, CA) with 1µg of total RNA and random hexamers. *Fmo3*, *Fmo1*, *Arnt*, *Ahr*, *Cyp1a2*, *Cyp3a2* and *Actb* genes were quantified by RT-qPCR with Applied Biosystems 7500 Fast Real-Time PCR System (Foster City, CA) using Taqman®

Gene Expression Master Mix (Applied Biosystems, Foster City, CA) and Taqman® specific primers. Template and reverse transcriptase controls were not included in every reaction. *AHR* and *Arnt* were selected because the AhR signaling pathway may be activated in CKD and partially regulate FMO expression [154, 155]. Hepatic mRNA concentrations were normalized to *Actb* (β-Actin) and expressed relative to the controls using absolute quantification. *Actb* was selected as an adequate housekeeping gene because it is not affected by CKD [156].

3.2.5 **Western Blot Analysis**

The protein expression of hepatic FMO3, CYP1A2, CYP3A2, AHR and β-Actin was determined with fluorescent Western blotting. Homogenized liver tissue from n=12 CKD and n=12 control rats (30 μg total protein) were separated by 4-15% Mini Protean TGX (Bio-Rad, Hercules, CA) gel electrophoresis and transferred onto PVDF membranes. Transferred membranes were blocked for 1 hour at room temperature using Odyssey Blocking Buffer. Membranes were incubated at 4°C overnight with specific primary antibodies diluted in 50:50 blocking buffer and TBS- 0.1% Tween (TBST) (1:1000-2500 mouse monoclonal anti- β-Actin, CYP1A2 and AhR; 1:1000 polyclonal rabbit anti- FMO3 and CYP3A2). Membranes were washed four times for 10 minutes with TBST and then for one hour with 50:50 blocking buffer and TBST. Membranes were then incubated for 1 hour in fluorescent secondary antibodies at room temperature (1:20000 donkey anti-rabbit; or 1:10000 donkey anti-mouse). Finally, membranes were washed with TBST, and antibody binding was measured by a LI-COR fluorescent (Lincoln, NE) detection system. CYP1A2 and CYP3A2 were selected as controls

because their expression in experimental CKD is unchanged and decreased, respectively [33]. Specificity was assessed by incubating blots that were loaded with control homogenized rat tissue with each primary antibody to check for overlapping fluorescent bands (Figure 8). The linear range of detection was determined for a range of total protein on these blots. Band intensity was quantified by densitometry using ImageJ software and normalized to β-Actin expression.

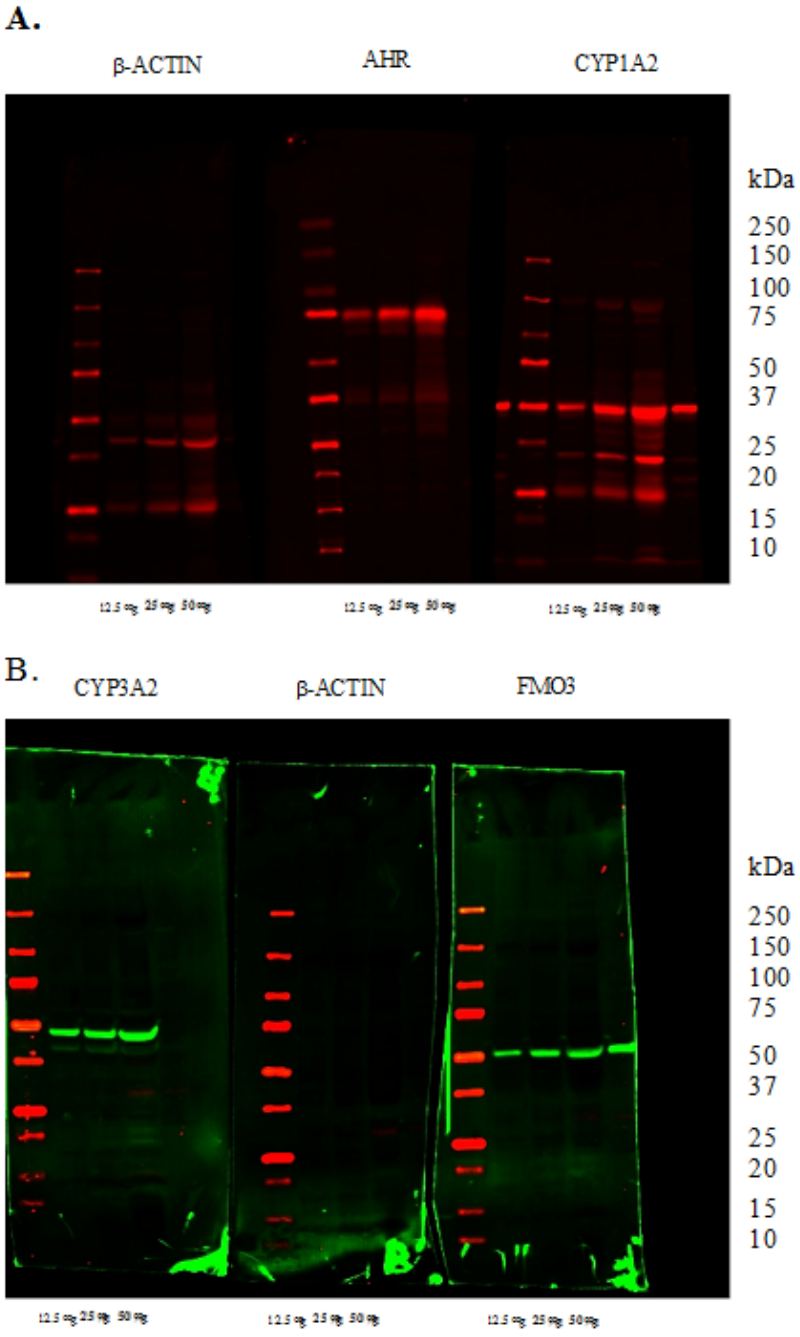


Figure 8. Specificity of antibodies and range of reactivity.

The specificity and range of reactivity was assessed for each primary antibody. Specificity was assessed by incubating blots that were loaded with control homogenized rat tissue with

each primary antibody to check for overlapping fluorescent bands. Linear ranges of detection were determined for a range (12.5, 25, and 50 μg) of total protein on these blots along with molecular size markers. (A.) Blots were individually incubated with primary antibodies for β-ACTIN, AHR, and CYP1A2, followed by an incubation with a fluorescent donkey anti-mouse secondary antibody. (B.) Blots were individually incubated with primary antibodies for CYP3A2, β-ACTIN, and FMO3, followed by an incubation with a fluorescent donkey anti-rabbit secondary antibody.

3.2.6 **Assessment of FMO Metabolic Activation and Inhibition**

Metabolic activation was explored as a potential mechanism of increased FMO activity (Figure 9). Metabolic activation was assessed using the experimental conditions described above for the determination of FMO activity. However, male Sprague-Dawley rat liver microsomes (purchased from Sekisui XenoTech, Kansas City, KS) were used in these experiments. The reference point of FMO activity in these microsomes was consistent with the experimental control rats. Also, potential activating compounds (0.01-2 mM octylamine or 0.5 and 2 mM L-arginine), and human serum were added to the microsomal incubations prior to the start of the reaction. The FMO inhibitor methimazole (1 mM) was added to 5 replicates of the 50 μM trimethylamine incubates. TMAO formation rates were assessed to measure changes in FMO metabolic activity.

In experiments with human serum, appropriate volumes were added to achieve the 5, 10 and 20% of total reaction volume. Baseline concentrations of trimethylamine and TMAO in the human serum were quantified before starting the incubation reactions (see Table 9). Final TMAO formation was calculated by taking the final TMAO concentration minus the baseline TMAO concentration. Concentrations of baseline trimethylamine in serum were below the limit of quantification, and therefore were negligible (Table 9). Baseline TMAO did not undergo any quantifiable metabolism or retro-reduction into trimethylamine in the microsomal incubations. Human serum samples were obtained from a healthy control donor and from a hemodialysis patient (pre-dialysis sample; serum creatinine, 14.6 mg/dL; BUN, 77 mg/dL) with appropriate Institutional Review Board(s) approval. All serum was ultra-filtered using 10 Kda Satorius Vivaspin® spin columns.

Table 9. Trimethylamine (TMA) and TMAO Concentrations in Human Serum Used in Microsomal Incubations

Sample	TMA (µg/mL) No Incubation	TMA (µg/mL) After Incubation	TMAO (µg/mL) No Incubation	TMAO (µg/mL) After Incubation
100% Uremic Serum	0.072	N/A	6.553	N/A
20% Uremic Serum	BLQ	BLQ	1.525	1.55
10% Uremic Serum	BLQ	BLQ	0.762	0.82
5% Uremic Serum	BLQ	BLQ	0.402	0.416
100% Healthy Serum	0.04	N/A	0.324	N/A
20% Healthy Serum	BLQ	BLQ	0.068	0.07
10% Healthy Serum	BLQ	BLQ	0.036	0.035
5% Healthy Serum	BLQ	BLQ	0.019	0.019

Abbreviations: BLQ, below lower limit of quantification; N/A, not applicable.

3.2.7 **Data and Statistical Analysis**

The formation rate of TMAO was determined from LC-MS/MS quantified TMAO concentrations in the microsomal incubates. Non-linear regression Michaelis-Menten kinetic models were used to fit FMO-mediated TMAO formation data, and the maximum velocity (V_{max}) and the affinity constant (K_m) were estimated and compared by extra-sum-of-squares F-tests with GraphPad Prism (Version 8.0.2; San Diego, CA). Hepatic mRNA levels were normalized to β-Actin and expressed relative to the controls using absolute quantification. Densitometry units of protein expression were normalized to that of β-Actin. Gene and protein expression were not performed in one control rat liver sample due to limited tissue quantity. Student's *t*-test or Mann-Whitney test for data exhibiting non-normal distribution were used to compare CKD to control rats. All results are presented as mean ± SD, unless otherwise stated.

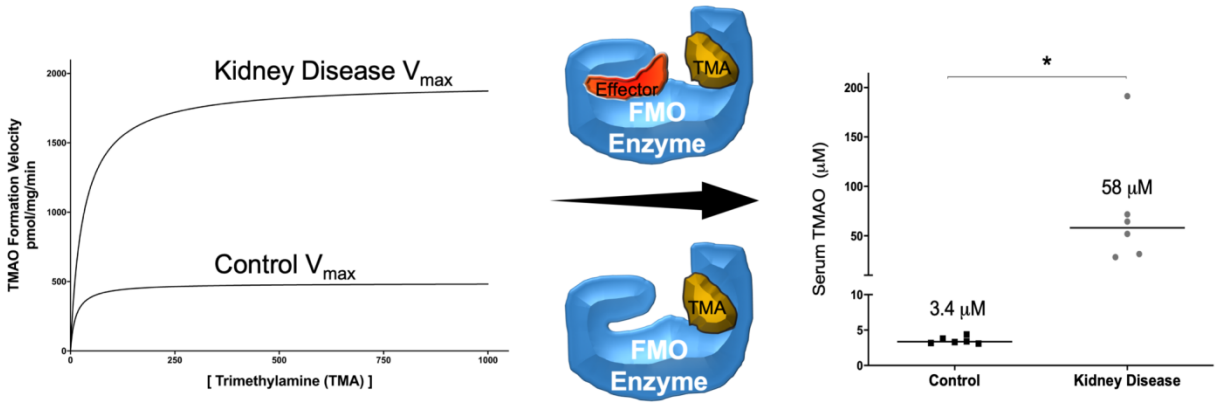


Figure 9. Scientific Premise of Metabolic Activation in Kidney Disease.

The scientific premise is that metabolic activation of FMOs may lead to increased TMAO formation from trimethylamine in kidney disease. FMO activity increases in experimental kidney disease as evidenced by increases in V_{max} compared to control. Metabolic activation involving effector compounds that increase enzymatic activity is a novel potential mechanism to explain increases in FMO activity. Finally, the increases in FMO activity may contribute, in part, to high systemic TMAO exposure observe in kidney disease along with decreased renal clearance.

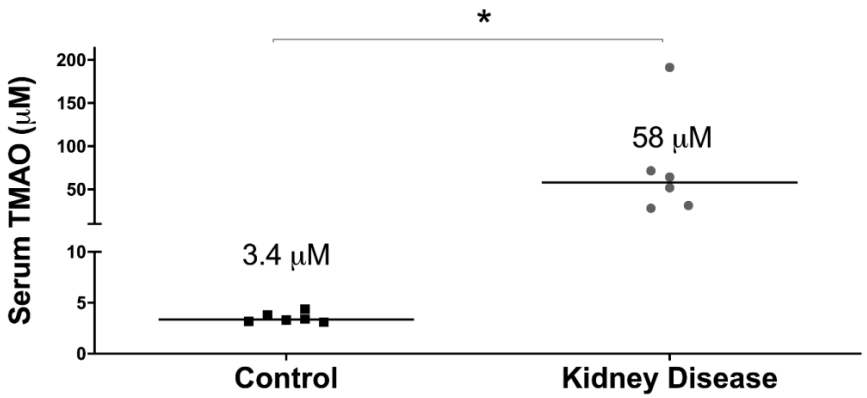
3.3.1 **Characteristics of CKD and Control Rats**

TMAO exposure was compared between CKD and control rats. The median (interquartile range) TMAO concentration in CKD versus control serum was 58 μM (31-102) and 3.4 μM (3.15-3.96), respectively ($P = 0.0022$; Figure 10A). Serum creatinine and BUN were also higher in CKD rats versus control ($P < 0.0001$; Figure 10B).

3.3.2 **FMO Activity in CKD and Control Rats**

The potential mechanism of increased FMO-mediated TMAO formation in CKD was assessed by comparing enzyme kinetic parameters observed in CKD rat liver microsomes with control rat microsomes. The V_{max} (i.e., the maximal rate at which the enzyme catalyzes the reaction) for TMAO formation was increased by 25% in CKD versus control (651.4 ± 18 versus 522.7 ± 22 pmol/mg protein/minute, $P < 0.0001$; Figure 10C). The K_m value was not different between groups (16.3 ± 2.3 versus 11.3 ± 2.5 μM, $P = 0.15$).

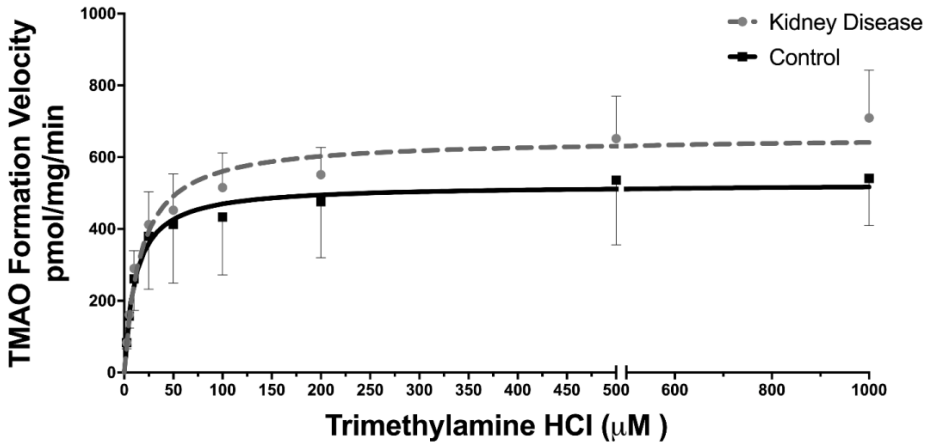
A.



B.

	Control	Kidney Disease	*P*
Serum creatinine (μM)	44.8 (10.6)	219 (95.7)	< 0.0001
BUN (mg/dL)	14.1 (5.54)	116 (81.9)	< 0.0001

C.



Michaelis-Menten Parameters	Control	Kidney Disease	*P*
Vmax (pmol/mg/min)	522.7 (21.7)	651.4 (18.2)	< 0.0001
Km (μM)	11.26 (2.29)	16.25 (2.18)	0.1520

Figure 10. FMO Activity and TMAO Exposure in CKD and Control Rats.

(A) Serum concentrations of TMAO compared between CKD (n=6) rats and controls (n=6). * $P < 0.05$ was observed for CKD compared to control with a Mann-Whitney test. (B)

Biochemical characteristics were compared between CKD (n=12) and controls (n=12) with a Student's *t*-tests and presented using mean ± SD. To convert μmol/L serum creatinine to standard units of mg/dL multiply by 0.0113. (C) Michaelis-Menten plot for the formation of TMAO in rat liver microsomes of both control and CKD rats. Liver microsomal protein was incubated with various concentrations of trimethylamine. Each point represents the mean ± SEM of 6 rats in each group. The parameters were statistically compared with an extra-sum-of-squares F-test.

3.3.3 **Gene and Protein Expression**

In order to evaluate whether any changes in FMO activity were due to changes in gene or protein regulation, hepatic FMO mRNA and protein expression were assessed (Figure 11A). No change in *FMO1* or *FMO3* mRNA was observed in CKD versus control. The positive control *CYP3A2* was downregulated in CKD versus control ($P < 0.0001$). *AhR* was upregulated in CKD versus control ($P < 0.05$) but *ARNT* and *CYP1A2* were not.

FMO3, CYP1A2, AhR and CYP3A2 protein expression is presented in Figure 11B. Decreases in AhR ($P < 0.01$) and CYP3A2 ($P < 0.001$) but no change in FMO3 and CYP1A2 protein expression were observed in CKD versus control.

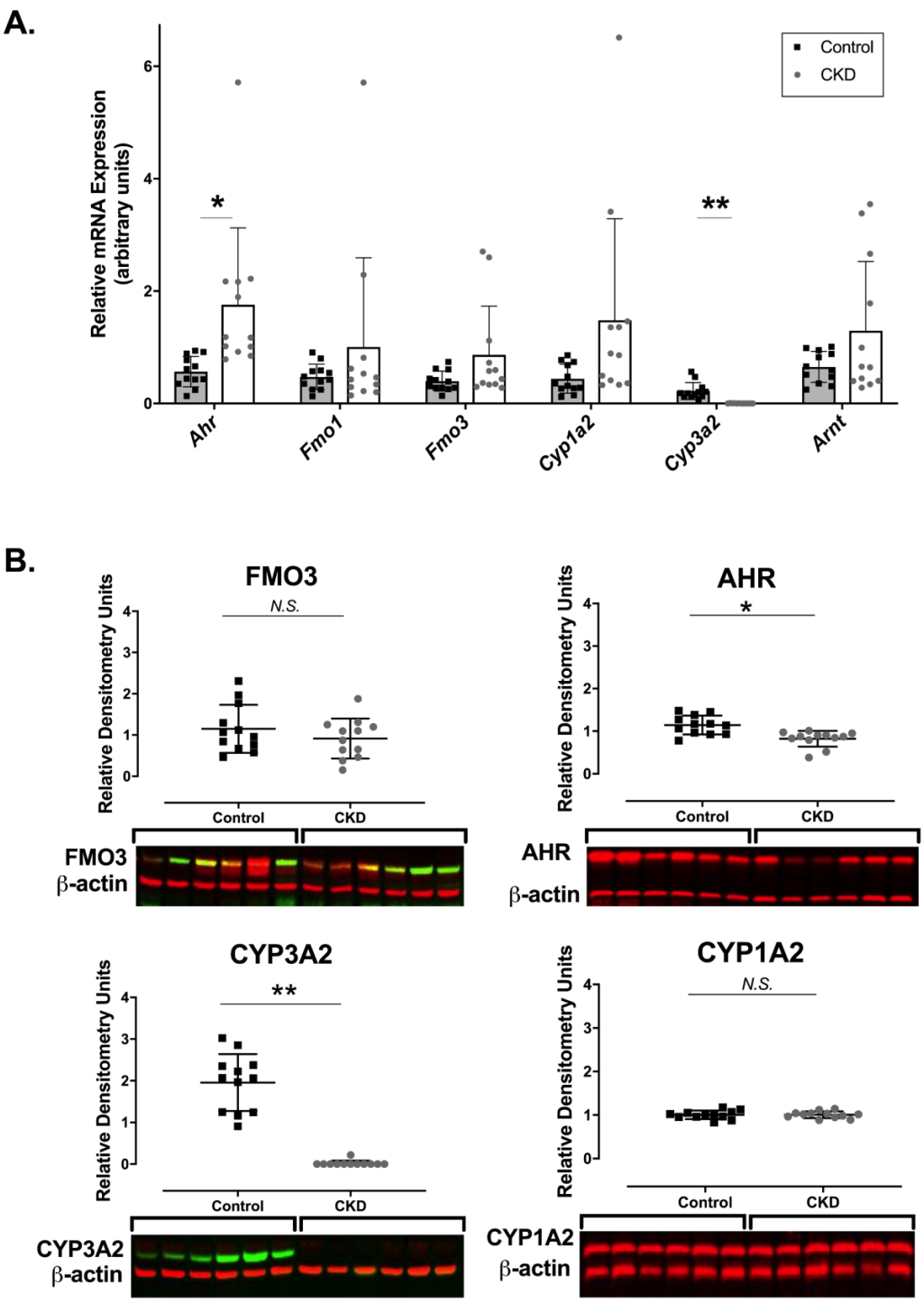


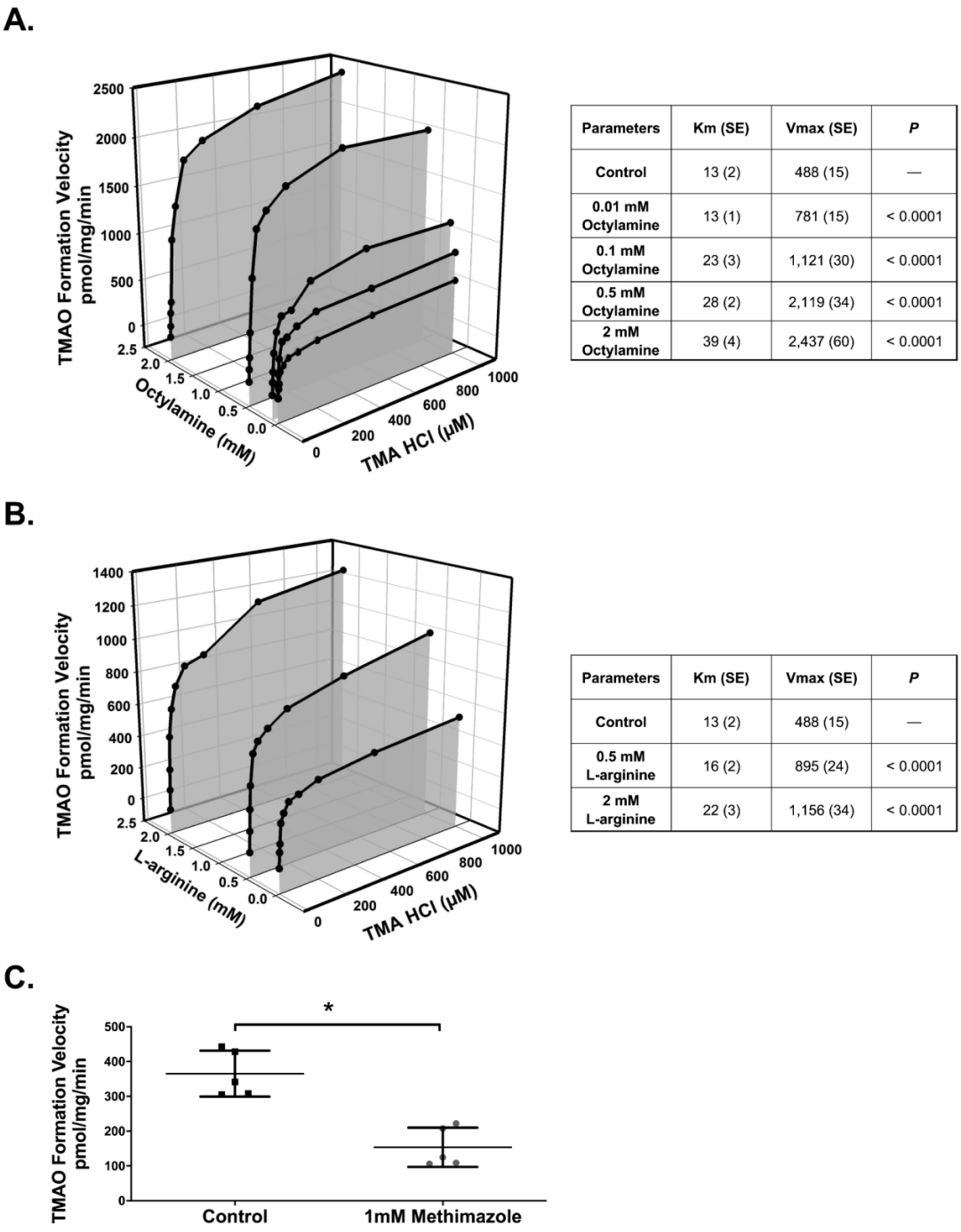
Figure 11. mRNA and Protein Expression

(A) The mRNA expression of hepatic drug metabolism enzymes and related genes (*FMO3*, *FMO1*, *ARNT*, *AHR*, *CYP1A2*, *CYP3A2* and *ACTB*) in control and CKD rats. Hepatic mRNA

levels were normalized to β-Actin and expressed relative to the controls using absolute quantification. Experiments were conducted in duplicates, and results are presented as mean ± SD of 12 rats in each group. * $P < 0.05$ and ** $P < 0.001$ compared with control by Student's *t*-tests. (B) Protein expression of drug metabolism enzymes and related proteins (FMO3, AhR, CYP1A2, CYP3A2 and β-Actin) in control and CKD rat livers. The densitometry units of protein expression were normalized to that of β-Actin. The results are presented as mean ± SD of 12 rats in each group. The lower panel represents blots of six control and six CKD rats. * $P < 0.05$ and ** $P < 0.001$ compared with control by Student's *t*-tests.

3.3.4 **Metabolic Activation and Inhibition of FMO**

An atypical kinetic reaction known as metabolic activation was explored as a potential mechanism of increased FMO activity. Concentration-dependent increases of up to 4-fold in metabolic activation were observed with octylamine as a positive activation control ($P < 0.0001$; Figure 12A). L-arginine was identified as an endogenous compound responsible for activation; up to a 1.4-fold increase in metabolic activation was observed in the presence of L-arginine ($P < 0.0001$; Figure 12B). FMO-mediated TMAO formation was decreased by 58% ($P < 0.0001$; Figure 12C) in the presence of the FMO inhibitor methimazole. Lastly, FMO activity was determined in the presence 5-20% ultra-filtered human serum to assess whether solutes retained in ESKD can activate FMO enzymes. TMAO formation velocity was increased up to 3-fold with uremic serum compared to 1.9-fold with healthy control serum ($P < 0.0001$; Figure 13).



Parameters	Km (SE)	Vmax (SE)	*P*
Control	13 (2)	488 (15)	—
0.01 mM Octylamine	13 (1)	781 (15)	< 0.0001
0.1 mM Octylamine	23 (3)	1,121 (30)	< 0.0001
0.5 mM Octylamine	28 (2)	2,119 (34)	< 0.0001
2 mM Octylamine	39 (4)	2,437 (60)	< 0.0001

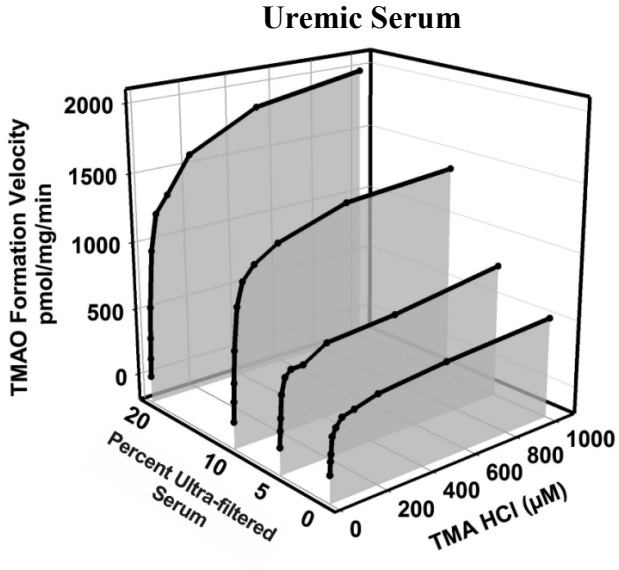
Parameters	Km (SE)	Vmax (SE)	*P*
Control	13 (2)	488 (15)	—
0.5 mM L-arginine	16 (2)	895 (24)	< 0.0001
2 mM L-arginine	22 (3)	1,156 (34)	< 0.0001

Figure 12. Metabolic Activation and Inhibition of FMO

(A) Michaelis-Menten plot for the formation of TMAO in rat liver microsomes in the presence of increasing concentrations of octylamine and (B) L-arginine. Liver microsomal

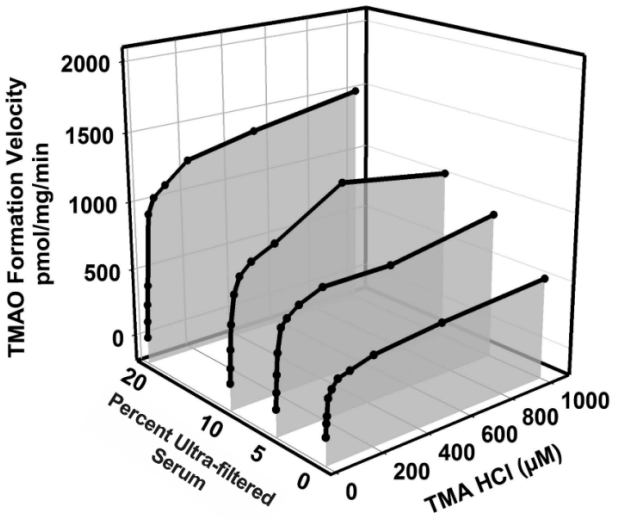
protein (0.5 mg/mL) was incubated with various concentrations of trimethylamine (2.5-1000 μM) for 60 min at 37 ºC in the presence of either octylamine (0.01-2mM) or L-arginine (0.5 and 2mM). Each point represents the mean of at least 3 replicates at each trimethylamine concentration in each group. The parameters were statistically compared with an F-test. Note that *P* value represents a comparison of Vmax for each octylamine concentration versus control. (C) FMO-mediated TMAO formation was also assessed with 50 μM of trimethylamine in the presence of 1mM of methimazole. Each point represents the mean ± SD of 5 replicates. * $P < 0.001$for treatment group compared to control by Student's *t*-test.

A.



Parameters	Km (SE)	Vmax (SE)	*P*
Control	13 (2)	488 (15)	—
5% Serum Kidney Disease	12 (2)	692 (19)	< 0.0001
10% Serum Kidney Disease	19 (1)	1,368 (20)	< 0.0001
20% Serum Kidney Disease	31 (3)	1,931 (49)	< 0.0001

B.



Parameters	Km (SE)	Vmax (SE)	*P*
Control	13 (2)	488 (15)	—
5% Serum Healthy Control	12 (1)	797 (19)	< 0.0001
10% Serum Healthy Control	21 (2)	1,097 (30)	< 0.0001
20% Serum Healthy Control	21 (2)	1,435 (21)	< 0.0001

Figure 13. Metabolic Activation of FMO-mediated TMAO Formation by Human Serum

Michaelis-Menten plots for the formation of TMAO in rat liver microsomes in the presence of increasing concentrations of human uremic serum (A) and healthy control serum (B). Liver

microsomal protein (0.5 mg/mL) was incubated with various concentrations of trimethylamine (2.5-1000 μM) for 60 min at 37 ºC in the presence of serum (5-20%). Each point represents the mean of at least 3 replicates at each trimethylamine concentration in each group. The parameters were statistically compared with an F-test. Note that *P* value represents a comparison of Vmax for each octylamine concentration versus control.

3.4 **DISCUSSION**

We show for the first time that metabolic activation of hepatic FMOs leads to increased formation of the non-traditional CVD risk factor TMAO, which may contribute to dramatically elevated serum concentrations in CKD rats. These findings corroborate our clinical observations of significantly elevated systemic TMAO concentrations in patients with advanced CKD and provide a novel mechanism for our recent observations of enhanced FMO-mediated TMAO formation in experimental CKD [10, 144].

Mechanistically, metabolic activation of FMO enzymes by uremic solutes may contribute to increased TMAO formation in CKD (Figure 9). In fact, metabolic activation likely contributes

to the increased systemic exposure of TMAO observed in CKD, evidenced by disproportionate increases of serum TMAO in advanced CKD relative to earlier stages of CKD. For instance, TMAO serum concentrations are increased 16-fold in CKD rats (Figure 10A), and 30-fold in ESKD patients compared to controls [10]. The V_{max} of TMAO formation was increased by 25% ($P < 0.0001$; Figure 10C) in CKD versus control tissue in the isolated microsomal incubation experiments. However, no changes in FMO gene or protein expression (Figure 11A and 11B) were observed, suggesting that increased FMO activity was unrelated to changes in expression. The latter finding is inconsistent with our previous observations in mice, in which gene expression changes may partially explain differences in FMO activity [144], but is not unexpected given well-known species differences in FMO3 regulation in mice compared to rats and humans. For instance, FMO3 expression varies by gender and is localized to the periportal region in mice, compared to no gender differences and perivenous localization in rats and humans [157, 158], Therefore, the current experimental rat model may better reflect FMO3 regulation and metabolic activity observed in humans with CKD. Nevertheless, current and previous findings collectively indicate that FMO-mediated TMAO formation is increased in CKD, and our present data suggest that it is mechanistically driven by metabolic activation.

Metabolic activation was elicited with octylamine, L-arginine, and uremic and healthy control serum. The up to 4-fold increase in V_{max} in the presence of octylamine clearly demonstrates that FMO-mediated TMAO formation can be activated (Figure 12A) [147]. The increased metabolic activation with human uremic serum versus healthy control serum suggests that increased concentrations of uremic solutes retained in uremic serum elicit greater metabolic activation (Figure 13A). Although the potential effector compounds remain unknown, the endogenous solute L-arginine elicits activation (Figure 12B), and other structurally similar

endogenous substances may behave in a similar manner. Interestingly, L-arginine serum concentrations are similar in CKD and healthy patients [159], and it is possible that L-arginine is contributing to the metabolic activation observed with healthy control serum (Figure 13B).

Metabolic activation is an atypical kinetic reaction. Activation of FMO-mediated TMAO formation indicates that 'effector' compounds are eliciting structural or electrostatic changes in the FMO catalytic site (Figure 9) [160]. In fact, FMO enzymes can be activated by a broad range of effector compounds that are known uremic retention solutes, including primary amines, guanidine derivatives and small peptides [147, 161, 162]. In CKD, these compounds may deposit and accumulate in liver tissue, specifically in the microsomal fraction where they can act as FMO effectors [1, 59], and this may explain the present findings of metabolic activation and increased TMAO formation in experimental CKD [163-167].

In accordance with enzyme kinetic principles, the increased V_{max} resulting from metabolic activation of FMOs would result in a corresponding increase in TMAO formation when substrate (trimethylamine) concentrations exceed the relatively low K_m value (enzyme affinity for substrate) of approximately 28 μM for FMO3 enzymes [168]. In this scenario, the reaction is rate limited and not substrate/supply limited, such that any increase in V_{max} leads to a corresponding increase in TMAO formation. Trimethylamine concentrations can exceed this K_m value in humans considering trimethylamine production is approximately 50 mg/day, especially after a precursor-nutrient rich meal [11, 169, 170]. For example, baseline portal vein concentrations of trimethylamine are about 46 μM and increase to 710 μM after giving ~35 mg trimethylamine to rats, these concentrations exceed the K_m and demonstrate the physiological relevance of metabolic activation increasing TMAO formation. [11, 169, 170] Furthermore, FMO3 gene polymorphisms are associated with increased TMAO concentrations, suggesting that FMO-mediated TMAO

formation is not supply limited [150]. Overall, these data support a novel biologically plausible mechanism involving metabolic activation that increases the V_{max} of FMO-mediated TMAO formation and contributes to well documented increases in systemic concentrations of TMAO in CKD patients.

Although activation of FMOs leads to increased TMAO formation in this experimental model of CKD, this requires validation in humans. We and several other investigators have reported elevated TMAO concentrations in CKD patients [10, 141, 143], but to date the mechanism has been unclear. Collectively, the observations that systemic TMAO concentrations increase disproportionately in advanced CKD relative to earlier stages of CKD [10], and that hemodialysis is relatively ineffective at lowering systemic exposure of TMAO (i.e., evidenced by pre-dialysis concentrations) [171], despite high intradialytic clearance [172], support the premise that TMAO formation is increased in CKD. It has been postulated that TMAO production is not changed in ESKD patients receiving chronic hemodialysis, and that the extraordinarily increased concentrations in these patients are due partly to the inability of hemodialysis to provide clearances of the magnitude achieved by tubular secretion [172]. While the latter point is valid, TMAO concentrations rise disproportionately even in non-dialyzed Stage 4 CKD patients with residual kidney function and presumably residual secretory clearance. One possible explanation is that the loss of secretory clearance in ESKD is outweighed by a simultaneous increase in TMAO production leading to the increased systemic exposure observed. Lastly, though decreased nonrenal clearance also may impact the systemic exposure of substrates predominantly cleared by the pathway in question [14], TMAO undergoes little nonrenal clearance [173] so this is unlikely to explain the increases in systemic exposure observed as kidney disease progresses. Overall, high

systemic TMAO exposure is likely due to a combination of decrease renal clearance,[174] and increased TMAO production in kidney disease.

The effects of CKD on AhR gene and protein expression are also intriguing findings. The increase in hepatic AhR mRNA expression and decreases in AhR protein expression suggest that AhR signaling pathways may be dysregulated. AhR is an important transcription factor that regulates Phase I and II drug metabolism genes such as CYP1A2 and may be involved in cardiovascular disease pathogenesis [175]. Increased AhR mRNA and decreased protein expression may be due to presence of uremic factor(s) that are ligands for cytosolic AhR proteins [176]. In addition, CYP3A2 was an experimental control, demonstrating reproducibility of previous results and robustness of the experimental kidney disease model [33]. These results indicate that AhR is dysregulated in CKD and consistent downregulation of CYP3A2 expression provides confidence in the reproducibility of this well-characterized experimental model.

There are several limitations of the current study. Post-translational modifications like phosphorylation may affect enzyme activity and this was not evaluated. The microbiome plays an integral role in the production of trimethylamine, the FMO substrate that is metabolized into TMAO, and we did not explore how it is changed in CKD or whether any changes are associated with altered TMAO formation. It is also possible that the potential effector compounds may be derived from dietary nutrients. The patients that contributed the uremic and healthy serum were not on a controlled diet, which may have influenced the metabolic activation results. In addition, serum from other donors was not evaluated to replicate these findings. The serum concentrations of flavin adenine dinucleotide were also not measured and may have affected FMO activity. We also acknowledge that the mechanism for increases in K_m values are unclear because metabolic activation has previously been shown to increase substrate affinity with lower K_m values [160];

this suggests that future mechanistic work is needed with recombinant FMO3 enzymes to evaluate the catalytic site structural characteristics that elicit FMO metabolic activation. Moreover, this future work should also assess intrinsic clearance (V_{max}/ K_m) because it reflects the net effects of kinetic changes. Lastly, there are hundreds of known uremic retention solutes that accumulate in CKD and future work is necessary to determine the potential effector compounds that may activate TMAO formation [5]. Future *in vitro* and *in vivo* studies will evaluate FMO activity in the presence of individual solutes (i.e., urea, primary amines, guanidine derivatives, etc.).

3.5 **CONCLUSION**

In conclusion, we show for the first time that metabolic activation of hepatic FMOs leads to increased formation of the non-traditional CVD risk factor TMAO. These data provide important mechanistic insight into the function of hepatic FMOs, as metabolic activation may contribute to the elevated TMAO concentrations observed as kidney function declines. FMO-mediated metabolism may be a therapeutic target to decrease TMAO exposure and thereby lower rates of CVD in patients with CKD.

CHAPTER 4: *EX-VIVO* STUDY OF THE EFFECT OF THE GUT-DERIVED UREMIC TOXIN INDOXYL SULFATE ON FMO EXPRESSION AND TMAO FORMATION IN PRIMARY HUMAN HEPATOCYTES

[Prokopienko AJ, Adams SM, Joshi A, Venkataramanan R, Nolin TD. In preparation]

4.1 **INTRODUCTION**

The metabolic activation of FMO-mediated TMAO formation described in Chapter 3 was a significant discovery. However, we were also intrigued by the findings of increased arylhydrocarbon receptor (AhR) transcription factor mRNA expression and decreased protein expression. This led us to speculate if dysregulated AhR transcription in KD could be involved in changing hepatic gene expression like *FMO3* and FMO-mediated TMAO formation [69].

Patients with kidney disease (KD) are at high risk for cardiovascular disease (CVD) events [119]. Unfortunately, reducing traditional CVD risk factors in KD does not improve CVD outcomes [3]. Interestingly, indoxyl sulfate and trimethylamine-*N*-oxide (TMAO) are strongly associated with CVD events [10, 177]. There may be a interaction between these two non-traditional CVD risk factors. For instance, FMO-mediated TMAO formation is increased in experimental KD [144] and indoxyl sulfate alters expression of several hepatic genes including AhR which can increase *FMO3* expression [155, 178]. The effect of indoxyl sulfate on FMO3 and other hepatic drug metabolism enzymes and drug transporters remains unclear. Therefore, we aimed to assess the effect of indoxyl sulfate on these pathways in primary cultures of human hepatocytes.

Indoxyl sulfate and TMAO are two examples of the over 100 uremic solutes that accumulate as kidney function declines and they are well-recognized as uremic toxins [5, 179]. They originate from breakdown of dietary nutrients by the gut microbiota and from hepatic metabolism [180]. For instance, indoxyl sulfate is formed from gut microbiota catabolism of tryptophan to indole, followed by CYP2E1-mediated hydroxylation to indoxyl and then conjugation with sulfotransferases. TMAO is formed through gut-derived trimethylamine

metabolism by hepatic FMO3 enzymes. Collectively, these gut-derived toxins can interact with drug metabolizing enzymes (i.e., FMO3) and drug transporters [1]. The "remote sensing and signaling" system also provides a physiological perspective of the impact of uremic toxins on inter-organ and inter-organism communication in KD [181]. For instance, these interactions have clinical implications such as indoxyl sulfate-mediated upregulation of P-glycoprotein (Pgp, ABCB1) expression and increased excretion of cyclosporine, requiring dose adjustments [12].

Indoxyl sulfate interacts with these pathways as a direct inhibitor and/or by dysregulating AhR transcription. Circulating indoxyl sulfate concentrations are 40-fold higher in KD than in the general healthy population. As a potent activator of the AhR, these high concentrations may alter normal AhR-mediated hepatic gene expression [5, 69]. AhR typically senses toxic compounds such as dioxin (TCDD) and responds by increasing transcription of several target genes like *CYP1A2* [182]. Interestingly, *FMO3* expression and function is induced by dioxin and dioxin-like pollutant exposure in mice suggesting that AhR partially regulates *FMO3* expression [155, 183]. Dioxin-like pollutant exposure also may contribute to increased serum TMAO concentrations in humans [184]. In fact, this potential indoxyl sulfate-AhR-FMO3 connection is especially relevant to CVD events in patients with KD because of the implications to FMO-mediated TMAO formation.

High concentrations of indoxyl sulfate may contribute to increased FMO-mediated TMAO formation in KD [26, 177]. Notably, systemic exposure of TMAO increases disproportionately in advanced KD compared to earlier stages [10], FMO-mediated TMAO formation is increased [144] and AhR is dysregulated in KD [178]. Given the dysregulation of AhR and the elevated concentrations of indoxyl sulfate in KD, we hypothesized that indoxyl sulfate changes hepatic gene expression (i.e., *FMO3*) through AhR or by other pathways and increases FMO-mediated

TMAO formation. The hypothesis was tested by exposing primary cultures of human hepatocytes to indoxyl sulfate concentrations observed in patients with KD. Gene expression changes were measured with innovative Nanostring technology and reverse transcriptase quantitative polymerase chain reactions (RT-qPCR). We also measured FMO enzyme activity by FMO-mediated TMAO formation from trimethylamine.

4.2 **METHODS**

4.2.1 **Chemical Reagents**

Dimethyl sulfoxide (DMSO), indoxyl sulfate potassium salt, trimethylamine HCl, TMAO, testosterone, 6β-hydroxytestosterone, rifampin, β-mercaptoethanol, PCB-77, and dioxin (TCDD) were purchased from Sigma-Aldrich (St. Louis, MO). Hepatocyte maintenance media (William's E Medium, no phenol red) and supplements were purchased from ThermoFisher Scientific (Waltham, MA). LC-MS acetonitrile and methanol were purchased from Fisher Scientific (Pittsburgh, PA). The *d*9-TMAO internal standard was purchased from Cambridge Isotope Laboratories (Tewksbury, MA).

4.2.2 **Primary cultures of human hepatocytes and TMAO formation**

Primary human hepatocytes (1.5×10^6 cells/well in 6-well collagen-coated tissue culture plates) were isolated from 3 donor livers, as described previously [185]. There were three

biological replicates and two technical replicates for each experimental group, except for TCDD that had two biological replicates. Viability of ≥86% of all isolated hepatocytes was verified with trypan blue cell exclusion assays. Hepatocytes were either purchased from Xenotech (Kansas City, KS) or obtained from the University of Pittsburgh. Hepatocyte incubation conditions were optimized prior to beginning this study, to ensure cell viability after treatments and quantifiable TMAO formation. All treatments and controls were prepared in phenol-free hepatocyte media supplemented with HMM hepatocyte maintenance medium (ThermoFisher Scientific, Waltham, MA) containing 10,000 U/mL penicillin/streptomycin. Cells were maintained at 37°C in a humidified incubator with 5% carbon dioxide.

To begin the experiments, hepatocytes were treated with 0.1% DMSO (vehicle control), rifampin (10 μM; positive induction control), PCB-77, TCDD (100nM and 10nM, respectively; positive controls), and indoxyl sulfate (1, 25, 100, and 250 μM) in duplicate for 72-hours with once-a-day media replacement. Next, fresh media containing 200 μM/L trimethylamine HCl or 250 μM/L testosterone was added to each well for 3-hour incubations to measure enzyme activity. At the end of the incubation, culture supernatant was sampled for TMAO formation and testosterone metabolism. The induction potential of all hepatocytes was measured using rifampin (a known CYP3A inducer) treated cells ability to form 6β-hydroxytestosterone compared to vehicle control. TMAO and 6β-hydroxytestosterone were measured by ultra-performance liquid chromatography-tandem mass spectrometry as previously described [40, 185]. The measured TMAO concentrations from each treatment group were used to calculate and compare TMAO formation. TCDD was added as a positive control in the second and third donor hepatocytes because it is a stronger activator of the AhR compared to PCB-77. Additionally, trypan blue dye exclusion was used to confirm indoxyl sulfate concentrations were non-cytotoxic and this is

consistent with other investigations [12, 63]. Experimental indoxyl sulfate concentrations are within ranges seen in end-stage KD [5].

4.2.3 **RNA extraction**

Total RNA was extracted from the human hepatocytes at the end of the experiments. Hepatocytes were placed on ice, scraped and suspended in ice-cold RLT buffer with 0.1% v/v β-mercaptoethanol and stored at -80°C prior to RNA extraction. RNeasy Mini Kit (QIAGEN, Hilden, Germany) was used for RNA extraction according to the manufacturer's protocol. Adequate RNA purity (260/280 ratios ranging from 1.8 to 2.0) and concentrations were measured using the optical density at 260nm and 280nm with NanoDrop (ThermoFisher Scientific, Waltham, MA). Reverse transcription was carried out with Invitrogen SuperScript III Reverse Transcriptase protocol using random hexamers (ThermoFisher Scientific, Waltham, MA) with a C1000™ Thermal Cycler (Bio-Rad, Hercules, CA). The resulting cDNA was carried forward for qPCR.

4.2.4 **Measurement of mRNA Expression with RT-qPCR**

Quantitative PCR was performed using an Applied Biosystems 7500 Fast Real-Time PCR System (Foster City, CA). *CYP1A2, FMO3, AHR* and *ACTB* cDNAs were amplified with TaqMan® Fast Universal PCR Master Mix and TaqMan® Gene Expression Assays (Hs00167927_m1, Hs00199368_m1, Hs00169233_m1, Hs99999903_m1) (Life Technologies, Carlsbad, CA) according to the Taqman® protocol. No reverse transcriptase and no template

controls were included in every reaction. The qPCR conditions included two initial denaturation steps at 50°C for 2 minutes, followed by 95°C for 10 minutes. Amplification was accomplished by a denaturation at 95°C for 15 seconds, followed by annealing and extension at 60°C for 60 seconds and fluorescence was quantified after each cycle for 45 cycles. Disassociation curves were evaluated for non-specific amplification and the CT cutoff was set at 0.2. All target gene expression was normalized to *ACTB*. The $2^{-\Delta\Delta Ct}$ method was used to quantify target gene expression in treatment groups relative to the vehicle controls [186].

4.2.5 **Nanostring Custom Codeset of Genes**

The genes incorporated into the Nanostring Custom Codeset (Nanostring Technologies, Seattle, WA) were selected to include a targeted panel of drug metabolism enzymes and transporters, vitamin D relevant genes and several transcription factors. The majority of these were chosen based on the International Transporter Consortium and FDA recommendations for relevance to drug therapy [187]. In addition, we included four housekeeping genes (*ACTB*, *GAPDH, HPRT1, TBP*) for normalization purposes.

4.2.6 **Measuring mRNA Expression with Nanostring Technologies**

mRNA expression was quantified using a Nanostring Custom CodeSet (Seattle, WA). Reagents and consumables were provided in the nCounter Master Kit. The high sensitivity Nanostring protocol was selected for accurate, precise and sensitive analysis of all genes in the codeset, including any low expressing genes. Briefly, 100ng of isolated mRNA (5 µl at 20ng/µl)

from each sample was added to tubes containing biotinylated Capture probes and color-coded Reporter probes and a hybridization master mix. The probes have complementary target-specific sequences covalently attached that hybridize to the 'target' single-stranded mRNA to form a double-stranded probe-target complex. This hybridization step occurred at 65°C for 16 hours. The hybridized samples were cooled to 4°C and loaded onto the nCounter Prep Station (multi-channel pipetting robot) (Seattle, WA). Samples are then purified, and the molecular labels are immobilized on an nCounter Cartridge. Finally, the cartridge is transferred to the nCounter Digital Analyzer (multi-channel epifluorescence scanner), that takes images to count the fluorescent reporters. These are processed and each lane generates a reporter code count for data analysis.

Table 10. Nanostring Custom Codeset

HUMAN GENE	COMMON NAME		HUMAN GENE	COMMON NAME
TRANSPORTERS			**TRANSCRIPTION FACTORS**	
ABCB11	BSEP		*AHR*	AHR
ABCB1	MDR1/PGP		*NR1I2*	PXR
ABCC2	MRP2		*NR1I3*	CAR
ABCC4	MRP4		*NR1H4*	FXR
ABCC3	MRP3		*VDR*	VDR
ABCC6	MRP6		*GC*	VDBP
ABCG2	BCRP		*NR1H2*	LXR
SLC22A1	OCT1		*NFE2L2*	NRF2
SLC22A3	OCT3		**BIOMARKERS**	
SLC22A7	OAT2		*CRP*	CRP
SLC22A9	OAT7		**HOUSKEEPING GENES**	
SLCO1B3	OATP8/OATP1B3		*B-ACTIN*	B-actin
SLCO2B1	OATP2B1		*GAPDH*	GAPDH
SLCO1B1	OATP1B1		*TBP*	TBP
SLC47A1	MATE1		*HPRT1*	HPRT1
SLC10A1	NTCP		**MISCELLANEOUS GENES**	
SLC29A1	ENT1		*ALPL*	ALPL
SLC29A2	ENT2			
SLC51A	OST-alpha			
SLC51B	OST-beta			
DRUG METABOLISM ENZYMES				
CYP1A2	CYP1A2			
CYP2C8	CYP2C8			
CYP2C9	CYP2C9			
CYP2C19	CYP2C19			
CYP2D6	CYP2D6			
CYP3A4	CYP3A4			
CYP3A5	CYP3A5			
CYP2B6	CYP2B6			
CYP2B6	UGT1A1			
UGT2B7	UGT2B7			
FMO3	FMO3			
FMO1	FMO1			
CYP2R1	CYP2R1			
CYP24A1	CYP24A1			
CYP27B1	CYP27B1			
SULT2A1	SULT2A1			
UGT1A4	UGT1A4			
UGT1A3	UGT1A3			
SULT1A1	SULT1A1			

4.2.7 **Nanostring Data Analysis**

Nanostring data was analyzed with R v.3.5.2. (R Development Core Team, Vienna, Austria) and the raw transcript counts were normalized using the R package NanoStringNorm. Background variation was controlled by subtracting the negative control mean plus two standard deviations from each probe count. Correction for technical variation was accomplished by normalizing individual counts to the geometric mean of the positive controls. Total RNA count was normalized to the housekeeper geometric means. All samples passed the normalization and quality control procedures, and therefore no samples were excluded from the analysis. The counts after normalization were log2 transformed for further statistical analysis.

4.2.8 **Statistical Analysis**

In the Nanostring analysis, technical replicates were averaged with the mean prior to use in analysis. Since this was a limited and targeted study, an alpha of 0.05 was used to determine statistical significance with no *P* value correction. A one-way analysis of variance (ANOVA) test used to compare expression across each group with the Tukey post-hoc test or applied to genes with ANOVA $P < 0.05$. Data is presented in log2 transcript count vs. the treatment groups. Data was visualized using ggplot2 packages [188].

In the RT-qPCR and FMO activity experiments, treatment groups were compared to control with a One-way ANOVA with Dunnett's multiple comparison tests were used for

analyses. mRNA expression of target genes was normalized to β-actin expression. Data is presented as mean ± SD. There were three biological replicates (3 donors) and two technical replicates for each experimental group, except for TCDD that had two biological replicates.

4.3 **RESULTS**

The effect of indoxyl sulfate on hepatic gene expression and TMAO formation in primary culture human hepatocytes was investigated. Demographics of the human liver donors are displayed in Table 11. All treatment groups were compared to vehicle-treated controls. Induction potential of each donor was verified by CYP3A-mediated hydroxylation of testosterone after exposure to rifampin. Overall, rifampin effectively increased hydroxylation of testosterone by over 9-fold compared to control ($P = 0.0063$).

Table 11. Patient Demographics of Hepatocyte Donors

DONOR	AGE (YR)	SEX	RACE	CAUSE OF DEATH	VIABILITY (%)
HH1341	25	M	Caucasian	Head Trauma	95
HH1349	38	M	African American	Anoxia	88
HHUP01	46	M	Caucasian	Colon Cancer	86

Changes in gene expression after indoxyl sulfate exposure were assessed by RT-qPCR shown in Figure 14. We observed significant dose-dependent increases in both *FMO3* and *CYP1A2* gene expression. *FMO3* mRNA expression increased up to 6-fold in response to indoxyl sulfate ($P < 0.0001$). *CYP1A2* mRNA expression increased up to 200-fold ($P < 0.0001$). *AHR* mRNA expression was not changed.

Increased *FMO3* gene expression was associated with higher FMO enzyme activity as measured by TMAO formation (Figure 15). FMO-mediated TMAO formation was increased up to 3-fold in the human hepatocytes compared to control (Figure 14A; $P < 0.001$). The mean TMAO formation for all three donors was increased up to 1-fold compared to control (Figure 14D; $P < 0.001$). TCDD was also associated with increased FMO-mediated TMAO formation up to 2-fold compared to control (Figure 15B; $P < 0.0001$).

Hepatic drug metabolism enzyme and drug transporter gene expression was also altered after exposure to indoxyl sulfate or TCDD. This was determined with a Nanostring Custom Codeset of 53 genes shown in Table 10. The targeted panel of genes included Phase I and II drug metabolism enzymes, drug transporters, transcription factors, and housekeepers. Expression of several genes were either increased or decreased (Figure 16). Indoxyl sulfate and TCDD increased the expression of *CYP1A2* ($P = 0.0187$). Another transcription factor gene, nuclear factor (erythroid-derived 2)-like 2, also known as *NFE2L2* or *NRF2*, was decreased ($P = 0.0014$) after indoxyl sulfate exposure. TCDD exposure decreased expression of *SLCO1B1* ($P = 0.0033$), also known as organic anion transporter polypeptide 1B1 (OATP1B1). TCDD also increased gene expression of *CYP24A1* ($P = 0.008$), and *UGT1A*4 ($P = 0.0076$).

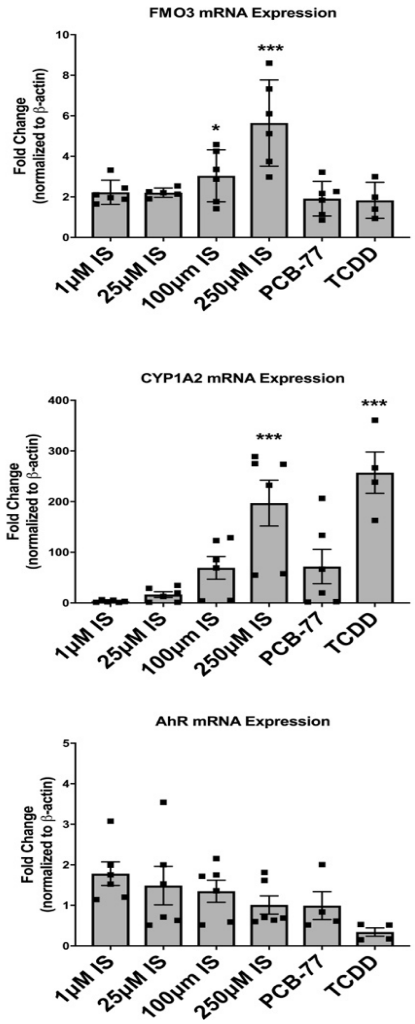


Figure 14. RT-qPCR Measured mRNA Expression in Human Hepatocytes After Exposure to Indoxyl Sulfate

RT-qPCR revealed significant increases in *FMO3* and *CYP1A2* expression but no change in AhR mRNA. There were three biological replicates and two technical replicates for each experimental group, except for TCDD that had two biological replicates. Statistical analysis of the relative expression values was conducted using One-way ANOVA with Dunnett's multiple comparison tests. Data is presented as mean ± SD. *$P < 0.05$, ***$P < 0.0001$ vs. vehicle controls.

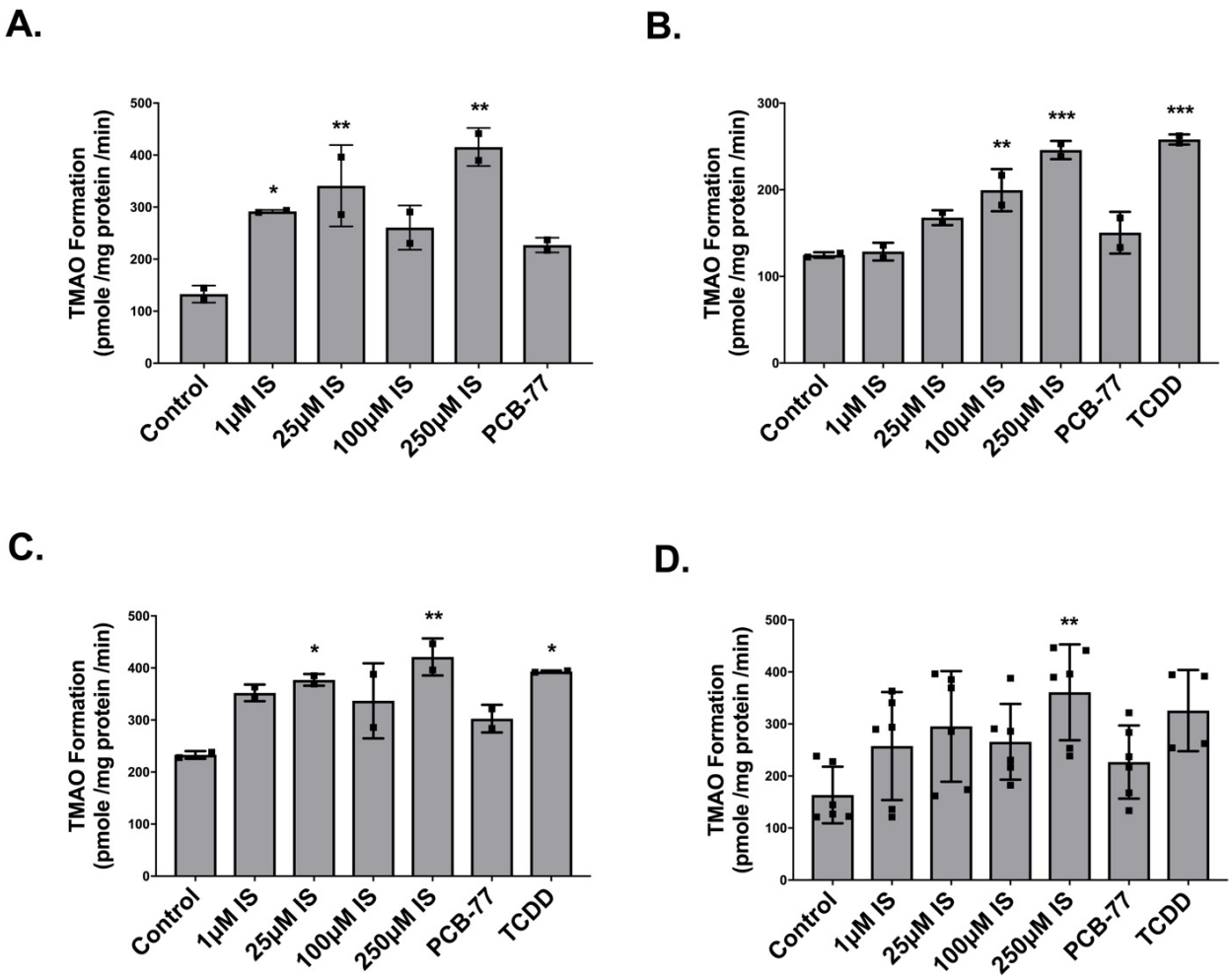


Figure 15. FMO-mediated TMAO Formation in Human Hepatocytes Exposed to Indoxyl Sulfate

There were two technical replicates for each human liver donor presented in (A), (B), and (C). (D) The combined data from three human liver donors. Statistical analysis was conducted using One-way ANOVA with Dunnett's multiple comparison tests. Data is presented as mean ± SD. *$P < 0.05$, **$P < 0.001$, ***$P < 0.0001$ vs. vehicle controls

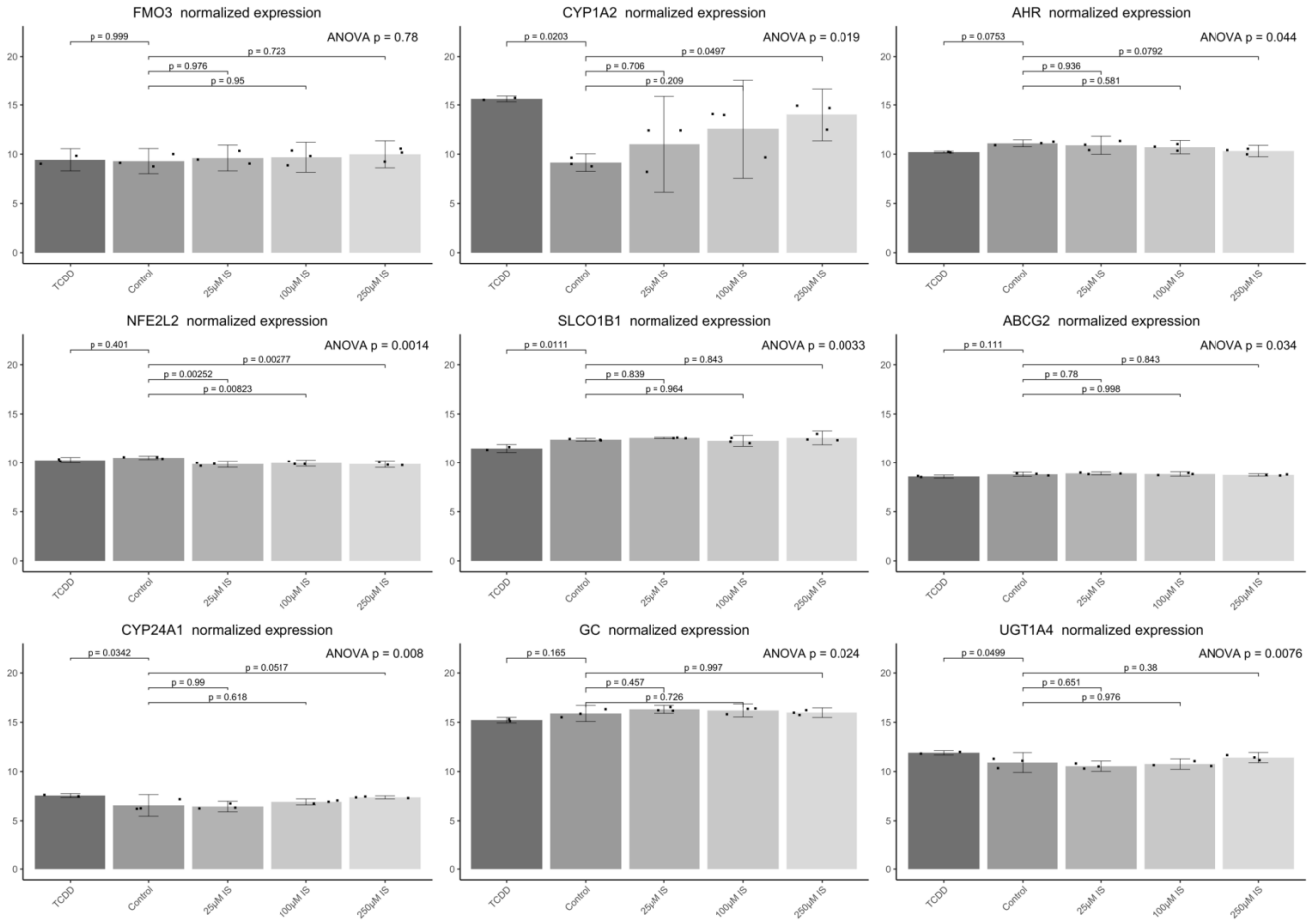


Figure 16. Nanostring Measured mRNA Expression of Hepatic Genes After Exposure to Indoxyl Sulfate

One-way ANOVA with the Tukey post-hoc test applied found statistically significant changes in at least one treatment group for *CYP1A2*, *CYP24A1*, *NFE2L2*, *SLCO1B1*, and *UGT1A4* mRNA expression. Bars represent the mean log2 transformed normalized transcript count for biological replicates (3 in each group except for TCDD, which had two) and error bars show the standard deviation. Biologic replicates comprise the mean of two technical replicates, which are shown independently with the data points. TCDD; dioxin, Control; vehicle control, IS; indoxyl sulfate. Ns = $P > 0.05$, * = $P < 0.05$, ** = $P < 0.01$.

4.4 **DISCUSSION**

The study findings demonstrate that indoxyl sulfate is associated with and might induce *FMO3* mRNA expression and corresponding FMO-mediated TMAO formation in primary cultures of human hepatocytes. Increased *FMO3* expression and corresponding FMO-mediated TMAO formation has potential clinical implications for CVD in KD. In this study, we hypothesized that indoxyl sulfate activates AhR transcription of *FMO3*, thereby increasing TMAO formation and changing expression of other hepatic genes. The novel link between indoxyl sulfate and TMAO may contribute in part, to increases in *FMO3* expression in experimental KD [144] and the significant increases in TMAO exposure in KD [10]. The hypothesis was tested using the *ex vivo* primary human hepatocyte model that is the gold-standard for hepatic induction studies because these hepatocytes overcome inter-species differences, retain complex biological functions, and recapitulate *in vivo* hepatic processes [189]. Indoxyl sulfate and TCDD changed the expression of 5 other hepatic genes in primary cultures of human hepatocytes.

Indoxyl sulfate and TMAO are non-traditional CVD risk factors in patients with KD. For instance, circulating TMAO concentrations are 30-fold higher in advanced KD patients compared to the general population [10]. Changes in kidney function undoubtedly contribute to increased TMAO exposure; however, we previously observed increased *FMO3* expression and hepatic FMO-mediated TMAO formation in experimental KD that also likely contribute to TMAO exposure in KD [144, 178]. The potential mechanism(s) of this increase was unclear, and this present data suggest that indoxyl sulfate exposure increases FMO-mediated TMAO formation in human hepatocytes. Specifically, we observed dose-dependent increases up to 6-fold in *FMO3* mRNA expression after indoxyl sulfate exposure. FMO-mediated TMAO formation was also

increased by up to 3-fold compared to control. Collectively, the findings suggest that indoxyl sulfate at concentrations observed in ESKD may increase *FMO3* transcription and TMAO formation.

AhR activation may increase FMO-mediated TMAO formation in humans. The well-known AhR activator TCDD also increases FMO3 expression in mice [155]. Here, we demonstrate that clinically relevant concentrations of the AhR activator indoxyl sulfate increases formation of the non-traditional CVD risk factor TMAO. Importantly, the AhR pathway is activated in KD patients but it is currently unclear if FMO3 expression is increased [154]. Interestingly, TCDD-like pollutants associate with increased serum TMAO concentrations in patients, suggesting a clinical link between AhR activation and increased FMO-mediated TMAO formation [184]. Overall, our *ex vivo* findings support the need to investigate underlying mechanisms of increased TMAO exposure in KD patients.

The targeted Nanostring analysis was conducted to assess the effect of indoxyl sulfate and TCDD on hepatic drug metabolism, drug transporter and transcription factor genes. Changes in the expression of 5 hepatic genes was observed. These data provide additional evidence of a disordered "remote sensing and signaling" system within KD [181]. Specifically, these changes are likely observed in KD because of high concentrations of disruptive uremic toxins like indoxyl sulfate [95]. For example, indoxyl sulfate exposure decreased expression of *NFE2L2* (NRF2) transcription factors that regulate drug metabolism enzyme and drug transporter gene expression. Indoxyl sulfate exposure also increased *CYP1A2* expression, but how closely this translates to *in vivo* expression is unclear. For instance, CYP1A2 expression is unchanged in animal models of advanced KD [33]; this is potentially due to compensatory feedback mechanisms like the upregulation of AhR repressors [154]. TCDD also changed the expression of *CYP1A2*, *SLCO1B1*,

CYP24A1 and *UGT1A4*, which suggests these genes may be affected by activation of AhR signaling.

Methodological differences are an important consideration in comparing Nanostring and RT-qPCR results. For example, custom Nanostring codesets are not validated and probes can have poor binding affinity, leading to much more conservative results and potential increases in false negatives. Whereas, RT-qPCR involves reverse transcription of mRNA to cDNA and then amplification of cDNA sequences and these steps may magnify underlying differences in expression. This can lead to Nanostring compressing or minimizing overall expression changes compared to RT-qPCR [190]. For instance, we observed differences in the magnitude of change of *CYP1A2* gene expression across both platforms. RT-qPCR measured roughly up to 200-fold increase in *CYP1A2* expression, whereas Nanostring measured roughly up to a 4.9-fold increase. This likely explains the significant increases in *FMO3* measured by RT-qPCR and non-significant differences determined by Nanostring. Considering the methodological differences and that we may have been underpowered to detect expression changes, we caution definitively stating the genes that were not changed as determined by Nanostring, are not affected by indoxyl sulfate.

There are limitations to this study. The Nanostring analysis was likely underpowered due to the limited sample size and this may explain non-significant findings for the other 44 target genes; although, the three hepatocyte donors met FDA recommendations for assessing our primary aim with induction studies [189]. We acknowledge that the increased *FMO3* gene expression after indoxyl sulfate exposure may not be strictly AhR-dependent as evidenced by TCDD treatment having no impact on *FMO3* mRNA (Figure 13), but there was increased TMAO formation (Figure 14) suggesting an increase in FMO activity. The reasons for this are unclear but other mechanisms that increase FMO activity could be occurring like metabolic activation [147].

Alternatively, TCDD is known to induce degradation of AhR protein by ubiquitination [191], so we speculate that TCDD may have initially increased FMO3 expression but after 72 hours the degraded and therefore lower AhR protein concentrations were not significantly increasing *FMO3* mRNA expression but this does not explain the increase in TMAO formation. Alternative regulatory mechanisms may also influence *FMO3* expression. In fact, regulation of FMO3 is complex, in mice there is clear evidence of an AhR Response Element in the regulatory region of the *FMO3* gene, but this may not be the case for rats or humans and requires further evaluation [192]. Finally, the Nanostring and RT-qPCR findings only describe gene expression and further validation at the protein level is needed.

4.5 **CONCLUSION**

In summary, these data suggest that indoxyl sulfate perpetrates interactions with drug metabolism enzyme and drug transporter pathways. For instance, we show for the first time that indoxyl sulfate increases *FMO3* expression and subsequent TMAO formation. These findings provide one mechanism to explain previously reported increases in hepatic FMO-mediated TMAO formation in experimental KD.

CHAPTER 5: TARGETING FMO-MEDIATED TMAO FORMATION AND EVALUATION OF GUT-DERIVED UREMIC TOXINS IN PATIENTS WITH KIDNEY DISEASE: A PILOT STUDY

5.1 **INTRODUCTION**

Chapter 3 and 4 provide two potential mechanisms that may contribute to increased serum TMAO concentrations in KD. The findings suggest FMOs are a potential therapeutic target to reduce TMAO formation. As such, we designed a pilot study in Stage 3-4 CKD patients with three aims. The first aim was to assess if the nutraceutical compound diindolylmethane could lower FMO-mediated TMAO formation as determined by serum TMAO concentrations. The second aim was to evaluate the effect of diindolylmethane on the gut microbiome. Finally, the third aim was to investigate the effect of alterations to the microbiome in Stage 3-4 kidney disease patients on serum concentrations of TMAO, choline, kynurenic acid, hippuric acid, indoxyl sulfate and p-cresol sulfate.

CKD is estimated to affect over 26 million Americans and these patients have accelerated rates of CVD progression [119, 139, 193]. Targeting traditional risk factors like hyperlipidemia has not reduced CVD in this population [3, 194]. Therefore, targeting non-traditional CVD risk factors such as trimethylamine-*N*-oxide (TMAO) may be an innovative strategy to reduce CVD. TMAO increases cholesterol transport into atherosclerotic plaques and is associated with increased risk of CVD events [7, 19, 195]. Microbiota production and subsequent oxygenation of trimethylamine by hepatic flavin-containing monooxygenases (FMO) leads to TMAO formation. In chapter 2, we identified a 25% increase of FMO activity in KD, suggesting that increased FMO production contributes to elevated TMAO concentrations (see Figure 10C) [196]. Metabolic activation that likely contributes to increased FMO activity (Figure 12). Compounds in human kidney disease serum act as effectors to activate FMO enzyme function and increase TMAO formation (Figure 9). Findings from our laboratory also demonstrate that TMAO concentrations

increase disproportionately in advanced kidney disease compared to earlier stages [10]. Overall, TMAO is an important non-traditional CVD risk factor and reducing FMO-mediated TMAO production is a potential therapeutic strategy in KD patients.

The nutraceutical compound diindolylmethane was selected as a potential FMO inhibitor because it is commercially available, there was pharmacokinetic/safety data available, and previous pre- and clinical data suggested it may be an FMO inhibitor [197]. In humans administered diindolylmethane-rich brussel sprouts, there was decreased urinary TMAO and increased trimethylamine suggesting that FMO-mediated TMAO formation was reduced [197]. The investigators went on to identify diindolylmethane as the active FMO inhibitor in enzyme activity experiments. Another potential mechanism of action is reducing FMO protein expression and decreasing FMO-mediated metabolism [198]. Collectively, diindolylmethane has been reported as a potent inhibitor of FMO activity and protein expression [197, 198].

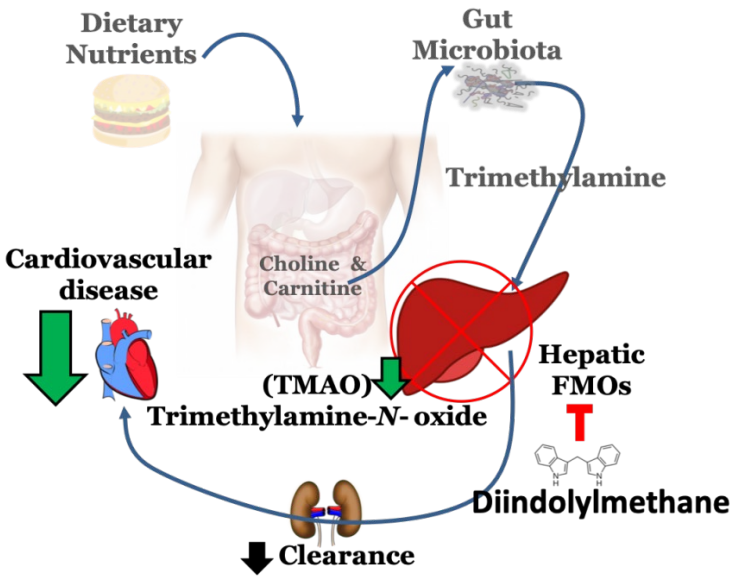


Figure 17. Scientific Premise of Targeting FMO-mediated TMAO Formation

The nutraceutical compound, diindolylmethane, was evaluated as a potential FMO inhibitor. The hypothesis was that diindolylmethane could reduce serum TMAO concentrations by partially inhibiting hepatic FMO-mediated TMAO formation.

The third aim sought to investigate the gut microbiome because it is a promising frontier of research for patients with kidney disease. Kidney disease is associated with alterations to the bacterial communities in the gut microbiome [25, 199]. Alterations to the microbiome have implications to patients because the gut microbiota catabolize dietary nutrients and form precursors to gut-derived toxins such as the non-traditional CVD risk factor TMAO. In fact, the

microbiome and the GDUT products are promising therapeutic targets [127]. However, to date, there is limited clinical and translational research providing direct evidence of whether these alterations and which specific genera taxa contribute to increased systemic toxin exposure.

Over 100 uremic retention solutes accumulate in serum as kidney disease progresses and GDUTs make up a significant portion of this uremic milieu in patients with kidney disease [5]. In particular, GDUTs such as TMAO, choline, kynurenic acid, hippuric acid, indoxyl sulfate and p-cresol sulfate associate with CVD [8, 124-126]. For example, TMAO is associated with CVD events in the general population and in CKD [10]. The exact biological mechanisms of toxicity are still an active area of research and may provide insights into novel therapeutic targets or strategies. Importantly, these GDUTs accumulate to supraphysiologic concentrations in advanced kidney disease such as 30-fold for TMAO [10] and 40-fold for indoxyl sulfate [5]. Declines in kidney function undoubtedly contributes to these striking increases in exposure, but there is speculation that the microbiome also increases formation of precursors [25].

The human microbiome contributes to human health and disease. The studies investigating the microbiome in kidney disease have provided important information with different approaches. Hostetter and colleagues have demonstrated that several uremic toxins are colon-derived by comparing metabolomics data of ESKD patients to those with resected colons [200]. Metabolomics approaches also demonstrated there are distinct metabolites generated in the colons of ESKD patients and rats compared to healthy controls [201]. The blood microbiome is also different between CKD and healthy patients [202]. Other investigators demonstrated that ESKD patients and rats microbiome diversity is different compared to healthy controls [25, 203-205]; follow-up *in silico* tests predicted an expansion of bacterial families that form precursors to indoxyl sulfate and p-cresol sulfate [51]. However, evidence of gut microbiome alterations in

CKD Stage 3-4 are limited [199]. Currently, findings suggest that the CKD microbiome potentially contributes to increased GDUT production but limited direct functional measure of systemic concentrations and correlation analyses exist [195, 206, 207]. Comparing the GDUT serum concentrations to the bacterial families within the microbiome may shed light on changes that occur as kidney disease progresses and may inform therapeutic strategies to reduce formation of gut-derived toxins.

There are three aims in this study. The first and second aims consisted of assessing the effect of diindolylmethane on inhibiting the formation of TMAO and on the microbiome. The third aim was to investigate direct impact of alterations to the microbiome in Stage 3-4 kidney disease patients on serum concentrations of TMAO, choline, kynurenic acid, hippuric acid, indoxyl sulfate and p-cresol sulfate. We hypothesized that changes to the gut microbiota would increase the systemic concentrations of these GDUTs. This is the first prospective controlled study to correlate the gut microbiome to systemic exposure of several gut-derived toxins in Stage 3-4 CKD. Thirteen CKD patients were recruited and had corresponding serum and microbiome samples collected. The study was a prospective, randomized, double blind, placebo controlled, crossover study with four sampling timepoints over 12 weeks. CKD microbiome profiles were compared to healthy controls. Analyses evaluating associations between these gut-derived toxins, covariates and bacterial families within the gut microbiome were conducted.

5.2 **METHODS**

5.2.1 **Participants**

The study was approved by the University of Pittsburgh Institutional Review Board and registered at clinicaltrials.gov [NCT03152097]. All participants were enrolled after providing written informed consent. The initial screening visit included evaluation of medical history, current medications, laboratory biochemistries, vital signs and weight assessments.

Inclusion criteria included adults' age 18-75 with a medical diagnosis of chronic kidney disease defined as an estimated glomerular filtration rate (eGFR) of $\leq$ 60 ml/min/1.73m^2. Exclusion criteria included unacceptable vital signs at the Screening visit, subjects who currently smoke, or any confirmed or suspected state of immunosuppression. Patients taking any of the following medications, methimazole, alosetron, duloxetine, ramelteon, tasimelteon, theophylline, tizanidine, clozapine, pirfenidone and ramosetron were excluded. In addition, use of any of the following drugs within the last 4 weeks prior to the Day 1 appointment: Systemic antibiotics, antifungals, antivirals or antiparasitics (intravenous, intramuscular, or oral); corticosteroids (oral, intravenous, or intramuscular); cytokines; methotrexate or immunosuppressive cytotoxic agents; anti-diarrheal agents and bile acid sequestrants was prohibited. Subjects were also excluded if they had any recent changes in consumption of commercial probiotics including tablets, capsules, lozenges, chewing gum or powders in which probiotic is a primary component. Chronic, clinically significant hepatic abnormality (i.e. elevated 3X ULN ALT/AST), as determined by medical history or physical examination, chronic alcohol consumption, or any recent history of cancer were exclusions. Allergies to corn or soy, or unstable dietary history as defined by major

changes in diet during the previous month, where the subject has eliminated or significantly increased a major food group in the diet, were exclusions. Participants were also excluded if they had any history of active uncontrolled gastrointestinal disorders or diseases including: Inflammatory bowel disease (IBD) including ulcerative colitis (mild-moderate- severe), Crohn's disease (mild-moderate-severe), or indeterminate colitis; irritable bowel syndrome (IBS) (moderate-severe); persistent, infectious gastroenteritis, colitis or gastritis, persistent or chronic diarrhea of unknown etiology, Clostridium difficile infection (recurrent) or Helicobacter pylori infection (untreated); chronic constipation or any major bowel resection at any time. Participants who may be pregnant or lactating were also excluded. All participants were instructed to abstain from consuming cruciferous vegetables for approximately 14 days before the start of the study and throughout the duration of the study.

Participants were recruited and enrolled between February 2018 and January 2019. In total, 54 people were assessed for eligibility, 15 participants were randomized, and 12 participants completed the entire study (Figure 18). In regard to the 3 patients who were randomized but did not complete the study, one participant was lost to follow-up and two patients withdrew for unrelated medical issues. Healthy control serum (n = 6) was obtained from a local blood bank and healthy control stool (n = 21) was obtained from fecal microbiome transplant donors.

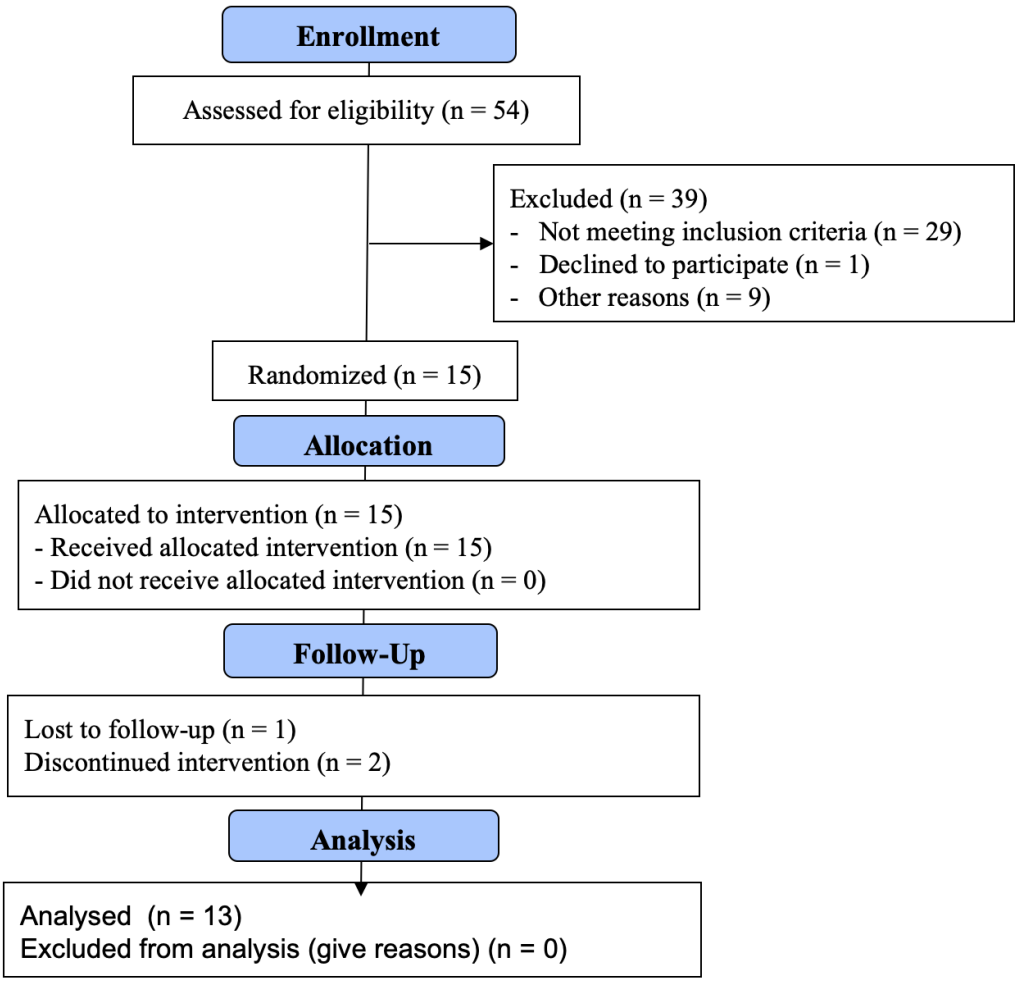


Figure 18. TMAO Study CONSORT Diagram

The TMAO Study assessed 54 participants for eligibility, of those, 15 were randomized to receive the intervention. Three participants were lost to follow up for various reasons. In total, there were 13 participants who completed the diindolylmethane treatment period and of those 12 patients that completed the entire study.

5.2.2 **Clinical Trial Design**

This study was a randomized, double-blind, placebo-controlled, cross-over, single-site clinical trial. All study visits occurred at the University of Pittsburgh Clinical and Translational Research Center in Montefiore Hospital. Participants were randomized to receive diindolylmethane and a placebo for four weeks with a four-week washout period in-between treatment phases. The study treatment was provided in identical bottles with encapsulated DIM or placebo. There were 5 study visits, including the screening visit, over the course of 12 weeks (Figure 19). The 12-week study duration was selected to assess changes to the gut microbiome over time. Randomization and blinding were performed by the University of Pittsburgh Medical Center Investigational Drug Service. At each visit blood was drawn for clinical biochemistries and gut-derived toxins (i.e., TMAO, choline, kynurenic acid, hippuric acid, indoxyl sulfate and p-cresol sulfate) concentrations. Participants also provided a stool sample at each visit for microbiome analysis. Females of child-bearing age had a urine pregnancy test at screening and again at Week 8, after the cross-over. Adherence was assessed with pill counts.

Diindolylmethane has been evaluated in clinical trials up to doses that may safely inhibit TMAO formation. Single dose escalation studies in 24 healthy male and female patients demonstrated tolerable safety data up to a 300 mg dose of diindolylmethane and the half-life to be approximately 3 hours [208]. Another Phase 1 dose escalation study in 12 males, also found 300 mg twice daily to be the maximum tolerated dose of diindolylmethane [209]. Several clinical studies using diindolylmethane suggests that the novel use of diindolylmethane in KD is safe and may effectively inhibit FMO formation of TMAO [210, 211]. The safe use of diindolylmethane in

these studies and consideration of the pharmacokinetic parameters informed the dose selection of diindolylmethane in this study at 150 mg twice daily.

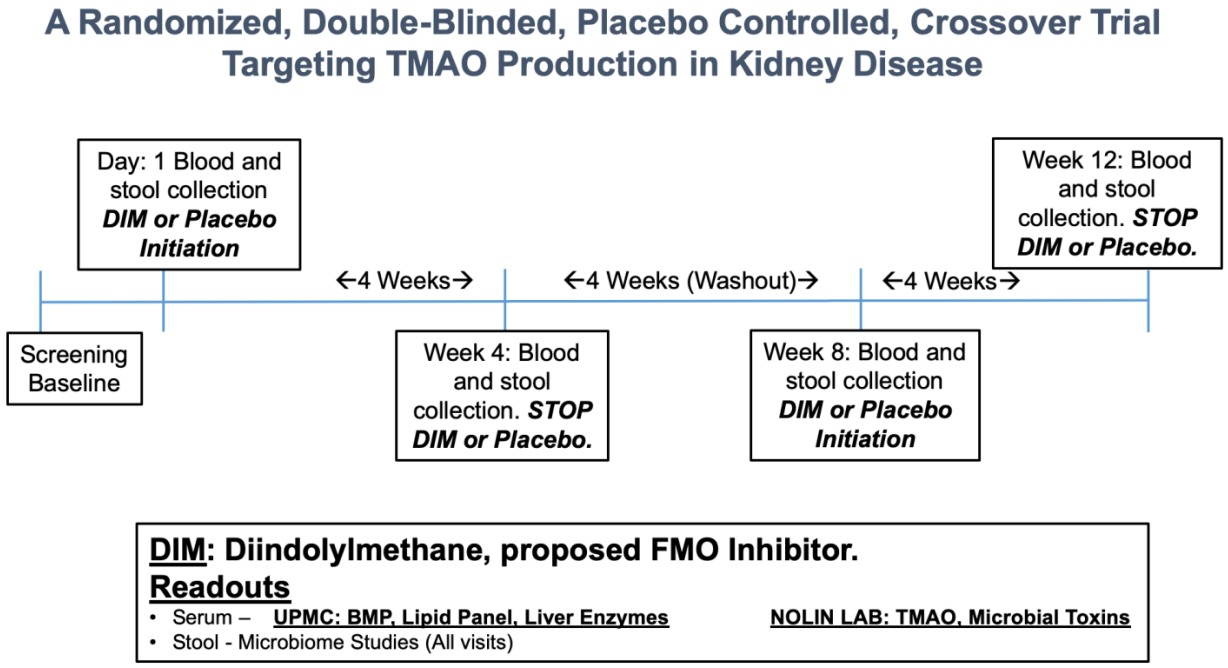


Figure 19. Study Design

The Targeting FMO-Mediated TMAO Formation Study (TMAO Study) was a randomized, double-blinded, placebo controlled, crossover trial in patients with Stage 3-4 kidney disease. The primary endpoint was to assess reductions in serum TMAO concentrations after diindolylmethane treatment. Secondary endpoints included the characterization of the gut microbiome and gut-derived toxin exposure in patients with Stage 3-4 kidney disease. The study was conducted at the University of Pittsburgh Medical Center (UPMC) Clinical and Translational Research Center between February of 2018 and March of 2019.

5.2.3 **Sample Analysis**

5.2.3.1 **Quantification of Gut-Derived Uremic Toxins**

We measured serum TMAO, choline, kynurenic acid, hippuric acid, indoxyl sulfate and p-cresol sulfate concentrations by ultra-high-performance liquid chromatography-tandem mass spectrometry. Heated electrospray ionization (positive and negative modes) and selected reaction monitoring with two separate analytical methods as previously described in Chapter 2 and published [40]. The standard curves for TMAO and choline ranged from 0.010 to 5.00 μg/mL, for hippuric acid and indoxyl sulfate 0.200 to 80.0 μg/mL, for p-cresol sulfate 0.250 to 80.0 μg/mL and for kynurenic acid 0.010 to 0.500 μg/mL. The inter-day and intra-day accuracy (standard deviation from nominal value) and precision (percent coefficient of variation) was < 15% for both analytical methods.

5.2.3.2 **Statistical Analysis**

Descriptive statistics were calculated for all continuous variables. All data analyses were assessed for normality. As a primary analysis, the effect of diindolylmethane treatment on changes in uremic solute concentrations was tested using Wilcoxon rank-sum tests as a formal cross-over analysis while evaluating sequence and period (i.e., carryover) effects [212]. As exploratory analyses, multivariate regression models were used to study whether changes in the diindolylmethane treatment phase were associated with their baseline values in the diindolylmethane treatment phase controlling for covariates selected based on stepwise regression analyses. The candidate variables for the stepwise regression analyses were baseline uremic solute concentration in the treatment phase, female, BMI, diabetes, and eGFR. Pearson correlation

analyses were conducted between all the study variables. A p-value < 0.05 was used as a cutoff for statistical significance. Statistical analyses were conducted using SAS 9.4 (SAS Institute Inc., Cary, NC). In stepwise selections, 0.15 was used in forward and backward selection methods as it is a specified default level in SAS.

5.2.3.3 **Microbiome Analysis**

To investigate the intestinal microbiome in kidney disease we conducted an analysis of 16S rRNA gene sequences from stool samples from 13 kidney disease subjects with a total of 50 samples and 8 healthy patients as controls. Microbiome samples were processed by the Center for Medicine and the Microbiome at the University of Pittsburgh. Samples were collected by patients with at home collection kits and mailed into the Center's laboratory. The stool samples were then processed, and bacterial DNA was extracted. The well-established experimental and analysis protocols have been previously described [213]. All statistical analyses were performed in R (version 3.5.3). Briefly, taxonomic descriptions and beta diversity analyses (Bray Curtis dissimilarity index) were performed with QIIME and compared distance matrices between groups with the PERMANOVA method. The top 25 most abundant genera taxa were added to a predictive model with age, sex, diabetes and BMI as covariates. The genera taxa were used as predictors of clinical variables (e.g., eGFR) and gut-derived toxin concentrations as univariate responses. Correlations could be identified based on the statistical model and individual genera taxa. This statistical modeling has been described previously [214].

5.3 **RESULTS**

5.3.1 **Participant Demographic Characteristics**

Demographics of all CKD study patients that completed the diindolymethane treatment phase (n=13) and completed the entire study (n=12) are shown in Table 12.

Table 12. Demographics

Demographics	**Total Treatment Cohort (n=13)**	**Completed Entire Study (n=12)**
Age (years)	63.2 ± 8.9	63.3 ± 9.3
Sex		
Female	6 (46.2 %)	6 (50%)
Male	7 (53.8%)	6 (50%)
Race		
African-American	1 (7.7%)	1 (8.3%)
Caucasian	12 (92.3%)	11 (91.7%)
BMI	32.9 ± 6.2	33.5 ± 6.0
Diabetes	5 (38.5%)	5 (41.7%)
eGFR	37.8 ± 12.3	37.4 ± 12.8

5.3.2 **Effect of Diindolylmethane on Serum TMAO Concentrations and Gut Microbiome**

We observed no significant difference between the serum concentration change of TMAO (μg/mL) after four weeks of diindolylmethane treatment in comparing the corresponding 4-week placebo phase (median change between the treatment phase vs. placebo phase [min, max]: -0.011 [-2.01, 0.51] vs. 0.23 [-5.3, 1.14], $P = 0.27$). Comparing the serum TMAO concentrations before and after diindolylmethane treatment there was no significant change in TMAO (Figure 18; $P = 0.29$). There was no sequence or period effect in any of the study variables. In the exploratory stepwise regression analysis on TMAO change in the diindolylmethane treatment period, sex was found to be a significant covariate ($P = 0.038$). Males had a -0.61 ± 0.73 μg/mL change in TMAO concentrations during the treatment period compared to 0.25 ± 0.27 μg/mL for females (Figure 19; $P = 0.02$). When sex was excluded from the set of candidate covariates in the stepwise regression analysis, baseline TMAO and eGFR were selected as significant covariates. In the subsequent multivariate regression analysis with baseline TMAO and eGFR, baseline TMAO was significantly associated with the treatment effect of diindolymethane on the change in TMAO in the treatment phase (parameter estimate -0.53, $P = 0.0095$). This indicates that a patient with a 1.0 (μg/mL) higher baseline TMAO compared to lower baseline TMAO concentrations showed 0.53 (μg/mL) greater decrease in TMAO during the treatment phase when controlling for baseline eGFR. Interestingly, males had 1.56 ± 1.57 μg/mL baseline TMAO concentrations compared to 0.58 ± 0.23 μg/mL for females, although the difference was not significant ($P = 0.19$).

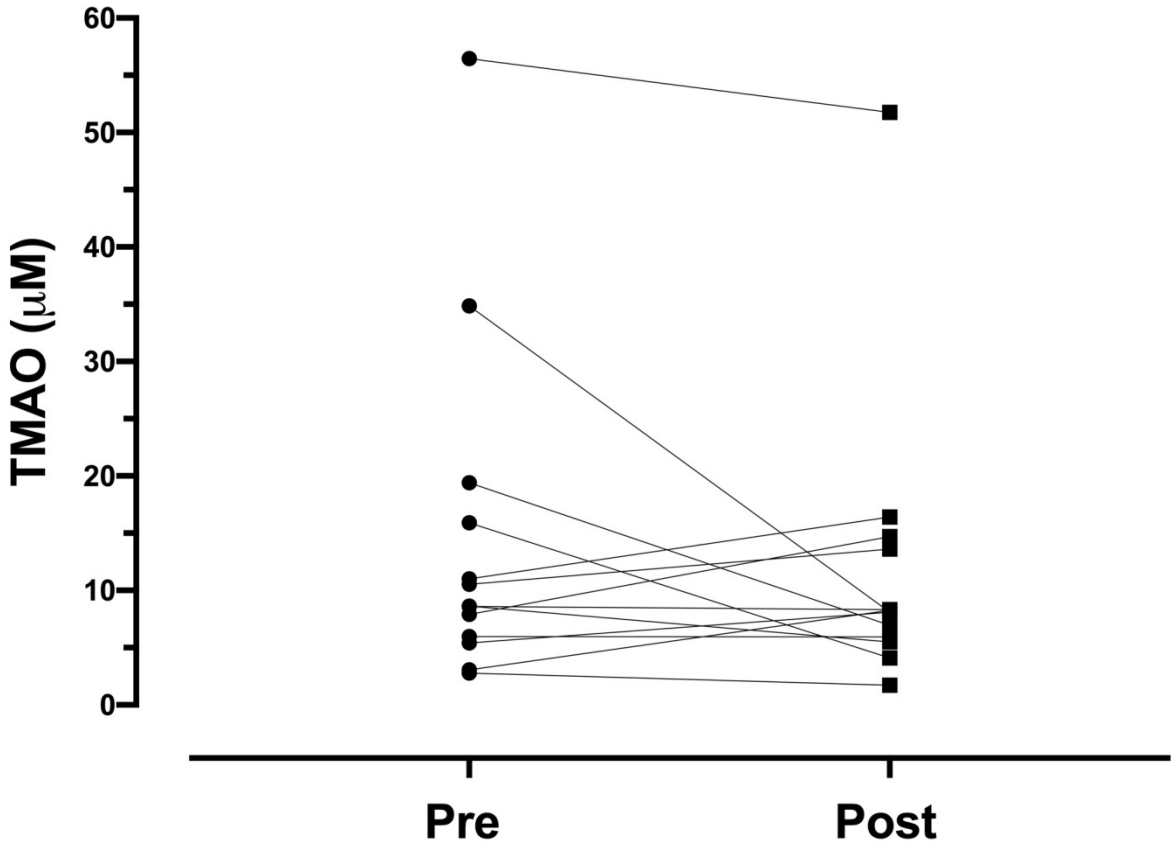


Figure 20. Serum TMAO Concentrations Pre and Post Diindolylmethane Treatment

The primary endpoint was reductions in serum TMAO concentrations before and after diindolylmethane treatment. Serum TMAO concentrations for each patient are presented before and after 4-weeks of diindolylmethane treatment (connecting line). There was no significant reduction in TMAO concentration in the total study population ($P = 0.29$).

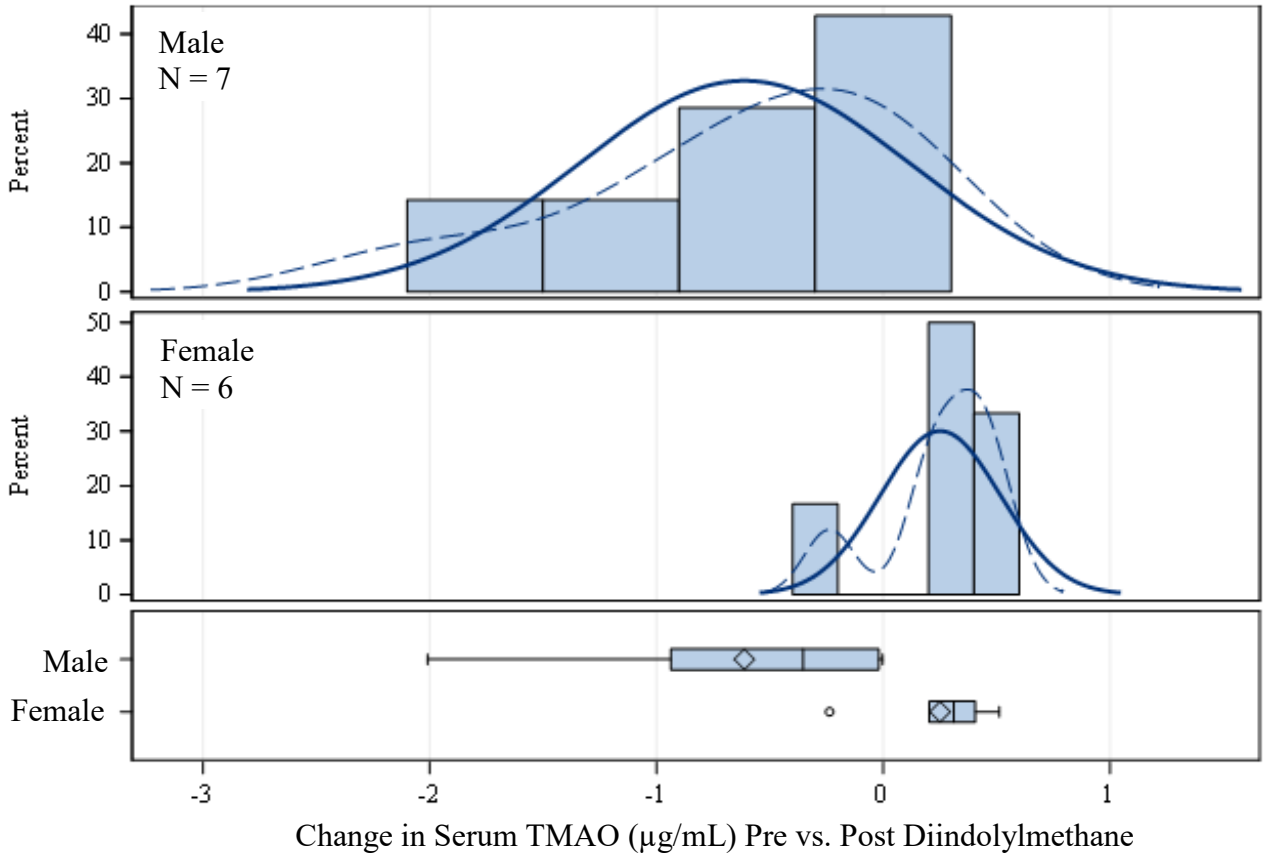


Figure 21. Change in TMAO Concentrations with Diindolylmethane Treatment in Males Compared to Females.

The primary endpoint was reductions in serum TMAO concentrations before and after diindolylmethane treatment. In an exploratory analysis, males had on average 8 µM reduction in serum TMAO concentrations compared to no significant change for females after diindolylmethane treatment. Here, the data represents the µg/mL change in serum TMAO concentrations. The top panel depicts the changes in concentration in males (n = 7), the middle panel shows the change in females (n = 6) and the bottom panel represents the distribution of the results for both males and females.

Additional findings included change in TMAO being positively correlated with change in indoxyl sulfate (0.628; P = 0.0289) and negatively correlated with change in hippuric acid (-0.693; P = 0.013) during the placebo period. Overall, diindolylmethane had no effect on the other gut-derived toxins or clinical variables such as low-density lipoprotein.

5.3.3 **Gut-derived Uremic Toxin Concentrations**

The concentrations of gut-derived toxins were measured in 6 healthy control patients, and 15 study patients at the Day 1 visit (Table 13). Stepwise increases were observed in all toxins as CKD stages progressed. In particular, indoxyl sulfate and p-cresol increased by 11.8-fold and 22.8-fold in Stage 4 CKD patients compared to healthy controls, respectively.

Table 13. Gut-derived Toxin Serum Concentrations in Study Patients

	Healthy controls (n = 6)	Stage 3a (n = 6)	Stage 3b (n = 6)	Stage 4 (n = 3)
	Concentration (µg/mL)			
Kynurenic acid	0.006 ± 0.002	0.016 ± 0.007*	0.020 ± 0.009*	0.036 ± 0.009*
Hippuric acid	0.406 ± 0.316	1.215 ± 1.317	1.440 ± 0.810*	2.954 ± 2.822
Indoxyl sulfate	0.424± 0.133	1.502 ± 1.092*	2.151 ± 0.499*	5.407 ± 0.701*
p-Cresol sulfate	1.15 ± 1.078	7.483 ± 5.853*	11.337 ± 5.567*	27.40 ± 11.732*
TMAO	—	0.368 ± 0.201	1.071 ± 0.850	4.213 ± 3.443†
Choline	—	1.163 ± 0.387	1.263 ± 0.406	2.144 ± 0.779†

* $P < 0.05$, compared to healthy volunteers.
† $P < 0.05$, compared to Stage 3a
Stages 3a, 3b, and 4, as eGFR values of >120, 59-45, 44-30, and 29-15 mL/min/1.73 m², respectively.

5.3.4 **Gut Microbiome in CKD Compared to Healthy Controls**

To determine if the gut microbiome is altered in stage 3-4 CKD the microbiota composition was compared between CKD patients (n = 13) and healthy control patients (n = 21)

(P = 0.0042; Figure 22). Multi-dimensional scaling (MDS) demonstrated significant separation of Bray-Curtiss dissimilarity indices between CKD and healthy control group centroids.

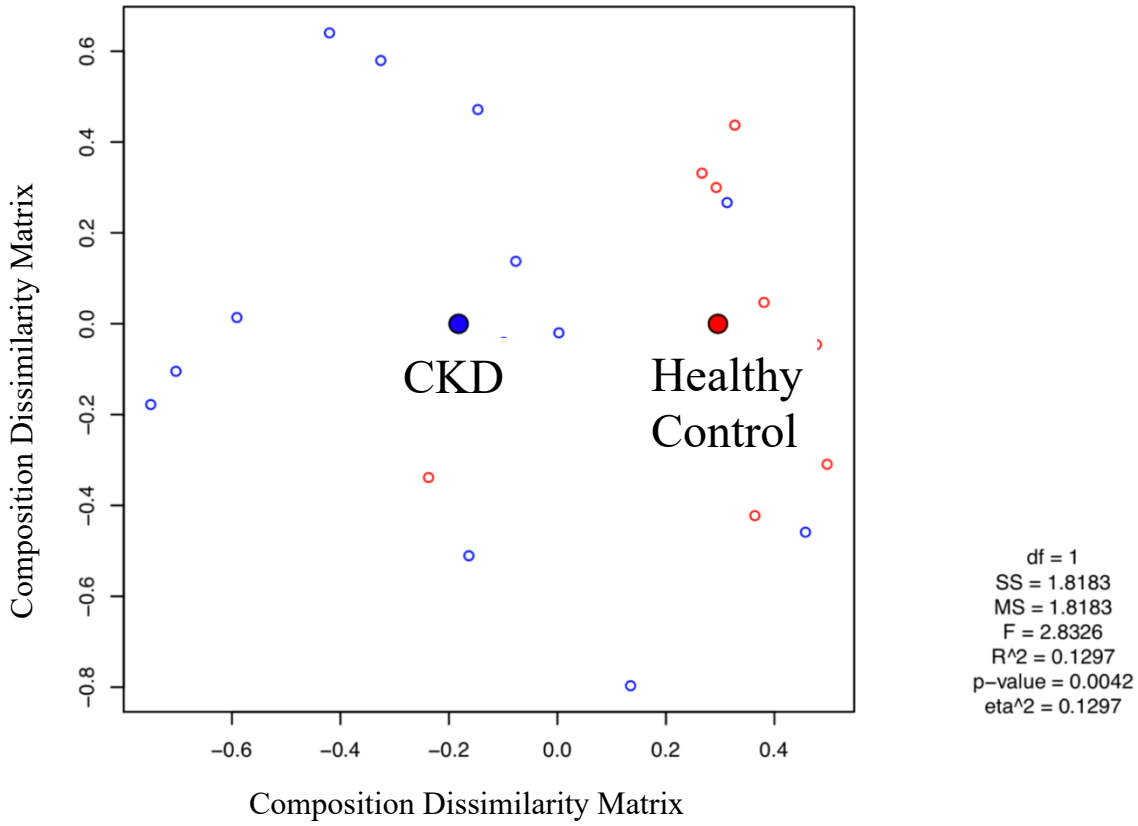


Figure 22. Multidimensional scaling (MDS) Plot of Bray-Curtis Dissimilarity Indices between CKD and Healthy Control Patients.

The gut microbiota composition was compared between CKD and healthy control patients using Bray-Curtis Dissimilarity Indices to compare the differences in genera taxa abundances. The centroids for CKD (blue) and healthy controls (red) are the averages of the differences between the individual samples (open circles). The data is displayed in a composition dissimilarity matrix. The gut microbiota composition of the CKD group was significantly different than the healthy control group (P = 0.0042).

5.3.5 **Gut Microbiota Diversity with BMI and Age**

The microbiota composition was compared between sub-groups over a range of BMIs and ages in the CKD patients (n = 13) (Figure 23). The composition of abundances of genera taxa was significantly different between the sub-groups of BMIs (P = 0.045) and age (P = 0.0129). The differences in composition were determined by Bray-Curtis Dissimilarity Indices and are presented in Figure 23.

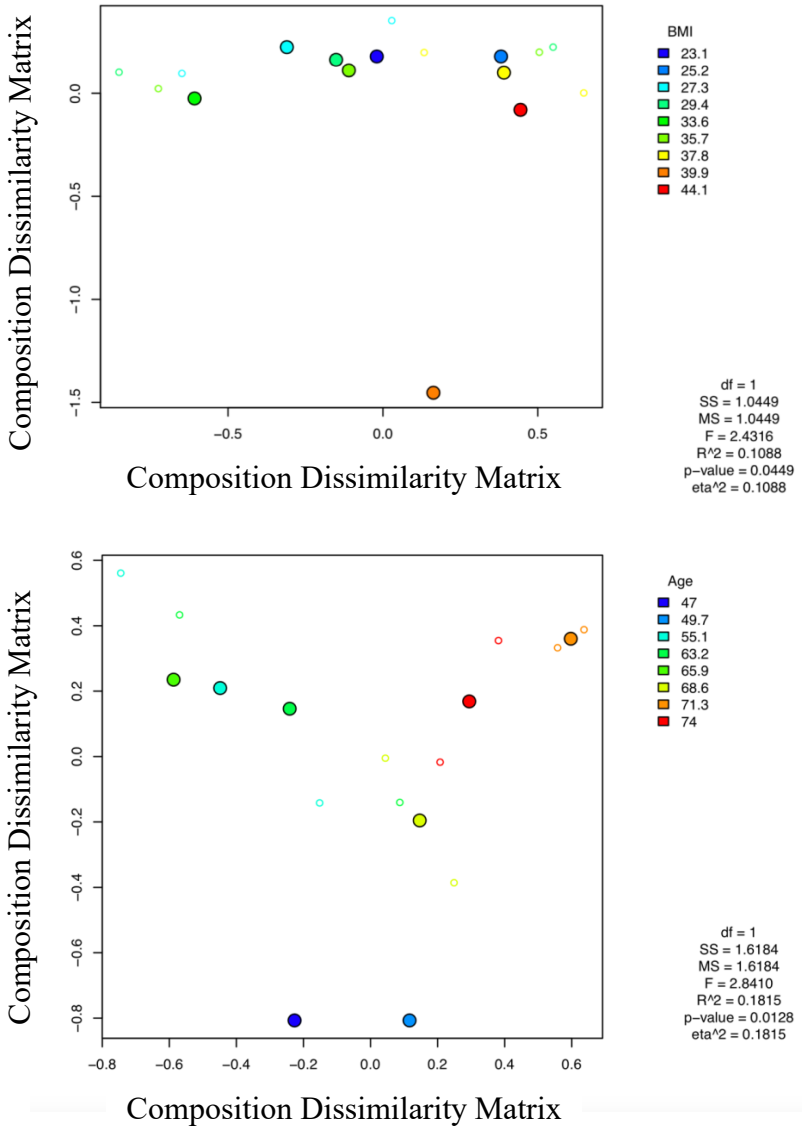


Figure 23. Microbiota Composition for Ranges of Age and BMI in CKD.

The gut microbiota composition was compared across BMI (top panel) and age (bottom panel) ranges using the Bray-Curtis Dissimilarity Indices. The centroids or averages of each individual group's composition are color coded and are displayed in a composition dissimilarity matrix. The microbiota composition was significantly different across ranges of BMI ($P = 0.045$) and ages ($P = 0.013$).

5.3.6 **Gut Microbiota Diversity with eGFR and Prediction**

The 25 most abundant genera were used as predictors of set of clinical variables as univariate responses: eGFR, AST and ALT. The model also included age, sex, diabetes and BMI as covariates. The eGFR, a measure of kidney function was moderately associated with the microbiota and specific genera taxa, although the relationship was non-significant (adjusted R^2 = 0.632, P = 0.433; Figure 24). In fact, lower eGFR was also associated with decreased diversity (P = 0.0182).

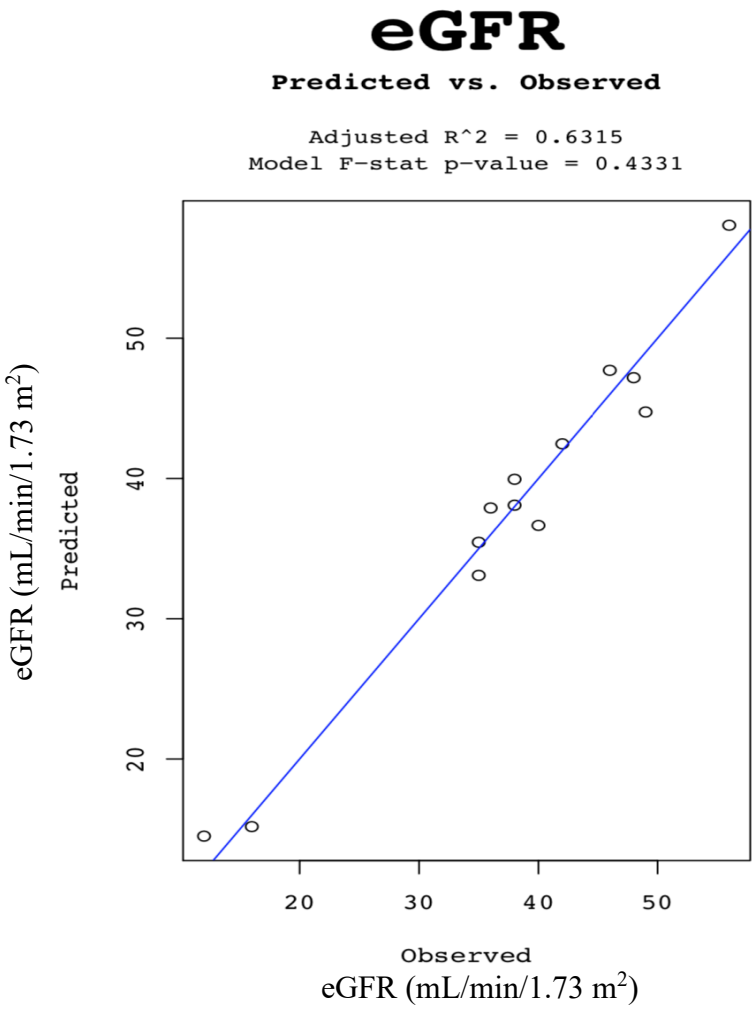


Figure 24. Microbiota as a Predictor of eGFR.

The 25 most abundant genera were used as predictors of eGFR as univariate responses. The model also included age, sex, diabetes and BMI as covariates. eGFR was moderately associated with the microbiota and specific genera taxa, although the relationship was non-significant (adjusted $R^2 = 0.632$, $P = 0.433$).

5.3.7 **CKD Gut Microbiome Predicts Gut-Derived Toxin Concentrations**

In a similar statistical model, the 25 most abundant genera were used as predictors of TMAO, choline, kynurenic acid, hippuric acid, indoxyl sulfate and p-cresol sulfate as univariate responses. The model also included age, sex, diabetes and BMI as covariates. The microbiota was a strong predictor when added to the full model for TMAO (adjusted $R^2 = 1$, $P = 0.0029$; Figure 23), hippuric acid (adjusted $R^2 = 0.955$, $P = 0.158$; Figure 24), and indoxyl sulfate (adjusted $R^2 = 0.987$, $P = 0.083$; Figure 24). The improvement of the univariate adjusted R^2 with adding the microbiota data to the model with covariates were a 1.49, 0.98 and 1.19 increase, respectively ($P < 0.001$).

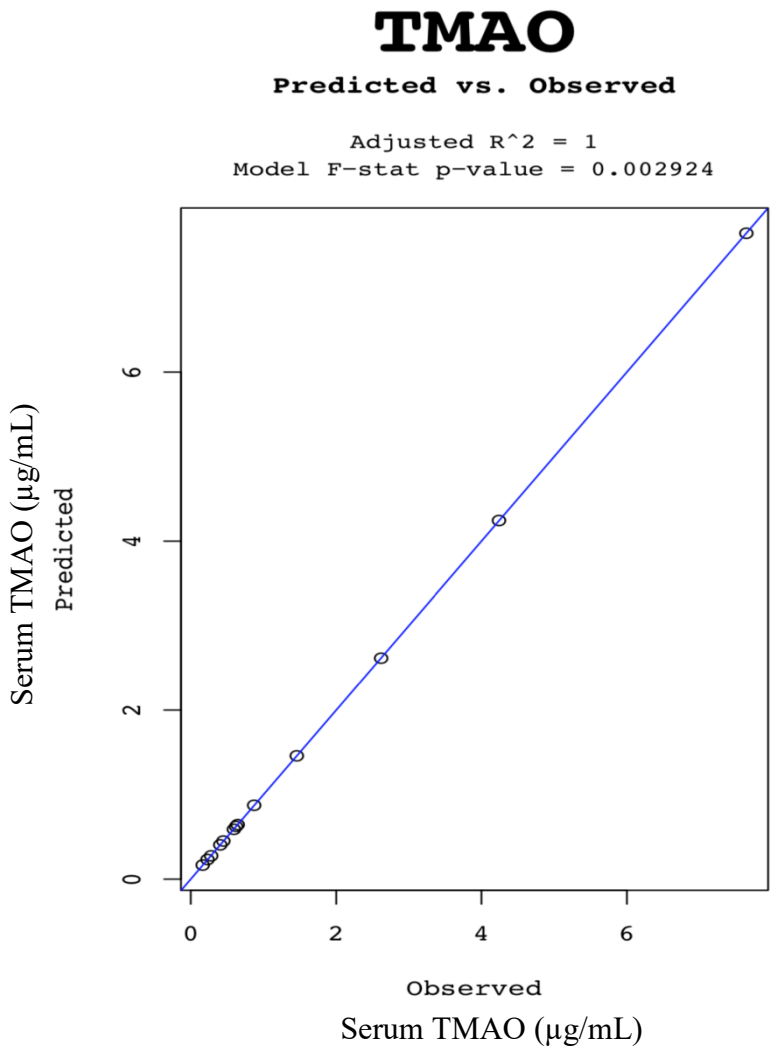


Figure 25. Microbiota as a Predictor of Serum TMAO Concentrations.

The 25 most abundant genera were used as predictors of TMAO as univariate responses. The model also included age, sex, diabetes and BMI as covariates. The microbiota was a strong predictor when added to the full model for TMAO (adjusted $R^2 = 1$, $P = 0.0029$).

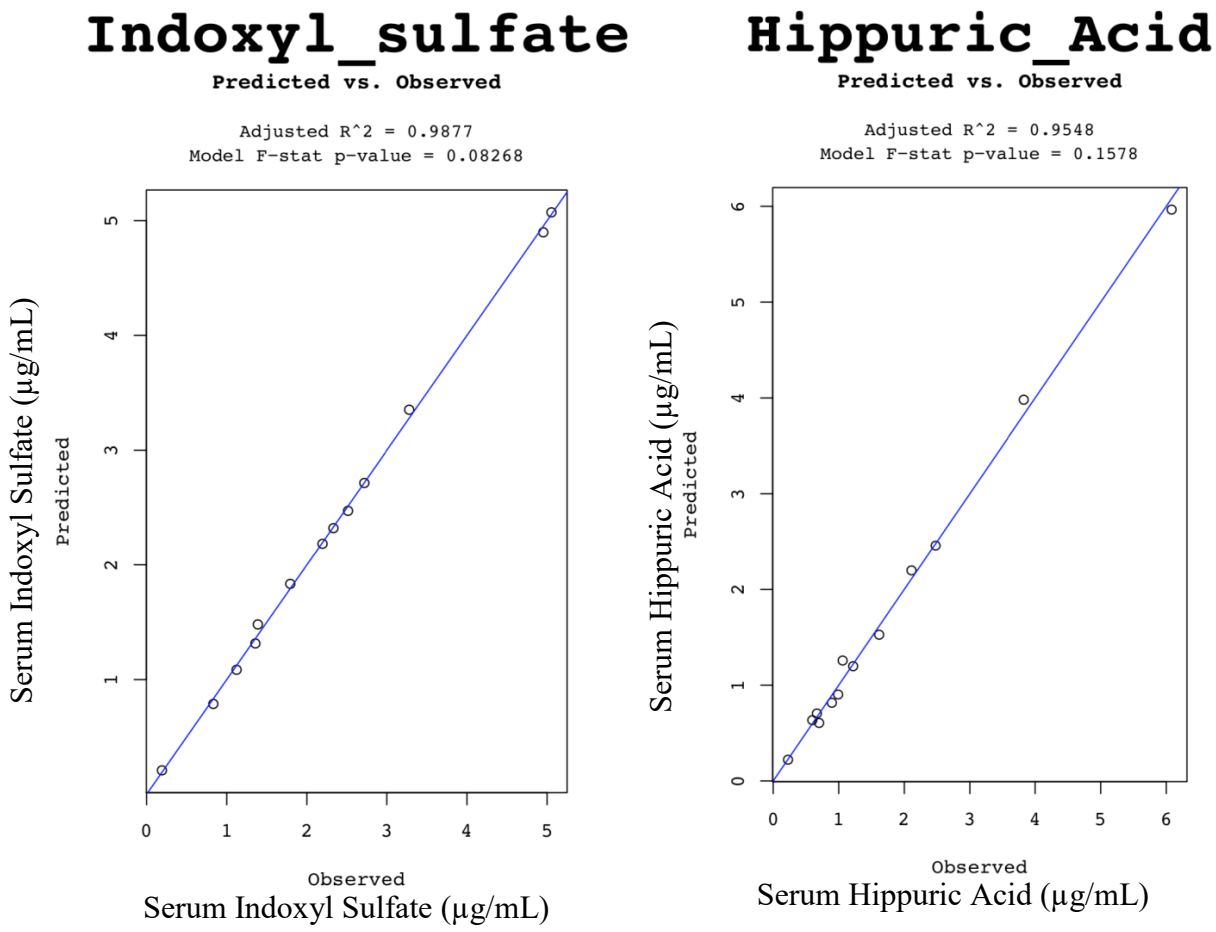


Figure 26. Microbiota as a Predictor of Indoxyl Sulfate and Hippuric acid Serum Concentrations.

The 25 most abundant genera were used as predictors of indoxyl sulfate and hippuric acid as univariate responses. The model also included age, sex, diabetes and BMI as covariates. The microbiota genera taxa were a predictors when added to the full model for indoxyl sulfate indoxyl sulfate (adjusted $R^2 = 0.987$, $P = 0.083$) and hippuric acid (adjusted $R^2 = 0.955$, $P = 0.158$), although the fits were not statistically significant.

TMAO also correlated with several genera taxa (Figure 26). In particular, TMAO was positively correlated with *Prevotella_9*, *Lachnospiraceae_uncl*, *Faecalibacterium, Bacteroides and Alistipes.* TMAO was also negatively correlated with *Lachnoclostridium.* The positive correlations indicate that as abundance of the genera taxa increases the serum concentrations of TMAO.

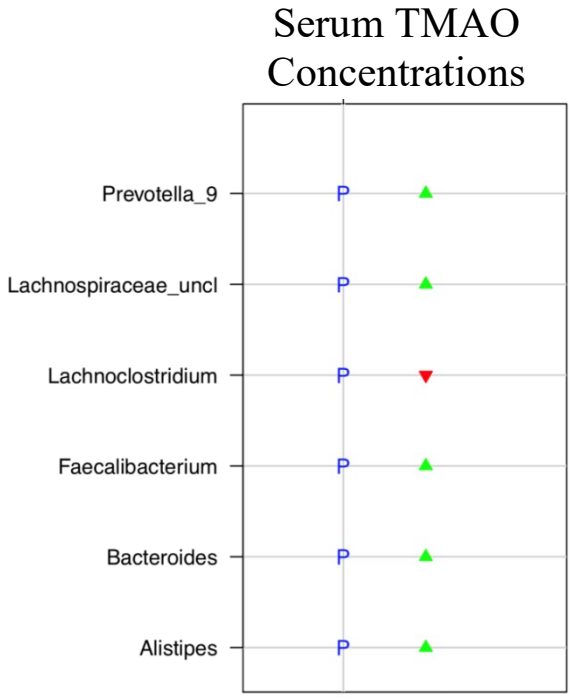


Figure 27. CKD Gut Microbiota and Genera Taxa as Predictors of TMAO Concentrations.

Changes in genera taxa abundance may predict TMAO concentrations. These correlations are based on the univariate linear regression and corresponding estimated values of coefficients (genera taxa) within the statistical model (Figure 25). For example, serum TMAO concentrations increase as *Prevotella_9* abundance increases. Alternatively, as abundance of *Lachnoclostridium* increases serum TMAO concentrations decrease.

5.4 **DISCUSSION**

The study demonstrates that the gut microbiome is altered in Stage 3-4 CKD patients and is directly correlated with and predictive of gut-derived toxin exposure. The gut microbiome was known to be altered in humans with ESKD [25, 203-205], but limited evidence existed about microbiome alterations in Stage 3-4 CKD and the systemic exposure of gut-derived toxins [199, 207]. Although diindolymethane did not significantly reduce TMAO concentrations in the study population, exploratory analyses suggest it may reduce TMAO concentrations in male CKD patients with high TMAO concentrations compared to females. Collectively, the findings provide insights into the altered CKD gut microbiome and novel strategies to reduce gut-derived toxin exposure.

Although diindolymethane treatment period compared to the placebo period had no significant effect in the total cohort, male CKD patients with high baseline TMAO concentrations had reductions in serum TMAO after diindolylmethane treatment compared to females. The differences in the treatment effect between males and females are displayed in Figure 21. Males had an 8 μM average reduction in serum TMAO concentrations after diindolylmethane treatment. The underlying reason for this sex difference is unclear, but sex differences in the regulation of FMO expression may have contributed to the findings. Specifically, the effect of sex and potential testosterone suppression of FMO3 expression is most pronounced in male mice [215]. In fact, in reviewing data from Figure 7 in Chapter 3, an Eadie-Hofstee transformation of the FMO activity

data demonstrates the contributions of a high affinity (i.e., FMO3) and low affinity (FMO1) enzymes to TMAO formation in mice (Figure 28). The contributions of only the low affinity FMO1 enzyme are observed in the non-CKD male mice. CKD seemed to enhance the activity of FMO3 in both males and females, to the point that the high affinity FMO3 appears to be the only isoform contributing to TMAO formation. Similar predispositions are observed in humans, as males also have lower FMO3 gene and protein expression compared to females, suggesting underlying differences in regulation [145, 146]. Serum TMAO concentrations have been found to be higher in males than females [216] and a higher proportion of males have increased serum TMAO concentrations in CKD [10]. It is possible that these elevated TMAO concentrations contribute to the higher mortality in men with Stage 3-4 CKD compared to females [217]. Testosterone suppresses *FMO3* expression and concentrations are low in males with CKD, which may also contribute to sex differences in the response to diindolylmethane [218]. Therefore, sex differences may partially explain the treatment effect only observed in males because diindolylmethane may work by decreasing FMO expression and there may be inherent differences in *FMO3* regulation [198]. Additionally, we did not measure serum diindolylmethane concentrations and there may be differences in exposure between males and females. Finally, the sample size of males (n = 7) and females (n = 6) who received diindolylmethane is relatively small, so this finding requires validation in larger cohorts.

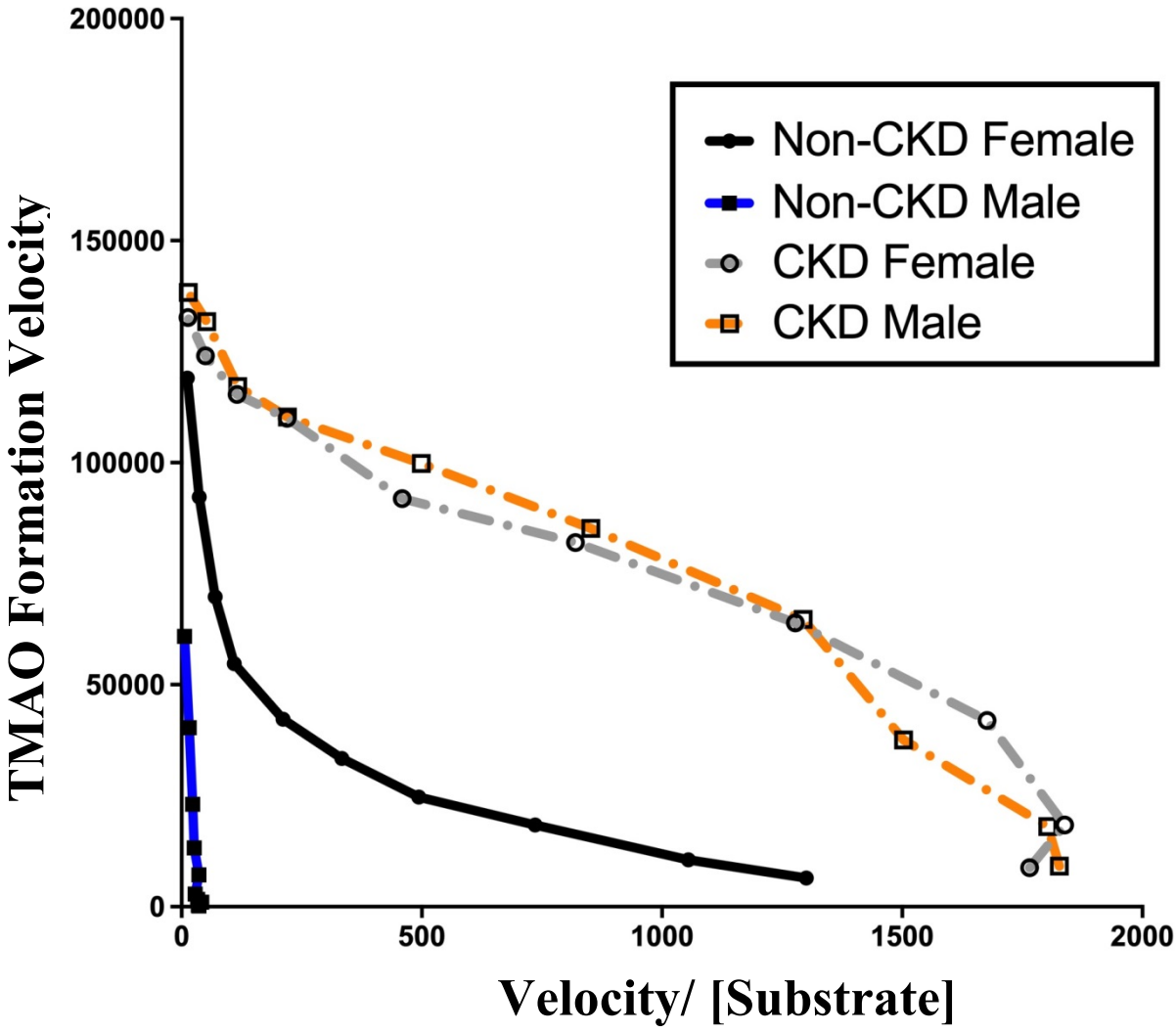


Figure 28. Eadie-Hofstee Transformation of FMO-mediated TMAO Formation in Mice.

Eadie-Hofstee transformation of FMO activity data presented in (Figure 7) to examine the contributions of FMO1 and FMO3 to TMAO formation in CKD. The lines are color coded to represent the different groups of mice.

The correlation analyses also identified some intriguing findings. The positive Pearson correlation with TMAO concentrations and indoxyl sulfate (0.628; $P = 0.0289$) supports the work presented in Chapter 4. The correlation provides potential clinical evidence to support the scientific premise that indoxyl sulfate increases TMAO formation. In brief, the high indoxyl sulfate concentrations may activate AhR-mediated *FMO3* expression and corresponding TMAO formation.

The findings also demonstrated significant increases in serum concentrations of gut-derived toxins long before the complete loss of kidney function (i.e., ESKD). The extent of GDUT accumulation is concerning because each of these toxins are associated with and may accelerate cardiovascular disease [8, 121, 124-126]. The exposure of some of these toxins is also disproportionate to the corresponding decreases in kidney function. For example, p-cresol sulfate increased by almost 23-fold compared in Stage 4 CKD compared to healthy controls (Table 13). Comparatively, there is only a corresponding 80% reduction in kidney function (i.e., eGFR) in Stage 4 CKD compared to healthy controls. Therefore, decreased renal clearance may not fully explain increases in toxin concentrations. GDUTs primarily undergo renal elimination so they would be expected to increase linearly as kidney function decreases as defined by the Dettli drug dosing equation (Equation 1) [219]. Changes in k_{NR} would also be negligible because these compounds have limited hepatic elimination. Therefore, if you have an increase in exposure that is not fully explained by decreased clearance, there must be increases in microbiota production and/or hepatic formation of these GDUTs. Moreover, the findings in this Chapter suggest that increased microbiota production also contributes to GDUT exposure. We have also demonstrated two potential mechanisms for increased hepatic TMAO formation in Chapters 3 and 4. In a

related note, decreased tubular secretion may also partially contribute to the disproportionate increases in GDUTs [220].

$$k = k_{NR} + \alpha * \text{CrCl}$$

Equation 1. Dettli Nomogram

k, elimination rate constant based on a first-order one compartment model
k_{NR}, non-renal clearance constant
α, constant relating the renal drug elimination rate constant, kr, to the creatinine clearance
CrCl, endogenous creatinine clearance (mL/min)

Another important finding of this study is that the microbiota composition was significantly altered in Stage 3-4 CKD compared to control. The findings suggest that as kidney disease progresses the microbiota is simultaneously changing, and the diversity of genera taxa decreases. The alterations and changes to specific genera taxa provide fundamental knowledge for patients with Stage 3-4 CKD. Alterations were better understood in ESKD [25] but our collective knowledge about the microbiome in earlier stages was previously limited [199, 207]. The statistical modeling of microbiota as a predictor of gut-derived toxins provides evidence that the microbiota alterations have functional implications. Investigations into the microbiota and the bidirectional relationships were previously conducted for TMAO [216]. These previous analyses are nicely complemented by our study findings that expand beyond TMAO to include similar analyses of 5 additional gut-derived toxins. The microbiota alteration findings are facilitating future work to develop therapeutic strategies to reduce CVD events.

This study had some limitations. The sample size was limited but we identified significant alterations to the microbiome and strong influences on gut-derived toxin exposure. The findings should be verified in larger prospective studies. In addition, the 16S sequencing provides limited

insight into the microbiota function and these findings suggest that future metatranscriptomics analyses should be pursued in order to shed light on microbial function by measuring active gene expression. Another limitation is that serum diindolylmethane concentrations were not measured and this would be necessary to evaluate if the twice daily dosing regimen achieved concentrations that would adequately inhibit FMOs. For instance, the 3-hour half-life of diindolylmethane may have required 3-4 doses daily to achieve steady state concentrations that would provide sustained FMO inhibition. Finally, the unexpected findings of the sex-dependent effect of diindolylmethane may not be clinically significant but further translational or clinical analyses can interrogate the underlying mechanism(s).

5.5 **CONCLUSION**

The study findings provide evidence that Stage 3-4 CKD patients have altered gut microbiomes compared to healthy patients. The microbiota alterations and specific genera taxa were correlated with and predicted to gut-derived toxin concentrations suggesting the microbiota contributes to the high systemic exposures of these non-traditional CVD risk factors. Characterizing changes in specific genera taxa improved our understanding of the altered CKD microbiota and influence on gut-derived toxins which may guide future therapeutic strategies. Diindolylmethane administration did not reduce serum TMAO concentrations in the total population but in an exploratory analysis, males had significant reductions compared to females during the treatment phase. Finally, therapeutic strategies to reduce gut-derived toxins are needed to decrease CVD outcomes, especially in earlier stages of CKD.

CHAPTER 6: CONCLUSIONS AND FUTURE DIRECTIONS

The objective of this dissertation was to evaluate the effect of kidney disease and gut-derived uremic toxins on flavin monooxygenase function. This was achieved by 1) development and validation of a robust analytical method to measure a panel of four gut-derived uremic toxins in human serum, 2) assessing the effect of kidney disease and uremic solutes on flavin monooxygenase expression, activity, and metabolic activation, 3) investigating the effect of indoxyl sulfate on flavin monooxygenase expression and activity, 4) evaluating the effect of the gut microbiome and diindolylmethane on gut-derived toxin exposure in a pilot study of patients with kidney disease. The individual chapter summaries and key findings, clinical implications, limitations and future directions are discussed in the following sections.

6.1 SUMMARY AND KEY RESEARCH FINDINGS

6.1.1 Development and Validation of a Robust LC-MS/MS Method to Measure GDUTs

The development and validation of LC-MS/MS method to measure kynurenic acid, hippuric acid, indoxyl sulfate and p-cresol sulfate is described in Chapter 2. These compounds accumulate to high concentrations in KD, as observed in our study patients in Chapter 5 (Table 13).

GDUTs contribute to the uremic syndrome and are gaining attention as potentially modifiable cardiovascular disease risk factors in patients with underlying chronic kidney disease. A simple, rapid, robust, accurate and precise ultra-performance liquid chromatography–tandem mass spectrometry method was developed and validated for the simultaneous determination of a

panel of four gut-derived uremic toxins in human serum. The panel was comprised of kynurenic acid, hippuric acid, indoxyl sulfate, and p-cresol sulfate. Serum samples were protein precipitated with acetonitrile containing deuterated internal standards. Chromatographic separation of analytes was accomplished with an Acquity BEH C18 (2.1 × 100 mm, 1.7 µm) column by isocratic elution at a flow rate of 0.3 mL/min with a mobile phase composed of solvent A (10 mM ammonium formate; pH 4.3) and solvent B (acetonitrile) (85:15, v/v). Analytes were detected using heated electrospray ionization and selected reaction monitoring. The total run-time was 4 minutes. Standard curves were linear and correlation coefficients (r) were ≥0.997 for concentration ranges of 0.01-0.5 µg/mL for kynurenic acid, 0.25-80 µg/mL for p-cresol sulfate, and 0.2-80 µg/mL for hippuric acid and indoxyl sulfate. Intra- and inter-day accuracy and precision were within 19.3% for the LLOQs and ≤10.9% for all other quality controls. Matrix effect from serum was <15% and recovery was ≥81.3% for all analytes. The method utilizes a short run-time, simple/inexpensive sample processing and has passed FDA validation recommendations. The method was developed to specifically support the work in Chapter 5 and to facilitate future work to reduce systemic GDUT concentrations in patients with KD.

6.1.2 **Metabolic Activation of FMO-mediated TMAO Formation in Experimental Kidney Disease**

Cardiovascular disease (CVD) remains the leading cause of death in chronic kidney disease (CKD) patients despite treatment of traditional risk factors, suggesting that non-traditional CVD risk factors are involved. Serum TMAO concentrations correlate with atherosclerosis burden in CKD patients and may be a non-traditional CVD risk factor. Serum TMAO concentrations are

significantly increased in CKD patients, which may be due in part to the increased hepatic flavin monooxygenase (FMO)-mediated TMAO formation. The objective of this work was to elucidate the mechanism of increased FMO activity in CKD. In this study**,** FMO enzyme activity was evaluated *in vitro* with liver microsomes isolated from experimental CKD and control rats. Trimethylamine was used as a probe substrate to assess FMO activity. The effect of FMO activator octylamine and human uremic serum were evaluated on FMO activity. FMO gene and protein expression were also determined. FMO-mediated TMAO formation was increased in CKD versus control. Although gene and protein expression of FMO were not changed, metabolic activation elicited by octylamine and human uremic serum increases FMO-mediated TMAO formation. The findings suggest that metabolic activation of FMO resulting in increased TMAO formation is a novel mechanism that contributes to increased TMAO formation in CKD and FMOs represent a therapeutic target to reduce TMAO exposure and CVD.

6.1.3 **Effect of Indoxyl Sulfate on Hepatic Gene Expression and FMO-mediated TMAO Formation**

Patients with kidney disease are at high risk for cardiovascular disease and investigating non-traditional risk factors may lead to better treatments. Indoxyl sulfate and TMAO are strongly associated with cardiovascular disease and may be modifiable non-traditional risk factors. Indoxyl sulfate may interact with several hepatic drug metabolism enzymes, such as FMOs. This interaction with FMOs may be a potential mechanism to explain our recent findings that FMO-mediated TMAO formation is increased in experimental kidney disease. In this study, primary

cultures of human hepatocytes from 3 donors were exposed to clinically relevant concentrations of indoxyl sulfate. The objective was to investigate the effect of indoxyl sulfate on the expression of hepatic drug metabolizing enzymes (i.e., *FMO3*) and transporters. We assessed the expression of 53 genes with innovative Nanostring technology and 4 genes were assessed by reverse transcriptase quantitative polymerase chain reactions, after exposure to indoxyl sulfate. Additionally, FMO-mediated TMAO formation was used as a measure of FMO3 protein function. Dose/exposure-dependent expression changes were observed for *FMO3, CYP1A2, AHR, NFE2L2, ABCG2, CYP24A1, GC,* and *UGT1A4*. In fact, *FMO3* mRNA expression was increased up to 6-fold ($P < 0.0001$) and FMO-mediated TMAO formation was also increased up to 3-fold ($P < 0.0001$) compared to control. This study demonstrates that indoxyl sulfate perpetrates interactions with drug metabolism enzyme and transporter pathways in primary human hepatocytes. Furthermore, indoxyl sulfate increases *FMO3* expression and FMO-mediated TMAO formation.

6.1.4 **Targeting FMO-mediated TMAO Formation and Investigating the Microbiome in Stage 3-4 Kidney Disease: A Pilot Study**

The human gut microbiome and gut-derived toxins contribute to cardiovascular disease in patients with kidney disease. Increased production of gut-derived toxins in kidney disease is theorized but there is limited clinical evidence. Here, we conducted a prospective, randomized, double-blinded, crossover study to investigate a novel strategy reduce formation of TMAO in Stage 3-4 kidney disease patients with diindolylmethane. The second aim was to evaluate gut microbiota alterations in earlier stages of kidney disease. The gut-derived toxins TMAO, choline,

kynurenic acid, hippuric acid, indoxyl sulfate and p-cresol sulfate and corresponding intestinal microbiome profiles were measured. CKD microbiome profiles were compared to healthy controls. Analyses evaluating associations between gut-derived toxins, covariates and bacterial families within the gut microbiome were conducted. The findings demonstrate that males had a significant reduction in serum TMAO concentrations in the diindolylmethane treatment phase compared to females (P = 0.02). The microbiota composition was altered in the CKD patients compared to control (P = 0.0042). Lower eGFR was associated with decreased diversity (P = 0.0182). The microbiota also predicted and responded to gut-derived toxin exposure as evidenced by several bacterial genera taxa being significantly correlated with the individual gut-derived toxins. The findings demonstrate the gut microbiota changes as kidney disease progresses and production of gut-derived toxins is likely increased. This may contribute to high concentrations of these non-traditional CVD risk factors in CKD patients. Finally, continuing to investigate gut microbiota alterations in early stage CKD will facilitate the development therapeutic strategies to minimize or prevent CVD. Future studies with diindolylmethane in male CKD patients may also be warranted.

6.2 **LIMITATIONS AND CLINICAL IMPLICATIONS**

A major strength of this work is that the clinical and translational studies performed were designed to test biologically plausible mechanisms of increased FMO activity in kidney disease.

This translational approach may bridge the gap between the bench and the bedside. The limitations will be summarized below along with the clinical implications for each chapter.

The LC-MS/MS method for measuring the panel of gut-derived uremic toxins is robust and reproducible. However, the method did not include additional uremic toxins that are also associated with CVD such as TMAO, 3-carboxy-4-methyl-5-propyl-2-furanpropanoic acid (CMPF), and indole-3-acetic acid. Inclusion of additional uremic toxins would have enhanced the utility of the method to investigate systemic exposures of these other compounds. During method development we encountered challenges that limited the selection of toxins in the panel. For example, TMAO was not retained on the C18 column that was most suitable for the majority of the other uremic toxins and would elute in the solvent front. This was not ideal, and it was decided to continue to use our previously validated method to separately measure TMAO with excellent accuracy and precision <10% [40], instead of incorporating it in the panel. Importantly, the current method allows for accurate and precise measurement of serum concentrations of kynurenic acid, hippuric acid, indoxyl sulfate, and p-cresol sulfate. The clinical implications of this study are already being realized. For example, the method has allowed us to measure these GDUTs in our clinical pilot study presented in Chapter 5. The method will continue to support preclinical and clinical studies evaluating the microbiome and interventions to reduce gut-derived toxin exposure.

There were limitations in the FMO metabolic activation study. The metabolic activation was observed with healthy and uremic human serum samples. Activation was not assessed with serum from other donors. In addition, we did not assess the microbiome and if potential differences in genera taxa were associated with altered TMAO formation. Post-translational modifications like phosphorylation could affect enzyme activity and this was not evaluated for the

proteins isolated from the experimental animal model. Conducting the metabolic activation experiments with human recombinant FMO3 enzymes would have also verified that activation occurs in human FMO3 enzymes. It is also possible that the potential effector compounds may be derived from dietary nutrients that were not monitored or controlled in the serum donors. Lastly, there are hundreds of known uremic retention solutes that accumulate in CKD and the potential effector compounds that may contribute to the metabolic activation of FMOs remain unclear.

The discovery that metabolic activation is responsible for increased FMO-mediated TMAO formation is a novel contribution of this work. This metabolic activation may contribute in part to the significantly elevated serum concentrations of TMAO in patients with kidney disease and has relevance to the accelerated CVD in this population. Collectively, the findings elucidate a biologically plausible mechanism that enhances TMAO formation and may have direct clinical implications for patients with KD.

The *ex vivo* studies assessing the effect of the gut-derived toxin indoxyl sulfate on hepatic gene expression and FMO-mediated TMAO also has limitations. For instance, the Nanostring analysis was likely underpowered to detect differences in gene expression after exposure to indoxyl sulfate due to the limited sample size of three hepatocyte donors. Although, the three hepatocyte donors met FDA recommendations for induction studies, so the sample size was appropriate for the primary aim of the study [189]. Future studies are also needed to evaluate if GDUTs are ligands for other transcription factors such as constitutive androstane receptor (CAR) or pregnane X receptor (PXR). In addition, the Nanostring and RT-qPCR findings only describe gene expression and further validation at the protein level is needed.

In regard to clinical implications, this work provided another plausible mechanism for increased TMAO formation. Indoxyl sulfate accumulates to about 40-fold higher in advanced KD

compared to healthy controls [5]. These high concentrations were capable of increasing *FMO3* expression and overall FMO-mediated TMAO formation in primary cultures of human hepatocytes. The clinical implications of this finding are unclear because this interaction may or may not be mediated by AhR transcription factor signaling. Indoxyl sulfate actions in kidney disease patients would be complex because there are feedback mechanisms such as AhR repressors that limit excessive activation and aberrant signaling [176]. Furthermore, future work with luciferase reporter assays is needed to evaluate the presence of an AhR Response Element in the regulatory region of the human *FMO3* gene.

The limitations to the pilot clinical study included a small sample size, uncontrolled diets that could influence the microbiome and serum TMAO concentrations, and only analyzing the microbiota with 16S sequencing. FMO3 gene polymorphisms were also not tested within the study patients. Larger more controlled clinical studies with metatranscriptomics analyses should be conducted to verify and expand upon the pilot study findings.

There are also clinical implications to the clinical pilot study. Assessing the effect of the altered CKD microbiome and diindolylmethane on systemic exposure of GDUTs demonstrated the predictive nature of the microbiota on GDUT concentrations. The clinical implication is that the altered microbiota may contribute to increased gut-derived toxin concentrations and this suggests that additional studies into the gut microbiota will improve our understanding of when in CKD progression bacterial communities are changing. The other intriguing finding was the reductions in serum TMAO concentrations in only male patients during the diindolylmethane treatment phase compared to females. The 8 μM average reduction in serum TMAO concentrations in males may not provide clinically significant reductions in CVD outcomes.

6.3 **FUTURE DIRECTIONS**

In this dissertation, we demonstrated that FMO activity is increased and may contribute to high systemic exposures of the CVD risk factor TMAO in kidney disease. Gut microbiota alterations in kidney disease also likely increase the production of other gut-derived uremic toxins. Gut-derived toxins accumulate in advancing stages of kidney disease and directly affect drug metabolizing enzymes, drug transport pathways, and CVD progression. Gut-derived toxin research in kidney disease presents unique opportunities to identify potential therapeutic targets to slow the accelerated rates of CVD.

To date, our findings demonstrate that FMO-mediated TMAO formation is increased in rat and mouse models of KD, suggesting FMO activity is likely increased in patients with kidney disease. Furthermore, we discovered two potential mechanisms including metabolic activation and indoxyl sulfate-mediated increases in FMO functional expression. In particular, metabolic activation with human uremic serum indicates that uremic solutes are potential effector compounds. Collectively, the findings provide evidence and a scientific rationale to investigate increases in FMO activity and other mechanisms of increased TMAO exposure in patients with kidney disease. Potential clinical studies include using a phenotypic probe of FMO activity such as ranitidine or *d*9-trimethylamine in kidney disease compared to healthy control patients. In fact, FMO-mediated formation of *d*9-TMAO and/or ranitidine-*N*-oxidation has been successfully used in prior clinical studies to assess FMO activity [7, 221]. Alternatively, similar *in vivo* studies in an experimental CKD animal model compared to controls will further evaluate potential changes in FMO activity. The effect of decreases in renal clearance on serum metabolite concentrations will necessitate the use of formation rate estimates. Formation rates for renally cleared metabolites are

calculated by the total amount of metabolite in urine divided by the AUC of the parent compound in serum. This calculation assumes that the metabolite (e.g., *d*9-TMAO) is excreted unchanged in the urine. These proposed *in vivo* FMO activity assessments will build of the foundation of the present work and provide insights into FMO as a novel therapeutic target in KD.

Translational and mechanistic studies to evaluate metabolic activation of FMO activity are also an immediate future direction of this work. Studies similar to those presented in chapter 3 should be done with recombinant human FMO3 enzymes to overcome species differences. Evaluating intrinsic clearance will also provide a more comprehensive kinetic assessment of the net effects of changes to V_{max} and K_m parameters. Furthermore, recombinant FMO3 enzymes should be used moving forward in these mechanistic studies. A roadmap towards future mechanistic studies is detailed in work on CYP2C9 activation by Tracy and colleagues. Mechanistic studies to evaluate the FMO-substrate-effector interactions may include using probe atomic force microscopy [222], NMR and spectral binding analyses [223, 224], and/or virtual chemical library screens to identify effector compounds [225]. This work may shed light on the observed changes in K_m with the metabolic activation in Chapter 3. Additionally, identifying potential endogenous activators/effectors with virtual library screens would allow for additional metabolic activation experiments to validate the selected effectors. In fact, we speculate that other intermediates in the urea cycle like L-arginine (Figure 12B) may be involved [226]. TMAO is present in high concentrations in fish to counteract the protein destabilization effect of urea. We propose that high urea concentrations in KD may cause intermediates of the urea cycle to act as effectors and increase metabolic activation of FMO in order for TMAO to counteract the deleterious effect of urea in KD patients. Collectively, identification of other effectors and

mechanistic assessments are pertinent future directions to expand upon this discovery of metabolic activation FMO-mediated TMAO formation.

FMOs may also be a therapeutic target to lower overall TMAO formation. Therapeutic strategies to inhibit FMO activity should be evaluated in future studies. In Chapter 5, we assessed the use of diindolylmethane as a potential inhibitor for FMO-mediated TMAO formation. There was no significant difference in serum concentrations of TMAO in the treatment period compared to placebo treatment periods. This particular strategy in the total patient cohort was ineffective possibly due to non-optimal dose selection and the serum concentrations of diindolylmethane should be measured to determine that the concentrations are high enough to achieve the desired FMO inhibition. The half-life of diindolylmethane is approximately 3 hours, and the daily dosing in the clinical study may have not been sufficient to maintain concentrations that could inhibit FMOs. Future studies should evaluate diindolylmethane dosing regimens to ensure adequate exposures in both males and females. Other strategies should be considered from pharmacological inhibition to some oligonucleotide approaches that have lowered serum TMAO concentrations in mice [149]. Future work is necessary both in translational studies using recombinant human FMO3 enzymes, and in prospective clinical studies. Indeed, there are still reasonable debates about whether TMAO is a CVD risk factor [227]. Ultimately, to prove that TMAO exposure predicts CVD risk and causes CVD, clinical trials are needed to assess if interventions that reduce TMAO concentrations lower CVD outcomes.

Additional biomarkers should also be evaluated in the clinical pilot study. Future directions include comparing serum concentrations of Zonulin and LPS, biomarkers of intestinal permeability, in CKD and healthy controls. This would provide additional evidence of CKD patients having disrupted intestinal wall tight junctions that facilitate increased translocation of

bacterial byproducts such as LPS [54, 228, 229]. As such, the issue of gut permeability in CKD may be a therapeutic target. Strategies to reduce gut permeability may lower the absorption of multiple GDUTs and other microbial byproducts.

There is also a gap in our knowledge about the clinical effect of the gut microbiota and gut-derived toxins on drug disposition. The comprehensive review in Chapter 1 provides evidence that gut-derived toxins perpetrate interactions with drug metabolism enzymes and drug transporters. In this era of personalized medicine, the gut microbiome must not be overlooked as a source of inter-individual variations in drug exposure and response. As such, systematic research is needed to investigate the effect of the altered gut microbiota on drug disposition in patients with kidney disease. In fact, microbiota alterations and increased gut-derived toxins may provide a mechanistic explanation for decreases in drug transporter function in patients with kidney disease [14]. This hypothesis can be tested in future *in vitro* and *in vivo* studies investigating gut-derived toxins effect on transporters such as OATPs. In future clinical studies, phenotypic probes of transporter function such as fexofenadine and/or olmesartan can be used along with characterization of the gut microbiome and gut-derived toxins. Determining if the gut microbiome and gut-derived toxins explain variations in the pharmacokinetics of these phenotypic probes would provide clinical evidence of potential mechanisms to altered non-renal clearance in kidney disease.

The findings also demonstrate that future research is warranted to investigate the effect of CKD progression on the gut microbiota and the clinical implications for CVD. Future studies will expand upon the current work to understand alterations to the gut microbiota in CKD and identify therapeutic targets. Human microbiome research is quickly progressing through discovery phases and investigators are poised to test clinical interventions in CKD patients. Prospective studies

should evaluate the gut microbiota across the spectrum of kidney function from healthy patients to end-stage kidney disease patients. Information about the changes in the gut microbiota will advance our understanding and guide future interventions. For instance, the exposure to some of these gut-derived toxins in end-stage kidney disease may oversaturate their harmful CVD effect [230]. As such, future microbiota research should focus on identifying therapeutic targets to lower CVD in earlier stages of CKD.

The gut microbiome is not only a therapeutic target, but also a potential source of novel therapeutic compounds to improve human health. Intestinal microorganisms generate unique compounds to communicate amongst community members and with human systems; these unique compounds might be a source for novel drug discoveries. For instance, from a historical perspective several antimicrobials have been discovered in unique ecological niches [231]. Future research will also focus on positive intestinal microbiome-host interactions. For example, there will be increases in discussion of the benefits of gut-derived short chain fatty acids [232]. The long-term therapeutic objective will be to harness the beneficial effects and limit the harmful attributes of our microbiomes to improve human health.

6.4 **CONCLUSION AND COMMENTARY**

The gut microbiota directly contributes to the accelerated CVD in patients with kidney disease. In this work, we focused on the non-traditional CVD risk factor TMAO. The major aim was to mechanistically evaluate the effect of gut-derived toxins and kidney disease on FMO-mediated TMAO formation. Collectively, the work provided insights into increased FMO activity

and also advanced our understanding of the gut microbiota in earlier stages of kidney disease. There are several highlights of this dissertation. First, we developed and validated a simple, rapid and robust UPLC-MS/MS method for the simultaneous determination of non-traditional CVD risk factors. Next, we demonstrated for the first time that metabolic activation of hepatic FMOs increases formation of the non-traditional CVD risk factor TMAO. We also discovered that indoxyl sulfate increases *FMO3* expression and subsequent TMAO formation. Finally, in a clinical pilot study we demonstrated that diindolylmethane reduced serum TMAO concentrations in males compared to females and that the altered CKD gut microbiota predicts the exposure of non-traditional CVD risk factors. In conclusion, the gut microbiota is a promising area for future research and innovate strategies are needed to slow CVD progression in earlier stages of kidney disease.

Appendix A ABBREVIATED IRB APPROVED PROTOCOL

Title of study: Targeting FMO-Mediated TMAO Formation in Kidney Disease [TMAO Study]

Short title: TMAO Study

Brief description:

Objective: What is the overall purpose of this research study?
The goal of this preliminary study is to identify a novel strategy to inhibit FMO- mediated TMAO formation in patients with kidney disease. We also seek to understand the contributions of the intestinal microbiota to microbial toxin exposure in this population.
Specific Aims: List the goals of the proposed study

Aim 1: To evaluate the effect of FMO inhibition on systemic exposure of TMAO in humans with kidney disease. Twelve patients with moderate kidney disease will receive a dietary diindolylmethane (DIM) nutraceutical (inhibitor) for four weeks. TMAO concentrations will be quantified at various study time-points. We predict a sustained reduction of systemic TMAO exposure while patients receive the FMO inhibitor. This will be the first study to evaluate this strategy to target TMAO formation.

Aim 2: To evaluate the contributions of the intestinal microbiota to the overall exposure to microbial toxins in patients with kidney disease. We will take microbiome samples at each visit over the course of twelve weeks in order to assess the relationship between intestinal microbial communities and microbial toxin exposure in serum. We predict that dysbiotic bacteria in the intestines will be correlated with differences in microbial toxin exposure.

We hypothesize that DIM can safely decrease serum TMAO in patients with kidney disease. We will evaluate the ability of DIM to reduce serum TMAO concentrations. Twelve patients with Stage 3 or 4 kidney disease (estimated glomerular filtration: 15-60 ml/min) will receive DIM. The primary endpoint will be a reduction of serum TMAO. We will also monitor common safety laboratory values to ensure the health and safety of the volunteers. We predict that DIM will be an inhibitor of FMO formation of TMAO. This may result in a safe lowering of the atherosclerosis risk factor TMAO in patients with kidney disease. Patients will also serve as their own controls for a 4-week treatment period either before or after DIM supplementation. This will occur after a 4-week washout between treatment periods. The reason for this crossover design is to control for high interpatient variability of TMAO concentrations and also to make study recruitment more feasible.

We are also interested in the contributions of the intestinal microbiota to the overall health of patients with kidney disease. Understanding these contributions is currently limited and we propose to collect stool from kidney disease patients to characterize the intestinal microbial communities. We will also quantify microbiota- derived toxins in the patient's serum. Linking and understanding the microbial communities to overall toxin exposure might contribute to improving our scientific knowledge in order to advance future research.

Background: Briefly describe previous findings or observations that provide the background leading to this proposal.

Trimethylamine-*N*-oxide (TMAO) has been identified as a pro-atherosclerotic metabolite of dietary nutrients. Dietary sources of precursors to TMAO (e.g. phosphatidylcholine, L-carnitine, and betaine) are metabolized by intestinal microbiota to trimethylamine and subsequently FMO enzymes metabolize trimethylamine to TMAO. In clinical studies, TMAO has been associated with increased risk of cardiovascular disease (CVD) events. Graded increases in serum levels of TMAO are correlated with higher rates of CVD events. Kidney disease is staged according to estimated glomerular filtration rate (eGFR) and TMAO concentrations are found to be incrementally increased in progressing stages of kidney disease. Data from our laboratory has shown that patients with stage IIIa to IV/V kidney disease and hemodialysis patients have significantly elevated median TMAO concentrations compared to healthy control patients. In these kidney disease patients, the highest concentrations of TMAO are associated with the lowest overall survival. This data suggests that TMAO is an important potentially modifiable proatherogenic risk factor in kidney disease patients. An integral component to overall TMAO exposure is FMO activity. FMO expression has also found to be upregulated in insulin resistant diabetes patients because insulin is a negative regulator of FMO. Diabetes is a leading cause of kidney disease and therefore FMO activity could be dysregulated in a significant number of patients with kidney disease. Preliminary data in our laboratory has also identified a 26% increase in FMO functional activity in experimental kidney disease.
This evidence of increases in FMO activity is an important finding that suggests an increased importance in pursuing FMO as a target to reduce TMAO formation. Diindolylmethane (DIM) has previously been identified as a potential inhibitor of FMO activity. We seek to investigate the use of DIM to reduce FMO-mediated formation of TMAO in this clinical study.

Significance:
Our pilot study will evaluate the effect of DIM on the FMO enzyme system. There are gaps in our knowledge about the role of FMO activity in kidney disease. This will inform future research on strategies to target FMO activity to potentially reduce proatherogenic TMAO concentrations.
We have identified a clinically tested formulation of DIM that has been proven safe in clinical trials up to levels that would safely inhibit the formation of TMAO. (PMID: 25025957, 25613194, 15623462, 20733950, 27069550, 26313229, 18843002, 22075942) Single dose escalation studies in 6 healthy female patients with this product and reported tolerable safety data up to 300mg of DIM. This group has also published single dose and multi-dose study of the DIM precursor indole-3-carbinol in healthy female patients using DIM as their pharmacokinetic plasma concentration analyte. A Phase 1 dose escalation study in 12 males found 225mg BID to be the

maximum tolerated dose of DIM. Overall, the plethora of clinical studies using DIM suggests that the novel use of DIM in kidney disease is safe and may inhibit FMO formation of TMAO.
In addition, there are gaps in our knowledge about the contributions of intestinal microbiota to the health of patients with kidney disease. This study will research these contributions.

Principal investigator: Thomas Nolin
What kind of study is this? Single-site study
Indicate all sources of support: Internal funding and external funding.
Identify each organization supplying funding for the study: American College of Clinical Pharmacy

Study Team Members

Thomas D. Nolin, PharmD, PhD, will serve as the Principal Investigator. He is an Associate Professor at the University of Pittsburgh School of Pharmacy and NIH-funded investigator. Dr. Nolin has led several clinical trials, including PK/PD studies in patients with kidney disease, and has extensive experience in the development and use of various quantitative analytical techniques. He will oversee the scientific portions of this research, including the conduct of the clinical research activities, quantitative analysis of TMAO, and the corresponding analyses, data review and interpretation of the results

Alexander J. Prokopienko, PharmD, will serve as a Co-Investigator and Study Coordinator. He has more than 7 years of clinical experience in nephrology clinical research and is a currently a Clinical and Translational Research Postdoctoral Fellow and a PhD Candidate at the University of Pittsburgh School of Pharmacy. Dr. Prokopienko will lead the clinical aspects of this research as the study coordinator, including subject recruitment, screening and evaluation, and assessment of the patient safety. He will also conduct the primary analysis and interpretation of all study findings.

Evan C. Ray, MD, PhD is an Assistant Professor of Medicine at the University of Pittsburgh School of Medicine. He is an academic nephrologist that sees patients in the Renal-Electrolyte Division. He has extensive experience in clinical research. He will participate in the recruitment of subjects and review of any safety laboratory results.

Alison Morris, MD, MS, is a Professor of Medicine, Immunology, and Clinical and Translational Research, the Director of the Center for Medicine and the Microbiome, the Vice Chair for Clinical Research in the Department of Medicine, and the UPMC Chair in Translational Pulmonary and Critical Care Medicine. As the Director of the Center for Medicine and the Microbiome, she works with collaborators in diverse areas studying the microbiome. Dr. Morris has extensive experience conducting NIH-funded clinical and translational research.

Barbara Methé, PhD, is a Visiting Professor of Medicine and the Co-Director for Basic Science in the Center for Medicine and the Microbiome. As part of this role, she works with collaborators in diverse areas studying the microbiome. Dr. Methé has extensive knowledge and experience with science and analytical techniques to assess the human microbiome.

Have you, Thomas Nolin**, verified that all members of the research team have the appropriate expertise, credentials, training, and if applicable, child clearances and/or hospital privileges to perform those research procedures that are their responsibility as outlined in the IRB application? Yes**

Study Scope

Research Sites
Choose all sites that apply: UPMC Clinical and Translational Research Center

Select the UPMC sites where research will be conducted: Presbyterian

Select the CTRC sites where research will be conducted:
Montefiore Hospital Clinical and Translational Research Center (MUH-CTRC)

Describe the availability of resources and the adequacy of the facilities to conduct this study:

We will be recruiting patients mainly from outpatient UPMC kidney disease clinics. We have standing collaborations with physicians in these clinics and we are currently conducting another clinical study with these same physicians. These clinics routinely see enough patients that would be eligible for our study.
We will also utilize the MUH-CTRC for blood draws and patient visits. This is a state- of-the-art research facility that has excellent clinical research experience, staff and resources. We are currently also using this excellent facility for ongoing research. Therefore, we have standing professional relationships with the administrators and research personnel.

Study Aims
Describe the purpose, specific aims, or objectives and state the hypotheses to be tested:
Specific Aims:

Aim 1: To evaluate the effect of FMO inhibition on systemic exposure of TMAO in humans with kidney disease. Twelve patients with moderate kidney disease will receive a dietary diindolylmethane nutraceutical (inhibitor) for four weeks. TMAO concentrations will be quantified at various study time-points. We predict a sustained reduction of systemic TMAO exposure after patients receive the FMO inhibitor. This will be the first study to evaluate this strategy to target TMAO formation.

Aim 2: To evaluate the contributions of the intestinal microbiota to the overall exposure to microbial toxins in patients with kidney disease. We will take microbiome samples at each visit over the course of twelve weeks in order to assess the relationship between intestinal microbial communities and microbial toxin exposure in serum. We predict that dysbiotic bacteria in the intestines will be correlated with differences in microbial toxin exposure.

We hypothesize that diindolylmethane (i.e. nutraceutical) can safely decrease serum TMAO in patients with kidney disease. We will evaluate the ability of DIM to reduce serum TMAO concentrations. Twelve patients with Stage 3 or 4 kidney disease (estimated glomerular filtration: 15-60 ml/min) will receive DIM. We plan to inhibit FMO formation of TMAO to provide sustained reduction of TMAO with this nutraceutical compound. After obtaining informed consent, patients will have a one-week run-in period to comply to study restrictions on potential confounding variables and then 4-week trial of 150mg of DIM twice daily. The primary endpoint will be a reduction of serum TMAO from baseline to the end of the study. We will also monitor common safety laboratory values to ensure the health and safety of the volunteers. We predict that DIM will be an inhibitor of FMO formation of TMAO. This will result in a safe lowering of the atherosclerosis risk factor TMAO in patients with kidney disease. Patients will also serve as their own placebo controls for a 4-week treatment period either before or after DIM supplementation. This will occur after a 4-week washout between treatment periods. The reason for this crossover design is to control for high interpatient variability of TMAO concentrations and also to make study recruitment more feasible.
We are also interested in the contributions of the intestinal microbiota to the overall health of patients with kidney disease. Understanding these contributions is currently limited and we propose to collect stool from kidney disease patients to characterize the intestinal microbial communities. We will also quantify microbiota-derived toxins in the patient's serum. Linking and understanding the microbial communities to overall toxin exposure might contribute to improving our scientific knowledge in order to advance future research.

Describe the relevant prior experience and gaps in current knowledge including preliminary data. Provide for the scientific or scholarly background for, rationale for, and significance of the research based on existing literature and how it will add to existing knowledge:
Cardiovascular disease (CVD) is one of the leading causes of death in the United States. In patients with kidney disease, CVD is the leading cause of death despite targeting traditional risk factors of atherosclerosis. This persisting CVD in patients with kidney disease has stimulated a search for modifiable non-traditional risk factors for CVD. Chronic kidney disease (CKD) is related to a progressive loss of kidney functions. In the general population, multiple prospective studies have identified that diets high in red meat are linked to increased CVD risk. Medical and scientific approaches have focused on high cholesterol levels as the most important modifiable biomarker for increasing CVD risk. Cholesterol lowering HMG-CoA reductase inhibitors or statins have effectively reduced overall incidence of CVD. However, there is a persisting high prevalence of major CVD events and CVD related mortality in CKD, further suggesting that additional non-traditional risk factors for CVD need to be explored for therapeutic intervention in patients with CKD. Trimethylamine-N-oxide (TMAO) is a potential modifiable risk factor of atherosclerosis. TMAO has been identified as a pro-atherosclerotic metabolite of dietary nutrients. Dietary sources of precursors to TMAO (e.g. phosphatidylcholine, L-carnitine, and betaine) are metabolized by intestinal microbiota to trimethylamine and subsequently flavin-containing monooxygenases (FMO) enzymes predominately metabolize trimethylamine to TMAO. In clinical studies, TMAO has been associated with increased risk of CVD events. Graded increases in serum levels of TMAO are correlated with higher rates of CVD events. CKD is staged according to estimated glomerular filtration rate (eGFR) and TMAO concentrations are found to

be incrementally increased in progressing stages of CKD. Data from our laboratory has shown that patients with stage IIIa to IV/V CKD and hemodialysis patients have significantly elevated median TMAO concentrations compared to healthy control patients. In these kidney disease patients, the highest concentrations of TMAO are associated with the lowest overall survival. This data is instrumental in highlighting the clinical relevance of TMAO as an important potentially targetable risk factor for CVD and mortality in CKD. Another integral component to overall TMAO exposure is FMO activity. FMO expression has also found to be upregulated in insulin resistant diabetes patients because insulin is a negative regulator of FMO. Diabetes is a leading cause of kidney disease and therefore FMO activity could be
dysregulated in a significant number of patients with kidney disease. Preliminary data in our laboratory has also identified a 26% increase in FMO functional activity in experimental kidney disease. This evidence of increases in FMO activity is an important finding that suggests an increased importance in pursuing FMO as a target to reduce TMAO formation.

Significance:
The link between DIM and FMO inhibition is established by a trial that was conducted with a glucosinolate treatment group with a 3-week treatment period with 300 grams of Brussel sprouts daily. The authors reported 2.6-3.2 times lower TMAO levels in the urine of patients after exposure to Brussel sprouts compared to their own baseline levels. Subsequent microsomal incubations identified the FMO inhibiting component of the Brussel sprouts as Dindolylmethane (DIM). DIM was identified as one of the most potent FMO inhibitors. Cruciferous vegetables including broccoli, Brussel sprouts, cabbage, and cauliflower contain these glucosinolates that become predominately DIM. We have identified a clinically tested formulation of DIM that has been proven safe in clinical trials up to levels that would safely inhibit the formation of TMAO. Single dose escalation studies in 6 healthy female patients with this product and reported tolerable safety data up to 300mg of DIM. This group has also published single dose and multi-dose study of the DIM precursor indole-3- carbinol in healthy female patients using DIM as their pharmacokinetic plasma concentration analyte. A Phase 1 dose escalation study in 12 males found 225mg BID to be the maximum tolerated dose of DIM. Overall, the plethora of clinical studies using DIM suggests that the novel use of DIM in kidney disease is safe and can be utilized effectively to inhibit FMO formation of TMAO.

Recruitment Methods
Will you be recruiting individuals for participation in this study? Yes

Describe who will be recruiting individuals for participation for this study: We will recruit patients that have been telephone screened or consented from previous studies and have agreed to be contacted about future studies.
We will also recruit patients from kidney disease clinics that are part of the UPMC system. Interested patients will be given a copy of the consent to read. The investigators and/or their research coordinator will carefully review the consent with each candidate before signature is obtained. All patients who meet eligibility criteria for the study will be asked to participate in the study. Written informed consent will be obtained from all study participants either by a licensed medical doctor, or the pharmacist-scientist investigators. The purpose of the study and its relevance to chronic kidney disease will be discussed with the patient in lay terminology.

Informed consent will be obtained prior to any screening study procedures being performed. Subjects will also be presented detailed information regarding the study at the time of screening so that they are able to assent to later participation. Patients will be asked to sign a consent form approved by the Institutional Review Board of the University of Pittsburgh. It will be emphasized to all study participants that the data collected will be for research purposes only, and that participation (or refusal to participate) in the investigation will have absolutely no effect on the patient's usual medical care. The person obtaining consent will inform the patient that there are no obligations to participate in the study and will provide his/her name and phone number where he/she can be reached if they have further questions or wish to withdraw from the study at any time.

Select all methods to be used for recruitment:
Flyers/Posters or Brochures
Pitt+Me
Telephone scripts

Describe all compensation/incentives offered to participants and timing of these offers:
Screening: $20
Day 1: $20
Week 4 (Day 28): $20 Week 8 (Day 56): $20
Week 12 (Day 84): $40
TOTAL COMPENSATION: $120

Study Design
Total number of subjects to be enrolled at this site (enter -1 for chart reviews, banking, registries): 35

Describe and explain the study design:
General classification: Experimental.
Methodological design: double-blind placebo-controlled crossover study.
Twelve patients with stage 3-4 CKD will be recruited. Patients serve as their own controls. Therefore, they will be randomly assigned to receive both study drug or matching placebo for four weeks with a four-week washout period in-between treatment phases. Patients will be randomly assigned to take DIM 150mg twice a day or matching placebo for four weeks and then have a four-week washout period followed by the other treatment allocation for 4 weeks. Patients will be recruited from outpatient kidney disease clinics. There will be 5 study visits, including the screening visit, over the course of 12 weeks.

Describe the primary and secondary study endpoints:
Primary endpoint is the change in TMAO concentrations.
Secondary endpoints include microbiome and microbiota-derived uremic toxin measurements

Provide a description of the following study timelines:
Duration of an individual subject's active participation: 12 weeks

Duration anticipated to enroll all subjects: 11 months

Estimated date for the investigator to complete this study (complete primary analyses): 5/31/2019

List the inclusion criteria:
1. Male or female subjects 18 years of age, but not more than 75 years of age at the time of enrollment.
2. Must be able to provide signed and dated informed consent.
3. Medical diagnosis of chronic kidney disease (eGFR $\leq$ 60 ml/min/1.73m^2)

List the exclusion criteria:
1. Vital signs outside of acceptable range at Screening Visit
2. Use of any of the following drugs within the last 4 weeks prior to the Day 1 appointment: Systemic antibiotics, antifungals, antivirals or antiparasitics (intravenous, intramuscular, or oral); corticosteroids (oral, intravenous, or intramuscular); cytokines; methotrexate or immunosuppressive cytotoxic agents; anti-diarrheal agents, bile acid sequestrants.
3. Recent changes in consumption of commercial probiotics including tablets, capsules, lozenges, chewing gum or powders in which probiotic is a primary component. Ordinary dietary components such as fermented beverages/milks, yogurts, foods do not apply.
4. Chronic, clinically significant hepatic abnormality (i.e. elevated 3X ULN ALT/AST), as determined by medical history or physical examination.
5. Recent history of cancer except for squamous or basal cell carcinomas of the skin that have been medically managed by local excision.
6. Unstable dietary history as defined by major changes in diet during the previous month, where the subject has eliminated or significantly increased a major food group in the diet.
7. Recent history of chronic alcohol consumption defined as more than five 1.5- ounce servings of 80 proof distilled spirits, five 12-ounce servings of beer or five 5- ounce servings of wine per day.
8. Any confirmed or suspected condition/state of immunosuppression or immunodeficiency.
9. History of active uncontrolled gastrointestinal disorders or diseases including: Inflammatory bowel disease (IBD) including ulcerative colitis (mild-moderate- severe), Crohn's disease (mild-moderate-severe), or indeterminate colitis; irritable bowel syndrome (IBS) (moderate-severe); persistent, infectious gastroenteritis, colitis or gastritis, persistent or chronic diarrhea of unknown etiology, Clostridium difficile infection (recurrent) or Helicobacter pylori infection (untreated); chronic constipation. Major surgery of the GI tract, with the exception of cholecystectomy and appendectomy, in the past five years. Any major bowel resection at any time. 10. Patient who may be pregnant or lactating.
11. Not willing to abstain from cruciferous vegetable (i.e. cabbage, brussels sprouts, garden cress, mustard greens, turnips, broccoli, collard greens, cauliflower, kale) consumption.
12. Current smoking.
13. Unwilling or unable to adhere to study procedures or instructions.
14. Patients taking any of the following medications, methimazole, alosetron, duloxetine, ramelteon, tasimelteon, theophylline, tizanidine, clozapine, pirfenidone and ramosetron.
15. Allergies to corn or soy.

Will children or any gender, racial or ethnic subgroups be explicitly excluded from participation? Yes

Identify the subgroups and provide a justification:
Children will be excluded. The intended population that may benefit from this research are adults with chronic kidney disease.

Describe the power analysis used and cite your method of statistical analysis. If a power analysis is not possible, thoroughly justify the sample size required for the study, including appropriate literature citation (alternatively provide page reference in attached protocol):
This is a pilot study and no information currently exist to inform any sample size calculations. However, we believe that the sample size of 12 subjects and the crossover design, which enables subjects to serve as their own controls, offers sufficient power to detect a difference in the primary outcome (TMAO concentrations), if DIM is indeed effective at lowering FMO function.

Research Activities

Provide a detailed description of all research activities (including screening and follow-up procedures) that will be performed for the purpose of this research study. This description of activities should be complete and of sufficient detail to permit an assessment of associated risks.
Provide a detailed description of all research activities (e.g., all drugs or devices; psychosocial interventions or measures) that will be performed for the purpose of this research study.
This description of activities should be complete and of sufficient detail to permit an assessment of associated risks.

All research activities:
Twelve patients with stage 3-4 CKD will be recruited. Patients serve as their own controls. Therefore, they will be randomly assigned to receive both study drug or matching placebo for four weeks with a four-week washout period in-between treatment phases. Patients will be randomly assigned to take DIM 150mg twice a day or matching placebo for four weeks and then have a four-week washout period followed by the other treatment allocation for 4 weeks. Patients will be recruited from outpatient kidney disease clinics. There will be 5 study visits over the course of 12 weeks. The screening visit will include IRB approved informed consent. The first study visits at the Clinical and Translational Research Center (CTRC) will include a blood draw and dispensing of a 4-week supply of DIM or placebo. The final visit will be at week 12 for a blood draw at the CTRC. The patients will also have labs conducted at each study visit. There will also be serum collected for TMAO concentrations, in addition to urine and stool collected at the study visits.

Personnel (by role) performing the procedures:
Study coordinator: Conduct any screening and recruitment in consultation with the study physician, will also oversee conduct of study visits and ensuring appropriate study sample collection.

Study Physician: Will order labs and study drug from IDS, in addition to overseeing the safety and well-being of the study participants.

Directors of Center for Microbiome and Medicine: Will participate in the processing and analysis of the microbiome in these patients.

Location of procedures:
UPMC outpatient kidney disease clinics
Montefiore Clinical and Translational Research Center

Duration of procedures:
Screening and consent: ~ 1 hour
Day 1(+/- 3 days): ~1 hour
Week 4(+/- 3 days): ~1 hour
Week 8(+/- 3 days): ~1 hour
Week 12(+/- 3 days): ~1 hour
Intermittent phone calls: ~ 15 minutes each.
Timeline of study procedures:
Day 0: Screening and consent. Screening labs.
Day 1: Study visit to MUH-CTRC. Stool collection, labs drawn, blood for TMAO and FMO gene polymorphisms. Dispensing of 4-week supply of nutraceutical or placebo.
Week 4 (Day 28 (+/- 3 days)): Visit to MUH-CTRC. Stool collection, labs drawn, blood for TMAO.
Week 8 (Day 56 (+/- 3 days)): Dispensing of 4-week supply of nutraceutical or placebo. Stool collection, labs drawn, blood for TMAO.
Week 12 (Day 84 (+/- 3 days)): End of study treatment visit to MUH-CTRC. Stool collection, labs drawn, blood for TMAO.

The subject will be instructed by the coordinator on how to collect a stool specimen at home. All supplies will be provided by the study. The stool will be brought to a research visit or mailed in in a postage-paid envelope. The purpose of this collection is to look at the microbiome of the gut. The de-identified sample will be processed for purification of DNA and aliquots of stool will be generated for future purification of RNA or other markers. The de-identified samples will be stored at –80C.
Females of child-bearing age will have a urine pregnancy test at screening and again at Week 8, after the cross-over.

Will blood samples be obtained for research purposes? Yes
Provide the volume per withdrawal, total volume and frequency, and qualifications of individual performing the procedure:
Screening visit: ~ 1 tablespoon (sodium, chloride, BUN, Creatinine, ALT/AST, Alk Phos, and Total bilirubin)

Day 1 (+/- 3 days): One blood draw. Total volume ~ 2 tablespoons. (1 tablespoon- Lipid Panel, sodium, chloride, ALT/AST, Alk Phos, and Total bilirubin and 1 tablespoon- TMAO measurement, FMO gene polymorphisms)
Week 4 (+/- 3 days): One blood draw. Total volume ~ 2 tablespoons. (1 tablespoon- Lipid Panel, sodium, chloride, ALT/AST, Alk Phos, and Total bilirubin and 1 tablespoon- TMAO measurement)
Week 8 (+/- 3 days): One blood draw. Total volume ~ 2 tablespoons. (1 tablespoon- Lipid Panel, sodium, chloride, ALT/AST, Alk Phos, and Total bilirubin and 1 tablespoon- TMAO measurement)
Week 12 (+/- 3 days): One blood draw. Total volume ~ 2 tablespoons. (1 tablespoon- Lipid Panel, sodium, chloride, ALT/AST, Alk Phos, and Total bilirubin and 1 tablespoon- TMAO measurement)
All procedures will be conducted by highly trained staff at the MUH-CTRC. This includes nurses and/or phlebotomists.

Consent Process

Indicate where the consent process will take place and at what point consent will be obtained:
The screening visit will include IRB approved informed consent during the patient's outpatient office visit or at a separate visit to the Montefiore CTRC. If the patient is considered eligible based on their screening, we will discuss the study and allow the patient sufficient time to review the informed consent form prior to signing and in advance of any study procedures. Screening will include measuring a set of safety labs including: Urine pregnancy test, sodium, chloride, BUN, Creatinine, ALT/AST, Alk Phos, and total bilirubin. We will also conduct a review of the patient's medical history, demographics, and current medications to ensure patients meet our inclusion criteria.

Describe the steps that will be taken to minimize coercion and undue influence, including assurance that there is sufficient time for subjects to make an informed decision:
The informed consent form will be reviewed in detail with each patient. All questions and concerns will be addressed by a knowledgeable study investigator. After the consent form is explained in detail, participants will be allowed to spend time reviewing the form independently. Patients will also be allowed to take a copy of the consent form home for review of the information.
The person obtaining consent will inform the patient that there are no obligations to participate in the study and will provide his/her name and phone number where he/she can be reached if they have further questions or wish to withdraw from the study at any time.

For studies that involve multiple visits, describe the process to ensure ongoing consent:
Participants will be contacted throughout the study to assess their ongoing consent. Study procedures will be reviewed prior to each visit and participants will be specifically asked if they have any questions or concerns at each visit.

Steps to be taken to ensure the subjects' understanding:

Participants' understanding will be assessed by their questions throughout the consenting process. They will also be asked specific questions to assess their understanding. For example, participants will be asked if they understand the study-related procedures or risks and benefits. Typically, engaging in a participant-investigator discussion about the study allows for assessment of understanding.

Consent Form: See Appendix B

Select all options that apply to the request to waive the requirement to obtain informed consent: Review of identifiable medical records

The research involves no more than minimal risk to the subjects;
Initial screening of patients' medical records will not have any physical or physiological risk. Dr. Ray and the study team members have secure access to subject's medical information granted by UPMC. The study team will only access medical records under Dr. Ray's direction. The study team all have their own passwords that enable them to review medical records in a confidential manner. To minimize the risk of breaching confidentially, each patient will be assigned a specific code number that will secure their identity. Information that we extract from the PHI used to determine eligibility will be secured in a password and firewall protected database. In addition, no patient information will be retained or disclosed if the eligible patient declines to participate.

The waiver or alteration will not adversely affect the rights and welfare of the subjects;
The information is used only to identify subjects who meet the inclusion/exclusion criteria and that will not affect the patient welfare. Dr. Ray has his secured access to subject's medical information as a member of the CKD clinic. If the eligible patient refuses to participate, that will never have any effect on their medical care and their relations with Ray or the other health care providers.

The research could not practicably be carried out without the waiver or alteration: Identifying subjects to participate in the research is crucial to completing the research. CKD is defined by the estimated GFR of patients, and this information can only be obtained by examining patient parameters in the medical record.

Whenever appropriate, the subjects will be provided with additional pertinent information after participation: There will be no pertinent information that will need to be provided to the patient.

Under what circumstances (if any) will you obtain consent from some of these subjects:
Patients will be identified and recruited from the nephrology clinics by Dr. Ray and study personnel. Dr. Ray or other physicians will present the study to them to gauge their level of interest and determine if they meet eligibility requirements. Study personnel will then formally present the study and consent interested subjects.
View: Pitt SF: Waiver to Document Informed Consent

Waiver to Document Informed Consent

Identify the specific research procedures and/or the specific subject populations for which you are requesting a waiver of the requirement to obtain a signed consent form:
We are requesting a waiver to document informed consent for the telephone screening script questions. Staff will perform pre-screening questions on the phone to determine eligibility. The research activity for which this waiver is being requested is limited to the phone screening questions for the purpose of asking inclusion/exclusion criteria to determine eligibility.

Address why the specific research procedures for which you are requesting a waiver of the requirement to obtain a signed consent form presents no more than minimal risk of harm to the research subjects: A phone screening will be conducted to include/exclude patients without having to bring in all potential subjects for screening.

Justify why the research involves no procedures for which written informed consent is normally required outside of the research context: We will be asking questions about diagnosis of certain diseases such as: chronic kidney disease, diabetes, and liver disease. Phone script was set up to be used for potential participants.

Medical Records
Describe the protected health information that will be collected from the covered entity and/or the research derived information that will be placed into the medical records:
Medication history
medical diagnoses (e.g. kidney disease, Cardiovascular disease, intestinal diseases)
routine laboratory measurements
Medical records will be reviewed for inclusion criteria to confirm medical diagnosis of chronic kidney disease, diabetes, and/or hypertension. Additional information that would be beneficial to the study would be to confirm eligibility.
Sodium, chloride, BUN, Creatinine, ALT/AST, Alk Phos, and Total bilirubin and Lipid Panels ordered during the study will be automatically uploaded to patient medical records.

Data Safety and Monitoring
Describe your plan to periodically evaluate the data collected regarding both harms and benefits to determine whether subjects remain safe. The plan might include establishing a data monitoring committee and a plan for reporting data monitoring committee findings to the IRB and the sponsor:
Dr. Nolin and Dr. Prokopienko will meet at least every month to discuss the data and safety monitoring throughout the duration of the study.
Study Personnel will meet on at least a quarterly basis to review data and safety metrics.

Describe your plan for sharing data and/or specimens: No plan to share data and/or specimens at this time.

If any research data is collected, stored, or shared in a paper format, address what precautions will be used to maintain the confidentiality of the data:
All paper documents are stored at the University of Pittsburgh School of Pharmacy in a locked cabinet, in a locked laboratory, that only members of the study team have access.

Paper-based records will be kept in a secure location and only be accessible to personnel involved in the study, computer-based files will only be made available to personnel involved in the study through the use of access privileges and passwords, prior to access to any study-related information, personnel will be required to sign statements agreeing to protect the security and confidentiality of identifiable information, whenever feasible, identifiers will be removed from study-related information, precautions are in place to ensure the data is secure by using passwords and encryption.

Data and Specimens will be stored: Long-term banking (e.g., indefinitely)

How the specimens will be accessed and who will have access to the specimens:
We will create a unique ID number that will be written only on the specimen tube and document the subject name and the unique ID number. The linkage code will be given to Dr. Nolin for storage in a locked cabinet. The computerized file linking the study number to patient identifiers will be accessible only to the principal investigator. A hard copy of the file will be placed in a locked file within the principal investigator's office.

Describe the procedures to release data or specimens, including the process to request a release, who can obtain data or specimens, the data to be provided with the specimens:
We will request a material transfer agreement with IRB approval prior to transferring any biological specimens. We do not plan to release any identifiable data with these specimens.

Risk and Benefits
Research Activity: Diindolylmethane
Common Risks: Darkened urine.
Infrequent Risks: Headaches and nausea have been reported as potential side effects. Elevated liver enzymes. Potential interactions with CYP1A2 medications.
Other Risks: There is one report in the literature of hyponatremia in an elderly male taking DIM, but this has not been documented in any larger studies.

Research Activity: Research Information
Common Risks:
Infrequent Risks:
There is a potential risk of loss/breach of confidentiality.
Other Risks: N/A

Research Activity: Genetic Research
Common Risks:
Infrequent Risks:
Other Risks: Genetic research raises certain questions about informing patients of any results. Possible risks of knowing results include: anxiety; other psychological distress; and the possibility of stigmatization or insurance and job discrimination. A possible risk of not knowing includes being unaware of the need for treatment. These risks can change depending on the results of the research and whether there is a treatment or cure for a particular disease. Sometimes patients have been required to furnish information from genetic testing for health insurance, life insurance,

and/or a job. A Federal law, the Genetic Information Nondiscrimination Act of 2008 (GINA), generally makes it illegal for health insurance companies, group health plans, and employers with 15 or more employees to discriminate against you based on their genetic information.

Research Activity: Blood Draw
Common Risks:
Infrequent Risks: Infection or phlebitis (with or without a blood clot).
Other Risks: Pain, slight discomfort, lightheadedness and fainting. A small amount of bleeding, discoloring or bruising at the site of the needle puncture.

Enter all reasonably foreseeable risks, discomforts, hazards, or inconveniences to the subjects related to subjects' participation in the research:
Describe the steps that will be taken to prevent or to minimize risks:
The medication lists of patients will be thoroughly reviewed prior to participation to reduce the risk of drug-drug and drug-herbal interactions. Patients taking known medications that would be potentially affected by or effect CYP1A2 or FMO drug metabolism pathway will be excluded. Sodium, chloride, BUN, ALT/AST, Alk Phos, and Total bilirubin will be assessed at each visit. This will include assessing patient's sodium levels to monitor for any hyponatremia. Any potential risk for hepatotoxicity will be monitored at each visit. The genetic information obtained because of participation in this research will not be included in patient's medical record. Information from which patients may be personally identified will be maintained in a confidential, secure location at the University of Pittsburgh, accessible only by authorized members of the study team, and will not be disclosed to third parties except as described in this consent form, with their permission, or as may be required by law.
We will also de-identify any patient samples and data collection in our laboratory.

Describe the steps that will be taken to protect subjects' privacy:
We will also de-identify any patient samples and data collection in our laboratory.

Describe the potential benefit that individual subjects may experience from taking part in the research or indicate if there is no direct benefit. Do not include benefits to society or others: No direct benefit is known at this time. This study will increase knowledge about novel strategies to reduce heart disease in patients with kidney disease.

Do you anticipate any circumstances under which subjects might be withdrawn from the research without their consent? Yes

Describe the circumstances and any procedures for orderly termination: If the patient it lost to follow up.

Describe procedures that will be followed when subjects withdraw from the research, including partial withdrawal from procedures with continued data collection and data already collected: Prior to collecting the blood, and stool samples, if consent is withdrawn, the sample will not be taken. After the blood draw and stool collection, the biological specimens will be retained as a part of the study.

Appendix B : Informed Consent Form

CONSENT TO ACT AS A PARTICIPANT IN A RESEARCH STUDY

STUDY TITLE:Targeting FMO-Mediated TMAO Formation in Kidney Disease (TMAO) Study

University of Pittsburgh

School of Pharmacy

Department of Pharmacy and Therapeutics

Principal Investigator
Thomas D. Nolin, PharmD, PhD
University of Pittsburgh School of Pharmacy
208 Salk Pavilion
Pittsburgh, PA 15261
Tel: 412-624-4683
Email: nolin@pitt.edu

Co-Investigator
Alexander J. Prokopienko, PharmD
University of Pittsburgh School of Pharmacy
222 Salk Pavilion
Pittsburgh, PA 15261
Tel: 412-624-8129
Email: ajprokop@pitt.edu

Co-Investigator
Evan C. Ray, MD, PhD
A915 Scaife Hall
3550 Terrace Street
Pittsburgh, PA 15261
Tel: 412-802-3043
Email: rayec@upmc.edu

Co-Investigator
Alison Morris, MD, MS
University of Pittsburgh School of Medicine
UPMC Montefiore Hospital,
Pittsburgh, PA 15213
Tel: 412-648-7784
Email: morrisa@upmc.edu

Co-Investigator
Barbara Methé, PhD
University of Pittsburgh School of Medicine
UPMC Montefiore Hospital,
Pittsburgh, PA 15213
Tel: 412-692-2210
Email: metheba@upmc.edu

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Source of Support: This study is supported by a NIH training grant and a grant from the American College of Clinical Pharmacy Research Institute.

You are being asked to be in a research study. This form provides you with information about the study. A member of the research team will describe this study to you and answer all of your questions. Please read the information below and ask questions about anything you don't understand before deciding whether or not to take part.

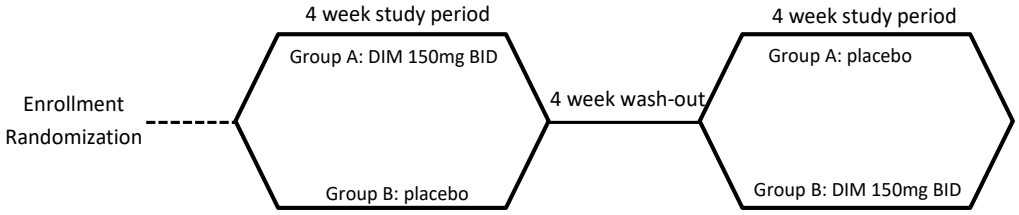
Why is this study being done?

Complications from heart disease are commonly seen in patients with kidney disease. In fact, heart disease is the most common cause of death in patients with kidney disease. Targeting traditional risk factors for heart disease like high cholesterol has not decreased complications for patients with kidney disease, and this suggests that there is a need to identify and target non-traditional risk factors. A substance in the blood called trimethylamine-N-oxide (TMAO) is commonly seen in high levels in patients who have heart complications. TMAO is formed from the breakdown of dietary nutrients in the gut and by liver enzymes known as flavin-containing monooxygenases (FMO). FMO enzyme function is decreased by a substance called diindolymethane (DIM) that is found in cruciferous vegetables such as Brussels sprouts and cabbage. DIM is available for sale over-the-counter in many health food stores that sell natural products and nutritional supplements. The goal of this study is to assess whether DIM decreases the ability of FMO enzymes to form TMAO in patients with kidney disease.

Other people in this study

Up to 12 people will participate in this research study. Participants will all receive the study medication; in addition, their blood, stool and urine will be collected. If you choose to participate, you will be enrolled in this clinical study being done at the University of Pittsburgh.

Study Design: 12-week randomized interventional placebo-controlled cross-over study.



Study screening

In order to be a participant in the study, you will meet with one of the study investigators for a screening visit to see if you are eligible for this study. If you are a woman of child-bearing potential, then you will have a urine pregnancy test done. In order to be included, you must be willing to comply with taking this dietary supplement and/or a placebo twice a day for two separate 1-month periods. You will also be asked to collect four stool samples with provided kits. We will collect information from your medical record including age, lab tests, and past medical and medication history to help determine eligibility. We are also interested in your levels of sodium, liver function tests, total bilirubin, and creatinine, which also require a blood draw. The approximate amount of blood we will take will be 1 tablespoon. Should you meet all requirements of the study and you express interest in participating, then we will set up a time to begin the study.

What happens if I join this study?

The study involves four study sessions over a total of 12 weeks. The first session will take place before taking the dietary supplement or placebo, there will be a visit at 4 weeks, followed by 4 weeks of no interventions. You will return at the Week 8 time-point to receive the either the dietary supplement or placebo for 4 weeks. The last visit will occur at 12 weeks where you will have a final blood draw and then be finished with the study.

First visit

For approximately fourteen days before this visit, we will ask you not to eat or drink certain things. These include alcohol (>5 alcoholic drinks/day), and cruciferous vegetables. Specifically, we will ask you not to eat cabbage, brussels sprouts, garden cress, mustard greens, turnips, broccoli, collard greens, cauliflower, and kale. You will need to be fasting for this visit. We ask that you do not consume any food for 3-4 hours before your first visit. You are welcome to drink water as needed. You will be asked to give an update to your medical history including any changes in medications or vitamin supplements since your screening visit.

We will collect blood from you from your arm during this visit. Some of the blood will be sent to the hospital lab for tests such as liver function tests, sodium, cholesterol tests, and bilirubin. We will also give you a stool sample kit to collect at home and mail back to our laboratory.

An additional teaspoon of blood will also be drawn to be used for DNA (genetic material) analysis of drug metabolism and transporters. The DNA will be used for future research to learn more about chronic kidney disease and how chronic kidney disease can affect the efficacy and toxicity of medicines. Blood will also be collected to measure toxins that are produced from your intestinal bacteria.

A total of approximately 2 tablespoons of blood will be collected at this first visit. At this visit you will be given a 1-month supply of the dietary supplement diindolymethane or placebo. You will be asked to take this dietary supplement or placebo twice daily for 1 month prior to coming in to the Week 4 follow up.

We will also ask you to avoid alcohol (>5 alcoholic drinks/day), and cruciferous vegetables during the study. Specifically, we will ask you not to eat cabbage, brussels sprouts, garden cress, mustard greens, turnips, broccoli, collard greens, cauliflower, and kale.

Second visit (WEEK 4)

You will return to the research center for your Week 4 visit. You will need to be fasting for this visit. We ask that you do not consume any food for 3-4 hours before your visit. You are welcome to drink water as needed. You will be asked to give an update to your medical history including any changes in medications or vitamin supplements since your first visit.

We will collect blood from you from your arm during this visit. Some of the blood will be sent to the hospital lab for tests such as sodium, liver function tests, cholesterol tests, and bilirubin. We will also give you a stool sample kit to collect at home and mail back to our laboratory.

Blood will also be collected to measure toxins that are produced from your intestinal bacteria. A total of approximately 2 tablespoons of blood will be collected at this visit.

No study drug will be taken between Week 4 - Week 8. We will also ask you to avoid alcohol (>5 alcoholic drinks/day), and cruciferous vegetables during the study. Specifically, we will ask you not to eat cabbage, brussels sprouts, garden cress, mustard greens, turnips, broccoli, collard greens, cauliflower, and kale.

Third visit (WEEK 8)

Study instructions and procedures are the same as described in the Week 4 visit.

At this visit you will be given a 1-month supply of the dietary supplement diindolymethane or placebo. You will be asked to take this dietary supplement or placebo twice daily for 1 month prior to coming in to the Week 12 follow up.

Fourth visit (WEEK 12)

Study instructions and procedures are the same as described in the Week 4 visit.

We will ask you some final closeout questions and you will be finished with the study.

What are the possible discomforts or risks?

Several blood samples are required for participation in this study. We will be doing a blood draw. You may feel some pain when the needle goes into your vein. You may experience slight discomfort, lightheadedness and fainting. You may experience a small amount of bleeding, discoloring or bruising at the site of the needle puncture. There is also the risk of infection or phlebitis (blood clot) however these occurrences are rare. The total amount of blood collected throughout the study will be approximately 9 tablespoons.

You will be receiving a dietary supplement called diindolymethane to reduce the formation of TMAO. Occasionally, diindolymethane or similar dietary supplements may slightly increase or decrease the function of liver enzymes. This could potentially affect the therapeutic effect or toxicity of certain medications. Your liver function will be checked at every visit to determine if you have elevations of liver function enzymes. It may be necessary to stop taking the dietary supplement if your other therapies are affected or your liver function tests are elevated above 3X the upper limits of normal values.

There is a potential risk of loss of confidentiality. Every effort will be made to protect your confidential information, but this cannot be guaranteed. The genetic information obtained because of your participation in this research will not be included in your medical record. Information from which you may be personally identified will be maintained in a confidential, secure location at the University of Pittsburgh, accessible only by authorized members of the study team, and will not be disclosed to third parties except as described in this consent form,

with your permission, or as may be required by law. Genetic research raises certain questions about informing you of any results. Possible risks of knowing results include: anxiety; other psychological distress; and the possibility of stigmatization or insurance and job discrimination. A possible risk of not knowing includes being unaware of the need for treatment. These risks can change depending on the results of the research and whether there is a treatment or cure for a particular disease. Sometimes patients have been required to furnish information from genetic testing for health insurance, life insurance, and/or a job. A Federal law, the Genetic Information Nondiscrimination Act of 2008 (GINA), generally makes it illegal for health insurance companies, group health plans, and employers with 15 or more employees to discriminate against you based on your genetic information.

The study may include risks that are unknown at this time.

Reproductive risks

Participants cannot be pregnant to participate in the study. You will undergo a urine pregnancy test at screening, and before the week 8 visit to the research center. Abstinence from sexual activity is the only certain method to prevent pregnancy. If you choose to be sexually active during this study, you accept the risk that pregnancy could still result, exposing you to potential loss of pregnancy as well as other unknown effects on a developing fetus. If you have reason to believe that you are pregnant, that there is a possibility that you might become pregnant, or that you stopped using adequate contraception, you agree that you will immediately inform the study doctor.

New Information

You will be promptly notified if any new information we learn during this research study may cause you to change your mind about continuing to participate in the study.

A description of this clinical trial will be available at http://www.clinicaltrials.gov, as required by US 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.

What are the possible benefits of the study?

There is no direct benefit to study participation. This study is designed for the researchers to learn more about formation of TMAO in patients with chronic kidney disease. It is hoped the study will generate new knowledge which may allow us to better treat cardiovascular disease in patients in the future.

Who is paying for this study?

The pilot study is sponsored primarily by a NIH training grant from the University of Pittsburgh, and a grant from the American College of Clinical Pharmacy Research Institute.

Will I have to pay for anything?

It will not cost you anything to be in the study. You will not incur any costs for the blood, stool or urine collection directly related to this research study.

Will I be paid for being in the study?

You will be compensated $20 for the screening and follow-up visits. At the final week 12 visit you will be paid $40. The total compensation for completion of all study visits is up to $120 and in addition parking will be provided or bus fare will be reimbursed.

Compensation for injury

If you believe that the research procedures have resulted in an injury to you, immediately contact the Principal Investigator who is listed on the first page of this form. Emergency medical treatment for injuries solely and directly related to your participation in this research study will be provided to you by the hospitals of UPMC. Your insurance provider may be billed for the costs of

this emergency treatment, but none of those costs will be charged directly to you. If your research-related injury requires medical care beyond this emergency treatment, you will be responsible for the costs of this follow-up care. At this time, there is no plan for any additional financial compensation.

Is my participation voluntary?

Taking part in this study is voluntary. You have the right to choose not to take part in this study. If you choose to take part, you have the right to stop at any time. If you refuse or decide to withdraw later, you will not lose any benefits or rights to which you are entitled. If you decide later that you do not want the specimens collected from you to be used for future research, you may tell this to Dr. Nolin, who will use his best efforts to destroy the DNA and other samples. If you decide that you no longer wish to continue in this study, you will be required to either write to the researcher or tell someone on the research team of your wishes. Written requests can be sent to:

Dr. Thomas Nolin
University of Pittsburgh, School of Pharmacy
208 Salk Pavilion
335 Sutherland Drive
Pittsburgh, PA 15261

Can I be removed from this study?

Drs. Nolin and Prokopienko may decide to stop your participation without your permission if we think that being in the study may cause you harm, or for any other reason. If you become pregnant during the study alert the study team as you may be removed from the study.

Genetic Information Nondiscrimination Act (GINA)

A Federal law, called the Genetic Information Nondiscrimination Act (GINA), generally makes it illegal for health insurance companies, group health plans, and most employers to

discriminate against you based on your genetic information. This law generally will protect you in the following ways:

- Health insurance companies and group health plans may not request your genetic information that we get from this research.
- Health insurance companies and group health plans may not use your genetic information when making decisions regarding your eligibility or premiums.
- Employers with 15 or more employees may not use your genetic information that we get from this research when making a decision to hire, promote, or fire you or when setting the terms of your employment.

All health insurance companies and group health plans must follow this law by May 21, 2010. All employers with 15 or more employees must follow this law as of November 21, 2009. Be aware that this new Federal law does not protect you against genetic discrimination by companies that sell life insurance, disability insurance, or long-term care insurance.

Who will see my research information?

The University of Pittsburgh and the hospital(s) it works with have rules to protect information about you. Federal and state laws including the Health Insurance Portability and Accountability Act (HIPAA) also protect your privacy. This part of the consent form tells you what information about you may be collected in this study and who might see or use it.

The institutions involved in this study include:

- University of Pittsburgh
- University of Pittsburgh Medical Center (UPMC)

We cannot do this study without your permission to see, use and give out your information. You do not have to give us this permission. If you do not, then you may not join this study.

We will see, use and disclose your information only as described in this form and in our Notice of Privacy Practices; however, people outside the University of Pittsburgh and its affiliate hospitals may not be covered by this promise.

We will do everything we can to keep your records a secret, but it cannot be guaranteed.

The use and disclosure of your information has no time limit. You can cancel your permission to use and disclose your information at any time by writing to the study's Primary Investigator, at the name and address listed below. If you do cancel your permission to use and disclose your information, your part in this study will end and no further information about you will be collected. Information already obtained will continue to be used for the study.

Both the research records that identify you and the consent form signed by you may be looked at by others who have a legal right to see that information.

- Federal offices such as the Food and Drug Administration (FDA) that protect research subjects like you.
- The study doctor and the rest of the study team.
- The University of Pittsburgh, which will analyze blood samples for presence of toxins, which cannot be identified with you.
- Authorized representatives from the University of Pittsburgh Research Conduct and Compliance Office may review your data for the purpose of monitoring the conduct of this study.

We might talk about this research study at meetings. We might also print the results of this research study in relevant journals. But we will always keep the names of the research subjects, like you, private. You have the right to request access to your personal health information from the Investigator. Per University of Pittsburgh policy, all research records must be maintained for at least 7 years following final reporting or publication.

Information about you that will be seen, collected, used and disclosed in this study:

- Name and Demographic Information (age, sex, ethnicity, address, phone number, etc.)

- Portions of previous and current Medical Records that are relevant to this study, including but not limited to Diagnosis(es), History and Physical, laboratory or tissue studies, radiology studies, procedure results, dialysis information, and hospital stay
- Research Test records
- The data with the samples

What happens to Data, Blood and Specimens that are collected in this study?

Scientists at the University of Pittsburgh and the hospitals involved in this study work to find the causes and cures of disease. The data, blood and specimens collected from you during this study are important to this study and to future research. If you join this study:

- The data, or the blood, or other specimens are given by you to the investigators for this research and so no longer belong to you.
- Both the investigators and any sponsor of this research may study your data, blood, or other specimens collected from you.
- If data, blood, or other specimens are in a form that identifies you, University of Pittsburgh or the hospitals involved in this study may use them for future research only with your consent or IRB approval.
- Any product or idea created by the researchers working on this study will not belong to you.
- There is no plan for you to receive any financial benefit from the creation, use or sale of such a product or idea.

Your data and samples used in this research study may contribute to a new discovery or treatment. In some instances, these discoveries or treatments may be of commercial value and may be sold, patented, or licensed by the investigators and the University of Pittsburgh for use in other research or the development of new products. You will not retain any property rights, nor will you share in any money that the investigators, the University of Pittsburgh, or their agents may realize.

Optional Consent for Data and Specimen Banking for Future Research

Dr. Thomas Nolin would like to keep some of the data, blood, stool, urine and DNA that is taken during the study but is not used for other tests. If you agree, the data and samples will be

kept and may be used in future research to learn more about chronic kidney disease and metabolism and transport of drugs. The research that is done with your data and samples is not designed to specifically help you. It might help people who have chronic kidney disease and other diseases in the future. Dr. Nolin will be responsible for deciding how the DNA and blood sample will be used. Reports about research done with your data and samples will not be given to you or your doctor. These reports will not be put in your health records. The research using your data and samples will not affect your care.

The choice to let Dr. Thomas Nolin keep the data and samples for future research is up to you. No matter what you decide to do, it will not affect the care that you will receive as part of the study. If you decide now that your data and samples can be kept for research, you can change your mind at any time and contact your study doctor to let him or her know that you do not want Dr. Thomas Nolin to use your data and samples any longer, and they will no longer be used for research. Otherwise, they may be kept until they are used up, or until Dr. Thomas Nolin decides to destroy them.

When your data and samples are given to other researchers in the future, Dr. Thomas Nolin will not give them your name, address, phone number or any other information that will let the researchers know who you are. Sometimes data and samples are used for genetic research (about diseases that are passed on in families). Even if your data and samples are used for this kind of research, the results will not be told to you and will not be put in your health records. Your data and samples will only be used for research and will not be sold. The research done with your data and samples may help to develop new products in the future, but there is no plan for you to be paid.

There is no direct benefit for you agreeing to allow data and sample storage. It is hoped that future research on your data and samples will allow researchers to learn more about what causes chronic kidney disease and how the disease affects medicines. The greatest risk to you is the release of your private information. Dr. Thomas Nolin will protect your records so that your name, address and phone number will be kept private. The chance that this information will be given to someone else is very small. There will be no cost to you for any data or sample collection and storage by Dr. Thomas Nolin.

Please read each sentence below and think about your choice. After reading each sentence, circle "yes" or "no." If you have questions, please talk to your doctor or nurse. Remember, no matter what you decide to do about the storage and future use of your data and samples, you may still take part in the study.

I give my permission for my data, blood, stool, urine, and DNA to be stored in a central tissue bank at Dr. Thomas Nolin's laboratory at the University of Pittsburgh for future use by the study investigators:

1. I give my permissions for my data, stool, blood, urine and DNA samples to be kept by Dr. Thomas Nolin for use in future research to learn more about chronic kidney disease and how chronic kidney disease can affect the efficacy and toxicity of medicines.

   ☐ Yes ☐ No_____Initials

2. I give my permissions for my data, stool, blood, urine, and DNA samples to be used for research about other health problems (for example: causes of heart disease, osteoporosis, diabetes).

   ☐ Yes ☐ No_____Initials

VOLUNTARY CONSENT

The above information has been explained to me and all of my current questions have been answered. I understand that I am encouraged to ask questions about any aspect of this research study during the course of this study, and that such future questions will be answered by a qualified individual or by the investigator(s) listed on the first page of this consent document at the telephone number(s) given. I understand that I may always request that my questions, concerns or complaints be addressed by a listed investigator.

I understand that I may contact the Human Subjects Protection Advocate of the IRB Office, University of Pittsburgh (1-866-212-2668) to discuss problems, concerns, and questions; obtain information; offer input; or discuss situations in the event that the research team is unavailable.

By signing this form, I consent to participate in this research study and provide my authorization to share my medical records with the research team. A copy of this consent form will be given to me.

_____ _____

Participant's Signature Date

_____

Printed Name of Participant

CERTIFICATION OF INFORMED CONSENT

I certify that I have explained the nature and purpose of this research study to the above-named individual(s), and I have discussed the potential benefits and possible risks of study participation. Any questions the individual(s) have about this study have been answered, and we will always be available to address future questions, concerns or complaints as they arise. I further certify that no research component of this protocol was begun until after this consent form was signed.

_____ _____

Printed Name of Person Obtaining Consent Role in Research Study

_____ _____

Signature of Person Obtaining Consent Date

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