Effects of Diet and Disease on Renal Oxylipins and Related Enzymes in the Han:SPRD-*Cy* rat Model of Cystic Kidney Disease

By

Naser H. M. Ibrahim

A Thesis Submitted to the Faculty of Graduate Studies of

The University of Manitoba

In Partial Fulfillment of the Requirements for the Degree of

DOCTOR OF PHILOSOPHY

Department of Human Nutritional Sciences

University of Manitoba

Winnipeg, Manitoba, Canada

2015

Abstract

Selective inhibition of cyclooxygenase (COX) derived oxylipins reduces disease progression in the Han:SPRD-*Cy* rat model of cystic kidney disease, but the roles of lipoxygenase (LOX) and cytochrome P450 (CYP) derived products in this disease are not known. Dietary soy protein (SP), fish oil (FO) and flax oil (FXO) are beneficial in retarding renal disease progression in this and other models of cystic kidney disease and can alter renal oxylipin production through different mechanisms. The general objectives of this thesis were to: 1) investigate the effects of disease on renal oxylipin levels (produced from the three enzymatic pathways); 2) investigate the synergistic and additive effects of combining dietary SP with FO or FXO on disease progression, renal fatty acid composition, and renal oxylipin levels; 3) compare the effects of COX and LOX inhibitors on oxylipin levels and disease progression, in the Han:SPRD-*Cy* rat model of cystic kidney disease.

Our research demonstrates that COX oxylipins are elevated and n-6 derived LOX metabolites are reduced in diseased kidneys in this model of cystic kidney disease. N-3 polyunsaturated fatty acid (PUFA) derived LOX oxylipins, including those derived from alpha-linolenic acid (ALA) and docosahexaenoic acid (DHA) were also lower in diseased kidneys, as were CYP derived oxylipins. The beneficial effect of SP on disease was associated with amelioration of several oxylipin alterations in parallel with a reduction in kidney disease progression, improvement in kidney function and blood pressure. However, adding dietary FO or FXO to the SP diet improved some but worsened other oxylipin alterations and did not provide further disease protection.

Since both COX1 and COX2 activities are elevated in diseased kidneys, the effect of aspirin was examined; this treatment slowed disease progression and the decline in kidney

function. On the other hand, inhibiting the LOX pathway had no effects on disease. Thus interventions that specifically inhibit COX while maintaining LOX and CYP may be more effective than general oxylipin inhibitors in slowing disease in this renal disorder.

Acknowledgements

I would like to express my profound gratitude to all those who have made it possible for me to complete this Ph.D. journey.

I wish to thank, first and foremost, my supervisor, Dr. Harold Aukema, for his help, valuable support, constant encouragement, tremendous patience, great attitude, continuous guidance, teaching, and constructive criticism. Without his guidance and persistent help and support this thesis would not have been possible.

I would like to extend my sincer appreciation to my dissertation committee members, Dr. Jeffrey Wigle, from the Department of Biochemistry and Medical Genetics, Dr. James House, and Dr. Miyoung Suh, from the Department of Human Nutritional Sciences, for providing their experience and knowledge, and for their valuble thoughts, endless encouragement, constant guidance, and support, throughout this exciting journey.

I would like to thank Dr. Tamio Yamaguchi, visiting scientist in Dr. Aukema's lab, Dr. Daisuke Yoshihara, and Dr. Shizuko Nagao, from the Education and Research Center of Animal Models for Human Diseases, Fujita Health University, Toyoake, Aichi, Japan, for their collaboration, providing experience and knowledge, and for their assistance with the histopathology analysis of the last two studies of this thesis.

I further wish to express my sincere appreciation to Dr. Gustaaf Sevenhuysen, former Dean of the Faculty of Human Ecology, for his endless support and encouragement to my family and me during our most difficult times. Additionally, I would like to thank all the staff at the former Human Ecology General Office and Jocelyne Gaudet, Janelle Ritchot, Pam Gauthier, and Taylor Friesen for their friendship, smiles, and help with all administrative needs.

It is with immense gratitude that I acknowledge the financial support provided by the Manitoba Agri-Food Research and Development Initiative, Natural Sciences and Engineering Research Council of Canada **(**NSERC**)**, the NSERC Food Advancement through Science and Training (FAST) program, the Libyan-North American scholarship program, and the University of Manitoba Graduate Fellowship. I also would like to thank the Manitoba Institute of Child Health, the Richardson Centre for Functional Foods and Nutraceuticals, and the St. Boniface Hospital Research Center for providing access to labs and equipment. In addition, I owe my deepest thankfulness to the staff at the animal holding facility at the Duff Roblin building, University of Manitoba.

I cannot find words to express my gratitude to my friends, colleague graduate students (Yong Jia, Hong Shi, Jessay Devassy, Stephanie Caligiuri, Melissa Gabbs, Shan Leng, Md Monirujjaman, Shyamchand Mayengbam , Mohammed Abdullah, and Erin Goldberg), summer students (Yinhong Wu, Melanie Grégoire, and Clara Lysecki), and technicians in Dr. Aukema's lab (Dennis Labossiere, Joy Gauthier, and Tanja Winter) for their friendship, technical assistance, and support with animal studies and experimental work.

Above all, my deepest expression of appreciation goes to my wife, Ghada Elgadi, her wonderful support, continuous encouragement, and incredible patience have made it possible for me to get this far in education and life. I will never forget the sacrifices that she and my kids (Sajed and Camillia) made in order for me to devote time for my studies and pursue this degree. Simple words of appreciation will never be enough to thank you. Additionally, this thesis would not have been possible without the support of my parents, my brothers and sisters, their endless love and support even during war and most difficult situations have never stopped.

Dedication

I dedicate this thesis to my family.

To my wife Ghada Elgadi, to my children Sajed and Camillia

To my great parents, Elhadar and Soad

Student Contribution

First, I would like to acknowledge the help and support of all colleague graduate and summer students and lab technicians (their names are listed in the acknowledgements section of this thesis) for helping with all animal studies and for providing technical assistance with experimental work.

The Candidate was the lead on all animal studies, completed all applications to obtain ethical approval for all animal studies, designed and conducted all studies, completed the related experimental work and was the principal author for all manuscripts. Dr. Harold Aukema helped designing all three animal studies and reviewed all manuscripts before submission for publications.

Table of Contents

List of Tables

List of Figures

List of abbreviations

AA	Arachidonic acid
ACE	Angiotensin converting enzyme
ALA	Alpha-linolenic acid
BUN	Blood urea nitrogen
CV	Coefficient of variation
CVD	Cardiovascular disease
CKD	Chronic kidney disease
COX	Cyclooxygenase
CYP45	Cytochrome P450
PGES	Prostaglandin E synthase
DGLA	Dihomo-γ-linolenic acid
DiHETrE	Dihydroxy-eicosatrienoic acid
DiHOME	Dihydroxy-octadecenoic acid
DHA	Docosahexaenoic acid
DNA	Deoxyribonucleic acid
DPA	Docosapentaenoic acid
EPA	Eicosapentaenoic acid
EpETrE	Epoxyeicosatrienoic acid
eNOS	Endothelial nitric oxide synthase
ETA	Eicosatetraenoic acid
FAST	Food Advancement through Science and Training
FC	Fluorchem

FO	Fish oil
FXO	Flax oil
GC	Gas chromatography
GFR	Glomerular filtration rate
HDoHE	Hydroxy-docosahexaenoic acid
H&E	Haematoxylin and eosin stain
HEPE	Hydroxy-eicosapentaenoic acid
HETE	Hdroxy-eicosatetraenoic acid
HETrE	Hydroxy-eicosatrienoic acid
HHTrE	Hydroxy-heptadecatrienoic acid
HODE	Hydroxy-octadecadienoic acid
HOTrE	Hydroxy-octadecatrienoic acid
HPETE	Hydroperoxy-eicosatetraenoic acid
HPLC	High performance liquid chromatography
LA	Linoleic acid
Lt	Leukotriene
L-PGDS	Lipocalin-type prostaglandin D synthase
LOX	Lipoxygenase
LX	Lipoxin
LC-MS/MS	Liquid chromatography-mass spectrometry
MAPK	Mitogen-activated protein kinase
mPGES-1	Microsomal PGE synthase-1
NDGA	Nordihydroguaiaretic acid

NHANES	National Health and Nutrition Examination Survey
NSAIDs	Nonsteroidal Anti-inflammatory Drugs
NSERC	Natural Sciences and Engineering Research Council of Canada
PKD	Polycystic kidney disease
PLA_2	Phospholipase A_2
PG	Prostaglandin
PGDS	Prostaglandin D synthase
PGES	Prostaglandin E synthase
PGFS	Prostaglandin F synthase
PGIS	Prostaglandin I synthase
PKD	Polycystic kidney disease
RBF	Renal blood flow
SA	Stearidonic acid
SP	Soy protein
SUN	Serum urea nitrogen
Tx	Thromboxane
TxS	Thromboxane synthase

Chapter 1

1. Overall Introduction

1.1 Introduction

Chronic kidney disease describes the gradual loss of kidney function that subsequently leads to end-stage renal disease (ESRD) and the need for expensive renal replacement therapy. Cystic kidney disease is a hereditary disorder characterized by abnormal cellular proliferation, growth of numerous cysts, interstitial inflammation, and fibrosis, which eventually leads to renal failure and death [1-3]. Genetic cystic kidney disease includes autosomal dominant polycystic kidney disease (ADPKD), autosomal recessive polycystic kidney disease (ARPKD), and nephronophthisis (NPHP) [1-3]. ADPKD is the most common of all hereditary cystic kidney diseases, affecting approximately 1:500 to 1:1000 individuals in the general population, it is the fourth leading cause of renal failure, and it is responsible for 10-15% of the cases of ESRD [4-6]. There is no cure for cystic kidney disease and there is no therapy that is clinically effective in slowing the progression of this disease [5,7]. Current management options are mostly restricted to treating complications or slowing disease progression by either using angiotensin II antagonists to control high blood pressure, dialysis, or kidney transplant in ESRD cases [1,5,8]. In addition, cystic kidney disease is often associated with co-morbidities, such as cardiovascular disease, stroke, and infectious complications that further increase healthcare cost and mortality rate [9]. It is therefore of interest to find ways to prevent the progression of cystic kidney disease and thereby reduce healthcare cost, morbidity, and mortality in patients with this renal disorder.

Oxylipins are biologically active oxygenated metabolites of fatty acids that are formed in all cells upon release of fatty acids from cell membranes by phospholipase A_2 (PLA_2) and conversion via the cyclooxygenase (COX), lipoxygenase (LOX), and cytochrome P450 (CYP)

pathways [10]. They represent a large biological class of lipids that are important regulators of kidney hemodynamics and may have a role in cystic kidney disease progression. In addition to regulating salt and water balance, renin release, vascular tone, and glomerular filtration rate (GFR), oxylipins are also involved in kidney disease progression by mediating cell proliferation, inflammatory, and fibrotic processes in response to kidney injury [10-13].

In the Han:SPRD-*Cy* rat model of cystic kidney disease, COX activity [14] and several prostanoids (a subclass of oxylipins formed via the COX pathway) including prostaglandin E_2 (PGE_2), PGI_2 and thromboxane A_2 (TXA_2), are higher in diseased compared to normal kidneys, and selective COX2 inhibition reduces kidney disease progression in this model and in other models of renal disease [15-17]. Furthermore, PGE_2, and PGI_2 have been found to stimulate cell proliferation, fluid secretion, and cyst formation in primary cultured ADPKD cells and in inner medullary collecting duct cells in *vitro* [18-20], illustrating the importance of these prostanoids in cystic kidney disease progression. Nevertheless, other oxylipins produced from the COX, LOX, and CYP pathways may also be important, since they have been shown to be altered in other models of kidney diseases [21,22], including the *pcy* mouse model of cystic kidney disease [23].

Formation of oxylipins is not homogenous within the whole kidney [24], For example, it has been reported that the main sites of PGE_2 formation are the medullary interstitial cells and collecting ducts [25]. In the kidney, COX1 is expressed in all segments of the collecting ducts (medulla) and to a lesser extent in glomeruli (cortex) [24]. In contrast, COX2 is expressed in the glomeruli and the cortical thick ascending limb of Henle [24].
Oxylipins are locally acting signaling molecules that modulate cellular function according to their site of synthesis [24,26]. Therefore, it would be very important to learn about the

variations in oxylipin levels within different regions of the kidney (cortex and medulla) and how they are altered by cystic kidney disease.

The type of oxylipin produced depends mainly on the polyunsaturated fatty acid (PUFA) being oxidized and the enzyme metabolizing the PUFA. The most well-known precursor of oxylipins is the 20 carbon, omega-6 PUFA arachidonic acid (AA). However, oxylipins can also be produced from the omega-3 PUFA 20:5n-3 (EPA) or 18:3n-3 (ALA) utilizing the same pathways of metabolism [27,28]. Dietary fish oil (FO), and flax oil (FXO), rich in omega-3 PUFA, have been shown to have reno-protective effects in experimental models of cystic kidney disease [23,29-32], as well as other renal diseases [33-35]. EPA, DHA, and ALA from FO and FXO are incorporated into the phospholipids of cell membranes in a dose-dependent manner depending on the amount provided in the diet [36,37]. EPA, DHA, (from FO) and ALA (From FXO) can inhibit the metabolism of AA to the more pro-inflammatory oxylipins through altering the cell membrane and fatty acid composition of the kidney and increasing the production of EPA, DHA, and ALA derived oxylipins such as 3-series prostanoids, hydroxy-eicosapentaenoic acids (HEPE), and hydroxy-docosahexaenoic acids (HDOHE). Indeed, it has been reported that treatment with n-3 PUFA for 8 weeks in patients with chronic kidney disease decreased the release of leukotriene B_4 (LtB_4) and 5-hydroxy-eicosatetraenoic acids (5-HETE) produced from AA and increased release of the less proinflammatory LtB_5 and 5-HEPE, produced from EPA, as tested in isolated stimulated neutrophil granulocytes [37]. Thus, the beneficial effects of FO and FXO in kidney diseases could be due to altering the renal oxylipin profile.

Dietary soy protein (SP) also reduces disease progression in different models of kidney diseases including the Han:SPRD-*Cy* model of cystic kidney disease [38-41]. Nevertheless,

the mechanism(s) by which SP exert its beneficial effects still needs to be elucidated. SP contains several biologically active compounds such as isoflavones, saponins, and bioactive peptides and has a very unique amino acid composition, with low ratios of methionine/glycine and lysine/arginine that may contribute to its protective effects (more details in chapter 2) [42,43]. Bioactive peptides from SP have been shown to inhibit inflammation, and reduce reactive oxygen species (ROS), which might further contribute to its anti-inflammatory activity and ability to reduce disease progression [44]. Bioactive peptides have also been shown to reduce high blood pressure through inhibiting the angiotensin I converting enzyme activity (ACE) which mediates its effects via changes in oxylipin levels as well as being itself regulated by COX products [12,45,46]. Also, SP alters renal prostanoid production and reduces disease progression in the obese fa/fa Zucker rats [34]. Similar to this, SP has been shown to reduce the elevated protein levels of PLA_2 and COX_2 in the diseased kidneys of rats with cystic kidney disease in the Han:SPRD-*Cy* model [47]. However, whether SP has any effect on other oxylipins produced from different pathways of oxylipins metabolism still need to be elucidated.

In the current thesis, we have demonstrated that COX products are elevated and LOX metabolites are lower in diseased compared to normal kidneys and dietary SP blunted the effects of disease on these oxylipins in association with a reduction in kidney disease progression in the Han:SPRD-*Cy* rat model [48]. However, whether the alteration in COX and LOX products is causing the disease or affecting the disease is not known. Furthermore, previous studies have shown that selective inhibition of COX2 reduces kidney disease progression, but both COX1 and COX2 activities have been shown to be elevated in cystic kidney disease [15] and the cardiovascular safety of selective COX2 inhibitors has been

questioned [49]. Therefore, testing the beneficial effects of dual COX inhibitor (Aspirin) on disease progression and oxylipins in the kidney of cystic rats may provide a better treatment option and insights about the role of oxylipins in disease progression in this renal disorder. Furthermore, since we have demonstrated that LOX products are higher in diseased kidneys [48], we also investigated the effect of a LOX inhibitor [nordihydroguaiaretic acid, (NDGA)] on oxylipin levels and disease progression in the Han:SPRD-*Cy* rat model of cystic kidney disease.

1. 2 General hypotheses and objectives

The objectives of the various studies in this thesis were defined based on the following general hypotheses:

Study-1 Hypotheses **# 1**

Disease will increase the levels of pro-inflammatory oxylipins (2-series prostanoids) produced from AA via the COX pathway and decrease oxylipins produced from the LOX pathway. Supplementing the diet with FO will result in a shift in tissue fatty acid composition and oxylipin production away from the more bioactive and pro-inflammatory oxylipins produced from the n-6 PUFA (AA), to the less bioactive oxylipins derived from the n-3 PUFA (EPA) in association with a reduction in kidney disease progression. SP will reduce kidney disease progression and blunt the effect of disease on oxylipin levels in the kidney, cortex and medulla. Adding FO to the SP diet will enhance the beneficial effect of SP on disease and oxylipins.

Based on these hypotheses, the **objective** of the first study was to examine the effects of dietary SP and FO (individually and combined) on kidney disease progression by measuring parameters of renal pathology and function in the Han:SPRD-*Cy* rat. To examine the importance of both COX and LOX products in cystic kidney disease, the cortical and medullary fatty acid composition, levels of oxylipins and oxylipin related enzymes were examined. Since dietary FO and SP alter oxylipin formation via different mechanisms, these beneficial dietary interventions (individually or combined) were used to further probe the importance of the oxylipins in cystic kidneys in this model (chapter 3).

Study-1 Hypotheses **# 1……***continued*

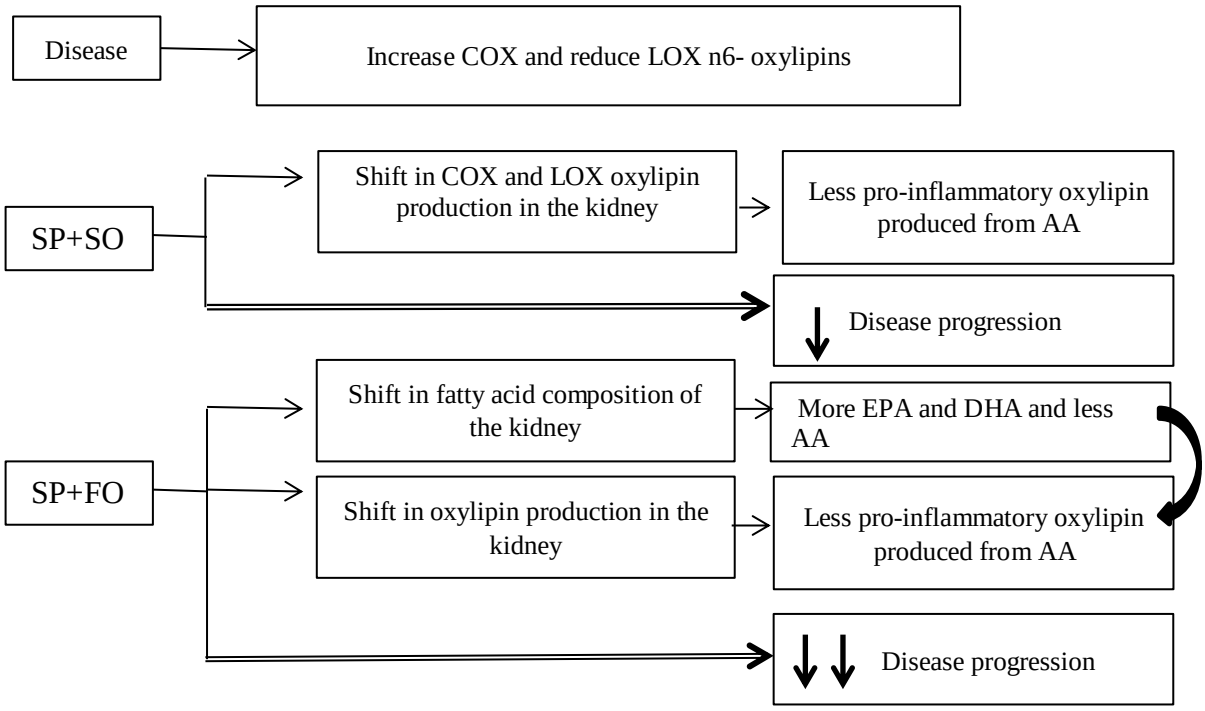


Figure 1.1 Study-1 Hypotheses **# 1**

Lead to association with

Indicate stronger effect

Study-2 Hypotheses # 2

Disease will increase the levels of pro-inflammatory oxylipins (2-series prostanoids) produced from AA via the COX pathway and decrease oxylipins produced from the LOX and CYP pathway. Adding FXO to SP will enhance the reno-protective effects of SP and result in a shift in tissue fatty acid composition and oxylipin production away from the more bioactive oxylipins produced from the n-6 PUFA, AA, to the less bioactive oxylipins derived from the n-3 PUFAs ALA and EPA.

Therefore, the **objective** of the second study was to examine the effects of combining SP with FXO on kidney disease progression, renal fatty acid composition, and oxylipin levels in the Han:SPRD-*Cy* rat model of cystic kidney disease to further probe the importance of oxylipins in cystic kidney disease in this model (chapter 4).

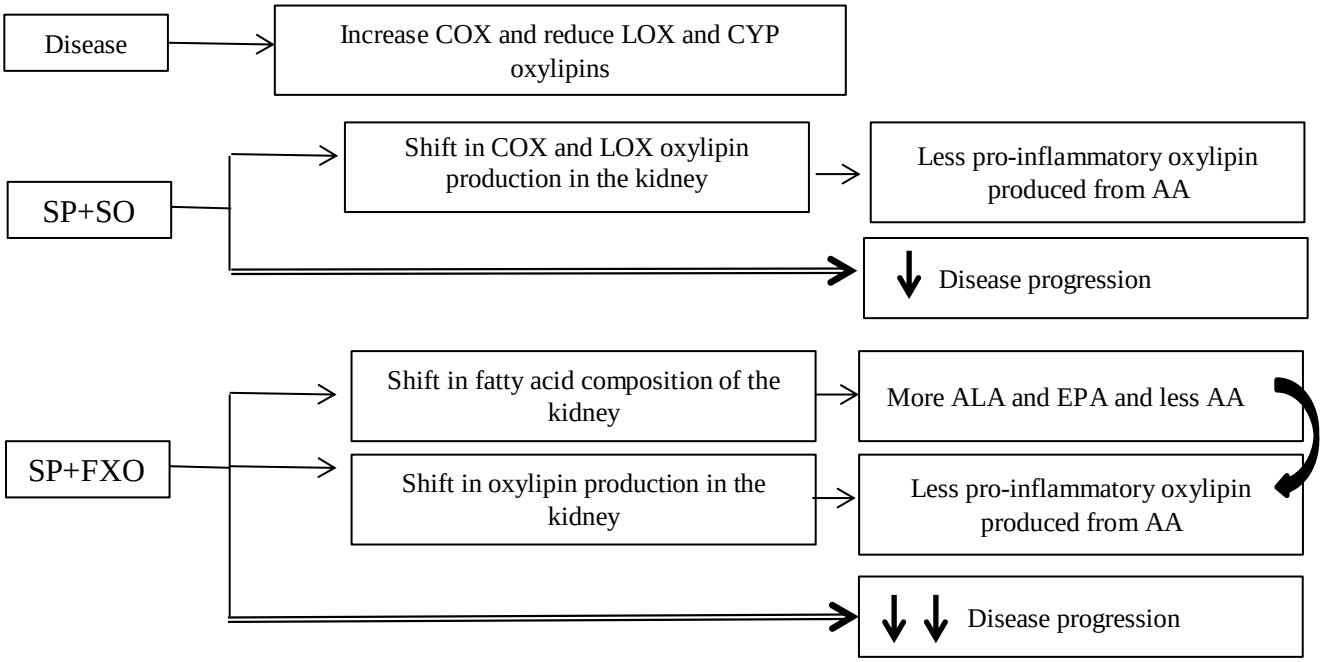


Figure 1.2 Study-2 Hypotheses # 2

Lead to association with

Indicate stronger effect

Study-3 Hypotheses **# 3**

A low dose of aspirin (dual COX isoform inhibitor) will ameliorate kidney disease progression through inhibiting renal COX products. On the contrary, a low dose of NDGA treatment will inhibit LOX production and worsen disease progression.

Therefore, to test these hypotheses and to directly explore the role of COX and LOX products in this disorder, the **objective** of the third study was to examine the impact of COX and LOX product inhibition (using aspirin and NDGA, respectively) on kidney disease progression and oxylipin levels in the kidney in Han:SPRD-*Cy* rats (chapter 5).

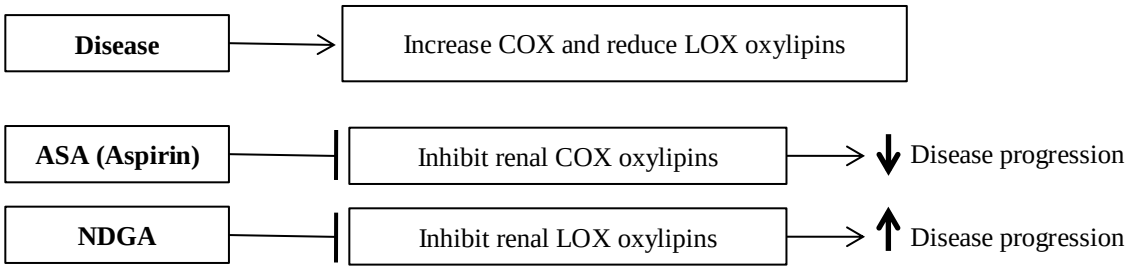


Figure 1.3 Study-3 Hypotheses **# 3**

1.3 References

[1] Corradi V, Gastaldon F, Virzi GM, de Cal M, Soni S, Chionh C et al. Clinical pattern of adult polycystic kidney disease in a northeastern region of Italy. Clin Nephrol 2009;72:259-67.

[2] Fick GM, Johnson AM, Strain JD, Kimberling WJ, Kumar S, Manco-Johnson ML et al. Characteristics of very early onset autosomal dominant polycystic kidney disease. J Am Soc Nephrol 1993;3:1863-70.

[3] Nagao S, Kugita M, Yoshihara D, Yamaguchi T. Animal models for human polycystic kidney disease. Exp Anim 2012;61:477-88.

[4] Niemczyk M, Niemczyk S, Paczek L. Autosomal dominant polycystic kidney disease and transplantation. Ann Transplant 2009;14:86-90.

[5] Wuthrich RP, Mei C. Pharmacological management of polycystic kidney disease. Expert Opin Pharmacother 2014;15:1085-95.

[6] Chen D, Ma Y, Wang X, Yu S, Li L, Dai B et al. Clinical characteristics and disease predictors of a large Chinese cohort of patients with autosomal dominant polycystic kidney disease. PLoS One 2014;9:e92232.

[7] Spithoven EM, Kramer A, Meijer E, Orskov B, Wanner C, Caskey F et al. Analysis of data from the ERA-EDTA Registry indicates that conventional treatments for chronic kidney disease do not reduce the need for renal replacement therapy in autosomal dominant polycystic kidney disease. Kidney Int 2014;86:1244-52.

[8] Collins AJ, Foley R, Herzog C, Chavers B, Gilbertson D, Ishani A et al. Excerpts from the United States Renal Data System 2007 annual data report. Am J Kidney Dis 2008;51:S1-320.

[9] Virzi GM, Corradi V, Panagiotou A, Gastaldon F, Cruz DN, de Cal M et al. ADPKD: Prototype of Cardiorenal Syndrome Type 4. Int J Nephrol 2010;2011:490795.

[10] Hao C, Breyer MD. Physiological regulation of prostaglandins in the kidney. Annual Review of Physiology 2008;70:357-77.

[11] Francois H, Facemire C, Kumar A, Audoly L, Koller B, Coffman T. Role of microsomal prostaglandin E synthase 1 in the kidney. Journal of the American Society of Nephrology 2007;18:1466-75.

[12] Jensen BL, Schmid C, Kurtz A. Prostaglandins stimulate renin secretion and renin mRNA in mouse renal juxtaglomerular cells. Am J Physiol 1996;271:F659-69.

[13] Matzdorf C, Kurtz A, Höcherl K. COX-2 activity determines the level of renin expression but is dispensable for acute upregulation of renin expression in rat kidneys. Am J Physiol 2007;292:F1782-90.

[14] Warford-Woolgar L, Peng CY, Shuhyta J, Wakefield A, Sankaran D, Ogborn M et al. Selectivity of cyclooxygenase isoform activity and prostanoid production in normal and diseased Han:SPRD-cy rat kidneys. Am J Physiol Renal Physiol 2006;290:F897-904.

[15] Sankaran D, Bankovic-Calic N, Ogborn MR, Crow G, Aukema HM. Selective COX-2 inhibition markedly slows disease progression and attenuates altered prostanoid production in

Han:SPRD-cy rats with inherited kidney disease. Am J Physiol Renal Physiol 2007;293:F821-30.

[16] Xu T, Wang N-, Fu L-, Ye C-, Yu S-, Mei C-. Celecoxib inhibits growth of human autosomal dominant polycystic kidney cyst-lining epithelial cells through the VEGF/Raf/MAPK/ERK signaling pathway. Mol Biol Rep 2012;39:7743-53.

[17] Cheng HF, Wang CJ, Moeckel GW, Zhang MZ, McKanna JA, Harris RC. Cyclooxygenase-2 inhibitor blocks expression of mediators of renal injury in a model of diabetes and hypertension. Kidney Int 2002;62:929-39.

[18] Elberg D, Turman MA, Pullen N, Elberg G. Prostaglandin E 2 stimulates cystogenesis through EP4 receptor in IMCD-3 cells. Prostaglandins and Other Lipid Mediators 2012;98:11-6.

[19] Elberg G, Elberg D, Lewis TV, Guruswamy S, Chen L, Logan CJ et al. EP 2 receptor mediates PGE 2-induced cystogenesis of human renal epithelial cells. American Journal of Physiology - Renal Physiology 2007;293:F1622-32.

[20] Mangoo-Karim R, Ye M, Wallace DP, Grantham JJ, Sullivan LP. Anion secretion drives fluid secretion by monolayers of cultured human polycystic cells. American Journal of Physiology - Renal Fluid and Electrolyte Physiology 1995;269:F381-8.

[21] Caligiuri SP, Blydt-Hansen T, Love K, Gregoire M, Taylor CG, Zahradka P et al. Evidence for the use of glomerulomegaly as a surrogate marker of glomerular damage and for

alpha-linolenic acid-rich oils in the treatment of early obesity-related glomerulopathy in a diet-induced rodent model of obesity. Appl Physiol Nutr Metab 2014;39:951-9.

[22] Yamaguchi T, Lysecki C, Reid A, Nagao S, Aukema HM. Renal Cyclooxygenase Products are Higher and Lipoxygenase Products are Lower in Early Disease in the pcy Mouse Model of Adolescent Nephronophthisis. Lipids 2014;49:39-47.

[23] Yamaguchi T, Devassy JG, Gabbs M, Ravandi A, Nagao S, Aukema HM. Dietary flax oil rich in alpha-linolenic acid reduces renal disease and oxylipin abnormalities, including formation of docosahexaenoic acid derived oxylipins in the CD1-pcy/pcy mouse model of nephronophthisis. Prostaglandins Leukot Essent Fatty Acids 2015;94:83-9.

[24] Vitzthum H, Abt I, Einhellig S, Kurtz A. Gene expression of prostanoid forming enzymes along the rat nephron. Kidney Int 2002;62:1570-81.

[25] Farman N, Pradelles P, Bonvalet JP. Determination of prostaglandin E2 synthesis along rabbit nephron by enzyme immunoassay. Am J Physiol 1986;251:F238-44.

[26] Hao CM, Breyer MD. Physiologic and pathophysiologic roles of lipid mediators in the kidney. Kidney Int 2007;71:1105-15.

[27] Camara NO, Martins JO, Landgraf RG, Jancar S. Emerging roles for eicosanoids in renal diseases. Curr Opin Nephrol Hypertens 2009;18:21-7.

[28] Xu ZG, Miao LN, Cui YC, Jia Y, Yuan H, Wu M. Angiotensin II type 1 receptor expression is increased via 12-lipoxygenase in high glucose-stimulated glomerular cells and type 2 diabetic glomeruli. Nephrol Dial Transplant 2009;24:1744-52.

[29] Sankaran D, Bankovic-Calic N, Cahill L, Peng CY, Ogborn MR, Aukema HM. Late dietary intervention limits benefits of soy protein or flax oil in experimental polycystic kidney disease. Nephron Experimental Nephrology 2007;106:E122-8.

[30] Sankaran D, Bankovic-Calic N, Peng CY-, Ogborn MR, Aukema HM. Dietary flax oil during pregnancy and lactation retards disease progression in rat offspring with inherited kidney disease. Pediatr Res 2006;60:729-33.

[31] Sankaran D, Lu J, Bankovic-Calic N, Ogborn MR, Aukema HM. Modulation of renal injury in pcy mice by dietary fat containing n-3 fatty acids depends on the level and type of fat. Lipids 2004;39:207-14.

[32] Sankaran D, Lu J, Ogborn MR, Aukema HM. COX-2 expression in cystic kidneys is dependent on dietary n-3 fatty acid composition. J Nutr Biochem 2007;18:806-12.

[33] Palanisamy N, Viswanathan P, Ravichandran MK, Anuradha CV. Renoprotective and blood pressure-lowering effect of dietary soy protein via protein kinase C βII inhibition in a rat model of metabolic syndrome. Can J Physiol Pharmacol 2010;88:28-37.

[34] Hwang SY, Taylor CG, Zahradka P, Bankovic-Calic N, Ogborn MR, Aukema HM. Dietary soy protein reduces early renal disease progression and alters prostanoid production in obese fa/fa Zucker rats. J Nutr Biochem 2008;19:255-62.

[35] Aukema HM, Lu J, Borthwick F, Proctor SD. Dietary fish oil reduces glomerular injury and elevated renal hydroxyeicosatetraenoic acid levels in the JCR:LA-cp rat, a model of the metabolic syndrome. Br J Nutr 2012:1-9.

[36] Clark WF, Parbtani A, Huff MW, Reid B, Holub BJ, Falardeau P. Omega-3 fatty acid dietary supplementation in systemic lupus erythematosus. Kidney Int 1989;36:653-60.

[37] Maaløe T, Schmidt EB, Svensson M, Aardestrup IV, Christensen JH. The effect of n-3 polyunsaturated fatty acids on leukotriene B4 and leukotriene B5 production from stimulated neutrophil granulocytes in patients with chronic kidney disease. Prostaglandins Leukotrienes Essential Fatty Acids 2011;85:37-41.

[38] Fair DE, Ogborn MR, Weiler HA, Bankovic-Calic N, Nitschmann EP, Fitzpatrick-Wong SC et al. Dietary soy protein attenuates renal disease progression after 1 and 3 weeks in Han:SPRD-cy weanling rats. J Nutr 2004;134:1504-7.

[39] Ogborn MR, Nitschmann E, Bankovic-Calic N, Weiler HA, Aukema HM. Dietary soy protein benefit in experimental kidney disease is preserved after isoflavone depletion of diet. Exp Biol Med 2010;235:1315-20.

[40] Aukema HM, Housini I. Dietary soy protein effects on disease and IGF-I in male and female Han:SPRD-cy rats. Kidney Int 2001;59:52-61.

[41] Asanoma M, Tachibana N, Hirotsuka M, Kohno M, Watanabe Y. Effects of soy protein isolate feeding on severe kidney damage in DOCA salt-treated obese Zucker rats. J Agric Food Chem 2012;60:5367-72.

[42] Watanabe G, Tomiyama H, Doba N. Effects of oral administration of L-arginine on renal function in patients with heart failure. J Hypertens 2000;18:229-34.

[43] Gabardi S, Munz K, Ulbricht C. A review of dietary supplement-induced renal dysfunction. Clin J Am Soc Nephrol 2007;2:757-65.

[44] Dia VP, Berhow MA, Gonzalez De Mejia E. Bowman-Birk inhibitor and genistein among soy compounds that synergistically inhibit nitric oxide and prostaglandin E2 pathways in lipopolysaccharide-induced macrophages. J Agric Food Chem 2008;56:11707-17.

[45] Stichtenoth DO, Wagner B, Frolich JC. Effect of selective inhibition of the inducible cyclooxygenase on renin release in healthy volunteers. J Investig Med 1998;46:290-6.

[46] Farzamirad V, Aluko RE. Angiotensin-converting enzyme inhibition and free-radical scavenging properties of cationic peptides derived from soybean protein hydrolysates. Int J Food Sci Nutr 2008;59:428-37.

[47] Peng CY, Sankaran D, Ogborn MR, Aukema HM. Dietary Soy Protein Selectively Reduces Renal Prostanoids and Cyclooxygenases in Polycystic Kidney Disease. Exp Biol Med 2009;234:737-43.

[48] Ibrahim NH, Jia Y, Devassy JG, Yamaguchi T, Aukema HM. Renal cyclooxygenase and lipoxygenase products are altered in polycystic kidneys and by dietary soy protein and fish oil treatment in the Han:SPRD-Cy rat. Mol Nutr Food Res 2014;58:768-81

[49] Trelle S, Reichenbach S, Wandel S, Hildebrand P, Tschannen B, Villiger PM et al. Cardiovascular safety of non-steroidal anti-inflammatory drugs: network meta-analysis. BMJ 2011;342:c7086.

Chapter 2
2. Literature Review

2.1 Cystic kidney disease (pathophysiology and animal models)

Cystic kidney disease is the most common of the inherited renal diseases, and encompasses a group of disorders characterized by the growth of numerous renal cysts, interstitial fibrosis, and the development of a variety of external manifestations, and often leads to ESRD and death [1-3]. The initiating event in cystic kidney disease is genetic mutation. In humans, ADPKD can be caused by a mutation in either *PKD1* or *PKD2* genes [3,4]. About 85% of cases of ADPKD are caused by a mutation in *PKD1* and 15% are caused by mutation in *PKD2* [4]. Regardless of the type of cystic kidney disease, studies using different experimental models have identified three essential pathophysiological mechanisms for the development and progression of cystic kidney disease: cell proliferation, fluid secretion, and extracellular matrix remodelling [3,4]. This increase in cyst number and size compresses the surrounding renal parenchyma, obstructs nephrons, and impairs kidney function, leading to renal failure [5,6]. Studies have also suggested a role for 3',5' cyclic adenosine monophosphate (cAMP) and prostanoids in the pathogenesis of this renal disorder [7-10]. Indeed, in human ADPKD, it has been demonstrated that PGE_2 induces cAMP and cyst formation through PGE_2 receptor-2 (EP_2) activity [9], illustrating the importance of oxylipins in the pathogenesis of cystic kidney disease.

To date, several treatment options have been shown to help in reducing cyst growth, inflammation, and fibrosis in different animal models of cystic kidney disease, such as glucocorticoids, immunosuppressants, peroxisome proliferator activated receptor gamma (PPARγ) agonist, ACE and COX2 inhibitors, vasopressin V2 receptor antagonist, and nuclear

factor kappa binding (NF-κB) inhibitors, reviewed in [5]. However, so far there is no actual treatment that has been shown to reduce the disease progression in human clinical trials.

Animal models of cystic kidney disease have played a very important role in understanding the mechanisms of cyst development and progression, identification of disease causing genes, and identification of potential treatment options [3,4,11,12]. There are two major types of cystic kidney disease animal models: spontaneous hereditary models identified by the distinctive manifestations of cystic kidney disease, and modified models established by mutation of human orthologous genes [3]. Spontaneous hereditary models of cystic kidney disease have many similar symptoms seen in humans but the responsible genes are not always orthologous to human genes [3,4]. Several models have been established for the study of cystic kidney disease including bpk, cpk, orpk, Pkd1 and Pkd2 mice and the PCK and Han:SPRD-*Cy* rats. Although the PCK rat and the *pcy* mouse are orthologous models of human cystic kidney disease with mutations in the *Pkhd1* and the *Nphp3* genes, respectively, they have a very slow progressive phenotype [3,6]. In 1991, Rittinghausen *et al.* described a strain of rats, the Han:SPRD-*Cy* rats that inherited cystic kidney disease as an autosomal incomplete dominant trait [13]. The model has a mutation in the *Pkdr1* gene (encoding the protein SamCystin). Recently, increased levels of both total and active β-catenin and mutation in ANKS6 associated cystic kidney disease have also been reported in this model [14].

In this model, homozygous rats usually show a markedly distended abdomen and die within the first 3 to 4 weeks of age, while heterozygous rats show signs of illness more slowly and die at about 7-12 months of age. Heterozygous male develop cystic kidney disease to a larger extent than the females. Both heterozygotes males and females develop renal cystic disease, however, only the males develop significant interstitial inflammation, interstitial

fibrosis, and azotemia [12,13]. The macroscopic and histological appearance of kidneys in heterozygous rats with cystic kidney disease was found to closely resemble that of human cystic kidney disease [12,13].

In addition, this rat model of kidney disease is widely accepted and it has been used in several studies of the pathogenesis and treatment of inherited renal cystic disease. In addition, our lab has extensive experience with it [7,15-18]. Both normal and diseased males of this model were used in our current studies.

2.2 Oxylipins and cystic kidney disease

Oxylipins, bioactive metabolites derived from the oxygenation of PUFA, play a very impotent role in inflammation and tissue homeostasis, and have recently been implicated in many diseases including cancer, cardiovascular, and kidney diseases [19-24]. However, the role of many oxylipins in the progression of cystic kidney disease is still uncertain or not known. The most well-known precursor of oxylipins in the body is the C_{20} omega-6 fatty acid arachidonic acid (AA) [25]. However, oxylipins can also be produced from other PUFA including dihomo-γ-linolenic acid (DGLA), EPA, DHA, and ALA. For more information about oxylipins, their source fatty acid, and the enzyme responsible for their production see Table 2.1. These fatty acids can be directly obtained from the diet or synthesized *de novo* through lipogenesis as illustrated in Figure 2.1. When tissues are exposed to different physiological and pathological stimuli, such as hormones, growth factors and cytokines, AA is released from membrane phospholipids by the action of phospholipase A_2 (PLA_2). AA can then be converted into bioactive oxylipins called the eicosanoids (subclass of oxylipins) through three enzymatic pathways: 1) the cyclooxygenase (COX), 2) the lipoxygenase (LOX), and 3) the cytochrome P450-mediated pathways (CYP) [25,26]. While, the COX pathway

results in the formation of prostaglandins (PG) and thromboxanes (Tx) (collectively called prostanoids), the LOX pathway results in the formation of hydroxy-eicosatetraenoic acids (HETE), and leukotrienes (Lt), and the CYP pathway results in the formation of epoxy-eicosatrienoic acids (EpETrE) via epoxygenase as well as 16-20- HETEs via ω-hydroxylase activity [25-27].

It is very well established that PLA_2 is the main enzyme responsible for the release of AA from the phospholipid membrane to produce oxylipins [28]. There are several types of PLA_2, nevertheless, $cPLA_{2\alpha}$ is the only PLA_2 that exhibits preference for hydrolysis of AA from phospholipid substrates in cells stimulated with diverse physiological and pathological factors [29,30].

It has been more than two decades since the discovery of PLA_2 in cells [31]. Cloning of $PLA_{2\alpha}$ revealed the C2 domain for $[Ca^{2+}]$ regulation; this was followed by the characterization of stimulus-dependent phosphorylation by mitogen-activated protein kinases (MAPKs) and the identification of the active site residues of this enzyme [28,32,33]. The discovery of PLA_2 played a very important role in understanding how oxylipins are produced from the cell membrane. Today, the role of PLA_2 in mediating AA release and oxylipin production is very well established. PLA_2 is expressed in different types of cells in multiple organs, including the kidney, and it can also be induced transcriptionally through a number of signaling pathways involving Ras and MAPKs, and transcriptional activators of the nuclear factor NF-kB [28,30,34]. Pro-inflammatory cytokines, such as interleukin-1β and tumor necrosis factor, induce the secretion of $cPLA_2$ from glomerular mesangial cells in parallel to enhanced PGE_2 production. Suppression of $cPLA_2$ has also been shown to reduce PGE_2 production in mesangial cells [35].

Several studies reviewed the regulation of $cPLA_{2\alpha}$ by posttranslational processes and reported that the translocation of $cPLA_{2\alpha}$ from the cytosol to intracellular membrane can be promoted by an increase in intracellular [Ca^{2+}] [36-38].. The phospholipid binding specificity of the C2 domain of $cPLA_{2\alpha}$ (phosphatidylcholine) plays a major role in determining its distinct subcellular targeting to intracellular membranes, respectively (reviewed in [30]). With respect to cystic kidney disease, polycytin-2, a protein encoded by a gene that is mutated in ADPKD, functions as an intracellular Ca^{2+} channel and PKD results from the loss of intracellular [Ca^{2+}] regulation [39]. cPLA2 is implicated in several pathophysiological processes in the kidney and it is involved in the pathogenesis of kidney disease as evidenced by alterations in renal disorders including PKD [34,40]. For example, higher levels of membrane bound $cPLA_2$ were observed in diseased kidneys in the *pcy* mouse and Han:SPRD-*Cy* rat models of cystic kidney disease [34].

After the release of AA from the membrane phospholipid by $cPLA_{2\alpha}$, COX1 and -2 enzymes further metabolize it to produce oxylipins [25,26]. COX catalyzes the rate-limiting step in the production of oxylipins from the COX pathways [25,26]. Both COX isoforms are integral membrane proteins, and are primarily localized to the endoplasmic reticulum membrane (where they are N-glycosylated) but they also occur on the Golgi (reviewed in [30])

5-LOX initiates the synthesis of Lt from AA [25,26]. In resting cells, 5-LOX can accumulate in either the cytoplasm or the nucleoplasm. Stimulation of the cell translocates 5-LOX to the nuclear envelope to induce Lt synthesis. Localization of 5-LOX within the nucleus of resting cells and the production of Lt requires 5-LOX activating protein (FLAP) (an integral nuclear envelope protein that functions as an AA binding protein). $cPLA_{2\alpha}$ derived AA has

been demonstrated to increase the ability of FLAP to recruit 5-LOX for complex formation and oxylipin production from the LOX pathway (reviewed in [30]).

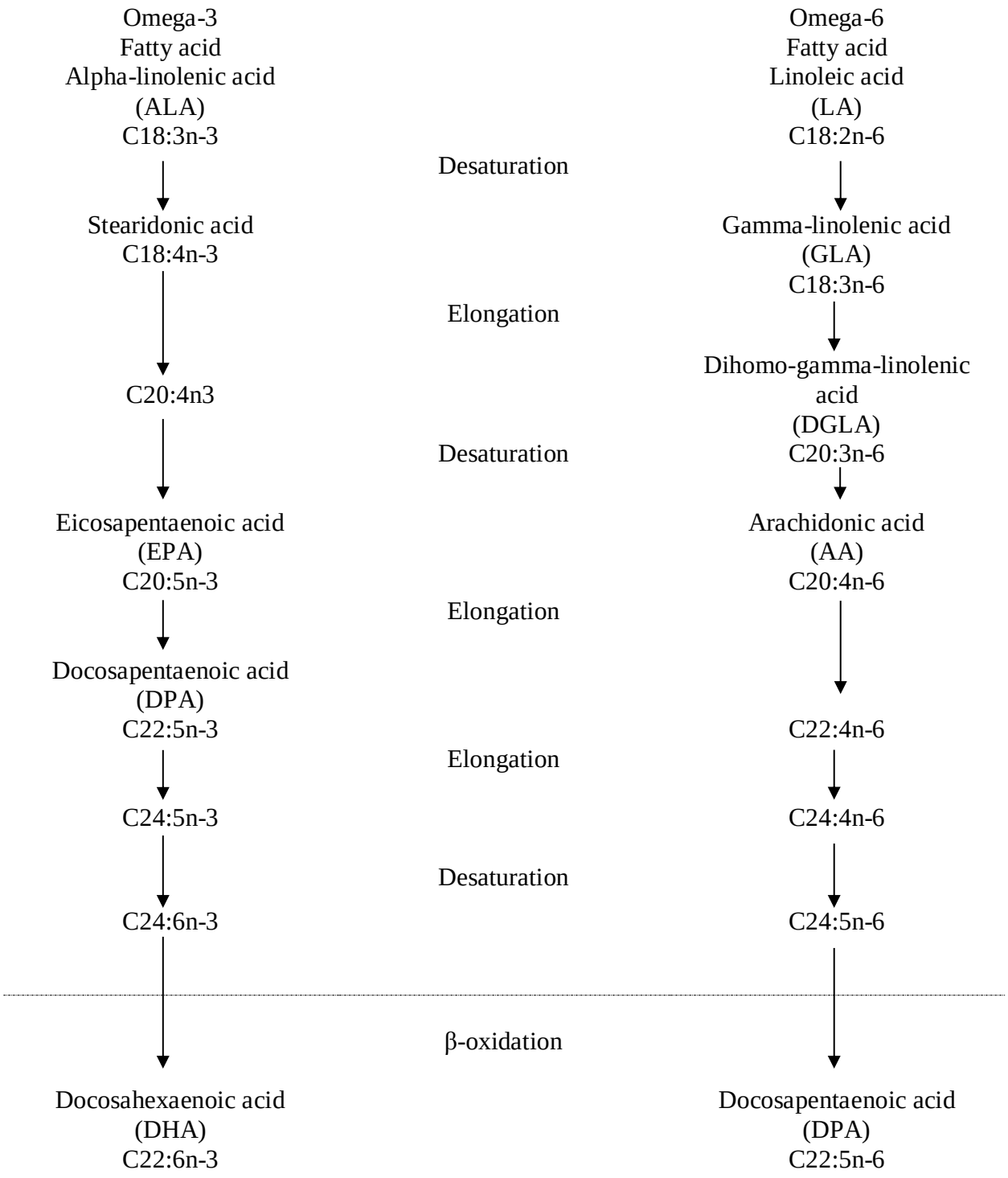
Within the normal kidney, oxylipins play an important role in maintaining kidney function and processes including blood flow, hemodynamics, water and solute transport, and renin secretion [7,41-43]. In the diseased kidney, oxylipins have been shown to have significant roles in maintaining glomerular filtration rate (GFR) by regulating hemodynamics and salt-water homeostasis, but they can also be involved in inflammatory and fibrotic processes in response to kidney injury [15,26,44,45]. More details about pathways of oxylipin metabolism and cystic kidney disease will be discussed next.

Table 2.1 Examples of oxylipins produced from different PUFA, and the pathway responsible for their production

Pathway (fatty acid)	Name of oxylipin
COX (AA)	6-keto-$PGF_{1\alpha}$
	PGD_2
	PGE_2
	$PGF_{2\alpha}$
	TXB_2
COX (EPA)	PGD_3
	PGE_3
	$PGF_{3\alpha}$
	TXB_3
LOX (AA)	5-HETE
	8-HETE
	9-HETE
	11-HETE
	12-HETE
	15-HETE
LOX (LA)	9-HODE
	13-HODE
	9-OxoODE
	15-HETrE
	9-HOTrE
LOX (DHA)	4-HDoHE
LOX (EPA)	5-HEPE
	12-HEPE
CYP (AA)	11,12-DiHETrE
	14,15-DiHETrE
	20-HETE
CYP (LA)	9,10-DiHOME
	12,13-DiHOME
CYP (EPA)	18-HEPE

AA, arachidonic acid, ALA, α-Linolenic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; DiHETrE, dihydroxy-eicosatrienoic acid; HETE, hydroxy-eicosatetraenoic acid; HODE, hydroxyoctadecadienoic acid; HOTrE, hydroxy-octadecatrienoic acid, HEPE, hydroxy-eicosapentaenoic acid; HDoHE, hydroxy-docosahexaenoic acid; LA, Linoleic acid; PG,prostaglandin; TX, thromboxane

Figure 2.1 Metabolic pathways of omega-3 and omega 6 fatty acids



2.2.1 The cyclooxygenase (COX) pathway

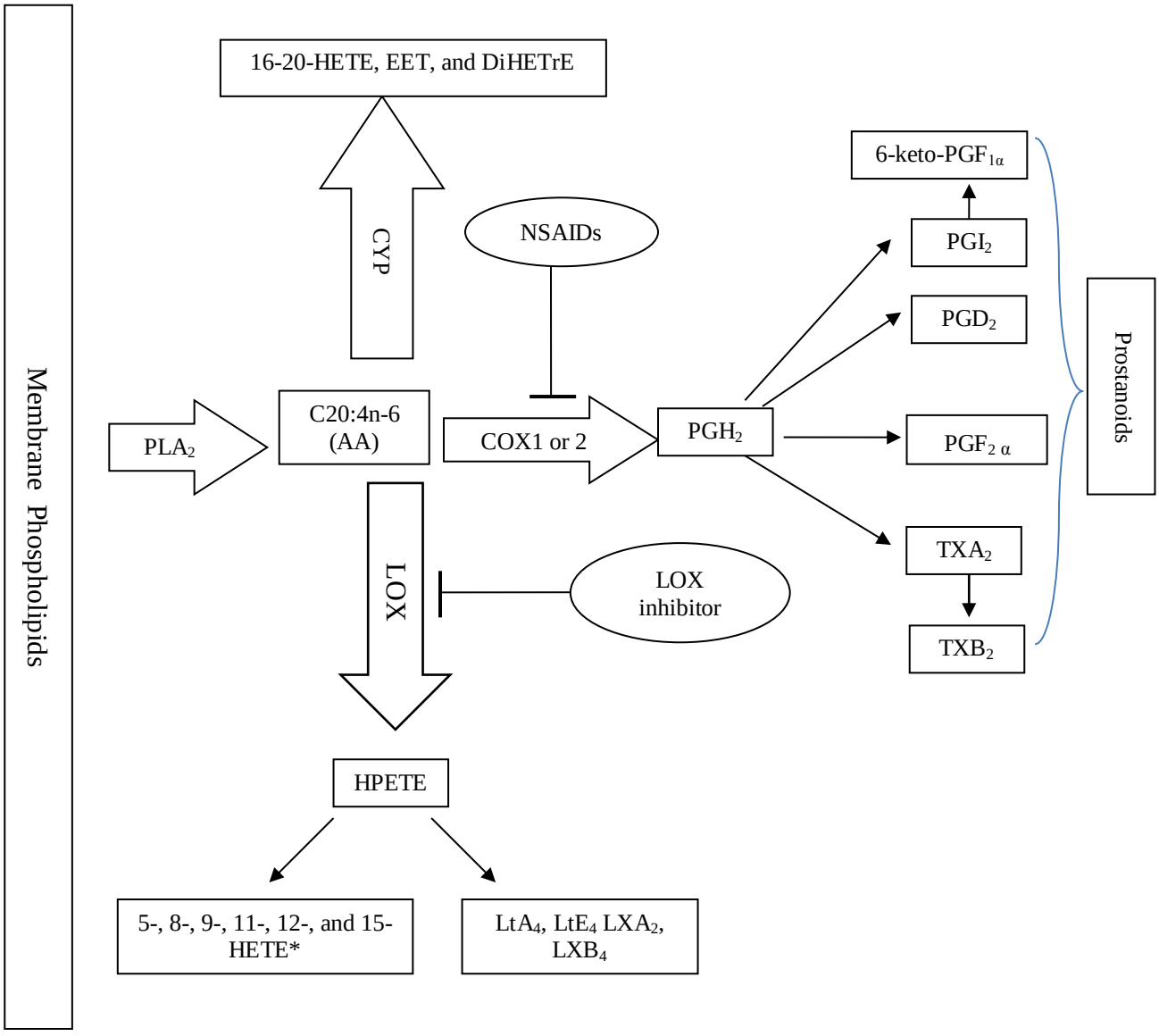
The COX pathway produces PG and Tx. These collectively are called the prostanoids. The biosynthesis of this subclass of oxylipins starts with the release of AA from phospholipids of cell membranes. This reaction is mainly catalyzed by phospholipase A_2 (PLA_2). The next step is the generation of prostanoids via COX or prostaglandin H synthase (PGHS) by oxidization of AA (oxidation reaction) to form the unstable intermediate hydroperoxy-endoperoxide PGH_2, which is subsequently metabolized by prostanoid synthases, prostaglandin E synthase (PGES**),** prostaglandin I synthase (PGIS), prostaglandin D synthase (PGDS), prostaglandin F synthase (PGFS), and thromboxane synthase (TXS), to form PGE_2, PGI_2, PGD_2, $PGF_{2\alpha}$, and TXA_2, respectively (Figure 2.2) [19,25]. COX also converts EPA to the three series prostanoids, PGI_3, PGD_3, $PGF_{3\alpha}$, PGE_3 and TXB_3, which are considered to be less proinflammatory compared to other AA metabolites [19,25].

Three isoforms of COX have been identified: COX1, COX2 and COX3 [46]. COX3, is the most recently identified isoform; it was discovered by Northern analysis of canine cerebral cortex RNA and it has been suggested to be the main target for the acetaminophen, nonsteroidal anti-inflammatory drug (NSAID), action [47]. However, COX3 protein is not expressed in human and rodents and we do not have any information about the role of this isoform of COX in the kidney [47]. Unlike COX3, we have more information about the two other isoforms. Both COX1 and -2 proteins are about 600 amino acids in size (COX1= 70 KDa, COX2=72 KDa) and share 60% homology at the amino acid level [48].

Nevertheless, initial studies showed that COX1 is constitutively expressed in virtually all cell types, and COX2 is thought to be mainly inducible and involved in inflammatory responses [49-51]. However, kidney disease was associated with elevated COX1 and COX2 enzyme activities in a number of renal disorders and nephritic models [7,8]. In addition, it has been proposed that PG derived from the constitutively expressed COX1 would predominantly perform homeostatic functions, whereas the higher levels of PG would be observed after the induction of COX2 would mediate the inflammatory responses and therefore can be therapeutically targeted. These findings contributed to the development of a new class of NSAID called COX2 inhibitors, which could selectively block COX2 and prevent the production of PGs that cause pain and inflammation without affecting the COX1 enzyme activity [7,52]. COX2 selective inhibitors are uniquely different from traditional NSAIDs; while the traditional NSAIDs block both COX1 and -2, COX2 selective inhibitors selectively block COX2 enzyme and not the COX1 enzyme. COX2 importantly modulates renal blood flow and glomerular filtration rate as well as sodium, potassium and water excretion by the kidney. Countering the previous findings, further studies have demonstrated that COX1 and -2 have both a constitutive and inductive role in the kidney. In 1994 Harris *et al* [53] demonstrated for the first time that COX2 is constitutively expressed in the juxtaglomerular apparatus, macula densa, cortical thick ascending limb of loop of Henle, and medullary interstitial cells of rats. Modulation of the renin-angiotensin system by renocortical COX2 metabolites has been demonstrated in studies using COX2 inhibitors [54,55], COX2 metabolites have been implicated in the mediation of renin release, regulation of sodium excretion, and maintenance of renal blood flow, indicating the important role of these enzymes in maintaining kidney functions under normal physiological and pathological conditions.

Figure 2.2 Simplified pathways of oxylipin production from the PUFA arachidonic acid (AA)

***** There is evidence that 11- and 15-HETE can also be produced from the COX pathway.
AA, arachidonic acid, COX, cyclooxygenase; DiHETrE, dihydroxy-eicosatrienoic acid; HETE, hydroxy-eicosatetraenoic acid; HPETE, hydroperoxy-eicosatetraenoic acid; LOX, lipoxygenase; NSAID, Nonsteroidal anti-inflammatory drug; PLA_2 phospholipase A_2, PG, prostaglandin; TX, thromboxane



Of the prostanoids, the most investigated with the best defined renal functions are PGE_2 and PGI_2. PGE_2 is a mediator of sodium reabsorption in the distal renal tubule and acts as a counter-regulatory factor under conditions of increased sodium reabsorption by limiting salt and water reabsorption [56,57].

In relation to cystic kidney disease, COX and phospholipase A_2 (PLA_2) levels are altered in the Han:SPRD-*Cy* rat and *pcy* mouse models of cystic kidney disease [7,15], and COX activities and same prostanoids (i.e. PGE_2, PGI_2, and TXA_2) are higher in diseased compared to normal kidneys in the Han:SPRD-*Cy* rat [8,58]. Consequently, pharmacologic inhibition of COX2 reduces kidney disease progression and results in a marked attenuation of renal cyst growth and interstitial fibrosis in this model [7,59], and suppresses proliferation of human polycystic kidney disease epithelial cells in cell culture [59].

In the *pcy* mouse model of cystic kidney disease, it has been demonstrated that COX derived oxylipins are higher in diseased compared to normal kidneys and feeding rats with FXO reduces the production of AA derived COX oxylipins and increases the levels of ALA (C18:3 n-3) and DHA (C22:6 n-3) derived LOX oxylipins in the diseased kidneys in parallel with a reduction in renal fibrosis and improvement in kidney function [60].

The elevated levels of renal COX metabolites in cystic kidney disease may accelerate disease progression via several mechanisms [9,10,23], such as stimulation of epithelial cell proliferation and transepithelial fluid secretion. The expansion of cysts in cystic kidney disease is determined to a major extent by these two processes [61,62], both of which are stimulated by cAMP [23,63]. cAMP formation is regulated in part via these COX metabolites [23]. PGE_2, PGI_2, and TXB_2 stimulate cell proliferation, fluid secretion, and cyst formation in renal epithelial cells from humans and mice with cystic kidney disease [9,64,65]. In addition

to promoting cAMP formation, PGs may also increase the cellular cAMP levels through inhibiting the enzyme cAMP phosphodiesterase whish selectively catalyzes the hydrolysis of the phosphodiester bond in cAMP [66]. These studies provide strong evidence regarding the importance of these oxylipins and their role in disease progression in different models of cystic kidney disease. Nevertheless, other oxylipins also may be important, as they are altered in other renal diseases [41,67-70] and they still need to be investigated**.**

2.2.2 The lipoxygenase (LOX) pathway

In the kidney, LOX products are primarily formed by leukocytes and include the leukotrienes (Lt), the monohydroxy-eicosatetraenoic acids (HETE), and the lipoxins [19,25]. Three LOX enzymes have been cloned, and expressed (5-, 12, and 15-LOX). These enzymes are capable of oxidizing AA and other PUFA including EPA, DHA, LA, and ALA. The initial oxygenation of AA by 5-LOX, 12-LOX, and 15-LOX will produce 5-hydroperoxy-eicosatetraenoic acid (5-HPETE), 8- HPETE, 12-HPETE, and 15-HPETE respectively [25]. These HPETEs are highly reactive, short-lived metabolites, which are subsequently converted to HETEs, primarily 5-, 8-, 12-, and 15-HETEs.

LtA_4 can be produced through the action of 5-LOX and can be further metabolized to form LtB_4, LtC_4, LtD_4, and LtE_4 [26]. In the kidney, LOX products play an important role in the regulation of blood pressure, vasoconstriction, renal blood flow and GFR regulation. Enhanced Lt production within the kidney could promote renal injury and inflammation by a variety of direct and indirect mechanisms. On the other hand, lipoxins, including lipoxin A_4 and B_4, represent a unique class of lipid mediators with potent anti-inflammatory actions; thus, it can oppose the actions of Lts and it can be formed by leukocytes during cell-cell interaction under a variety of conditions including inflammation [71]. It has been shown that the LOX

product (12-HETE) activates COX2 protein expression in pancreatic β cells [72] and in mesangial cells in a time and dose dependent manner [73]. In diabetic nephropathy, it is has been demonstrated that COX and the 12/15-LOX products cross- activate the corresponding enzymes and thereby greatly augment the process involved in disease progression [73].

Nevertheless, most of the previous studies focused on particular COX products without investigating the effect of disease and diet on oxylipins produced from the other pathways of oxylipin metabolism. In order to address the question as to whether higher or lower LOX products are directly associated with disease progression in cystic kidney disease, specific pharmacological LOX inhibitors still need to be tested.

2.2.3 The cytochrome P450 (CYP) pathway

The cytochrome P450 (CYP)-derived AA metabolites, including the four regioisomeric epoxyeicosatrienoic acids, EpETrE, (5,6-, 8,9-, 11,12-, and 14,15-EpETrE) and the hydroxy-eicosatetraenoic acid, HETES, (18-, 19-, and 20-HETEs), have been shown to have major autocrine and paracrine actions through which they mediate cell functions [20,74]. These metabolites have been implicated in the regulation of ion transport mechanisms, vascular tone, inflammation, cell proliferation and differentiation, renal hemodynamics and salt and water reabsorption and secretion [20,74].

In the kidney, the major site of synthesis of 20-HETE and EpETrE is the renal microcirculation [20]. Several vascular CYP proteins including CYP2C epoxygenases and CYP4A α-hydroxylases (The primary enzyme responsible for 20-HETE synthesis in the renal vasculature) are expressed on the vascular wall and have a large capacity to produce EpETrE and 20-HETE. While EpETrE are primarily localized to the endothelium, 20-HETE synthesis

is localized to the smooth muscle cell. EpETrE and 20-HETE have opposing effects on the vasculature; EpETrE are primarily vasodilators, whereas 20-HETE is a potent vasoconstrictor [75]. EpETrE produce vascular relaxation by activating smooth muscle large-conductance Ca^{2+}-activated K^{+} channels (BK_{ca}). In addition, it is believed that EpETrE contribute to the maintenance of vascular homeostasis by upregulating endothelial nitric oxide (eNOS) activity and increasing thereby the production of the vasodilator nitric oxide (NO) [76]. In small resistance arteries, 20-HETE acts as a functional antagonist of EpETrE [75]. Therefore, the relative amounts of each of these bioactive oxylipins may determine vascular tone and thereby kidney haemostasis and blood pressure. It has been reported that 20-HETE promotes hyperplasia of renal epithelial cells leading to the formation of cysts in a murine model of ARPKD [77] and chronic inhibition of 20-HETE reduces kidney size and cyst formation in the same model of cystic kidney disease [77].

Nevertheless, it has been demonstrated that some of the oxylipins produced via CYP pathway have many protective cardiovascular and renal properties, including vasodilatory, anti-inflammatory, anti-fibrotic, and anti-apoptotic effects [78-80]. Therefore, any reduction in these oxylipins may alter kidney disease progression and affect blood pressure in these rats. Recently it has been reported that plasma levels of epoxy-eicosatrienoic acid, a CYP oxylipin, are lower in patients with cystic kidney disease [81].

There is very little information about the role of these oxylipins in cystic kidney disease and whether diet can affect the production of these oxylipins still needs to be investigated.

2.3 Inhibition of COX and LOX products

2.3.1 COX inhibition, benefits and concerns

Traditional NSAIDs are among the most commonly used drugs and it is estimated that 5%-10% of all adults in the U.S use NSAIDs regularly [82,83]. More than 98 million prescriptions of NSAIDs were filled in 2012 in the U.S. and NSAIDs have accounted for more than 70 million prescriptions and 30 billion over the-counter purchases [82,83]. NSAIDs act by inhibiting COX1 and 2, which impedes the production of COX metabolites. This discovery provided a unifying explanation for their therapeutic actions and strongly established certain PG as important mediators of inflammatory diseases [7,25,50,54,84].

Selective COX2 inhibitors such as celecoxib, rofecoxib, and valdecoxib (coxibs), are widely used for the treatment of inflammatory conditions, including arthritis [82]. However, there have been some safety concerns about the use of these drugs. They have been found to be associated with increased risk of cardiovascular events and toxicity in patients with and with no history of cardiovascular diseases (CVD) [85-87]. In 2004, Rofecoxib was withdrawn from the market after it has been shown to be associated with increased risk of cardiovascular events in a placebo controlled clinical trial [85]. These findings have been further confirmed by systematic reviews of observational studies [86-88]. Trelle and *et al*. (2011) [88] reviewed thirty-one randomised controlled trials evaluating seven different NSAIDs and reported that coxibs were investigated the most (15 trials) and were associated with the highest risk of cardiovascular events [88].

Aspirin is among the most prescribed NSAIDs and one of the most frequently used over-the counter drugs worldwide. Its therapeutic effect has been demonstrated by several studies in a number of disease conditions and it is recognized for its role in the primary

prevention of cardiovascular events including myocardial infarction and stroke [89-92]. Concerns were also raised regarding the chronic use of NSAIDs is their potential to cause renal damage in people with renal insufficiency [93-95]. However, studies showed no adverse renal effects for aspirin use; a population based case control study targeted mainly to people with end-stage renal disease (ESRD) reported that there is no increased risk of renal damage or failure with aspirin use compared to other NSAIDs [96]. This observation was also supported by clinical trials; the first UK Heart and Renal Protection Study reported that low-dose aspirin (100 mg/d) did not significantly impair kidney function or increase the risk for major bleeding [97].

Elevated COX-2 enzyme activity has been reported in a number of renal disorders and nephritic models [7,8,17,48] including the Han:SPRD-*Cy* rat model of cystic kidney disease [8]. In this model, it has been demonstrated that renal disease selectively alters the prostanoid profile and ratio in the kidney. For example, results from our lab show that select prostanoids such as renal thromboxane B_2 (TXB_2), PGI_2, and PGE_2 were higher in diseased as compared to normal rats [7]. In addition, kidney disease was associated with elevated COX1 and COX2 enzyme activities in a number of renal disorders, and nephritic models [8]. In Han:SPRD-*Cy* rats, selective inhibition of COX2 (by NS-398) reduces the disease associated elevation in renal COX2 activity, ameliorates the alteration in prostanoid production related to the disease and results in marked attenuation of renal cyst growth, interstitial fibrosis, and macrophage counts [7]. Consistent with the selectivity of the drug, NS-398 did not significantly affect renal COX1 activity. Another study showed that selective inhibition of COX2 inhibited the growth of human PKD cyst-lining epithelial cells through the Raf/MAPK/ERK signaling pathway

[98], indicating that COX plays a significant role in the progression of disease in this renal disorder.

Considering the concerns around the cardiovascular safety of selective COX2 inhibitors [99], the absence of effective treatment for cystic kidney disease to date [100,101], and the fact that both COX1 and COX2 are elevated in this renal disorder, the benefits of a dual COX isoform inhibitor such as aspirin in the Han:SPRD-*Cy* rat model still needs to be investigated.

2.3.2 LOX inhibition

As for LOX inhibitors, the main enzyme in the production of Lt is 5-LOX. Therefore, it has been targeted for the development of anti-Lt drugs. U-60257 was found to be an effective in *vitro* inhibitor of allergen induced Lt biosynthesis in lung tissues from asthmatics [102]. Many 5-LOX inhibitors have been developed and some of them have been found to be effective in different asthma models. One drug, Zileuton (Zyflo ®) has been shown to have clinical efficacy and it is a US registered drug for asthma treatment [102]. In addition, inhibition of 5-LOX is documented as a main therapeutic target for drug development for joint inflammation (rheumatic diseases), and vascular diseases, as well as for certain cancers [103-105]. It was recognized that combination therapy with agents that block both COX2 and 5-LOX was preferred for arthritic inflammation because they would minimize the adverse effects on inhibition of PGD_2 needed for gastric protection (an anti-inflammatory product of COX1) and minimize any vascular impacts of COX2 inhibition [106,107].

In the kidney, the implications of a reduction in LOX oxylipins are less clear. However, similar to COX products, LOX metabolites also may be important, as they have been shown to

be altered in other renal diseases [60,69,70]**.** Lt play a significant role in the regulation of blood pressure, vasoconstriction, renal blood flow and GFR regulation [44,108,109]. Therefore, enhanced Lt production within the kidney could promote renal injury and inflammation by a variety of direct and indirect mechanisms. Recently, it has been demonstrated that LOX metabolites are lowered in cystic kidneys in the *pcy* mouse model [60]. Furthermore, the production of oxylipins from n-3 fatty acids is greater than the formation of n-6 oxylipins by the LOX pathway [110,111]. Furthermore, several protective metabolites such as lipoxins, HDoHE, and HEPE are produced by LOX [110]. Therefore, inhibition of LOX could be potentially harmful for the kidney. However, the effects of LOX inhibitors in the cystic kidney disease still need to be investigated.

2.4 Recent advances in oxylipin profiling

Radio- and enzyme immunoassay techniques have been used for the detection and quantitation of oxylipins. While very sensitive, these approaches have two limitations: 1) usually, only a single oxylipin can be detected at a time and 2) cross reactivity can cause variability in sample quantification [112]. Several other quantitative analytical techniques have been developed to analyse oxylipins. Historically, gas chromatography (GC), GC/mass spectrometry (MS) and high performance liquid chromatography (HPLC) with UV detection and MS. GC has long been the method of choice for routine separation of fatty acids and oxylipins [113,114]. Nevertheless, it has been reported that among these techniques, LC/MS/MS is considered to be one of the most reliable methods. Unlike GC/MS, LC/MS/MS does not require lengthy derivatization procedures which results in many advantages including: shorter time for sample pre-treatment, increased recovery, analyzing a large number of

oxylipins at the same time, and lack of formation of incomplete derivatization by-products [113].

A few limitations of LC-MS/MS have been reported. For example, while sample throughput for LC-MS/MS is higher than for GC-MS, it is less than automated immunoassays [115]. However, several techniques have been developed to improve throughput in LC-MS/MS including direct sample injection, LC-multiplexing and sample multiplexing [115]. Another concern is specificity and sensitivity. Methods to improve specificity and sensitivity include sample clean-up and optimizing chromatography to avoid interferences and ion suppression due to sample-matrix components. Nevertheless, newer generation LC-MS/MS offers both very strong specificity and sensitivity [115].

In addition, LC-compatible ionization methods such as electrospray ionization (ESI) offer tremendous sensitivity and efficient ionization of fatty acid oxidation products. Unterwurzacher *et al*. (2008) validated an LC-MS/MS assay to detect prostanoids, LOX-derived fatty acid metabolites and PUFA in a small sample of human plasma (20 µl). The technique was found to be rapid, reliable and efficient and it can be used for various biological samples [114]. In another study, Deems *et al*. (2007) successfully developed a protocol for identifying and quantifying more than 50 oxylipins in a single ESI-based LC-MS/MS run [116]. These advances in the method of detection and quantitation of oxylipins allowed us to scan for a large number of oxylipins in the kidney and determine the endogenous levels of renal oxylipins produced from the three pathways for the first time. In the present studies, we used this technique with some modification to make it applicable for kidney tissues; these modifications included homogenization of kidneys, oxylipin extraction and separation techniques, the use of antioxidant cocktail, and the scanning and detection of a larger number

of oxylipins. This technique employs a solid-phase extraction procedure to isolate oxylipins and an LC combined with MS/MS method to separate species and to unambiguously identify a large number of oxylipins. For quantitating oxylipins, deuterated internal standard, were selected that had a different precursor ion mass than the target analyte, but were chemically and structurally as similar to the target analyte as possible [113,114,116].

2.5 Diet, cystic kidney disease, and oxylipins

Dietary interventions in cystic kidney disease are very important not only to reduce the rate of disease progression from stage to stage (GFR declining rate) but also to prevent complications associated with cystic kidney disease, including but not restricted to, cardiovascular disease (CVD), hypertension, protein energy malnutrition, dyslipidemia, anemia, and bone loss [117,118].

Multiple studies support nutritional management as a cornerstone in the treatment of cystic kidney disease [60,119-121]. Several dietary interventions have been tentatively investigated, most importantly the modification of the type and quantity of dietary protein and fat [16,17,60,70,117-124]. Among these, dietary conjugated linoleic acid (CLA) has been shown to reduce interstitial inflammation and fibrosis through reducing the elevated levels of prostanoids, COX2 and, PLA_2 activities in diseased kidneys of the Han:SPRD-*Cy* rats [123,125]. On the other hand, a high fat diet increases kidney disease progression in the same model [126] and low fat diets slow progression of renal injury in the *pcy* mice model [16]. Flaxseed, soy protein (SP), fish oil (FO), and flax oil (FXO) appeared to have renoprotective effects in several epidemiological studies and experimental animal models of chronic kidney disease. In type 2 diabetic subjects with macroalbuminuria, dietary replacement of red meat

with chicken, or the replacement of protein of animal origin with SP, with a sustained amount of total protein intake, reduces urinary albumin excretion rate and improves lipid profile [127]. Dietary SP has been shown to reduce disease progression and alter renal hemodynamics, compared to animal protein in a number of models of renal disease, including the Han:SPRD-*Cy* rat [119,120].

In animal models, dietary supplementation with n-3 LCPUFA at low doses slows kidney disease progression [16]. Further details about the effect of SP, FO, and FXO on cystic kidney disease and oxylipin production will be discussed next.

2.5.1 The beneficial effect of soy protein

Dietary SP has been shown to retard disease progression and to have reno-protective effects in several experimental models of kidney diseases, including rats with early diabetic nephropathy [18,128], rats with nephritic syndrome [128], and rats and mouse models of cystic kidney disease [129,130]. In the obese fa/fa Zucker rats, SP ameliorated renal disease progression and improved kidney function by restoring renal NO production and renal nitric oxide synthase alterations, which play a very important role in controlling renal hemodynamics and oxylipin production [129]. In addition, SP attenuated early renal development of glomerular hypertrophy and altered the levels of several renal prostanoids (e.g. 6-keto-PGF1$_{\alpha}$) in the same rat model [119]. In the Han:SPRD-*Cy* rat, SP reduced the elevated levels of specific prostanoids (6-keto PGF$_{1\alpha}$, TXB$_2$, and PGE$_2$), and PLA$_2$ and COX protein levels in diseased kidneys [58]. However, the effects of dietary SP on other metabolites produced from the three pathways of oxylipin metabolism are not known. In addition, whether

SP mediates its beneficial effects on disease via altering oxylipin metabolism within the kidney still needs to be investigated.

It is not clear how SP alters kidney disease progression and prostanoid production, since it does not alter the fatty acid composition of the kidney. SP has a unique amino acid composition and contains several biologically active compounds such as isoflavones, saponins, and peptides that may contribute to its protective effects [131,132]. The biological properties of isoflavones are associated with the ability to prevent cancer, osteoporosis, and cardiovascular disease [133,134]. Kao *et al*. [135] reported that isoflavone powder inhibited lipopolysaccharide-induced inflammation in BALB/c mice via reducing the release of pro-inflammatory cytokines interleukin-1β (IL-1β), and interleukin-6 (IL-6), and reducing PGE_2 production [135]. However, as for cystic kidney disease, it has been demonstrated that the beneficial effects of SP were preserved in experimental kidney disease even after isoflavones depletion of the diet [124]. As for the unique amino acid composition, SP has more arginine and glycine and less methionine (sulphur amino acid) compared to casein protein (Table 2.2). L-arginine is an important substrate for the synthesis of endogenous renal NO [136]. It has been demonstrated that renal L-arginine and NO synthesis are significantly reduced in patients with chronic kidney disease or ESRD [136] and infusion of L-arginine has been shown to increase renal blood flow (RBF), and GFR, and decrease proteinuria in experimental animals [137]. In cystic kidney disease, it has been suggested that impaired release of NO by endothelial cell accelerates kidney disease progression in ADPKD patients [138]. However, the administration of L-arginine has been shown to have only a very modest beneficial effect on the development of renal cystic disease in the Han:SPRD-*Cy* rat [139]. In another study, hemp and pea protein had the same levels of methionine, glycine, and higher levels of L-

arginine compared to the SP diet but had much different effects on disease progression and blood pressure in the Han:SPRD-*Cy* rat model [121]. While hemp and SP were beneficial, pea protein was actually detrimental. Hence, although the amino acid composition and isoflavones content may contribute to the individual effects of plant protein sources, none of these appears to be common factor in the beneficial effects of hemp and SP compared to casein and pea protein in the reported study [121].

Table 2.2 Amino acid composition of casein and soy protein isolate (%)

	Casein	SP
Alanine	2.87	4.33
Arginine	3.51	7.41
Aspartic acid	6.71	11.66
Cysteine	0.46	1.23
Glutamic acid	20.23	19.71
Glycine	1.70	4.25
Histidine	2.87	2.57
Isoleucine	5.22	4.39
Leucine	8.94	7.91
Lysine	7.56	6.16
Methionine	2.77	1.34
Phenylalanine	4.79	4.99
Proline	10.65	5.34
Serine	4.90	5.30
Threonine	3.94	3.97
Tryptophan	1.17	1.26
Tyrosine	5.32	3.86
Valine	6.39	4.31
Total	100	100

The amino acid composition of soy protein isolate compared to casein (%). The SP has a unique amino acid composition with low ratios of methionine/glycine and lysine/arginine that may contribute to its protective effects.

A plausible mechanism is that the beneficial effect of SP might be related to the bioactive peptides produced via digestion of the proteins. SP contains peptides and proteins such as Bowman-Birk inhibitor (BBI), Kunit inhibitor, and lunasin. These peptides have been reported to reduce total cholesterol levels in the blood, prevent lung tumorigenesis [140], reduce high blood pressure [141], and stimulate the immune system [142]. BBI, genisteine and spogenol B from SP have been shown to inhibit inflammation, reduce PGE_2 production, and reduce reactive oxygen species (ROS) that might contribute to its anti-inflammatory activity and ability to reduce disease progression [143].

Bioactive peptides have also been shown to inhibit angiotensin I converting enzyme activity (ACE), which mediates its effects via changes in oxylipin levels as well as being itself regulated by COX products [55,144,145]. In spontaneously hypertensive rats (SHR), it has been demonstrated that ACE inhibitory peptides derived from SP at a dose of 100-1000 mg/kg of BW/day reduced high blood pressure and serum sodium concentration in a dose dependent manner [146]. Also, SP alters renal prostanoid production and reduces disease progression in the obese *fa/fa* Zucker rat [119]. Similar to this, SP has been shown to reduce the elevated protein levels of PLA_2 and COX_2 in the diseased kidneys of rats with cystic kidney disease in the Han:SPRD-*Cy* model [58]. However, whether SP has any effect on other oxylipins produced from different pathways of oxylipins metabolism still need to be elucidated.

2.5.2 The beneficial effects of fish and flax oils

The reno-protective effects of FO and FXO (rich in n-3 fatty acids) have been reported in several studies in different models of kidney disease [16,60,122]. Supplementing the diet with FO or FXO could cause a shift in oxylipin production, with 20:5n-3 (EPA) or C18:3n-3

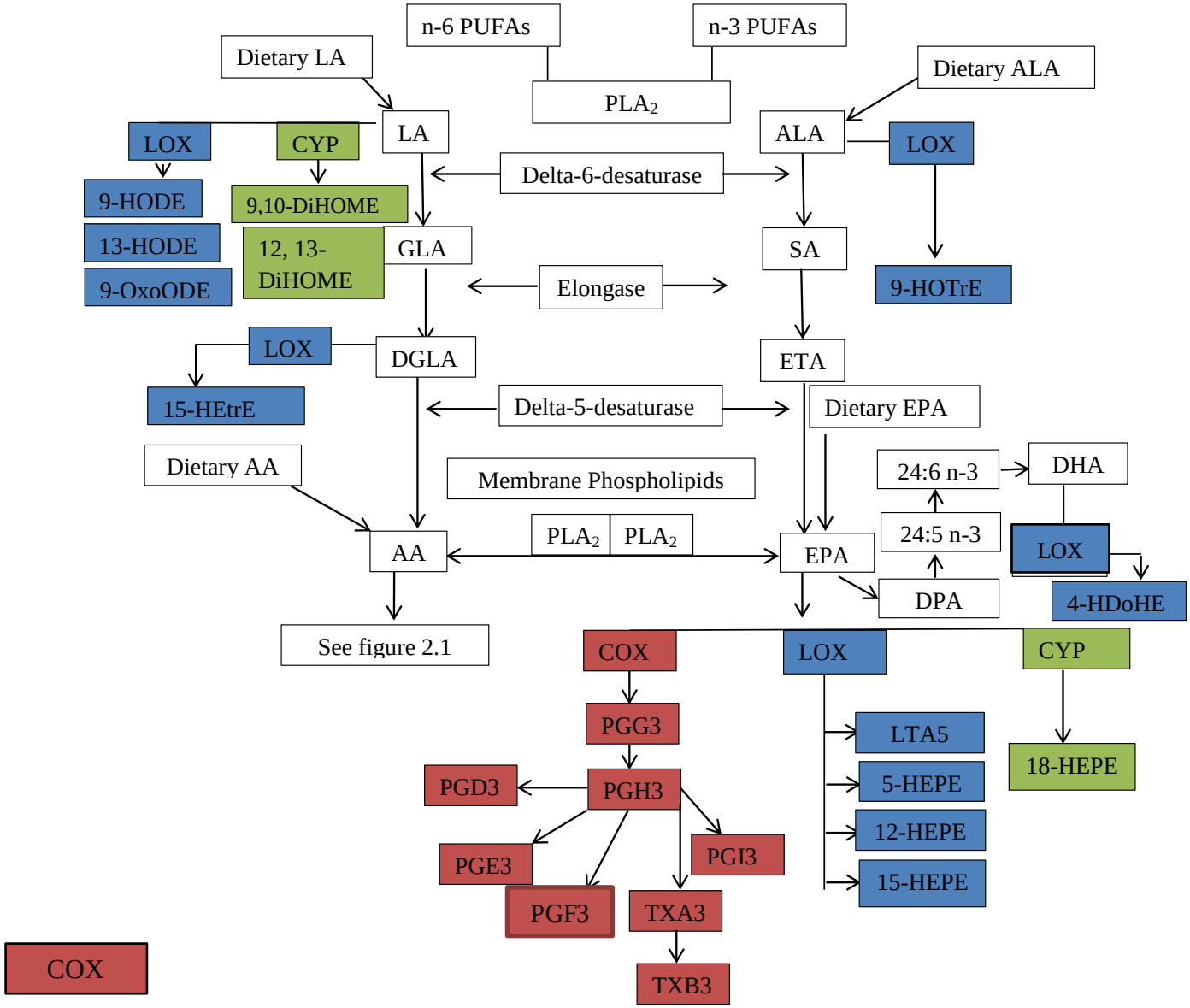
(ALA) inhibiting the metabolism of 20:4n-6 (AA) to the more inflammatory oxylipins through increasing the production of EPA and ALA derived oxylipins as shown in Figure 2.3, thereby exerting an anti-inflammatory effect in renal disease [122]. Thus, the beneficial effects of FO and FXO in early renal disease could be due to a reduction in renal inflammation by altering the renal oxylipin profile. Furthermore, the production of oxylipins from n-3 fatty acids is greater than the formation of n-6 (AA) oxylipins via the LOX pathway, although the study did not measure all oxylipins [110,111]. Furthermore, several protective metabolites such as 3-series prostanoids, lipoxins, HDoHE, and HEPE are produced by the COX and LOX pathways [110]. Therefore, supplementing the diet with FO, and FXO will enhance the production of these n-3 oxylipins, reduce inflammation and kidney disease progression.

It has been demonstrated that supplementing the diet with FXO reduces renal injury, oxidized LDL content, and tissue n6/n3 ration in the Han:SPRD-*Cy* rat model of cystic kidney disease. [147]. Recently, in the *pcy* mouse model of cystic kidney disease, it has been demonstrated that feeding mice with FXO does not generally reduce the production of AA derived LOX oxylipins but increases the levels of ALA and DHA derived COX oxylipins in the diseased kidneys in parallel with a reduction in renal fibrosis and improvement in kidney function [60]. In another study, dietary supplementation with n-3 LCPUFA at low doses slows kidney disease progression [16,148] and reduces the severity of kidney disease in dogs exposed to nephrotoxic agents [149]. In KKAY/Ta mice with type 2 diabetic nephropathy, EPA administration attenuated oxidative stress, and ameliorated tubular interstitial fibrosis and albuminuria [150]. Furthermore, in rats with renal mass reduction, Barcelli *et al*. (1986) reported that FO attenuated renal disease progression and favourably affected kidney histology [151].

As for cystic kidney disease, a high fat diet accelerated the progression of renal disease compared to low fat diet in the Han-SPRD-cy rat. Interestingly, rats fed high fat diets (20 g fat/100g diet) had a greater cystic expansion, larger kidneys, higher creatinine levels and lower creatinine clearances, compared to rats fed the low fat diets [152]. However, FO (a rich source of n-3 fatty acids) supplementation mitigated the adverse renal effects of the high fat diet [152]. In this study, long-term supplementation of rats with n-3 PUFAs resulted in amelioration of interstitial fibrosis and attenuation of profibrotic pathways, which was accompanied by significant reversal in the upregulation of the inflammatory and oxidative pathways.

Modification of lipid mediators formed from fatty acids might influence progression of kidney disease through its important role in regulation of renal hemodynamic processes. Prostanoids have been shown to participate in both hemodynamic and inflammatory events in the kidney. Omega 3 fatty acid supplementation has been associated with decreased oxidative stress and 5-LOX expression in human subjects [153] and administration of oil rich in long chain fatty acids resulted in a nonsignificant LtB_4 reduction and nonsignificant improvement in clinical symptoms (pruritus) in hemodialysis patients [154]. Data on the effect of FO and FXO on other oxylipins are very limited and therefore were investigated in this study.

Figure 2.3 Oxylipin production from n-3 fatty acids



AA, arachidonic acid, ALA, α-Linolenic acid; COX, cyclooxygenase; CYP, cytochrome p450; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; DiHETrE, dihydroxy-eicosatrienoic acid; HETE, hydroxy-eicosatetraenoic acid; HODE, hydroxyoctadecadienoic acid; HOTrE, hydroxy-octadecatrienoic acid, HEPE, hydroxy-eicosapentaenoic acid; HDoHE, hydroxy-docosahexaenoic acid; LA, Linoleic acid; LOX, lipoxygenase; PG, prostaglandin; TX, thromboxane

2.6 Lipid metabolism in cystic kidney disease

Abnormalities in renal lipid and fatty acid composition in cystic kidney disease have been reported [155]. Altered levels of PLA_2 and COX protein levels have also been demonstrated in models of cystic kidney disease [7,15]. Consequently, levels of oxylipins have been shown to be altered as well [7,15,17,60]. Furthermore, Aukema and *et al*. compared the fatty acid composition of *pcy* mice having cystic kidney disease to normal DBA mice and found that ratios of cholesterol/phospholipid, choline glycerophospholipid (GPC)/ethanolamine glycerophospholipid (GPE) and alkenylacyl GPE/diacyl GPE were higher in cystic kidneys, while total phosphatidylinositol was lower [155]. The same study showed that there were significantly lower levels of DHA and higher levels of adrenic acid ADA, (22:4n6) in the phospholipids of *pcy* mouse kidneys [155]. Using the same model, it has been demonstrated that supplementing the diet with FXO increased the levels of renal ALA, EPA, and reduced the elevated levels of AA but it did not change the levels of DHA and LA in cystic mice [60].

In addition, chronic kidney disease is also associated with alterations in different lipoprotein classes; both triglyceride (TG) and low-density lipoprotein (LDL) have been shown to be elevated, and high-density lipoprotein (HDL) has been shown to be lowered in ESRD patients, including cystic kidney disease patients [156]. This alteration in lipid metabolism may influence membrane-mediated events such as receptor activation, signal transduction, oxylipins production, and enzyme activities [110,155]. Therefore, change in lipid metabolism might be critical in cystic kidney disease. Hence, investigating the effect of disease and different dietary interventions on fatty acid composition of the kidney and renal oxylipins might open the door for new therapeutic opportunities.

2.7 Rationale

Oxylipins play a very important role in maintaining kidney function under normal physiological conditions but they can also contribute to the pathogenesis of disease by mediating inflammatory and fibrotic processes in diseased kidneys. However, we are still a long way from understanding the nature and presence of oxylipins produced from different pathways and how they are altered by disease and diet within the kidney.

We know which enzymes can potentially make oxylipins, but we do not known how changes in dietary n3 and n6 PUFA alter oxylipins production in the kidney under normal and pathological conditions. Since dietary FO, FXO and SP alter oxylipin formation via different mechanisms (as explained in chapter 1 and 2), these beneficial dietary interventions (individually or combined) will be used to further probe the importance of oxylipins in cystic kidneys in the Han:SPRD-*Cy* rat.

Currently, cystic kidney disease is the 4th leading cause of ESRD and there is no cure for this renal disorder. Therefore, new treatment strategies are urgently needed. While SP has been shown to be a very promising functional food in reducing disease progression in different models of kidney disease, the effect of combining SP with FO and FXO on kidney disease progression and the associated mechanisms in a model of cystic kidney disease have not been investigated. Novel findings from this research will determine if the reno-protective effects of these dietary interventions are modulated via regulation of oxylipins and their related enzymes. This will provide insights regarding the mechanisms through which dietary SP, FO, and FOX exert their reno-protective effects in cystic kidney disease and will contain supporting information on the potential use of these dietary interventions as effective measures in the management of cystic kidney disease. This will be the first study to explore the effect of disease and diet on renal oxylipin levels produced from the three pathways of metabolism with

more than 40 oxylipins scanned for. Furthermore, since formation of oxylipins is not homogenous within the whole kidney, this will be the first study to test the effect of disease and diet on oxylipin levels in the cortex and the medulla.

Selective COX2 inhibition reduces kidney disease progression in the Han:SPRD-*Cy* rat model, but it has been shown that both COX1 and COX2 activities are elevated in diseased kidneys in the same model. Considering the concerns surrounding the use of COX2 inhibitors, the lack of effective therapies to date, and the fact that both COX1 and COX2 are elevated, the potential benefits of a commonly used dual COX isoform inhibitor such as aspirin will be investigated. Furthermore, to directly explore the role of LOX products in cystic kidney disease progression, a LOX inhibitor (NDGA) will be also tested.

The overall outcomes of the current research will provide insights regarding the mechanisms through which dietary SP, FO, and FXO exert their reno-protective effects. Findings from this study will be crucial in reducing the incidence of ESRD, improve patient quality of life, and reduce healthcare cost.

2.7 References

[1] Corradi V, Gastaldon F, Virzi GM, de Cal M, Soni S, Chionh C et al. Clinical pattern of adult polycystic kidney disease in a northeastern region of Italy. Clin Nephrol 2009;72:259-67.

[2] Fick GM, Johnson AM, Strain JD, Kimberling WJ, Kumar S, Manco-Johnson ML et al. Characteristics of very early onset autosomal dominant polycystic kidney disease. J Am Soc Nephrol 1993;3:1863-70.

[3] Nagao S, Kugita M, Yoshihara D, Yamaguchi T. Animal models for human polycystic kidney disease. Exp Anim 2012;61:477-88.

[4] Cowley BD,Jr. Recent advances in understanding the pathogenesis of polycystic kidney disease: therapeutic implications. Drugs 2004;64:1285-94.

[5] Ta MH, Harris DC, Rangan GK. Role of interstitial inflammation in the pathogenesis of polycystic kidney disease. Nephrology (Carlton) 2013;18:317-30.

[6] Torres VE, Harris PC. Polycystic kidney disease: genes, proteins, animal models, disease mechanisms and therapeutic opportunities. J Intern Med 2007;261:17-31.

[7] Sankaran D, Bankovic-Calic N, Ogborn MR, Crow G, Aukema HM. Selective COX-2 inhibition markedly slows disease progression and attenuates altered prostanoid production in Han:SPRD-cy rats with inherited kidney disease. Am J Physiol Renal Physiol 2007;293:F821-30.

[8] Warford-Woolgar L, Peng CY, Shuhyta J, Wakefield A, Sankaran D, Ogborn M et al. Selectivity of cyclooxygenase isoform activity and prostanoid production in normal and diseased Han:SPRD-cy rat kidneys. Am J Physiol Renal Physiol 2006;290:F897-904.

[9] Elberg D, Turman MA, Pullen N, Elberg G. Prostaglandin E 2 stimulates cystogenesis through EP4 receptor in IMCD-3 cells. Prostaglandins and Other Lipid Mediators 2012;98:11-6.

[10] Elberg G, Elberg D, Lewis TV, Guruswamy S, Chen L, Logan CJ et al. EP 2 receptor mediates PGE 2-induced cystogenesis of human renal epithelial cells. American Journal of Physiology - Renal Physiology 2007;293:F1622-32.

[11] Fonseca JM, Bastos AP, Amaral AG, Sousa MF, Souza LE, Malheiros DM et al. Renal cyst growth is the main determinant for hypertension and concentrating deficit in Pkd1-deficient mice. Kidney Int 2014.

[12] Cowley BD, Gudapaty S, Kraybill AL, Barash BD, Harding MA, Calvet JP et al. Autosomal-Dominant Polycystic Kidney-Disease in the Rat. Kidney Int 1993;43:522-34.

[13] J. Kaspareit-Rittinghausen FD. Animal model of human disease hereditary polycystic kidney disease. 1991;139.

[14] Taskiran EZ, Korkmaz E, Gucer S, Kosukcu C, Kaymaz F, Koyunlar C et al. Mutations in ANKS6 cause a nephronophthisis-like phenotype with ESRD. J Am Soc Nephrol 2014;25:1653-61.

[15] Aukema HM, Adolphe J, Mishra S, Jiang J, Cuozzo FP, Ogborn MR. Alterations in renal cytosolic phospholipase A2 and cyclooxygenases in polycystic kidney disease. FASEB J 2003;17:298-300.

[16] Sankaran D, Lu J, Bankovic-Calic N, Ogborn MR, Aukema HM. Modulation of renal injury in pcy mice by dietary fat containing n-3 fatty acids depends on the level and type of fat. Lipids 2004;39:207-14.

[17] Sankaran D, Lu J, Ogborn MR, Aukema HM. COX-2 expression in cystic kidneys is dependent on dietary n-3 fatty acid composition. J Nutr Biochem 2007;18:806-12.

[18] Sankaran D, Bankovic-Calic N, Cahill L, Peng CY, Ogborn MR, Aukema HM. Late dietary intervention limits benefits of soy protein or flax oil in experimental polycystic kidney disease. Nephron Experimental Nephrology 2007;106:E122-8.

[19] Camara NO, Martins JO, Landgraf RG, Jancar S. Emerging roles for eicosanoids in renal diseases. Curr Opin Nephrol Hypertens 2009;18:21-7.

[20] Singh H, Schwartzman ML. Renal vascular cytochrome P450-derived eicosanoids in androgen-induced hypertension. Pharmacol Rep 2008;60:29-37.

[21] Dolegowska B, Pikula E, Safranow K, Olszewska M, Jakubowska K, Chlubek D et al. Metabolism of eicosanoids and their action on renal function during ischaemia and reperfusion: the effect of alprostadil. Prostaglandins Leukot Essent Fatty Acids 2006;75:403-11.

[22] Imig JD. Eicosanoids and renal vascular function in diseases. Clin Sci (Lond) 2006;111:21-34.

[23] Lone AM, Taskén K. Proinflammatory and immunoregulatory roles of eicosanoids in T cells. Frontiers in Immunology 2013;4.4:130

[24] Greene ER, Huang S, Serhan CN, Panigrahy D. Regulation of inflammation in cancer by eicosanoids. Prostaglandins Other Lipid Mediat 2011;96:27-36.

[25] Buczynski MW, Dumlao DS, Dennis EA. Thematic Review Series: Proteomics. An integrated omics analysis of eicosanoid biology. J Lipid Res 2009;50:1015-38.

[26] Imig JD. Eicosanoid regulation of the renal vasculature. Am J Physiol Renal Physiol 2000;279:F965-81.

[27] O'Donnell VB, Maskrey B, Taylor GW. Eicosanoids: generation and detection in mammalian cells. Methods Mol Biol 2009;462:5-23.

[28] Dennis EA, Cao J, Hsu YH, Magrioti V, Kokotos G. Phospholipase A2 enzymes: physical structure, biological function, disease implication, chemical inhibition, and therapeutic intervention. Chem Rev 2011;111:6130-85.

[29] Ghosh M, Tucker DE, Burchett SA, Leslie CC. Properties of the Group IV phospholipase A2 family. Prog Lipid Res 2006;45:487-510.

[30] C. C. Leslie. Cytosolic phospholipase A 2 : physiological function and role in disease. JLR 2015; Papers in Press.

[31] Alonso F, Henson PM, Leslie CC. A cytosolic phospholipase in human neutrophils that hydrolyzes arachidonoyl-containing phosphatidylcholine. Biochim Biophys Acta 1986;878:273-80.

[32] Sharp JD, Pickard RT, Chiou XG, Manetta JV, Kovacevic S, Miller JR et al. Serine 228 is essential for catalytic activities of 85-kDa cytosolic phospholipase A2. J Biol Chem 1994;269:23250-4.

[33] Rukoyatkina N, Mindukshev I, Walter U, Gambaryan S. Dual role of the p38 MAPK/cPLA2 pathway in the regulation of platelet apoptosis induced by ABT-737 and strong platelet agonists. Cell Death Dis 2013;4:e931.

[34] Aukema HM, Adolphe J, Mishra S, Jiang J, Cuozzo FP, Ogborn MR. Alterations in renal cytosolic phospholipase A2 and cyclooxygenases in polycystic kidney disease. FASEB J 2003;17:298-300.

[35] Huwiler A, Feuerherm AJ, Sakem B, Pastukhov O, Filipenko I, Nguyen T et al. The omega3-polyunsaturated fatty acid derivatives AVX001 and AVX002 directly inhibit cytosolic phospholipase A(2) and suppress PGE(2) formation in mesangial cells. Br J Pharmacol 2012;167:1691-701.

[36] Channon JY, Leslie CC. A calcium-dependent mechanism for associating a soluble arachidonoyl-hydrolyzing phospholipase A2 with membrane in the macrophage cell line RAW 264.7. J Biol Chem 1990;265:5409-13.

[37] Nalefski EA, Slazas MM, Falke JJ. Ca2+-signaling cycle of a membrane-docking C2 domain. Biochemistry 1997;36:12011-8.

[38] Evans JH, Gerber SH, Murray D, Leslie CC. The calcium binding loops of the cytosolic phospholipase A2 C2 domain specify targeting to Golgi and ER in live cells. Mol Biol Cell 2004;15:371-83.

[39] Miyakawa A, Ibarra C, Malmersjo S, Aperia A, Wiklund P, Uhlen P. Intracellular calcium release modulates polycystin-2 trafficking. BMC Nephrol 2013;14:34,2369-14-34.

[40] Perez-Torres I, Ibarra B, Soria-Castro E, Torrico-Lavayen R, Pavon N, Diaz-Diaz E et al. Effect of glycine on the cyclooxygenase pathway of the kidney arachidonic acid metabolism in a rat model of metabolic syndrome. Can J Physiol Pharmacol 2011;89:899-910.

[41] Breyer MD, Harris RC. Cyclooxygenase 2 and the kidney. Curr Opin Nephrol Hypertens 2001;10:89-98.

[42] Harris RC, Breyer MD. Physiological regulation of cyclooxygenase-2 in the kidney. Am J Physiol Renal Physiol 2001;281:F1-11.

[43] Sanchez PL, Salgado LM, Ferreri NR, Escalante B. Effect of cyclooxygenase-2 inhibition on renal function after renal ablation. Hypertension 1999;34:848-53.

[44] Certikova Chabova V. The role of arachidonic acid metabolites in the regulation of renal function and pathogenesis of hypertension. Cesk Fysiol 2008;57:44-52.

[45] Lopez-Parra M, Claria J, Planaguma A, Titos E, Masferrer JL, Woerner BM et al. Cyclooxygenase-1 derived prostaglandins are involved in the maintenance of renal function in rats with cirrhosis and ascites. Br J Pharmacol 2002;135:891-900.

[46] Davies NM, Good RL, Roupe KA, Yanez JA. Cyclooxygenase-3: axiom, dogma, anomaly, enigma or splice error?--Not as easy as 1, 2, 3. J Pharm Pharm Sci 2004;7:217-26.

[47] Hersh EV, Lally ET, Moore PA. Update on cyclooxygenase inhibitors: has a third COX isoform entered the fray? Curr Med Res Opin 2005;21:1217-26.

[48] Fujihara CK, Antunes GR, Mattar AL, Andreoli N, Malheiros DM, Noronha IL et al. Cyclooxygenase-2 (COX-2) inhibition limits abnormal COX-2 expression and progressive injury in the remnant kidney. Kidney Int 2003;64:2172-81.

[49] Zhang MZ, Wang JL, Cheng HF, Harris RC, McKanna JA. Cyclooxygenase-2 in rat nephron development. Am J Physiol 1997;273:F994-1002.

[50] Wang JL, Cheng HF, Shappell S, Harris RC. A selective cyclooxygenase-2 inhibitor decreases proteinuria and retards progressive renal injury in rats. Kidney Int 2000;57:2334-42.

[51] Smith WL, Bell TG. Immunohistochemical localization of the prostaglandin-forming cyclooxygenase in renal cortex. Am J Physiol 1978;235:F451-7.

[52] James MJ, Cook-Johnson RJ, Cleland LG. Selective COX-2 inhibitors, eicosanoid synthesis and clinical outcomes: a case study of system failure. Lipids 2007;42:779-85.

[53] Harris RC, McKanna JA, Akai Y, Jacobson HR, Dubois RN, Breyer MD. Cyclooxygenase-2 is associated with the macula densa of rat kidney and increases with salt restriction. J Clin Invest 1994;94:2504-10.

[54] Kammerl MC, Nusing RM, Seyberth HW, Riegger GA, Kurtz A, Kramer BK. Inhibition of cyclooxygenase-2 attenuates urinary prostanoid excretion without affecting renal renin expression. Pflugers Arch 2001;442:842-7.

[55] Stichtenoth DO, Wagner B, Frolich JC. Effect of selective inhibition of the inducible cyclooxygenase on renin release in healthy volunteers. J Investig Med 1998;46:290-6.

[56] Brater DC, Harris C, Redfern JS, Gertz BJ. Renal effects of COX-2-selective inhibitors. Am J Nephrol 2001;21:1-15.

[57] Harris RC. COX-2 and the kidney. J Cardiovasc Pharmacol 2006;47 Suppl 1:S37-42.

[58] Peng CY, Sankaran D, Ogborn MR, Aukema HM. Dietary Soy Protein Selectively Reduces Renal Prostanoids and Cyclooxygenases in Polycystic Kidney Disease. Exp Biol Med 2009;234:737-43.

[59] Xu T, Wang N-, Fu L-, Ye C-, Yu S-, Mei C-. Celecoxib inhibits growth of human autosomal dominant polycystic kidney cyst-lining epithelial cells through the VEGF/Raf/MAPK/ERK signaling pathway. Mol Biol Rep 2012;39:7743-53.

[60] Yamaguchi T, Devassy JG, Gabbs M, Ravandi A, Nagao S, Aukema HM. Dietary flax oil rich in alpha-linolenic acid reduces renal disease and oxylipin abnormalities, including

formation of docosahexaenoic acid derived oxylipins in the CD1-pcy/pcy mouse model of nephronophthisis. Prostaglandins Leukot Essent Fatty Acids 2015;94:83-9.

[61] Mochizuki T, Tsuchiya K, Nitta K. Autosomal dominant polycystic kidney disease: Recent advances in pathogenesis and potential therapies. Clinical and Experimental Nephrology 2013;17:317-26.

[62] Patel V, Chowdhury R, Igarashi P. Advances in the pathogenesis and treatment of polycystic kidney disease. Curr Opin Nephrol Hypertens 2009;18:99-106.

[63] Belibi FA, Reif G, Wallace DP, Yamaguchi T, Olsen L, Li H et al. Cyclic AMP promotes growth and secretion in human polycystic kidney epithelial cells. Kidney Int 2004;66:964-73.

[64] Breyer MD, Breyer RM. G protein - Coupled prostanoid receptors and the kidney. Annual Review of Physiology 2001;63:579-605.

[65] Schmitz PG, Zhang K, Dalal R. Eicosapentaenoic acid suppresses PDGF-induced DNA synthesis in rat mesangial cells: involvement of thromboxane A2. Kidney Int 2000;57:1041-51.

[66] Michalski J, Kanaji N, Liu X, Nogel S, Wang X, Basma H et al. Attenuation of inhibitory prostaglandin E2 signaling in human lung fibroblasts is mediated by phosphodiesterase 4. Am J Respir Cell Mol Biol 2012;47:729-37.

[67] Park F, Sweeney WE,Jr, Jia G, Akbulut T, Mueller B, Falck JR et al. Chronic blockade of 20-HETE synthesis reduces polycystic kidney disease in an orthologous rat model of ARPKD. Am J Physiol Renal Physiol 2009;296:F575-82.

[68] Muller DN, Schmidt C, Barbosa-Sicard E, Wellner M, Gross V, Hercule H et al. Mouse Cyp4a isoforms: enzymatic properties, gender- and strain-specific expression, and role in renal 20-hydroxyeicosatetraenoic acid formation. Biochem J 2007;403:109-18.

[69] Aragno M, Parola S, Brignardello E, Manti R, Betteto S, Tamagno E et al. Oxidative stress and eicosanoids in the kidneys of hyperglycemic rats treated with dehydroepiandrosterone. Free Radic Biol Med 2001;31:935-42.

[70] Aukema HM, Lu J, Borthwick F, Proctor SD. Dietary fish oil reduces glomerular injury and elevated renal hydroxyeicosatetraenoic acid levels in the JCR:LA-cp rat, a model of the metabolic syndrome. Br J Nutr 2012:1-9.

[71] Jozsef L, Zouki C, Petasis NA, Serhan CN, Filep JG. Lipoxin A4 and aspirin-triggered 15-epi-lipoxin A4 inhibit peroxynitrite formation, NF-kappa B and AP-1 activation, and IL-8 gene expression in human leukocytes. Proc Natl Acad Sci U S A 2002;99:13266-71.

[72] Han X, Chen S, Sun Y, Nadler JL, Bleich D. Induction of cyclooxygenase-2 gene in pancreatic beta-cells by 12-lipoxygenase pathway product 12-hydroxyeicosatetraenoic acid. Mol Endocrinol 2002;16:2145-54.

[73] Xu ZG, Li SL, Lanting L, Kim YS, Shanmugam N, Reddy MA et al. Relationship between 12/15-lipoxygenase and COX-2 in mesangial cells: potential role in diabetic nephropathy. Kidney Int 2006;69:512-9.

[74] Cheng HF, Wang CJ, Moeckel GW, Zhang MZ, McKanna JA, Harris RC. Cyclooxygenase-2 inhibitor blocks expression of mediators of renal injury in a model of diabetes and hypertension. Kidney Int 2002;62:929-39.

[75] Miyata N, Roman RJ. Role of 20-hydroxyeicosatetraenoic acid (20-HETE) in vascular system. J Smooth Muscle Res 2005;41:175-93.

[76] Wang H, Lin L, Jiang J, Wang Y, Lu ZY, Bradbury JA et al. Up-regulation of endothelial nitric-oxide synthase by endothelium-derived hyperpolarizing factor involves mitogen-activated protein kinase and protein kinase C signaling pathways. J Pharmacol Exp Ther 2003;307:753-64.

[77] Park F, Sweeney WE, Jia G, Roman RJ, Avner ED. 20-HETE mediates proliferation of renal epithelial cells in polycystic kidney disease. J Am Soc Nephrol 2008;19:1929-39.

[78] Makita K, Takahashi K, Karara A, Jacobson HR, Falck JR, Capdevila JH. Experimental and/or genetically controlled alterations of the renal microsomal cytochrome P450 epoxygenase induce hypertension in rats fed a high salt diet. J Clin Invest 1994;94:2414-20.

[79] Imig JD, Zhao X, Zaharis CZ, Olearczyk JJ, Pollock DM, Newman JW et al. An orally active epoxide hydrolase inhibitor lowers blood pressure and provides renal protection in salt-sensitive hypertension. Hypertension 2005;46:975-81.

[80] Chaudhary KR, Batchu SN, Das D, Suresh MR, Falck JR, Graves JP et al. Role of B-type natriuretic peptide in epoxyeicosatrienoic acid-mediated improved post-ischaemic recovery of heart contractile function. Cardiovasc Res 2009;83:362-70.

[81] Lorthioir A, Joannides R, Remy-Jouet I, Freguin-Bouilland C, Iacob M, Roche C et al. Polycystin deficiency induces dopamine-reversible alterations in flow-mediated dilatation and vascular nitric oxide release in humans. Kidney Int 2015;87:465-72.

[82] Wehling M. Non-steroidal anti-inflammatory drug use in chronic pain conditions with special emphasis on the elderly and patients with relevant comorbidities: management and mitigation of risks and adverse effects. Eur J Clin Pharmacol 2014;70:1159-72.

[83] Jang SM, Cerulli J, Grabe DW, Fox C, Vassalotti JA, Prokopienko AJ et al. NSAID-avoidance education in community pharmacies for patients at high risk for acute kidney injury, upstate New York, 2011. Prev Chronic Dis 2014;11:E220.

[84] Smith WL. Prostanoid biosynthesis and mechanisms of action. Am J Physiol 1992;263:F181-91.

[85] Bresalier RS, Sandler RS, Quan H, Bolognese JA, Oxenius B, Horgan K et al. Cardiovascular events associated with rofecoxib in a colorectal adenoma chemoprevention trial. N Engl J Med 2005;352:1092-102.

[86] Roumie CL, Choma NN, Kaltenbach L, Mitchel EF,Jr, Arbogast PG, Griffin MR. Non-aspirin NSAIDs, cyclooxygenase-2 inhibitors and risk for cardiovascular events-stroke, acute myocardial infarction, and death from coronary heart disease. Pharmacoepidemiol Drug Saf 2009;18:1053-63.

[87] McGettigan P, Henry D. Cardiovascular risk and inhibition of cyclooxygenase: a systematic review of the observational studies of selective and nonselective inhibitors of cyclooxygenase 2. JAMA 2006;296:1633-44.

[88] Trelle S, Reichenbach S, Wandel S, Hildebrand P, Tschannen B, Villiger PM et al. Cardiovascular safety of non-steroidal anti-inflammatory drugs: network meta-analysis. BMJ 2011;342:c7086.

[89] Tougeron D, Sha D, Manthravadi S, Sinicrope FA. Aspirin and colorectal cancer: Back to the Future. Clin Cancer Res 2014; 1; 20: 1087-94.

[90] Goren A, Liu X, Gupta S, Simon TA, Phatak H. Warfarin and Aspirin Use for Stroke Prevention Among Patients With Atrial Fibrillation: The US National Health and Wellness Survey. Am J Ther 2015; 22:248-56.

[91] Bartolucci AA, Tendera M, Howard G. Meta-analysis of multiple primary prevention trials of cardiovascular events using aspirin. Am J Cardiol 2011;107:1796-801.

[92] Veitonmäki T, Tammela TLJ, Auvinen A, Murtola TJ. Use of aspirin, but not other non-steroidal anti-inflammatory drugs is associated with decreased prostate cancer risk at the population level. Eur J Cancer 2013;49:938-45.

[93] Ciabattoni G, Cinotti GA, Pierucci A, Simonetti BM, Manzi M, Pugliese F et al. Effects of sulindac and ibuprofen in patients with chronic glomerular disease. Evidence for the dependence of renal function on prostacyclin. N Engl J Med 1984;310:279-83.

[94] Whelton A, Scott H, Stout RL. Effects of Misoprostol on Nonsteroidal Anti-Inflammatory Drug-Induced Renal Insufficiency in Patients with Stable Chronic Renal Failure: A Double-Blind, Crossover Study. Am J Ther 1995;2:858-63.

[95] Plantinga L, Grubbs V, Sarkar U, Hsu CY, Hedgeman E, Robinson B et al. Nonsteroidal anti-inflammatory drug use among persons with chronic kidney disease in the United States. Ann Fam Med 2011;9:423-30.

[96] Perneger TV, Whelton PK, Klag MJ. Risk of kidney failure associated with the use of acetaminophen, aspirin, and nonsteroidal antiinflammatory drugs. N Engl J Med 1994;331:1675-9.

[97] Baigent C, Landray M, Leaper C, Altmann P, Armitage J, Baxter A et al. First United Kingdom Heart and Renal Protection (UK-HARP-I) study: biochemical efficacy and safety of simvastatin and safety of low-dose aspirin in chronic kidney disease. Am J Kidney Dis 2005;45:473-84.

[98] Xu T, Wang S-, Ye C-, Mei C-. Celecoxib inhibits proliferation of Han: SPRD rat renal cyst through inhibiting COX-2 activity. Academic Journal of Second Military Medical University 2009;30:1140-4.

[99] Morales E, Mucksavage JJ. Cyclooxygenase-2 inhibitor-associated acute renal failure: case report with rofecoxib and review of the literature. Pharmacotherapy 2002;22:1317-21.

[100] Chen D, Ma Y, Wang X, Yu S, Li L, Dai B et al. Clinical characteristics and disease predictors of a large Chinese cohort of patients with autosomal dominant polycystic kidney disease. PLoS One 2014;9:e92232.

[101] Spithoven EM, Kramer A, Meijer E, Orskov B, Wanner C, Caskey F et al. Analysis of data from the ERA-EDTA Registry indicates that conventional treatments for chronic kidney disease do not reduce the need for renal replacement therapy in autosomal dominant polycystic kidney disease. Kidney Int 2014;86:1244-52.

[102] Hansel TT B. 5-Lipoxygenase Inhibitors. 2001;31:115-120.

[103] Kulkarni SK, Singh VP. Positioning dual inhibitors in the treatment of pain and inflammatory disorders. Inflammopharmacology 2008;16:1-15.

[104] Chen X, Sood S, Yang CS, Li N, Sun Z. Five-lipoxygenase pathway of arachidonic acid metabolism in carcino-genesis and cancer chemoprevention. Curr Cancer Drug Targets 2006;6:613-22.

[105] Back M. Inhibitors of the 5-Lipoxygenase Pathway in Atherosclerosis. Curr Pharm Des 2009;15:3116-32.

[106] Puhlmann U, Schafer D, Ziemann C. Update on COX-2 inhibitor patents with a focus on optimised formulation and therapeutic scope of drug combinations making use of COX-2 inhibitors. Expert Opinion on Therapeutic Patents 2006;16:403-30.

[107] Martel-Pelletier J, Lajeunesse D, Reboul P, Pelletier JP. Therapeutic role of dual inhibitors of 5-LOX and COX, selective and non-selective non-steroidal anti-inflammatory drugs. Ann Rheum Dis 2003;62:501-9.

[108] Williams JM, Sharma M, Anjaiahh S, Falck JR, Roman RJ. Role of endogenous CYP450 metabolites of arachidonic acid in maintaining the glomerular protein permeability barrier. American Journal of Physiology-Renal Physiology 2007;293:F501-5.

[109] Wang. Downregulation of renal CYP-derived eicosanoid synthesis in rats with diet-induced hypertension (vol 42, pg 594, 2003). Hypertension 2003;42:E19-.

[110] Serhan CN, Petasis NA. Resolvins and protectins in inflammation resolution. Chem Rev 2011;111:5922-43.

[111] Caligiuri SP, Aukema HM, Ravandi A, Pierce GN. Elevated levels of pro-inflammatory oxylipins in older subjects are normalized by flaxseed consumption. Exp Gerontol 2014;59:51-7.

[112] Lundstrom SL, D'Alexandri FL, Nithipatikom K, Haeggstrom JZ, Wheelock AM, Wheelock CE. HPLC/MS/MS-based approaches for detection and quantification of eicosanoids. Methods Mol Biol 2009;579:161-87.

[113] Blaho VA, Buczynski MW, Brown CR, Dennis EA. Lipidomic analysis of dynamic eicosanoid responses during the induction and resolution of Lyme arthritis. J Biol Chem 2009;284:21599-612.

[114] Unterwurzacher I, Koal T, Bonn GK, Weinberger KM, Ramsay SL. Rapid sample preparation and simultaneous quantitation of prostaglandins and lipoxygenase derived fatty acid metabolites by liquid chromatography-mass spectrometry from small sample volumes. Clin Chem Lab Med 2008;46:1589-97.

[115] Grebe SK, Singh RJ. LC-MS/MS in the Clinical Laboratory - Where to From Here? Clin Biochem Rev 2011;32:5-31.

[116] Deems R, Buczynski MW, Bowers-Gentry R, Harkewicz R, Dennis EA. Detection and quantitation of eicosanoids via high performance liquid chromatography-electrospray ionization-mass spectrometry. Methods Enzymol 2007;432:59-82.

[117] Aparicio M. Protein Intake and Chronic Kidney Disease: Literature Review, 2003 to 2008. Journal of Renal Nutrition 2009;19:S5-8.

[118] Fouque D, Aparicio M. Eleven reasons to control the protein intake of patients with chronic kidney disease. Nature Clinical Practice Nephrology 2007;3:383-92.

[119] Hwang SY, Taylor CG, Zahradka P, Bankovic-Calic N, Ogborn MR, Aukema HM. Dietary soy protein reduces early renal disease progression and alters prostanoid production in obese fa/fa Zucker rats. J Nutr Biochem 2008;19:255-62.

[120] Cahill LE, Peng CY, Bankovic-Calic N, Sankaran D, Ogborn MR, Aukema HM. Dietary soya protein during pregnancy and lactation in rats with hereditary kidney disease attenuates disease progression in offspring. Br J Nutr 2007;97:77-84.

[121] Aukema HM, Gauthier J, Roy M, Jia Y, Li H, Aluko RE. Distinctive effects of plant protein sources on renal disease progression and associated cardiac hypertrophy in experimental kidney disease. Molecular Nutrition and Food Research 2011;55:1044-51.

[122] Lefkowith JB, Klahr S. Polyunsaturated fatty acids and renal disease. Proc Soc Exp Biol Med 1996;213:13-23.

[123] Wakefield AP, Ogborn MR, Ibrahim N, Aukema HM. A dietary conjugated linoleic acid treatment that slows renal disease progression alters renal cyclooxygenase-2-derived prostanoids in the Han: SPRD-cy rat. J Nutr Biochem 2012;23:908-14.

[124] Ogborn MR, Nitschmann E, Bankovic-Calic N, Weiler HA, Aukema HM. Dietary soy protein benefit in experimental kidney disease is preserved after isoflavone depletion of diet. Exp Biol Med 2010;235:1315-20.

[125] Ogborn MR, Nitschmann E, Bankovic-Calic N, Weiler HA, Fitzpatrick-Wong S, Aukema HM. Dietary conjugated linoleic acid reduces PGE2 release and interstitial injury in rat polycystic kidney disease. Kidney Int 2003;64:1214-21.

[126] Jayapalan S, Saboorian MH, Edmunds JW, Aukema HM. High dietary fat intake increases renal cyst disease progression in Han:SPRD-cy rats. J Nutr 2000;130:2356-60.

[127] de Mello VDF, Zelmanovitz T, Perassolo MS, Azevedo MJ, Gross JL. Withdrawal of red meat from the usual diet reduces albuminuria and improves serum fatty acid profile in type 2 diabetes patients with macroalbuminuria. Am J Clin Nutr 2006;83:1032-8.

[128] Pedraza-Chaverri J, Barrera D, Hernandez-Pando R, Medina-Campos ON, Cruz C, Murguia F et al. Soy protein diet ameliorates renal nitrotyrosine formation and chronic nephropathy induced by puromycin aminonucleoside. Life Sci 2004;74:987-99.

[129] Trujillo J, Ramirez V, Perez J, Torre-Villalvazo I, Torres N, Tovar AR et al. Renal protection by a soy diet in obese Zucker rats is associated with restoration of nitric oxide generation. American Journal of Physiology-Renal Physiology 2005;288:F108-16.

[130] Ogborn MR, Nitschmann E, Weiler HA, Bankovic-Calic N. Modification of polycystic kidney disease and fatty acid status by soy protein diet. Kidney Int 2000;57:159-66.

[131] Watanabe G, Tomiyama H, Doba N. Effects of oral administration of L-arginine on renal function in patients with heart failure. J Hypertens 2000;18:229-34.

[132] Gabardi S, Munz K, Ulbricht C. A review of dietary supplement-induced renal dysfunction. Clin J Am Soc Nephrol 2007;2:757-65.

[133] Baglia ML, Gu K, Zhang X, Zheng Y, Peng P, Cai H et al. Soy isoflavone intake and bone mineral density in breast cancer survivors. Cancer Causes Control 2015;26:571-80.

[134] Gil-Izquierdo A, Penalvo JL, Gil JI, Medina S, Horcajada MN, Lafay S et al. Soy isoflavones and cardiovascular disease epidemiological, clinical and -omics perspectives. Curr Pharm Biotechnol 2012;13:624-31.

[135] Kao TH, Wu WM, Hung CF, Wu WB, Chen BH. Anti-inflammatory effects of isoflavone powder produced from soybean cake. J Agric Food Chem 2007;55:11068-79.

[136] Schmidt RJ, Yokota S, Tracy TS, Sorkin MI, Baylis C. Nitric oxide production is low in end-stage renal disease patients on peritoneal dialysis. Am J Physiol 1999;276:F794-7.

[137] Klahr S, Morrissey J. L-arginine as a therapeutic tool in kidney disease. Semin Nephrol 2004;24:389-94.

[138] Devuyst O. Variable renal disease progression in autosomal dominant polycystic kidney disease: a role for nitric oxide? J Nephrol 2003;16:449-52.

[139] Yoshida I, Bengal R, Torres VE. Gender-dependent effect of L-NAME on polycystic kidney disease in Han:SPRD rats. Am J Kidney Dis 2000;35:930-6.

[140] Kennedy AR, Kritchevsky D, Shen WC. Effects of spermine-conjugated Bowman-Birk inhibitor (spermine-BBI) on carcinogenesis and cholesterol biosynthesis in mice. Pharm Res 2003;20:1908-10.

[141] Mallikarjun Gouda KG, Gowda LR, Rao AG, Prakash V. Angiotensin I-converting enzyme inhibitory peptide derived from glycinin, the 11S globulin of soybean (Glycine max). J Agric Food Chem 2006;54:4568-73.

[142] Tsuruki T, Kishi K, Takahashi M, Tanaka M, Matsukawa T, Yoshikawa M. Soymetide, an immunostimulating peptide derived from soybean beta-conglycinin, is an fMLP agonist. FEBS Lett 2003;540:206-10.

[143] Dia VP, Berhow MA, Gonzalez De Mejia E. Bowman-Birk inhibitor and genistein among soy compounds that synergistically inhibit nitric oxide and prostaglandin E2 pathways in lipopolysaccharide-induced macrophages. J Agric Food Chem 2008;56:11707-17.

[144] Jensen BL, Schmid C, Kurtz A. Prostaglandins stimulate renin secretion and renin mRNA in mouse renal juxtaglomerular cells. Am J Physiol 1996;271:F659-69.

[145] Farzamirad V, Aluko RE. Angiotensin-converting enzyme inhibition and free-radical scavenging properties of cationic peptides derived from soybean protein hydrolysates. Int J Food Sci Nutr 2008;59:428-37.

[146] Wu J, Ding X. Hypotensive and physiological effect of angiotensin converting enzyme inhibitory peptides derived from soy protein on spontaneously hypertensive rats. J Agric Food Chem 2001;49:501-6.

[147] Ogborn MR, Nitschmann E, Bankovic-Calic N, Weiler HA, Aukema H. Dietary flax oil reduces renal injury, oxidized LDL content, and tissue n-6/n-3 FA ratio in experimental polycystic kidney disease. Lipids 2002;37:1059-65.

[148] Hagiwara S, Makita Y, Gu L, Tanimoto M, Zhang M, Nakamura S et al. Eicosapentaenoic acid ameliorates diabetic nephropathy of type 2 diabetic KKAy/Ta mice: involvement of MCP-1 suppression and decreased ERK1/2 and p38 phosphorylation. Nephrol Dial Transplant 2006;21:605-15.

[149] Brown SA, Brown CA, Crowell WA, Barsanti JA, Kang CW, Allen T et al. Effects of dietary polyunsaturated fatty acid supplementation in early renal insufficiency in dogs. J Lab Clin Med 2000;135:275-86.

[150] Zhang M, Hagiwara S, Matsumoto M, Gu L, Tanimoto M, Nakamura S et al. Effects of eicosapentaenoic acid on the early stage of type 2 diabetic nephropathy in KKA(y)/Ta mice:

involvement of anti-inflammation and antioxidative stress. Metabolism-Clinical and Experimental 2006;55:1590-8.

[151] Barcelli UO, Miyata J, Ito Y, Gallon L, Laskarzewski P, Weiss M et al. Beneficial-Effects of Polyunsaturated Fatty-Acids in Partially Nephrectomized Rats. Prostaglandins 1986;32:211-9.

[152] Lu J, Bankovic-Calic N, Ogborn M, Saboorian MH, Aukema HM. Detrimental effects of a high fat diet in early renal injury are ameliorated by fish oil in Han:SPRD-cy rats. J Nutr 2003;133:180-6.

[153] Friedman A, Moe S. Review of the effects of omega-3 supplementation in dialysis patients. Clin J Am Soc Nephrol 2006;1:182-92.

[154] Begum R, Belury MA, Burgess JR, Peck LW. Supplementation with n-3 and n-6 polyunsaturated fatty acids: effects on lipoxygenase activity and clinical symptoms of pruritus in hemodialysis patients. J Ren Nutr 2004;14:233-41.

[155] Aukema HM, Yamaguchi T, Takahashi H, Celi B, Holub BJ. Abnormal lipid and fatty acid compositions of kidneys from mice with polycystic kidney disease. Lipids 1992;27:429-35.

[156] Attman PO, Alaupovic P, Tavella M, Knight-Gibson C. Abnormal lipid and apolipoprotein composition of major lipoprotein density classes in patients with chronic renal failure. Nephrol Dial Transplant 1996;11:63-9.

Chapter 3

3. Renal cyclooxygenase and lipoxygenase products are altered in cystic kidneys and by dietary soy protein and fish oil treatment in the Han:SPRD-Cy rat

This manuscript has been published in Molecular Nutrition and Food Research, 2014 Apr;58(4):768-81. doi: 10.1002/mnfr.201300332.

Naser H.M. Ibrahim[1,2], Yong Jia[1,2], Jessay G. Devassy[1,2], Tamio Yamaguchi[1,2], and Harold M. Aukema[1,2,3]

[1]Department of Human Nutritional Sciences, University of Manitoba, Winnipeg, Manitoba, Canada

[2]Canadian Centre for Agri-Food Research in Health and Medicine, St. Boniface Hospital Research Centre, Winnipeg, Manitoba, Canada

[3] Manitoba Institute of Child Health, Manitoba, Winnipeg, Manitoba, Canada

Keywords:

Cystic kidney disease; oxylipin; dietary fish oil; dietary soy protein

3.1 Abstract

Dietary fish oil (FO) and soy protein (SP) are two interventions that slow disease progression in the Han:SPRD-*Cy* rat model of cystic kidney disease. Inhibition of cyclooxygenase (COX) derived oxylipins also reduces disease progression, but the role of lipoxygenase (LOX) products in this disease is not known. Since dietary FO and SP have been shown to alter oxylipin formation via differing mechanisms, Han:SPRD-*Cy* rats were given diets containing either casein protein (CP) or SP, and soy oil (SO) or FO. Analysis of oxylipins revealed that renal COX products were higher and LOX products were lower in diseased kidneys. SP (with SO) feeding resulted in lower COX products, activity and COX1 protein and higher LOX products in the diseased kidneys in parallel with reduced renal cyst growth and fibrosis. By comparison, FO reduced both COX and LOX products produced from n-6 fatty acids and increased 3-series prostanoids in both normal and diseased cortex and medulla, but these differences were not associated with changes in disease progression. In conclusion, renal COX derived oxylipins are elevated and LOX products are reduced in this model of kidney disease. The effects of dietary SP, but not FO, on renal oxylipins parallel the effects on disease.

3.2 Introduction

Oxylipins are produced from arachidonic acid (AA) when tissues or cells are exposed to diverse physiologic and pathologic stimuli [1]. The first step in their formation is the release of AA from membrane phospholipids by phospholipase A_2, followed by the formation of prostanoids [prostaglandins (PG) and thromboxanes (TX)] via the cyclooxygenase (COX) pathway, mono-, di- and tri-hydroxy-eicosatetraenoic acids (HETE) and leukotrienes via the lipoxygenase (LOX) pathway, and epoxyeicosatrienoic acids and HETE via the cytochrome P450 pathway [1-3]. Oxylipins are formed by cells in all parts of the kidney and nephron [4] and play important roles in the preservation of kidney functions, particularly under pathophysiological states, regulating hemodynamics, water and solute transport, inflammatory processes, and renin secretion [5-14]. However, they also can be involved in the detrimental effects of disease by mediating inflammatory processes as a result of renal injury [5,9].

Phospholipase A_2 and COX levels are altered in the Han:SPRD-*Cy* rat and *pcy* models of renal cystic disease [15,16], and COX activities and select prostanoids (i.e. PGE_2, TXA_2, PGI_2) are higher in diseased (Cy/+) compared to normal (+/+) kidneys in the Han:SPRD-*Cy* rat [17,18]. Pharmacologic inhibition of COX2 results in marked attenuation of renal cyst growth, interstitial fibrosis, and macrophage counts in this model [16,19], and suppresses proliferation of human polycystic kidney disease (PKD) epithelial cells in cell culture [19], indicating the importance of these prostanoids in cystic kidney disease. However, other COX (PGD_2, $PGF_{2\alpha}$) or LOX metabolites also may be important, as they have been shown to be altered in other renal diseases [20-24].

Dietary fish oil (FO), rich in omega-3 polyunsaturated fatty acids, has beneficial effects in a number of renal disorders [24-28], including in the Han:SPRD-*Cy* rat [29].

Supplementing the diet with FO results in a shift in tissue fatty acid composition and oxylipin production away from the more bioactive oxylipins produced from AA, to the less bioactive oxylipins derived from eicosapentaenoic acid [24,30]. Thus, the beneficial effects of FO in early renal disease could be due to alterations in the renal oxylipin profile.

Dietary soy protein (SP) consistently displays a protective effect in experimental models of renal cyst disease [17,31-35], as well as other renal diseases [36,37]. It is different from FO in that dietary SP only has minor effects on tissue fatty acid compositions, but it also may mediate its beneficial effects on renal diseases via alterations in oxylipin production. SP reduces glomerular filtration rate (GFR) and renal plasma flow in models of experimental renal disease and in humans [38-40], similar to the effects of low compared to high protein diets which are renoprotective and reduce prostanoid levels, GFR and renal plasma flow [41-43]. In one human study, the reduction in GFR with a soy based diet was associated with a reduction in PGI_2 [39]. In the Han:SPRD-*Cy* rat, dietary SP slows disease progression and reduces elevated COX activities and PGE_2, TXB_2 and 6-keto-$PGF_{1\alpha}$ levels in diseased kidneys [17]. However, effects on other renal oxylipins are not known.

To examine the importance of both COX and LOX products in cystic kidney disease, the levels of oxylipins and oxylipin related enzymes were examined in the Han:SPRD-*Cy* rat. Since dietary FO and SP alter oxylipin formation via different mechanisms, these beneficial dietary interventions (individually or combined) were used to further probe the importance of the oxylipins in cystic kidneys in this model.

3.3 Materials and methods

3.3.1 Animals and diet

Han:SPRD-*Cy* rats were derived from our breeding colony, which originated from breeders obtained from Dr. Benjamin Cowley (at the University of Kansas Medical Center) [16]. Male weanling Han:SPRD-*Cy* rats (*N*=72) were genotyped [44] and at 3 wk of age 13 *Cy/+* and 5 +/+ rats (18/group) were randomized into each of four groups given diets based on the AIN-93G diet [45], but varying only in the protein and lipid source as follows: 1) CP (Dyets, cat. #210025) and SO (Dyets, cat. #404360) (this is the standard AIN-93G diet); 2) SP (Dyets, cat. #400050) and SO; 3) CP and FO (Dyets, cat. #402940) ; and 4) SP and FO, as described [17,29]. The protein level was 17.4 g/100g diet and the oil level was 7g/100g diet for all diets. The FO diet contained 5.25g menhaden oil plus 1.75g soy bean oil to ensure essential fatty acid adequacy, diet details in supplementary Table S1. All diet ingredients and oils were purchased from Dyets (Bethlehem, PA), items # in the appendix section. TBHQ was added at a level of 0.02% to all oils by Dyets and all diet ingredients were stored at 4 ^{0}C in sealed containers. Diet was freshly prepared every week, were stored at 4 ^{0}C in sealed containers, and fresh diet was provided to rats three times per week. Daily examination of diet texture, odour and colour indicated that the oils were not oxidized. At weeks 4 and 6 rats were placed in metabolic cages for 24 h before measuring feed intake over the subsequent 2 day period. Feed intake was similar in all rats in all groups, indicating no aversion to the diets.

Rats were given free access to water and diet. The study continued for 8 wk, a time period that results in significant dietary effects on disease [29,29]. Rats were singly housed in a temperature and humidity controlled environment with a 12 hour day/night cycle. After 8 wk rats were anesthetized with isoflurane and terminated by cardiac puncture to obtain blood for

serum, and kidneys were weighed and removed. Left kidneys were cut longitudinally across the hilum and one half was fixed in 10% buffered formalin for morphological and histological analysis, while the other half was separated into cortex and medulla, snap frozen in liquid nitrogen and stored in -80^{0}C. The right kidneys were weighed and snap frozen in liquid nitrogen, and lyophilized prior to further analysis. The difference between the wet and dry weights was used to calculate the water content. All animal procedures were approved by the University of Manitoba Animal Care Committee (protocol F09-012) and adhered to the guidelines of the Canadian Council on Animal Care.

3.3.2 Histologic and morphometric assessment

Formalin fixed kidneys were embedded in paraffin, sectioned at 5 µm and processed using our previously described methods [16,46]. Briefly, transverse tissue sections were stained with Masson's trichrome stain and 13 random images from each of the cortex and medulla were captured for analysis. All measurements were carried out in an observer-blinded fashion (by N.I.). Renal interstitial fibrosis was measured by densitometry of the blue stained areas whereas the portion of the tissue section occupied by open area was quantified for cyst area; proportional cyst and fibrosis areas were multiplied by kidney weight to estimate volume as described [16,46]. To avoid underestimation of fibrosis due to empty cystic areas in the sections, measurements of fibrosis were corrected to solid tissue areas of these sections [16,46].

3.3.3 Serum biochemistry

Serum creatinine concentrations were determined calorimetrically using a modified Jaffe reaction. Serum cystatin C was measured using a commercial ELISA kit (Bio Vendor Research and Diagnostic Products, Candler, NC, USA). Intra-assay coefficient of variations (CV) were calculated for all assays; if the CV for any sample or standard was ≥ 10 the sample was repeated.

3.3.4 Oxylipin and fatty acid analyses

For analyses of renal oxylipin, fatty acid and total protein levels, 45 mg of kidney cortex and medulla lyophilates were homogenized on ice in 30 volumes of Tyrode's. After homogenization, Triton X-100 was added and mixed to achieve a final concentration of 0.01%. Protein concentrations of the homogenates were determined using the Bradford method [47]. Fatty acids were expressed in ng/mg protein to match the unite of oxylipins.

For determination of endogenous renal oxylipin levels and in *vitro* production for COX activity assessments, aliquots were incubated for 0 and 5 min, respectively, at 37^0C, as described [18]. Samples were then processed and analyzed by HPLC-tandem mass spectrometry as described [24] based on the method of Deems *et al* [48]. Quantification of oxylipins was determined using the stable isotope dilution method [49]. Intra-assay variability was determined by processing replicates of two samples and the inter-assay variability was determined by processing 4 replicates in 3 batches. Excellent linearity with r^2 >0.999 was obtained for an accuracy of 99%. Table S7 provides information on HPLC-tandem mass spectrometry parameters for oxylipins scanned for.

For analysis of renal phospholipid content, total lipids were extracted as described [50]. Phospholipid fractions from kidney fractions were purified by TLC plates using

heptane/isopropyl ether/ acetic acid 60/40/3 (v/v) as the mobile phase. Phospholipid was identified by comigration with authentic standards purchased from Nu-Chek Prep (MN). Fatty acids in renal phospholipid were transmethylated using methanolic HCl and analyzed by gas-liquid chromatography as described [50].

3.3.5 Western immunoblotting

Western immunoblotting of oxylipin related enzymes was performed as described [15,18] using the following primary antibodies: COX1, COX2, microsomal PGE synthase-1 (mPGES-1), TX synthase (TXS) (1:250 dilutions; Cayman Chemical, Ann Arbor, Michigan) and 5-LOX (1:500 dilution; Cell Signaling Technology, Beverly, MA, USA). Membranes were stained with Ponceau S Solution (Sigma, St Louis, MO) to be used as a protein loading control, as described [51]. A reference kidney homogenate was loaded on each gel in duplicate so that results could be compared across gels.

3.3.6 Statistical analysis

Data were analyzed by 3-way ANOVA (dietary protein × dietary lipid × genotype), except for histologic analysis, which employed a 2-way ANOVA since only diseased kidneys were analyzed. The GLM procedure of SAS (SAS, version 9.2, Cary, NC) was used.

The Shapiro-Wilk's Statistic was used to test normality of the data ($W>0.05$ for normally distributed data). If the data did not follow a normal distribution even if transformed, a non-parametric test was used (Kruskal-Wallis). Tukey's test was used for simple effect comparisons when interactions were present. Pearson's correlation was used to test the relationship between renal pathology and oxylipin levels and COX activity. All data were

presented as mean ± standard error of mean (SE). Significance was set at $P<0.05$ for main and interaction effects.

3.4 Results

3.4.1 Renal pathology and function

Diseased rats had significantly larger kidneys and higher water content compared to normal rats (Table 3.1). Rats given the CP diets and the FO diets have slightly higher body weights than those given the SP and SO diets. Despite apparently similar feed intake in all groups, there may have been small variations due to differences in protein quality and/or palatability, which may have resulted in these small body weight differences. Providing SP compared to CP diets resulted in less enlarged kidneys whether expressed in absolute terms or relative to body weights as well as less kidney water content. In comparison, FO did not lessen kidney enlargement and even resulted in higher kidney water content in the rats given SP diets. With respect to histologic changes, diseased rats that were given SP compared to CP had less cortical cyst volume and fibrosis, and less medullary cyst volume and fibrosis (Figure 3.1). In FO compared to SO fed diseased rats, fibrosis volume was lower only in the rats given CP diets, with the effect being significant only in the medulla. Cyst volume in either cortex or medulla was unaffected by FO feeding.

With respect to kidney function, in rats given the CP SO diets, diseased compared to normal rats had more than two times higher serum cystatin C and creatinine (Table 3.1). In the SO fed rats, SP compared to CP fed diseased rats had 72% and 56% lower serum creatinine and cystatin C, respectively. FO compared to SO only had an effect in the CP fed rats, with 38% lower serum creatinine and no effect on serum cystatin C levels. No additive effects of SP and FO on renal pathology or function were observed.

3.4.2 Phospholipid fatty acid composition

While almost all cortical phospholipid fatty acids were lower in diseased compared to normal kidneys, no fatty acids were significantly lower in disease in the medulla (Tables 3.2 and 3.3). Dietary SP compared to CP did not have significant effects on either cortical or medulla fatty acid composition. FO compared to SO, on the other hand, altered fatty acid composition, including effects on n-6 and n-3 fatty acids: kidneys from rats given FO generally had higher n-3 and lower n-6 fatty acids in both the cortex and medulla.

3.4.3 Oxylipin levels

In the cortex, diseased compared to normal kidneys in rats given the CP SO diet had higher levels of COX products $PGF_{2\alpha}$, 6-keto-$PGF_{1\alpha}$, PGE_2, and TXB_2, and had lower levels of LOX products 5- and 15-HETE and 9-hydroxy-octadecadienoic acid (9-HODE) (Figure 3.2). In diseased kidneys of rats given SO diets, SP compared to CP feeding resulted in reversal of these differences for all oxylipins except $PGF_{2\alpha}$, resulting in levels that were not different than normal levels. $PGF_{2\alpha}$ and other COX and LOX products (PGD_2, 8- and 12-HETE, 13-HODE) followed similar patterns, but did not always reach statistical significance. For the lipid effect in the cortex, FO resulted in lower levels of 6-keto-$PGF_{1\alpha}$, PGE_2 and PGD_2, in diseased kidneys and TXB_2, $PGF_{2\alpha}$, 5-, 8-, 12- and 15-HETE in both normal and diseased kidneys. Dietary FO also increased cortical levels of the 3-series prostanoids including TXB_3 in diseased kidneys and $PGF_{3\alpha}$, PGD_3, and PGE_3 in both diseased and normal kidneys. The importance of these oxylipin changes was further evidenced by correlation analysis across dietary groups. Theses analyses showed that cortical cyst and fibrosis volumes positively correlated with total COX products and activity and that cyst volumes correlated negatively

with total LOX products in the cortex (Table 3.4). The strongest correlation was observed for the COX/LOX ratio with cyst and fibrosis volumes.

In comparison to the cortex, the medulla had much higher oxylipin concentrations. Even though there is more cortex tissue, the level of oxylipins in the medulla was 2.2 times higher in the medulla (995 ± 140 µg) compared to the cortex (446 ± 45 µg). Disease did not result in elevated levels of COX products in the medulla, but 5- and 15-HETE, and 9-HODE were similarly lower in disease (Figure 3.3). The source of dietary protein did not have any effects on medullary oxylipins, but dietary FO compared to SO resulted in lower levels of most COX and LOX products derived from n-6 fatty acids, and higher levels of the 3-series prostanoids, in both normal and diseased kidneys. Correlation of oxylipins to renal pathology in the medulla followed similar patterns as in the cortex, but fewer of these correlations were significant. COX/LOX and total COX activity correlated to cyst volume and fibrosis volume, respectively.

3.4.4 Cyclooxygenase activities

Cortical and medullary COX activities were higher in diseased compared to normal kidneys in rats given the CP SO diet by 62-95% and 68-91% in the cortex and medulla, respectively (Figure 3.4 and 3.5). As with the higher oxylipin levels found in the medulla, COX activity also was much higher in medulla compared to cortex. In diseased kidneys of rats given SO diets, SP compared to CP reduced cortical COX activity by 53-85% when measured by production of $PGF_{2\alpha}$, PGE_2, 6-keto-$PGF_{1\alpha}$ and TXB_2, and medulla COX activity (by 45 and 55%) for production of 6-keto-$PGF_{1\alpha}$ and TXB_2, respectively. FO compared to SO also

reduced the cortical (by 63-78%) and medulla COX (by 58-64%) activities for production of all prostanoids.

3.4.5 Enzyme levels

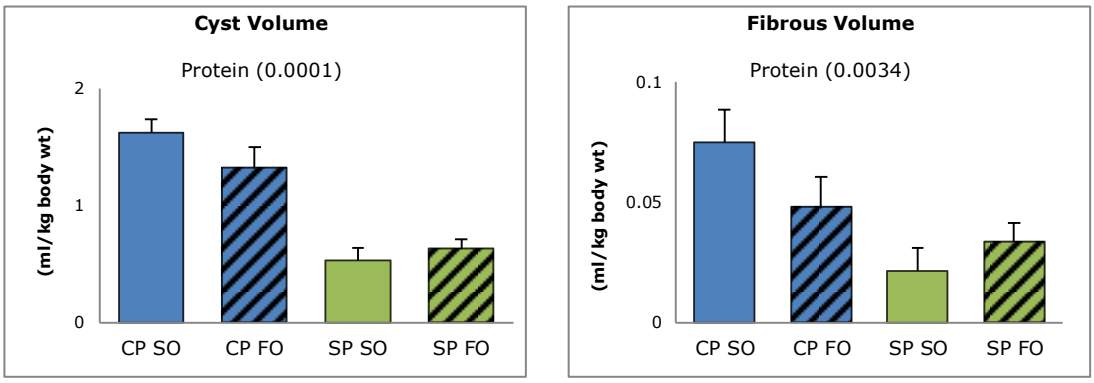
Levels of COX1, COX2, TXS, mPGES-1 and 5-LOX were examined since they are associated with the dietary intervention (SP compared to CP) that resulted in parallel changes in disease and oxylipin levels. Cortical TXS was 21% higher overall in diseased compared to normal kidneys, while cortical mPGES-1 was not altered by disease (Figure 3.6). Cortical COX1 and 5-LOX were more than two times higher in diseased compared to normal rats given CP diets. Providing SP to diseased rats resulted in lower cortex COX-1 and 5-LOX, resulting in levels not different from those observed in normal kidneys. The only other dietary effect was on cortical COX2, which was higher overall in kidneys from normal and diseased rats given SP compared to CP diets. In the medulla, COX2 was lower in diseased kidneys (Figure 3.7).

Table 3.1. Physiologic parameters in normal and diseased Han:SPRD-*Cy* rats given dietary CP or SP and SO or FO

Genotype	**Normal (+/+) rats**				**Diseased (Cy/+) rats**				**Effects (P-values)**
Protein	**Casein**		**Soy**		**Casein**		**Soy**		
Lipid	**Soy**	**Fish**	**Soy**	**Fish**	**Soy**	**Fish**	**Soy**	**Fish**	
Body weight (g)	389 (2.50)	409 (7.60)	372 (9.10)	378 (3.40)	370 (4.50)	402 (4.70)	368 (3.70)	386 (4.90)	Protein (0.0002) & lipid (<0.0001)
Kidney weight (g)	2.67 (0.04)^{c}	2.88 (0.10)^{c}	2.65 (0.06)^{c}	2.75 (0.02)^{c}	9.04 (0.37)^{a}	8.99 (0.44)^{a}	5.66 (0.55)^{b}	6.68 (0.41)^{b}	Interaction (0.0001)
Kidney/body weight (g/100 g)	0.68 (0.11)^{c}	0.70 (0.11)^{c}	0.71 (0.14)^{c}	0.72 (0.07)^{c}	2.44 (0.96)^{a}	2.33 (1.29)^{a}	1.54 (1.48)^{b}	1.73 (1.09)^{b}	Interaction (<0.0001)
Kidney water (g)	1.00 (0.01)^{d}	1.12 (0.04)^{d}	1.04 (0.02)^{d}	1.45 (0.31)^{d}	3.54 (0.29)^{ab}	3.73 (0.19)^{a}	2.31 (0.26)^{c}	3.03 (0.13)^{b}	Interaction (0.002)
Serum creatinine (µg/dl)	3.33 (0.47)^{bc}	2.06 (0.29)^{c}	2.15 (0.26)^{c}	2.87 (0.14)^{bc}	7.95 (0.48)^{a}	4.93 (0.58)^{b}	2.22 (0.25)^{c}	2.48 (0.26)^{c}	Interaction (0.0001)
Serum cystatin C (ng/ml)	2.66 (0.36)^{bc}	2.75 (0.36)^{bc}	3.04 (0.19)^{bc}	2.72 (0.13)^{bc}	5.50 (0.53)^{a}	4.13 (0.36)^{ab}	2.42 (0.21)^{c}	2.03 (0.09)^{c}	Interaction (0.0001)

Values are means (SE), (n=5 for +/+ and 13 for *Cy*/+ groups). Average CV for serum creatinine and serum cystatin C =1.5. Where interactions were present, values in the same row with different superscript letters are significantly different (P<0.05).

A: Cortex



B: Medulla

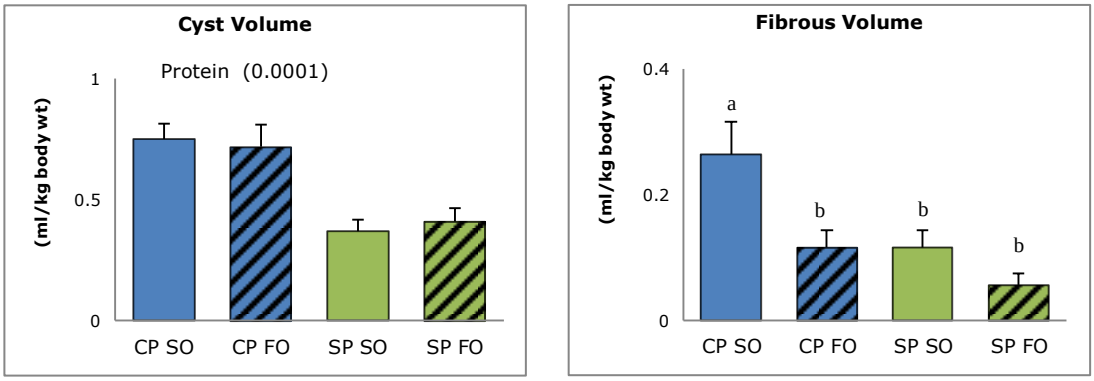


Figure 3.1. a. Morphometric analysis of the cortex (A) and medulla (B) in diseased Han:SPRD-*Cy* rats given dietary CP or SP and FO or SO. Cyst areas were captured from 13 random fields (×40 magnification) of H&E stained kidney sections and indices were measured and calculated by a naive observer. The product of proportional cyst area and kidney weight was used to estimate cyst volume. Fibrosis volume was calculated in the same way using Masson's trichrome stained sections. b. Images of representative kidney sections are shown in Figure 3.1b. Values are means ± SE (n=5 for +/+ and 13 for *Cy*/+ groups). Where interactions were present, values on the same graph with different letters are significantly different ($P<0.05$).

A: Cortex **B: Medulla**

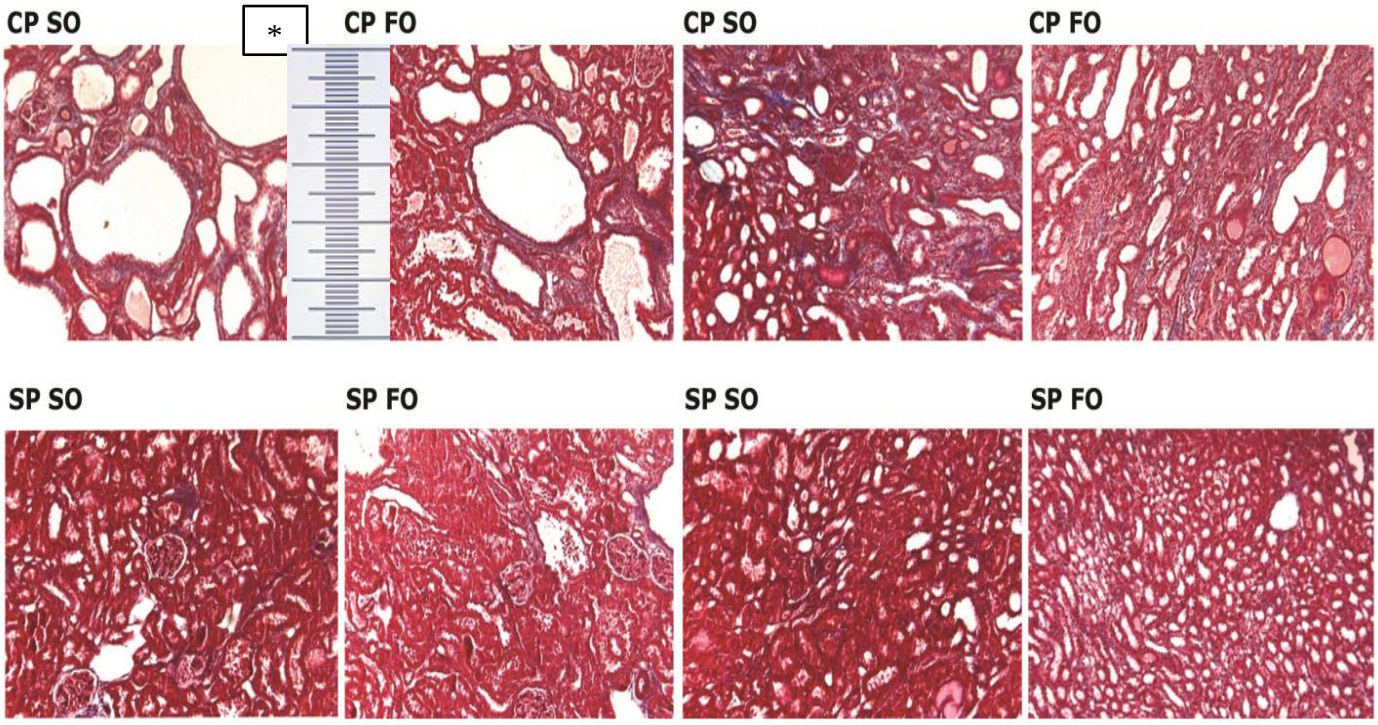


Figure 3.1b. Morphometric analysis of the cortex (A) and medulla (B) in diseased Han:SPRD-*Cy* rats given dietary CP or SP and FO or SO. Cyst areas were captured from 13 random fields (×40 magnification) of H&E stained kidney sections and indices were measured and calculated by a naive observer. The product of proportional cyst area and kidney weight was used to estimate cyst volume. Fibrosis volume was calculated in the same way using Masson's trichrome stained sections. (n=5 for +/+ and 13 for *Cy*/+ groups). *1 space of a stage micrometer = 10 µm.

Table 3.2. Renal cortex phospholipid fatty acid compositions in normal and diseased Han:SPRD-*Cy* rats given dietary CP or SP and SO or FO

Cortex	Normal (+/+)				Diseased (*Cy*/+)				
Protein	Casein		Soy		Casein		Soy		Effects (P-values)
Lipid	Soy	Fish	Soy	Fish	Soy	Fish	Soy	Fish	
C16:0	3468 (517)	4847 (642)	3231 (178)	5850 (189)	2680 (620)	3262 (614)	2445 (884)	3668(483)	Genotype (0.003) and lipid (0.001)
C16:1	83 (10)^{c}	251 (40)^{ab}	75 (7)^{c}	307 (15)^{a}	63 (18)^{c}	159 (43)^{bc}	49 (16)^{c}	141 (14)^{bc}	Interaction (0.008)
C18:0	3601 (461)	4845 (759)	3647 (202)^{b}	6060 (225)	3216 (637)	3504 (477)	2852 (1009)	4424 (395)	Genotype (0.019) and lipid (0.002)
C18:1	1064 (159)	1462 (116)	1032 (56)	1881 (185)	959 (201)	1111 (191)	759 (240)	1093 (90)	Genotype (0.004) and lipid (0.001)
C18:2n6	1891 (190)	2474 (527)	2007 (201)	3212 (167)	1432 (467)	1478 (356)	1312 (440)	2234 (362)	Genotype (0.008) and lipid (0.017)
C18:3n3	36 (5)	37 (4)	37 (4)	46 (2)	33 (7)	30 (5)	26 (6)	37 (5)	Genotype (0.033)
C18:3n6	17 (3)^{cd}	43 (6)^{ab}	17 (1)^{cd}	50 (2)^{a}	17 (3)^{cd}	31 (5)^{bc}	12 (4)^{d}	30 (4)^{bc}	Interaction (0.024)
C20:3n3	23 (1)	48 (6)	25 (2)	53 (2)	20 (5)	35 (10)	15 (4.27)	41 (5.43)	Genotype (0.023) and lipid (0.0001)
C20:3n6	104 (5)	197 (29)	105 (9)	219 (17)	79 (26)	111 (28)	61 (17)	138 (5)	Genotype (0.0004) and lipid (0.0001)
C20:4n6	4092 (471)	3397 (460)	4329 (283)	4123 (212)	3362 (1035)	2394 (597)	2516 (767)	3536 (913)	Genotype (0.039)
C20:5n3	33.89 (6)^{c}	1061 (136)^{ab}	28 (2)^{c}	1141 (107)^{a}	31 (9)^{c}	755 (185)^{ab}	23 (5)^{c}	579 (181)^{ab}	Interaction (0.018)
C22:0	110 (22)	115 (18)	108 (14)	148 (10)	104 (26)	94 (24)	77 (22)	100 (14)	No effect
C22:4n6	167 (81)	19 (3)	150 (38)	38 (7)	190 (63)	44 (15)	90 (19)	62 (35)	Lipid (0.0001)
C22:5n3	77 (27)	190n (27)	69 (13)	257 (35)	79 (24)	218 (67)	42 (10)	143 (23)	Lipid (0.0001)
C22:6n3	241 (23)	700 (88)	240 (13)	835 (82)	213 (68)	533 (129)	130 (38)	463 (49)	Genotype (0.004)

									lipid (0.0001)
C24:0	671 (100)	1139 (188)	676 (50)	1225 (111)	546 (177)	614 (138)	532 (191)	840 (69)	Genotype (0.007) and lipid (0.002)
C24:1	171 (29)	190 (28)	178 (17)	209 (10)	139 (46)	109 (27)	134 (47)	162 (34)	Genotype (0.036)

Values are means (SE), expressed in ng/mg protein. (n=8/group). Where interactions were present, values in the same row with different superscript letters are significantly different ($P<0.05$).

Table 3.3. Renal medulla phospholipid fatty acid compositions in normal and diseased Han:SPRD-*Cy* rats given dietary CP or SP and SO or FO

Medulla	**Normal (+/+)**				**Diseased (*Cy*/+)**				**Effects (P-values)**
Protein	**Casein**		**Soy**		**Casein**		**Soy**		
Lipid	**Soy**	**Fish**	**Soy**	**Fish**	**Soy**	**Fish**	**Soy**	**Fish**	
C16:0	4304 (752)	5360 (491)	3008 (1129)	2561 (1137)	2018 (411)	2180 (615)	4918 (701)	2402 (951)	Interaction (0.004)
C16:1	135 (13)^{ab}	322 (20)^{a}	65 (31)^{b}	102 (70)^{b}	44 (10)^{b}	107 (37)^{b}	111 (14)^{b}	211 (84)^{ab}	Interaction (0.002)
C18:0	4190 (985)	4706 (719)	3051 (999)	2477 (1239)	2239 (376)	2823 (1050)	5019 (681)	2529 (759)	No effect
C18:1	1495 (285)	1834 (217)	996 (288)	875 (262)	955 (166)	1116 (320)	1717 (199)	892 (272)	Interaction (0.023)
C18:2n6	2315 (494)	2640 (326)	1752 (748)	1453 (826)	1020 (228)	670 (223)	2680 (519)	1185 (566)	Interaction (0.013)
C18:3n3	43 (10)^{ab}	44 (6)^{ab}	32 (8)^{ab}	23 (12)^{ab}	29 (6)^{ab}	9 (8)^{b}	54 (9)^{a}	23 (10)^{ab}	Interaction (0.011)
C18:3n6	19 (4)	45 (6)	8 (7)	24 (13)	9 (6)	8 (7)	22 (3)	17 (12)	Interaction (0.018)
C20:3n3	24 (6)	48 (7)	10 (9)	23 (11)	8 (5)	32 (21)	26 (7)	12 (11)	No effect
C20:3n6	118 (28)	214 (31)	101 (48)	100 (52)	61 (14)	60 (20)	132 (27)	92 (47)	Interaction (0.026)
C20:4n6	4501(1161)	3594 (378)	3379 (1460)	1925 (883)	2611 (614)	1348 (489)	4570 (977)	1750 (927)	Lipid (0.027)
C20:5n3	32 (8)	1047 (168)	26 (13)	473 (269)	14 (6)	290 (102)	31 (6)	419 (231)	Lipid (<0.0001)
C22:0	114 (31)	119 (26)	84 (34)	68 (28)	121 (26)	84 (34)	159 (24)	76 (38)	No effect
C22:4n6	120 (30)	36 (7)	88 (35)	20 (7)	251 (70)	46 (20)	212 (36)	29 (18)	Lipid (<0.0001)
C22:5n3	61 (18)	255 (46)	40 (16)	110 (45)	74 (16)	148 (54)	80 (16)	133 (78)	Lipid (0.002)
C22:6n3	222 (71)	799 (132)	152 (69)	318 (182)	118 (27)	205 (68)	194 (42)	273 (162)	Lipid (0.026)
C24:0	610 (217)	890 (241)	447 (219)	419 (298)	291 (59)	220 (80)	730 (181)	364 (206)	Interaction (0.031)
C24:1	171 (58)	209 (53)	132 (60)	82 (48)	98 (17)	62 (22)	228 (53)	86 (46)	Interaction (0.034)

Values are means (SE), expressed in ng/mg protein. (n=8/group). Where interactions were present, values in the same row with different superscript letters are significantly different (P<0.05).

Table 3.4. Correlations of renal COX and LOX products with pathology in diseased kidneys from rats on all diet combined

Variables	**Cyst volume (r)**	**Fibrosis volume (r)**
Cortical COX products	0.472*	0.291
Cortical LOX products	-0.431*	- 0.267
Cortical COX/LOX products	0.720***	0.522**
Cortical total COX activity	0.500**	0.714***
Medullary COX products	0.024	0.116
Medullary LOX products	-0.279	-0.109
Medullary COX/LOX products	0.345*	0.192
Medullary total COX activity	0.014	0.381*

Pearson's correlation was used, n=5 for +/+ and 13 for *Cy*/+ groups. *$p < .05$. **$p < .001$. ***$p < .0001$

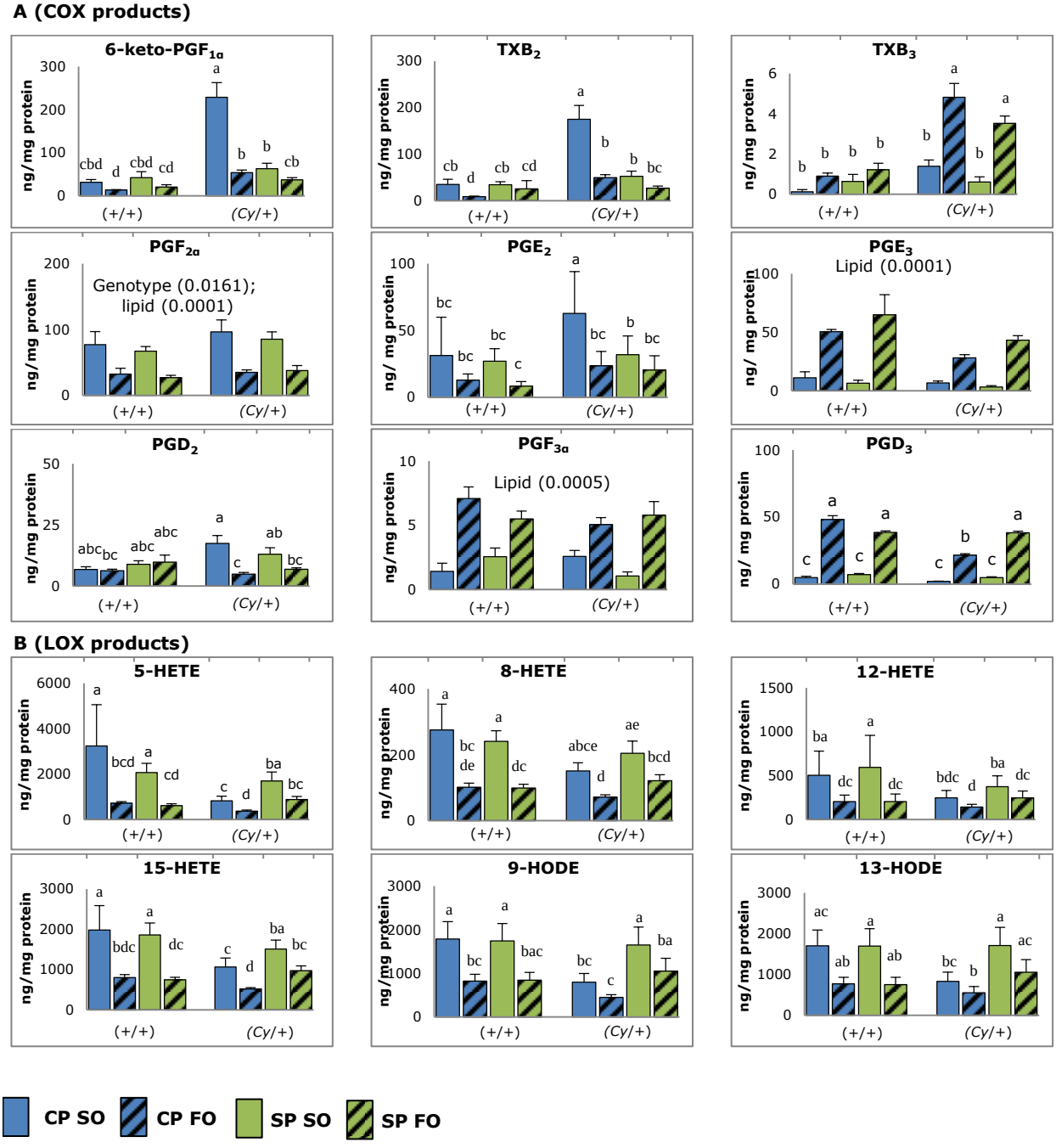


Figure 3.2. COX (A) and LOX (B) products in the cortex of diseased Han:SPRD-*Cy* rats given dietary CP or SP and FO or SO. Cortical oxylipin levels were analyzed by HPLC-tandem mass spectrometry Values are means ± SE (n=5 for +/+ and 13 for *Cy*/+ groups). Main effects (P value) are indicated on individual graphs; where interactions (P<0.05) were present, any differences are indicated with differing letters.

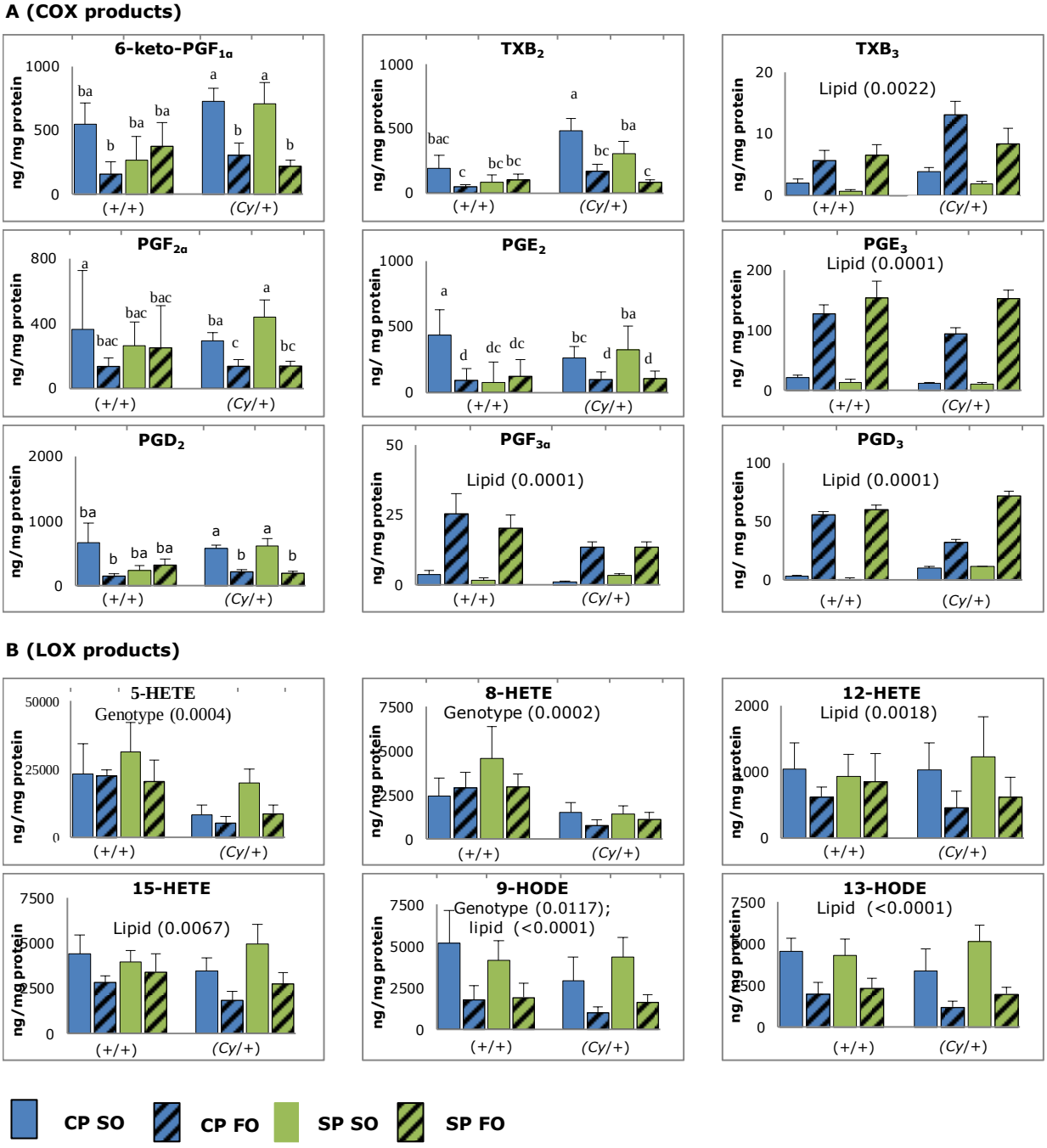


Figure 3.3. COX (A) and LOX (B) products in the medulla of diseased Han:SPRD-*Cy* rats given dietary CP or SP and FO or SO. Medullary oxylipin levels were analyzed by HPLC-tandem mass spectrometry. Values are means ± SE (n=5 for +/+ and 13 for *Cy*/+ groups). Main effects (P value) are indicated on individual graphs; where interactions (P<0.05) were present, any differences are indicated with differing letters.

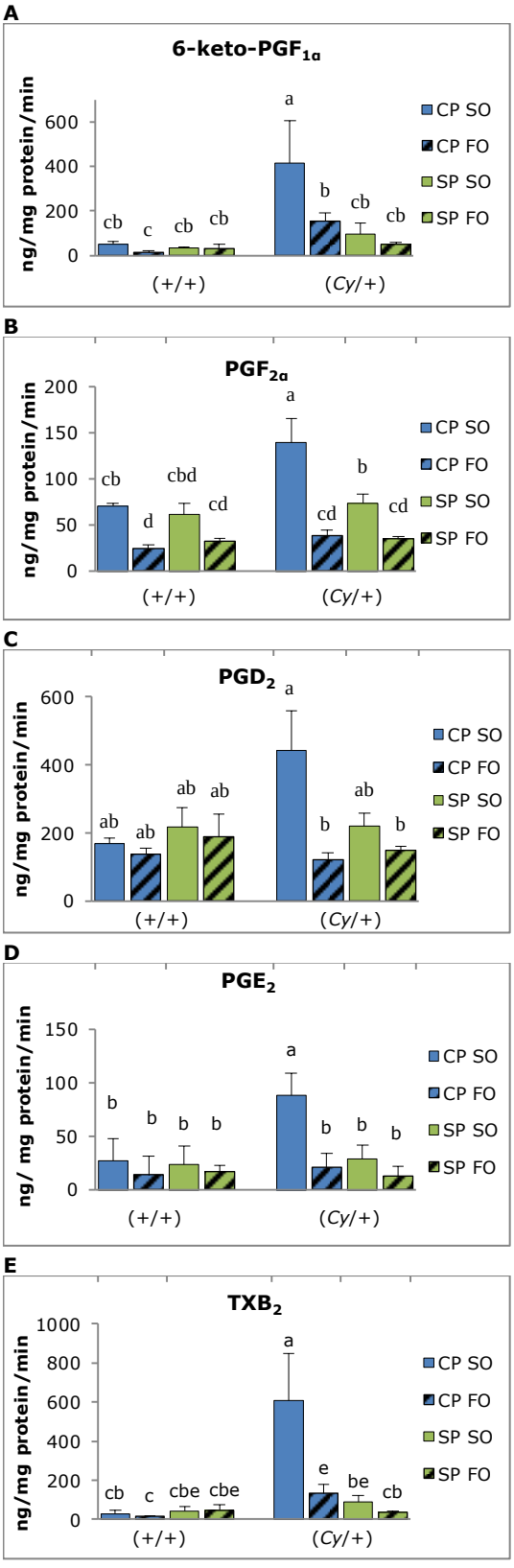


Figure 3.4. Cortex COX activity as measured by production of individual prostanoids. 45 mg of kidney cortex lyophilates were homogenized on ice in 30 volumes of Tyrode's. After homogenization, Triton X-100 was added and mixed to achieve a final concentration of 0.01%. Aliquots were incubated for 0 and 5 min, respectively, at 37^0C. Samples were then processed and analyzed by HPLC-tandem mass spectrometry. Values are means ± SE (n=5 for +/+ and 13 for *Cy*/+ groups). Interactions ($P<0.05$) were present for all; differences are indicated with differing letters.

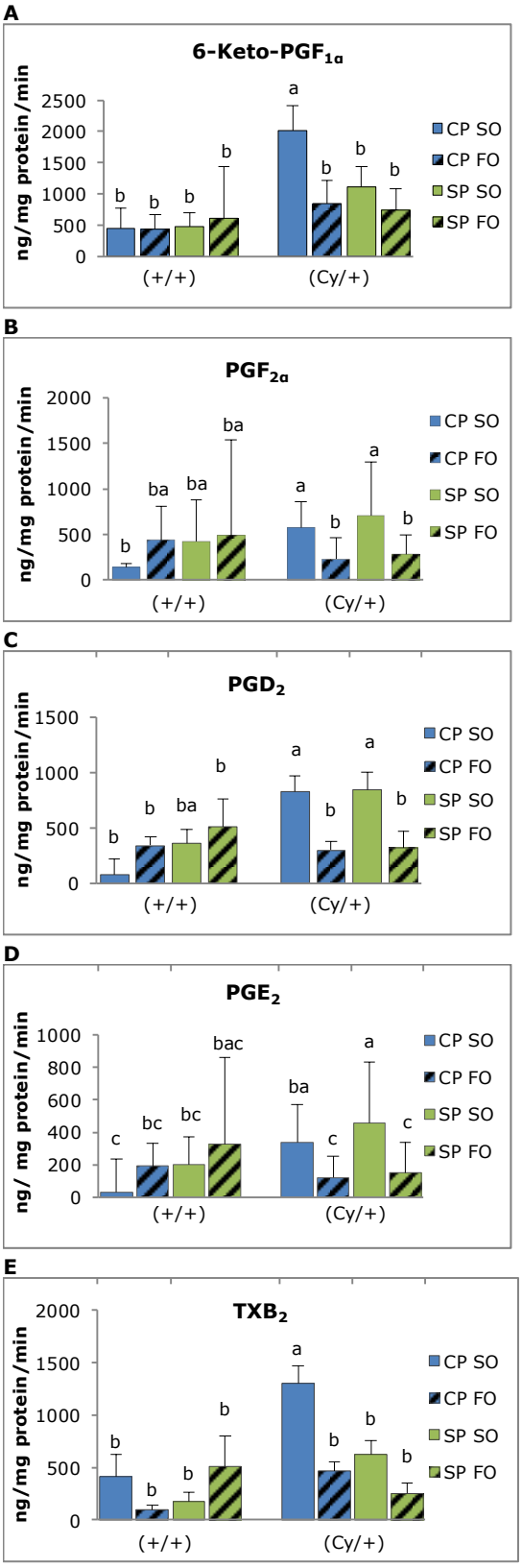
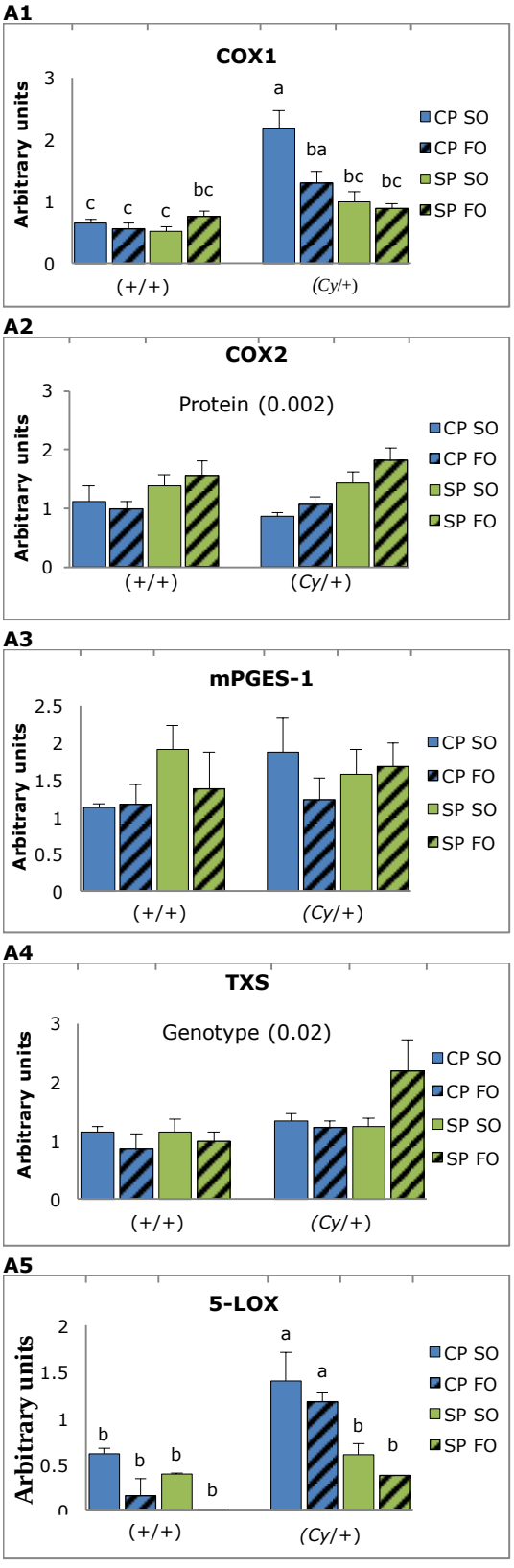


Figure 3.5. Medulla COX activity as measured by production of individual prostanoids. 45 mg of kidney medulla lyophilates were homogenized on ice in 30 volumes of Tyrode's. After homogenization, Triton X-100 was added and mixed to achieve a final concentration of 0.01%. Aliquots were incubated for 0 and 5 min, respectively, at 37^0C. Samples were then processed and analyzed by HPLC-tandem mass spectrometry. Values are means ± SE (n=5 for +/+ and 13 for *Cy*/+ groups). Values are means ± SE. Interactions ($P<0.05$) were present for all; differences are indicated with differing letters.



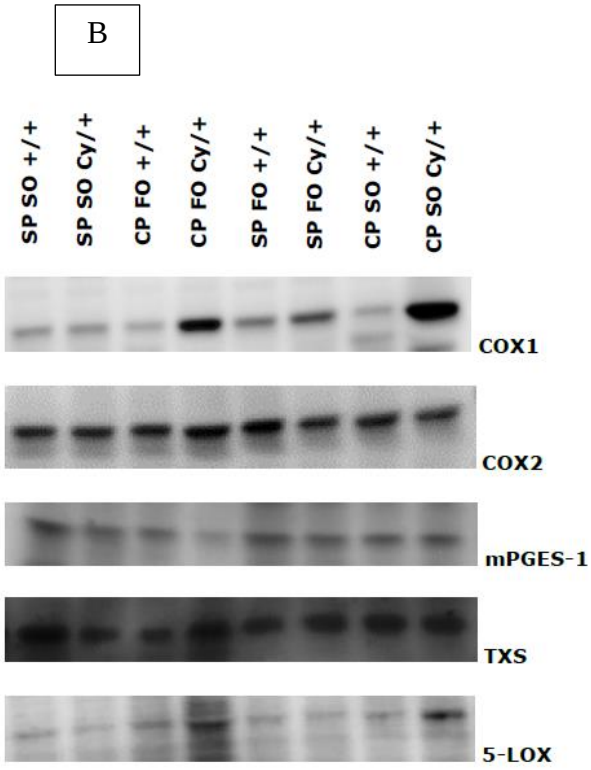
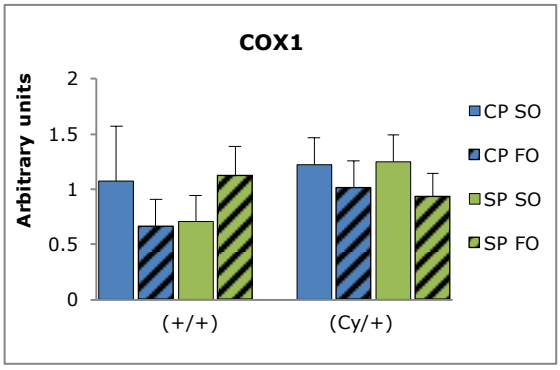
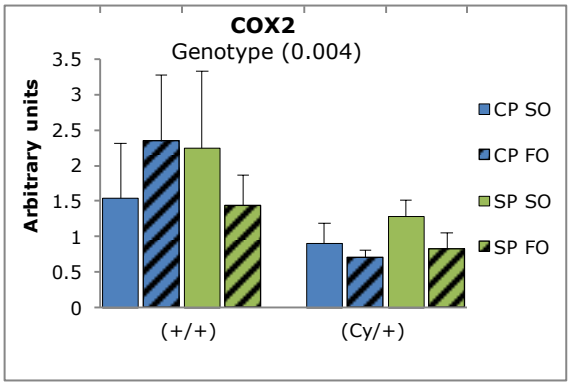


Figure 3.6. Protein levels (A) and representative western blots (B) of enzymes in cortex. Membranes were stained with Ponceau S Solution (Sigma, St Louis, MO) to be used as a protein loading control, as described [51]. A reference kidney homogenate was loaded on each gel in duplicate so that results could be compared across gels. Values are means ± SE. Main effects (P value) are indicated on individual graphs; where interactions ($P<0.05$) were present, any differences are indicated with differing letters.

A1



A2



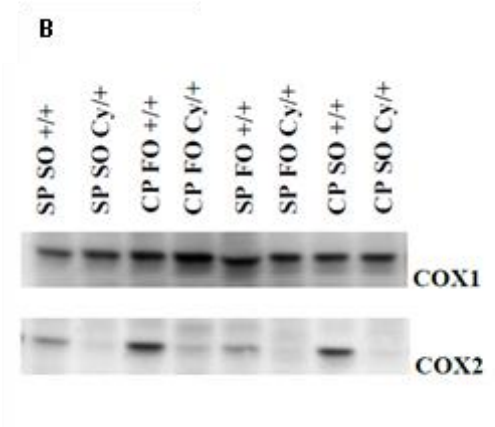


Figure 3.7. Protein levels (A) and representative western blots (B) of enzymes in medulla. Membranes were stained with Ponceau S Solution (Sigma, St Louis, MO) to be used as a protein loading control, as described [51]. A reference kidney homogenate was loaded on each gel in duplicate so that results could be compared across gels. Values are means ± SE. Main effects (P value) are indicated on individual graphs.

3.5 Discussion

This study demonstrates that renal COX metabolism is elevated, but that production of LOX metabolites is lower in diseased compared to normal Han:SPRD-*Cy* rats. Our previous studies showed that the levels of oxylipin forming enzymes are altered and select COX products (PGE_2, TXB_2 and 6-keto $PGF_{1\alpha}$) are elevated in the whole kidney of diseased rats at 10 weeks of age [7-10]. The current findings show that prostanoid alterations are present primarily in the cortex, the region in which most cysts are found in Cy/+ kidneys [52]. Much higher concentrations (ng/mg protein) of oxylipins were present in the medulla of these kidneys, as has been previously shown [53,54], even though there are similar concentrations (ng/mg protein) of AA in the cortex and medulla. In contrast to COX products, LOX metabolites from both AA and linoleic acid were lower in diseased compared to normal kidneys in both the cortex and medulla.

The majority of COX activity in the kidney is due to COX2 activity [18] and selective COX2 inhibition reduces kidney disease progression in the Han:SPRD-*Cy* rat [16,19], illustrating the importance of COX metabolites in the progression of this disorder. We therefore hypothesized that reducing prostanoids via dietary FO intervention may have similar effects. Dietary FO can reduce oxylipin levels by increasing tissue n-3 fatty acids, which compete with AA for the conversion to oxylipins [30,55]. Hence, oxylipin production via both COX and LOX pathways were reduced with FO feeding in both normal and diseased kidneys in the current study. The lower levels of COX metabolites in kidneys of rats given the FO diets would have been expected to reduce disease progression, as this effect is similar to pharmacological COX2 inhibition [16,19]. Indeed, reduced COX activity was correlated to reduced fibrosis and cyst volume. However, the concomitant lowering of the LOX pathway metabolites in rats given the FO diets may have counteracted any potential beneficial effect on cyst volume of reducing COX

metabolites, since LOX products were correlated negatively with cyst volume. It is unlikely that the increase in 3-series prostanoids mitigated the beneficial effect of lowering the 2-series prostanoids in disease, as the 3-series prostanoids tend to have much less bioactivity [12,56,57], and the increase in n-3 series prostanoids is generally less than the reduction in 2-series prostanoids. Specific LOX inhibitors that do not affect COX activity need to be tested in this model in order to address the question as to whether lower LOX metabolites are directly associated with disease progression.

In contrast to FO effects, SP was more effective in slowing disease progression and the changes in disease were associated with amelioration of most of the alterations in renal oxylipins. The mechanism (for more details about the unique amino acid composition of SP and its bioactive compounds that may play a role in its reno-protective effect, please see chapter 6) by which SP affects oxylipin metabolism is different than for FO, as this dietary intervention did not alter the fatty acid composition of either normal or diseased kidneys. It is not clear how SP alters oxylipin production or whether SP alters disease via another mechanism that resulted in oxylipins being restored to levels similar to normal. A plausible mechanism may be via the bioactive peptides produced via digestion of the proteins, which have been reported to alter the renin-angiotensin system which mediates its effects via changes in prostanoid levels as well as being itself regulated by COX products [47-50]. The SP effects may be similar to low protein effects on kidneys, as dietary protein level also affects renal oxylipins via an unexplained mechanism [38-40]. The timing of the effects of diet on oxylipins also is different with dietary protein compared to lipid interventions. While dietary protein effects on oxylipin production are almost immediate [29,58], dietary lipids must be incorporated into tissue phospholipids before oxylipin formation is affected. Fatty acid turnover in phospholipid in response to dietary lipid is

in the range of 1-3 weeks [59,60]. Nevertheless, whether the effect of SP on oxylipins is direct or indirect, or is immediate or delayed, the findings with SP and FO provide further evidence that both COX and LOX metabolites are important in this disease and may be useful targets for dietary or pharmacologic treatment. Including inhibitors/activators of specific oxylipins and their receptors.

The roles of oxylipins in the kidney are multifactorial and sometimes opposing, depending on the oxylipin in question and the specific receptors present [5,61]. They are involved in hemodynamics and GFR, water and salt homeostasis, as well as inflammatory and fibrotic processes in response to kidney damage [5-14]. In the context of the current results, prostanoids stimulate cyclic AMP production and TXA_2 is a stimulator of renal cell proliferation [62], two key events in Cy/+ kidney disease progression [63]. In the current report, renal cystic change and fibrosis is associated with lower HETE levels, the significance of which remains to be elucidated. It is possible that the reduced LOX metabolites in the diseased kidneys simply reflect competition for the AA substrate by the COX enzymes. This increased demand by the COX pathway also could explain the reduction in linoleic acid LOX metabolites, as both AA and linoleic acid were reduced in the cortex of diseased compared to normal kidneys. However, direct inhibition of LOX enzymes by the elevated prostanoids, or another independent disease effect on LOX, also could have resulted in the lower LOX metabolites in diseased kidneys.

The only other oxylipin that is altered in other models of renal cyst disease is 20-HETE, which is formed from AA by cytochrome P450 ω-hydroxylase activity. 20-HETE promotes cyst formation in the Balb/C polycystic kidney (BPK) mouse model of autosomal recessive PKD both in *vitro* and in *vivo* [64], and chronic inhibition of 20-HETE in this model, as well as the PCK rat, reduces disease progression [20,64]. This oxylipin was scanned for, but not detected in the

current study, and therefore if present is below the limit of detection (<1 ng/mg dry tissue) in the Han:SPRD-*Cy* rat kidney.

We have previously shown that COX1 and COX2 protein levels are higher and lower, respectively, in diseased kidneys [18]. The current study shows that the higher COX1 levels occur primarily in the cortex, while the reduction in COX2 protein appears to occur in the medulla. The lower COX2 protein levels likely reflect feedback inhibition on gene expression of this enzyme, as mRNA levels of COX2 also are lower, but COX2 activity is markedly higher [18]. Thus enzyme protein levels, mRNA levels and activity are not always the same. This also may be the case for 5-LOX, which was higher in diseased kidneys, despite its products 5-HETE and 9-HODE being lower.

Since dietary SP and FO affect renal oxylipins via different mechanisms, it was of interest in the current study to examine whether they would have additive and/or synergistic effects on disease progression. The results of the current study demonstrate that both SP and FO individually reduced kidney disease progression, as has previously been demonstrated [17,31-33]. However, the current study demonstrated that dietary SP is more effective than FO, and adding FO to the SP diet did not increase the effect. A beneficial effect of SP on cyst growth and fibrosis was observed in the cortex and the medulla, and these were associated with mitigation of oxylipin alterations. On the other hand, the effect of FO was restricted to reducing fibrosis, which was reduced by FO oil feeding in the cortex and medulla, but only significantly in the medulla. This was associated with a reduction in COX products. However, further reduction of LOX products by FO may counteract the benefits of COX reduction on cyst volume. It remains to be determined whether lower doses of these interventions might show additive or synergistic

effects, as the level of SP and FO in human diets would be lower than what was achieved in the current rat study.

In conclusion, we show that COX products are elevated, but LOX metabolites are lower in diseased kidneys of Han:SPRD-*Cy* rats. Intervention with dietary SP is associated with amelioration of these oxylipin alterations and disease in parallel, while FO intervention lowers both COX and LOX products derived from n-6 fatty acids and is much less effective in slowing disease. These findings indicate that pharmacologic or dietary interventions that specifically inhibit COX without reducing LOX have potential for treating this disorder. Further studies examining the direct effects of oxylipins on cystogenesis and disease progression are warranted.

3.6 Acknowledgements

This work was supported by a grant from The Natural Sciences and Engineering Research Council of Canada (NSERC). Support to N.I. was provided by the NSERC Food Advancement through Science and Training (FAST) program, and by the Libyan-North American scholarship program and a University of Manitoba Graduate Fellowship. The authors thank the Manitoba Institute of Child Health and the Richardson Centre for Functional Foods and Nutraceuticals for providing access to equipment, and Joy Gauthier, Dennis Labossiere , Tanja Winter, Amy Wu and Melanie Grégoire for providing technical assistance with the oxylipin and fatty acid analyses.

3.7 References

[1] Buczynski MW, Dumlao DS, Dennis EA. Thematic Review Series: Proteomics. An integrated omics analysis of eicosanoid biology. J Lipid Res 2009;50:1015-38.

[2] O'Donnell VB, Maskrey B, Taylor GW. Eicosanoids: generation and detection in mammalian cells. Methods Mol Biol 2009;462:5-23.

[3] Imig JD. Eicosanoid regulation of the renal vasculature. Am J Physiol Renal Physiol 2000;279:F965-81.

[4] Campean V, Theilig F, Paliege A, Breyer M, Bachmann S. Key enzymes for renal prostaglandin synthesis: site-specific expression in rodent kidney (rat, mouse). Am J Physiol Renal Physiol 2003;285:F19-32.

[5] Hao C-, Breyer MD. Physiological regulation of prostaglandins in the kidney. Annual Review of Physiology 2008;70:357-77.

[6] Hodeify RF, Kreydiyyeh SI. PGE2 reduces net water and chloride absorption from the rat colon by targeting the Na+/H+ exchanger and the Na+ K+ 2Cl- cotransporter. Prostaglandins Leukot Essent Fatty Acids 2007;76:285-92.

[7] Mori A, Saito M, Sakamoto K, Narita M, Nakahara T, Ishii K. Stimulation of prostanoid IP and EP(2) receptors dilates retinal arterioles and increases retinal and choroidal blood flow in rats. Eur J Pharmacol 2007;570:135-41.

[8] Dolegowska B, Pikula E, Safranow K, Olszewska M, Jakubowska K, Chlubek D et al. Metabolism of eicosanoids and their action on renal function during ischaemia and reperfusion: the effect of alprostadil. Prostaglandins Leukot Essent Fatty Acids 2006;75:403-11.

[9] Kitahara M, Eitner F, Ostendorf T, Kunter U, Janssen U, Westenfeld R et al. Selective cyclooxygenase-2 inhibition impairs glomerular capillary healing in experimental glomerulonephritis. J Am Soc Nephrol 2002;13:1261-70.

[10] Vukicevic S, Simic P, Borovecki F, Grgurevic L, Rogic D, Orlic I et al. Role of EP2 and EP4 receptor-selective agonists of prostaglandin E(2) in acute and chronic kidney failure. Kidney Int 2006;70:1099-106.

[11] Stitt-Cavanagh EM, Faour WH, Takami K, Carter A, Vanderhyden B, Guan Y et al. A maladaptive role for EP4 receptors in podocytes. J Am Soc Nephrol 2010;21:1678-90.

[12] Darlametsos IE, Varonos DD. Role of prostanoids and endothelins in the prevention of cyclosporine-induced nephrotoxicity. Prostaglandins Leukot Essent Fatty Acids 2001;64:231-9.

[13] Xu ZG, Miao LN, Cui YC, Jia Y, Yuan H, Wu M. Angiotensin II type 1 receptor expression is increased via 12-lipoxygenase in high glucose-stimulated glomerular cells and type 2 diabetic glomeruli. Nephrol Dial Transplant 2009;24:1744-52.

[14] Xie C, Wang DH. Inhibition of renin release by arachidonic acid metabolites, 12(s)-HPETE and 12-HETE: role of TRPV1 channels. Endocrinology 2011;152:3811-9.

[15] Aukema HM, Adolphe J, Mishra S, Jiang J, Cuozzo FP, Ogborn MR. Alterations in renal cytosolic phospholipase A2 and cyclooxygenases in polycystic kidney disease. FASEB J 2003;17:298-300.

[16] Sankaran D, Bankovic-Calic N, Ogborn MR, Crow G, Aukema HM. Selective COX-2 inhibition markedly slows disease progression and attenuates altered prostanoid production in Han:SPRD-cy rats with inherited kidney disease. Am J Physiol Renal Physiol 2007;293:F821-30.

[17] Peng CY, Sankaran D, Ogborn MR, Aukema HM. Dietary Soy Protein Selectively Reduces Renal Prostanoids and Cyclooxygenases in Polycystic Kidney Disease. Exp Biol Med 2009;234:737-43.

[18] Warford-Woolgar L, Peng CY, Shuhyta J, Wakefield A, Sankaran D, Ogborn M et al. Selectivity of cyclooxygenase isoform activity and prostanoid production in normal and diseased Han:SPRD-cy rat kidneys. Am J Physiol Renal Physiol 2006;290:F897-904.

[19] Xu T, Wang N-, Fu L-, Ye C-, Yu S-, Mei C-. Celecoxib inhibits growth of human autosomal dominant polycystic kidney cyst-lining epithelial cells through the VEGF/Raf/MAPK/ERK signaling pathway. Mol Biol Rep 2012;39:7743-53.

[20] Park F, Sweeney WE,Jr, Jia G, Akbulut T, Mueller B, Falck JR et al. Chronic blockade of 20-HETE synthesis reduces polycystic kidney disease in an orthologous rat model of ARPKD. Am J Physiol Renal Physiol 2009;296:F575-82.

[21] Muller DN, Schmidt C, Barbosa-Sicard E, Wellner M, Gross V, Hercule H et al. Mouse Cyp4a isoforms: enzymatic properties, gender- and strain-specific expression, and role in renal 20-hydroxyeicosatetraenoic acid formation. Biochem J 2007;403:109-18.

[22] Breyer MD, Harris RC. Cyclooxygenase 2 and the kidney. Curr Opin Nephrol Hypertens 2001;10:89-98.

[23] Aragno M, Parola S, Brignardello E, Manti R, Betteto S, Tamagno E et al. Oxidative stress and eicosanoids in the kidneys of hyperglycemic rats treated with dehydroepiandrosterone. Free Radic Biol Med 2001;31:935-42.

[24] Aukema HM, Lu J, Borthwick F, Proctor SD. Dietary fish oil reduces glomerular injury and elevated renal hydroxyeicosatetraenoic acid levels in the JCR:LA-cp rat, a model of the metabolic syndrome. Br J Nutr 2013; 110(1):11-9

[25] Lefkowith JB, Klahr S. Polyunsaturated fatty acids and renal disease. Proc Soc Exp Biol Med 1996;213:13-23.

[26] Rangel-Huerta OD, Aguilera CM, Mesa MD, Gil A. Omega-3 long-chain polyunsaturated fatty acids supplementation on inflammatory biomakers: A systematic review of randomised clinical trials. Br J Nutr 2012;107:S159-70.

[27] Fassett RG, Gobe GC, Peake JM, Coombes JS. Omega-3 polyunsaturated fatty acids in the treatment of kidney disease. American Journal of Kidney Diseases 2010;56:728-42.

[28] Himmelfarb J, Phinney S, Ikizler TA, Kane J, McMonagle E, Miller G. Gamma-tocopherol and docosahexaenoic acid decrease inflammation in dialysis patients. J Ren Nutr 2007;17:296-304.

[29] Lu J, Bankovic-Calic N, Ogborn M, Saboorian MH, Aukema HM. Detrimental effects of a high fat diet in early renal injury are ameliorated by fish oil in Han:SPRD-cy rats. J Nutr 2003;133:180-6.

[30] Dumlao DS, Cunningham AM, Wax LE, Norris PC, Hanks JH, Halpin R et al. Dietary fish oil substitution alters the eicosanoid profile in ankle joints of mice during Lyme infection. J Nutr 2012;142:1582-9.

[31] Cahill LE, Peng CY, Bankovic-Calic N, Sankaran D, Ogborn MR, Aukema HM. Dietary soya protein during pregnancy and lactation in rats with hereditary kidney disease attenuates disease progression in offspring. Br J Nutr 2007;97:77-84.

[32] Ogborn MR, Nitschmann E, Weiler HA, Bankovic-Calic N. Modification of polycystic kidney disease and fatty acid status by soy protein diet. Kidney Int 2000;57:159-66.

[33] Aukema HM, Housini I. Dietary soy protein effects on disease and IGF-I in male and female Han:SPRD-cy rats. Kidney Int 2001;59:52-61.

[34] Aukema HM, Housini I, Rawling JM. Dietary soy protein effects on inherited polycystic kidney disease are influenced by gender and protein level. J Am Soc Nephrol 1999;10:300-8.

[35] Tomobe K, Philbrick DJ, Ogborn MR, Takahashi H, Holub BJ. Effect of dietary soy protein and genistein on disease progression in mice with polycystic kidney disease. American Journal of Kidney Diseases 1998;31:55-61.

[36] Palanisamy N, Viswanathan P, Ravichandran MK, Anuradha CV. Renoprotective and blood pressure-lowering effect of dietary soy protein via protein kinase C βII inhibition in a rat model of metabolic syndrome. Can J Physiol Pharmacol 2010;88:28-37.

[37] Asanoma M, Tachibana N, Hirotsuka M, Kohno M, Watanabe Y. Effects of soy protein isolate feeding on severe kidney damage in DOCA salt-treated obese Zucker rats. J Agric Food Chem 2012;60:5367-72.

[38] Dhaene M, Sabot J-, Philippart Y, Doutrelepont J-, Vanherweghem J-. Effects of acute protein loads of different sources on glomerular filtration rate. Kidney Int 1987;32:S,25-S-28.

[39] Kontessis P, Jones S, Dodds R, Trevisan R, Nosadini R, Fioretto P et al. Renal, metabolic and hormonal responses to ingestion of animal and vegetable proteins. Kidney Int 1990;38:136-44.

[40] Kitazato H, Fujita H, Shimotomai T, Kagaya E, Narita T, Kakei M et al. Effects of chronic intake of vegetable protein added to animal or fish protein on renal hemodynamics. Nephron 2002;90:31-6.

[41] Bernstein AM, Treyzon L, Li Z. Are High-Protein, Vegetable-Based Diets Safe for Kidney Function? A Review of the Literature. J Am Diet Assoc 2007;107:644-50.

[42] Don BR, Blake S, Hutchison FN, Kaysen GA, Schambelan M. Dietary protein intake modulates glomerular eicosanoid production in the rat. American Journal of Physiology - Renal Fluid and Electrolyte Physiology 1989;256.

[43] Stahl RAK, Kudelka S, Helmchen U. High protein intake stimulates glomerular prostaglandin formation in remnant kidneys. American Journal of Physiology - Renal Fluid and Electrolyte Physiology 1987;252.

[44] Brown JH, Bihoreau MT, Hoffmann S, Kranzlin B, Tychinskaya I, Obermuller N et al. Missense mutation in sterile alpha motif of novel protein SamCystin is associated with polycystic kidney disease in (cy/+) rat. J Am Soc Nephrol 2005;16:3517-26.

[45] Reeves PG, Nielsen FH, Fahey GC,Jr. AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. J Nutr 1993;123:1939-51.

[46] Ogborn MR, Nitschmann E, Bankovic-Calic N, Weiler HA, Aukema H. Dietary flax oil reduces renal injury, oxidized LDL content, and tissue n-6/n-3 FA ratio in experimental polycystic kidney disease. Lipids 2002;37:1059-65.

[47] Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248-54.

[48] Deems R, Buczynski MW, Bowers-Gentry R, Harkewicz R, Dennis EA. Detection and quantitation of eicosanoids via high performance liquid chromatography-electrospray ionization-mass spectrometry. Methods Enzymol 2007;432:59-82.

[49] Hall LM, Murphy RC. Electrospray mass spectrometric analysis of 5-hydroperoxy and 5-hydroxyeicosatetraenoic acids generated by lipid peroxidation of red blood cell ghost phospholipids. J Am Soc Mass Spectrom 1998;9:527-32.

[50] Sankaran D, Lu J, Bankovic-Calic N, Ogborn MR, Aukema HM. Modulation of renal injury in pcy mice by dietary fat containing n-3 fatty acids depends on the level and type of fat. Lipids 2004;39:207-14.

[51] Romero-Calvo I, Ocon B, Martinez-Moya P, Suarez MD, Zarzuelo A, Martinez-Augustin O et al. Reversible Ponceau staining as a loading control alternative to actin in Western blots. Anal Biochem 2010;401:318-20.

[52] Schafer K, Gretz N, Bader M, Oberbaumer I, Eckardt KU, Kriz W et al. Characterization of the Han:SPRD rat model for hereditary polycystic kidney disease. Kidney Int 1994;46:134-52.

[53] Yanagisawa H, Moridaira K, Nodera M, Wada O. Ureteral obstruction enhances eicosanoid production in cortical and medullary tubules of rat kidneys. Kidney and Blood Pressure Research 1997;20:398-405.

[54] Bonvalet JP, Pradelles P, Farman N. Segmental synthesis and actions of prostaglandins along the nephron. Am J Physiol 1987;253:F377-87.

[55] Maaløe T, Schmidt EB, Svensson M, Aardestrup IV, Christensen JH. The effect of n-3 polyunsaturated fatty acids on leukotriene B4 and leukotriene B5 production from stimulated neutrophil granulocytes in patients with chronic kidney disease. Prostaglandins Leukotrienes Essential Fatty Acids 2011;85:37-41.

[56] Grimminger F, Mayer K, Kramer HJ, Stevens J, Walmrath D, Seeger W. Differential vasoconstrictor potencies of free fatty acids in the lung vasculature: 2-versus 3-series prostanoid generation. J Pharmacol Exp Ther 1993;267:259-65.

[57] Kulkarni PS, Srinivasan BD. Eicosapentaenoic acid metabolism in human and rabbit anterior uvea. Prostaglandins 1986;31:1159-64.

[58] Nakamura H, Ito S, Ebe N, Shibata A. Renal effects of different types of protein in healthy volunteer subjects and diabetic patients. Diabetes Care 1993;16:1071-5.

[59] Connor WE, Neuringer M, Lin DS. Dietary effects on brain fatty acid composition: the reversibility of n-3 fatty acid deficiency and turnover of docosahexaenoic acid in the brain, erythrocytes, and plasma of rhesus monkeys. J Lipid Res 1990;31:237-47.

[60] Suarez A, Faus MJ, Gil A. Dietary long-chain polyunsaturated fatty acids modify heart, kidney, and lung fatty acid composition in weanling rats. Lipids 1996;31:345-8.

[61] Camara NO, Martins JO, Landgraf RG, Jancar S. Emerging roles for eicosanoids in renal diseases. Curr Opin Nephrol Hypertens 2009;18:21-7.

[62] Schmitz PG, Zhang K, Dalal R. Eicosapentaenoic acid suppresses PDGF-induced DNA synthesis in rat mesangial cells: involvement of thromboxane A2. Kidney Int 2000;57:1041-51.

[63] Nagao S, Nishii K, Yoshihara D, Kurahashi H, Nagaoka K, Yamashita T et al. Calcium channel inhibition accelerates polycystic kidney disease progression in the Cy/+ rat. Kidney Int 2008;73:269-77.

[64] Park F, Sweeney WE, Jia G, Roman RJ, Avner ED. 20-HETE mediates proliferation of renal epithelial cells in polycystic kidney disease. J Am Soc Nephrol 2008;19:1929-39.

Chapter 4

4. Dietary soy protein and flax oil effects on cystic kidney disease and oxylipin alterations in the Han:SPRD-Cy rat

This manuscript has been submitted for publication to Prostaglandins and Other Lipid Mediators; March, 2015

Naser H.M. Ibrahim[1,2,4], *Daisuke Yoshihara*[5], *Shizuko Nagao*[5], *Tamio Yamaguchi*[1] , *and Harold M. Aukema*[1,2,3,4,*]

[1]Department of Human Nutritional Sciences, University of Manitoba, Winnipeg, Manitoba, Canada

[2]Canadian Centre for Agri-Food Research in Health and Medicine, St. Boniface Hospital Research Centre, Winnipeg, Manitoba, Canada

[3]Children's Hospital Research Institute of Manitoba, Winnipeg, Manitoba, Canada

[4]Richardson Center for Functional Foods and Nutraceuticals

[5]Education and Research Center of Animal Models for Human Diseases, Fujita Health University, Toyoake, Aichi, Japan

Keywords:

Oxylipins; α-Linolenic acid; Eicosapentaenoic acid; Docosahexaenoic acid; Flax oil; Soy protein; Cystic kidney disease;

4.1 Abstract

Dietary soy protein (SP) and flax oil (FXO) slow renal disease progression in the Han:SPRD-*Cy* rat model of cystic kidney disease. While it has been shown that some cyclooxygenase (COX) and lipoxygenase (LOX) oxylipins are altered in this model, cytochrome P450 (CYP) products have not been examined. Since dietary SP and FXO can alter oxylipins via differing mechanisms, the current study examined whether combining these two dietary treatments would further slow disease progression and mitigate these and other oxylipin abnormalities. Dietary SP reduced the loss of renal function and lowered the elevated blood pressure in the diseased rats. Renal COX oxylipins were elevated with disease and providing dietary SP reduced thromboxane B_2. Oxylipins formed via the LOX and CYP pathways were lower in disease, and SP elevated those derived via CYP from linoleic acid. Adding dietary FXO to the SP diet further improved the levels of some COX oxylipins and some derived from n-3 fatty acids, but worsened levels of several other LOX and CYP oxylipins derived from n-6 fatty acids, and did not provide further disease protection. Thus dietary SP reduced disease and ameliorated several oxylipin abnormalities, but adding FXO did not improve disease or overall oxylipin levels.

4.2 Introduction

Oxylipins are oxidized fatty acids formed upon release of fatty acids from cell membranes by phospholipase A_2 and conversion via the cyclooxygenase (COX), lipoxygenase (LOX), and cytochrome P450 (CYP) pathways [1]. They are essential for sustaining kidney functions and processes such as renin secretion, renal hemodynamics, and water and solute transport [1-10]. Equally, under pathophysiological conditions, they mediate several pathogenic mechanisms, such as hypertension and inflammatory and fibrotic processes, contributing to kidney damage and deterioration of function [1,5].

In the Han:SPRD-*Cy* rat model of cystic kidney disease, renal COX oxylipins are elevated and LOX oxylipins are lower in disease [11,12]. Consequently pharmacological COX inhibition reduces disease progression, slowing the development of renal cyst growth, interstitial fibrosis, and macrophage infiltration in this model [12-14]. Dietary interventions that slow disease progression in this model also may mediate their effects by reducing these oxylipin changes. Dietary soy protein (SP) is protective in this model, as well as other models of renal disease [11,15-18]. In the Han:SPRD-*Cy* rat, the beneficial effects of SP on disease are associated with reduction in the elevated COX oxylipins and blunting of the decrease in LOX oxylipins. While the mechanisms by which SP mediates these oxylipin changes is not known, it is clear that it does not alter their levels by changing fatty acid levels [11].

Dietary fat interventions that change fatty acid composition offer another potential approach to modulating oxylipin levels and disease progression. Both dietary fish oil and flax oil (FXO) interventions have been used to slow disease progression in the Han:SPRD-*Cy* rat [11,19,20]. Dietary fish oil results in reduced levels of COX oxylipins produced from n-6 fatty acids and is associated with some protection from disease, but it is not as effective as dietary SP,

possibly because it also further lowers the already reduced LOX products in diseased kidneys [11]. Dietary FXO also reduces disease progression in this and in another model of cystic kidney disease (CD1-*pcy/pcy* mice) [20,21]. In the latter model, the beneficial effects of dietary FXO were associated with a reduction in the elevated COX oxylipins, but there were no alterations of LOX oxylipins produced from n-6 fatty acids, suggesting that FXO may be more effective than fish oil in this regard [11,21]. FXO contains α-linolenic acid (ALA), while fish oil contains both eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), so FXO may not have as strong a LOX oxylipin lowering effect as fish oil. FXO supplementation in the CD1-*pcy/pcy* mouse also increased the depressed levels of LOX oxylipins produced from n-3 fatty acids [21]; these oxylipins has not been examined in the Han:SPRD-*Cy* rat.

In addition to the COX and LOX pathways, oxylipins produced via the CYP pathway play a role in maintaining kidney function and processes under normal physiological conditions, and altered levels of these oxylipins also may play a role in cystic kidney disease progression. CYP oxylipins have antihypertensive, anti-inflammatory, anti-fibrotic, natriuretic, and anti-apoptotic properties, and contribute to maintenance of renal hemodynamic function [22-24]. In patients with cystic kidney disease, plasma levels of the CYP oxylipin, epoxy-eicosatrienoic acid, are lower, indicating their potential importance in this renal disorder [25]. In the CD1-*pcy/pcy* mouse, CYP oxylipins are lower in diseased kidneys, and dietary FXO restored many of those derived from n-3 fatty acids back to normal levels, while not altering the CYP oxylipins derived from n-6 fatty acids [21]. These oxylipins have not been characterized in the Han:SPRD-*Cy* rat.

Since dietary SP and FXO both individually slow disease progression and both potentially improve the oxylipin profile via different mechanisms, the purpose of the current study was to examine whether combining these two treatments in the Han:SPRD-*Cy* rat would

increase the protective effect on disease progression and oxylipins derived via the COX, LOX and CYP pathways.

4.3 Materials and methods

4.3.1 Animals and diet

Han:SPRD-*Cy* rats were derived from our breeding colony, which originated from breeders obtained from Dr. Benjamin Cowley (at the University of Kansas Medical Center at the time) [13]. Normal and diseased male weanling rats (*N*=54) were genotyped [26] and randomly distributed into three groups and fed control, SP or SP+FXO diets: 1) the control diet, which is the standard AIN-93G diet, contains casein as protein source and soy oil as lipid source ; 2) the SP diet differed from the control only by substituting casein (Dyets, cat. #210025) with an equivalent amount of protein from SP (Dyets, cat. #400050); 3) the SP+FXO diet contained SP and substituted 5.25g of the 7.0g of soy oil with FXO. Details of the diet composition are provided in previous publications [11,27] and Supplementary Tables 4.1 and 4.2. Ca and P levels varied by <2% between diets. Each group consisted of 12 diseased (*Cy*/+) and 6 normal (+/+) rats (18/group). Diets were formulated and then stored at 4^0C and feed intake was measured at the 6^{th} week of the study using metabolic cages as described in [11]. Feed intake was similar between groups, indicating no aversion to the diets. Although water intake was not measured in the current study, no differences in water intake between rats provided the control compared with a SP diet were observed in a previous study [28]. All animal procedures performed in this study were conducted in accordance with the Canadian Council on Animal Care and were approved by the Animal Care Committee at the University of Manitoba.

The study continued for 8 weeks, a time period that results in significant dietary effects on disease [11,19,19]. Rats were singly housed in a temperature and humidity controlled environment with a 12 hour day/night cycle and were given free access to water and diet. During the 8^{th} week of the study rats were lightly anesthetized with isoflurane gas and systolic and

diastolic blood pressure were measured by the tail-cuff plethysmography method as described in [29]. At the end of 8 weeks rats were anesthetized with isoflurane and terminated by cardiac puncture to obtain blood for serum, and kidneys were weighed and removed. The left and the right kidneys were cut longitudinally across the hilum and one half of the left kidney was fixed in 10% buffered formalin for morphological and histological analysis, while the other half was separated into cortex and medulla, snap frozen in liquid nitrogen and stored in -80^{0}C. The right kidneys were processed similarly and lyophilized prior to further analysis. The difference between the wet and dry weights of the kidneys was used to calculate the water content.

4.3.2 Histologic and morphometric assessment

Fresh kidneys were fixed in formalin, embedded in paraffin, sectioned at 5 µm and processed using our previously described methods [11,12,20]. Briefly, transverse tissue sections were stained with Hematoxylin and Eosin or Masson's trichrome stain and 20 random images (×40 magnification) from each of the cortex and medulla were taken for analysis. Renal interstitial fibrosis was measured in the Masson's trichrome stained kidneys by densitometry of the blue stained areas, and the portion of the tissue section occupied by open area was quantified for cyst area using the Hematoxylin and Eosin stained tissues using LUZEX AP software (NIRECO CO.LTD, Tokyo, Japan) [12,30]. Proportional cyst and fibrosis areas were multiplied by kidney weight to estimate volume [11,31]. To avoid underestimation of fibrosis due to empty cystic areas in the sections, measurements of fibrosis were corrected to solid tissue areas of these sections [11,12,20,32].

4.3.3 Serum biochemistry

Serum creatinine concentrations were determined calorimetrically using a modified Jaffe reaction [33] and serum cystatin C was measured using a commercial ELISA kit (Bio Vendor Research and Diagnostic Products, Candler, NC, USA). Intra-assay CVs were calculated for all assays; if the CV for any sample or standard was ≥ 10 the sample was repeated.

3.3.4 Oxylipin analyses

Analyses of renal oxylipin, fatty acid and protein levels were as previously described [11,12,34]. Since cyst formation and oxylipin changes in the Han:SPRD-*Cy* rat occur primarily in the cortex [11,35], oxylipins were examined in this part of the kidney. Briefly, 45 mg of renal cortex lyophilates were homogenized on ice in 30 volumes of Tyrode's buffer. Triton X-100 then was added and mixed to achieve a final concentration of 0.01%. Protein concentrations of the homogenates were determined using the Bradford method [36].

Renal oxylipin levels were analyzed by HPLC-tandem mass spectrometry as described [11,12,37] based on the method of Deems et al [38]. Quantification of oxylipins was determined using the stable isotope dilution method [39] and expressed as ng per mg protein. Intra-assay variability was determined by processing replicates of two samples and the inter-assay was determined by processing 4 replicates in 3 batches. Excellent linearity with r^2 >0.999 was obtained for an accuracy of 99%. Details of the deuterated internal standards and analytes, *m/z* transitions, retention times and detector response factors used to measure the targeted oxylipins are described in Table S7 [11].

For analysis of fatty acids, total lipids were extracted and the phospholipid fraction was purified by TLC as described [11,34]. Fatty acids in renal phospholipid were transmethylated using methanolic HCl and analyzed by gas-liquid chromatography [34].

4.3.5 Statistical analyses

All data except histological data were analyzed using 2-way (diet, disease) ANOVA using the GLM procedure of SAS (SAS, version 9.2, Cary, NC) and expressed as mean ± standard error (SE). Histological data were analyzed by 1-way ANOVA, since only diseased kidneys were assessed. Normality of the data was assessed using the Shapiro-Wilk's test ($W>0.05$ for normally distributed data) and the data were normalized by logarithmic transformation if necessary. When no interactions (diet x disease) were present, only significant main (diet, disease) effects were reported. When the overall diet or interaction effects were significant, Tukey's test was used for simple effect comparisons and these effects were reported. Statistical significance for main and interaction effects was set at $P<0.05$. Pearson's correlation was used to test the relationship between biomarkers of kidney function and oxylipin levels and between blood pressure and renal CYP metabolites.

4.4 Results

4.4.1 Renal function and pathology

Providing Han:SPRD-*Cy* rats with SP in place of casein resulted in a 10% lower level of serum creatinine and 40% lower level of cystatin C in the diseased rats, resulting in levels that were not different than those in normal rats given the same diets. In the rats given FXO in addition to SP, similar reductions in serum creatinine and cystatin C were observed (Figure 4.1). Similarly, rats given the SP diet had lower diastolic and mean arterial blood pressure compared to the rats given the control diet, and further providing FXO resulted in a similar effect (Figure 4.2). These improvements in renal function and blood pressure were present in the absence of any significant differences in body weight, kidney size, kidney water content (Table 4.1) or renal pathology in the diseased rats given the differing diets (Supplementary Table 4.3).

4.4.2 COX oxylipins

Consistent with previous findings [11,12,21], COX oxylipins are significantly elevated in diseased compared to normal kidneys. Overall, genotype effects were observed for prostaglandin (PG) E_2, PGD_2, 6-keto-$PGF_{1\alpha}$, $PGF_{2\alpha}$, and thromboxane B_2 (TXB_2). Providing SP in the diet resulted in lower levels of TXB_2, while adding FXO to the SP diet also resulted in lower TXB_2, PGE_2, PGD_2 and $PGF_{2\alpha}$ when compared to the control diet, but not to the SP diet alone (Figure 4.3). No 3-series COX oxylipins were detected in kidneys of any of the rats on any of the diets (detection limit =1 ng/mg protein).

4.4.3 LOX oxylipins

In contrast to the COX oxylipins, those derived via the LOX pathway, including those derived from dihomo-γ-linolenic acid (DGLA), ALA and DHA, were generally lower in diseased compared to normal kidneys. Providing SP in the diet generally did not alter LOX oxylipins, with the exception of renal 12- and 15-HETE (hydroxy-eicosatetraenoic acid), which were higher in normal kidneys of rats given SP compared to the control diet. Several LOX oxylipins derived from n-3 fatty acids [9-hydroxy-octadecatrienoic acid (HOTrE), 5- and 12-hydroxy-eicosapentaenoic acid (HEPE), 4- hydroxy-docosahexaenoic acid (HDoHE)] were detected in the kidneys, with higher levels being present in kidneys from rats given dietary FXO. Adding FXO to the SP diet resulted in generally lower levels of the n-6 fatty acid derived oxylipins, while the n-3 fatty acid derived oxylipins were generally higher. Notably, the levels of the n-3 oxylipins derived via the LOX pathway in diseased kidneys were restored to levels that were higher (9-HOTrE) or not different (5-HEPE, 12-HEPE, 4-HDoHE) from levels in normal kidneys in rats given the control diet (Figure 4.4).

4.4.4 CYP oxylipins

As with the LOX oxylipins, those derived via the CYP450 pathway were lower in diseased compared to normal kidneys (Figure 4.5). Providing SP compared to the control diet did not alter LOX oxylipins derived from arachidonic acid (AA) [11,12- and 14,15-dihydroxy-eicosatrienoic acid (DiHETrE)] and EPA (18-HEPE), but resulted in higher levels of the two linoleic acid (LA) oxylipins [9,10- and 12,13- dihydroxy-octadecenoic acid (DiHOME)] derived via this pathway. Adding FXO to the SP diet lowered DiHETrE levels in normal but not diseased

kidneys, lowered 9,10-DiHOME but not 12,13-DiHOME, and increased the level of the n-3 oxylipin, 18-HEPE.

4.4.5 Correlations

Correlation analyses revealed that serum cystatin C correlated positively with total COX oxylipins (r=0.50, P=0.0135) and negatively with total CYP oxylipins (r=-0.41, P=0.0321), and serum creatinine correlated negatively with total LOX oxylipins (r=-0.49, P=0.0040) and total CYP oxylipins (r=-0.54, P=0.0010) (Supplementary table 4.4). Furthermore, blood pressure correlated negatively with the levels of individual and total CYP oxylipins, with the correlations being the strongest for diastolic blood pressure (Table 4.2).

4.4.6 Renal phospholipid fatty acid composition

Consistent with our previous report [11], almost all fatty acids were markedly lower in diseased compared to normal kidneys. Providing SP in the diet did not alter fatty acid levels, but adding FXO increased the levels of renal ALA (C18:3n3), EPA (C20:5n3), and DPA (C22:5n3), but not DHA (Table 4.3).

Table 4.1. Physiologic parameters in normal and diseased Han:SPRD-*Cy* rats given control, SP, or SP+FXO diets

Diet	Control		SP		SP+FXO		
Genotype	**+/+**	**Cy/+**	**+/+**	**Cy/+**	**+/+**	**Cy/+**	**Effects (P-value)**
Body weight (g)	401 (10.35)	379 (5.62)	382 (15.20)	391 (4.03)	391 (9.77)	394 (3.56)	No effect
Kidney weight (g)	2.68 (0.09)	9.49 (0.43)	2.56 (0.11)	9.38 (0.73)	2.56 (0.05)	10.78 (0.35)	Genotype (<0.0001)
Kidney/body weight (g/100 g)	0.67 (0.01)	2.50 (0.09)	0.67 (0.02)	2.39 (0.17)	0.67 (0.01)	2.74 (0.09)	Genotype (<0.0001)
Kidney water (g)	1.07 (0.06)	3.69 (0.19)	0.92 (0.09)	3.68 (0.36)	1.09 (0.06)	4.16 (0.13)	Genotype (<0.0001)

Values are means (SE), (n=6 for +/+ and 12 for *Cy*/+ groups).

Table 4.2. Correlation analysis of blood pressure and CYP oxylipins

Oxylipin (substrate fatty acid)	Systolic blood pressure r (P value)	Diastolic blood pressure r (P value)	Mean arterial pressure r (P value)
11,12-DiHETrE (AA)	-0.31 (0.0661)	-0.44 (0.0050)	-0.41 (0.0105)
14,15-DiHETrE (AA)	-0.25 (0.1396)	-0.39 (0.0128)	-0.41 (0.0107)
9,10-DiHOME (LA)	-0.25 (0.1337)	-0.42 (0.0083)	-0.38 (0.0185)
12,13-DiHOME (LA)	-0.41 (0.0107)	-0.35 (0.0284)	-0.34 (0.0378)
18-HEPE (EPA)	-0.15 (0.3838)	-0.35 (0.0312)	-0.31 (0.0590)
Total DiHETrE (AA)	-0.36 (0.0288)	-0.45 (0.0045)	-0.43 (0.0071)
Total DiHOME (LA)	-0.27 (0.1024)	-0.43 (0.0063)	-0.40 (0.0131)
Total AA and LA CYP products	-0.31 (0.0288)	-0.46 (0.0035)	-0.43 (0.0073)
Total CYP products (AA, LA, and EPA)	-0.20 (0.2397)	-0.40 (0.0106)	-0.37 (0.0234)

$P < 0.05$ is significant.

Table 4.3. Renal cortex phospholipid fatty acid compositions in normal and diseased Han:SPRD-*Cy* given control, SP, or SP+FXO diets

Diet	**Control**		**SP**		**SP+FXO**		
Genotype	**+/+**	**Cy/+**	**+/+**	**Cy/+**	**+/+**	**Cy/+**	**Effects (P-values)**
C18:0	8809 (2280)	2246 (295)	11463 (1182)	3193 (855)	7909 (2700)	3290 (482)	Genotype (0.0001)
C18:1	2256 (600)	673 (90)	2743 (352)	846 (271)	2377 (815)	779 (105)	Genotype (0.0004)
C18:2n6	4477 (1299)	979 (155)	5644 (628)	1387 (516)	5773 (1979)	1077 (142)	Genotype (0.0002)
C18:3n3	56 (15)[b]	13 (6)	62 (24)[b]	27 (8)	330 (121)[a]	83 (8)	Genotype and diet (0.0016 and 0.0002)
C18:3n6	34 (8)	4 (1)	11 (7)	27 (1)	30 (10)	6 (4)	Genotype (0.0002)
C20:3n3	65 (26)	3 (2)	57 (11)	14 (8)	68 (26)	29 (11)	Genotype (0.0033)
C20:3n6	259 (75)	54 (10)	268 (24)	71 (26)	292 (97)	59 (9)	Genotype (0.0002)
C20:4n6	9508 (2635)	2156 (318)	10153 (764)	3164 (122)	5822 (2044)	1507 (259)	Genotype (0.0001)
C20:5n3	66 (19)[b]	4 (4)	60 (9)[b]	21 (10)	742 (231)[a]	201 (43)	Genotype and diet (0.0028 and <0.0001)
C22:0	219 (65)	74 (13)	272 (31)	93 (32)	179 (62)	67 (11)	Genotype (0.0007)
C22:4n6	42 (23)	37 (6)	48 (14)	74 (26)	48 (16)	27 (10)	No effect
C22:5n3	162 (250)[b]	78 (24)	126 (10)[b]	76 (32)	341 (113)[a]	143 (33)	Genotype and diet (0.0081 and 0.0144)
C22:6n3	502 (189)	76 (28)	504 (41)	180 (72)	458 (164)	93 (60)	Genotype (0.0011)
C24:0	1460 (431)	330 (92)	2091 (222)	486 (123)	1480 (538)	413 (186)	Genotype (0.0002)
C24:1	404 (118)	57 (9)	498 (55)	99 (35)	395 (144)	64 (24)	Genotype (<0.0001)

Values are means (SE), expressed in ng/mg protein (n=8/group). Values in the same row with different superscript letters indicate significant differences in diet effects (P<0.05).

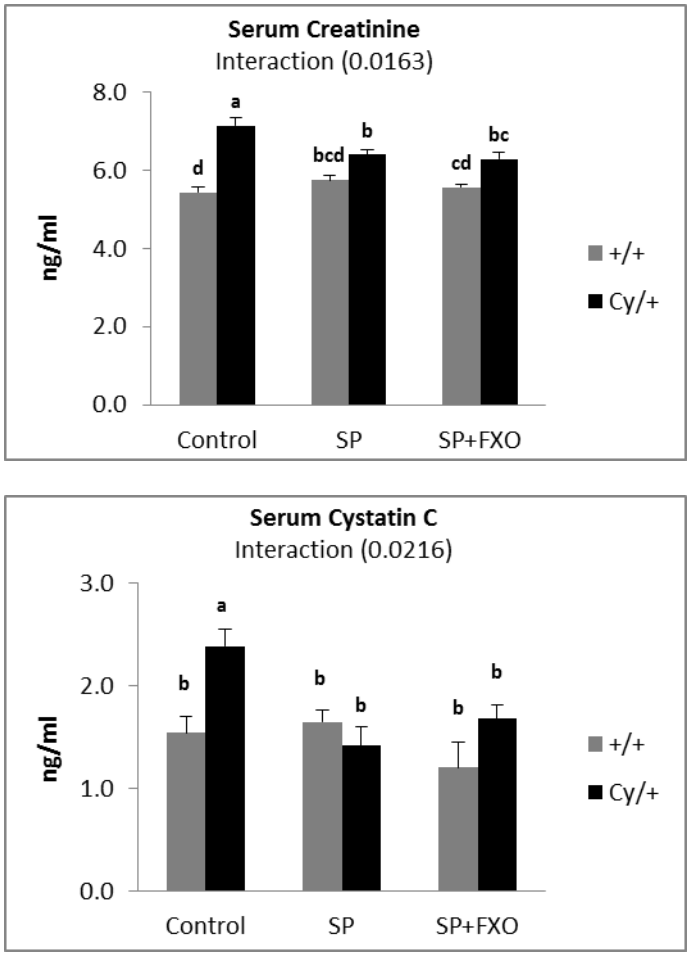


Figure 4.1. Serum cystatin C and creatinine levels in normal and diseased Han:SPRD-*Cy* rats given control, SP, or SP+FXO diets. Average CV for serum creatinine and serum cystatin C =1.5. Values are means ± SE (n=6 for +/+ and 10 for *Cy*/+ groups). Values with different letters indicate significant differences in diet and disease effects ($p<0.05$).

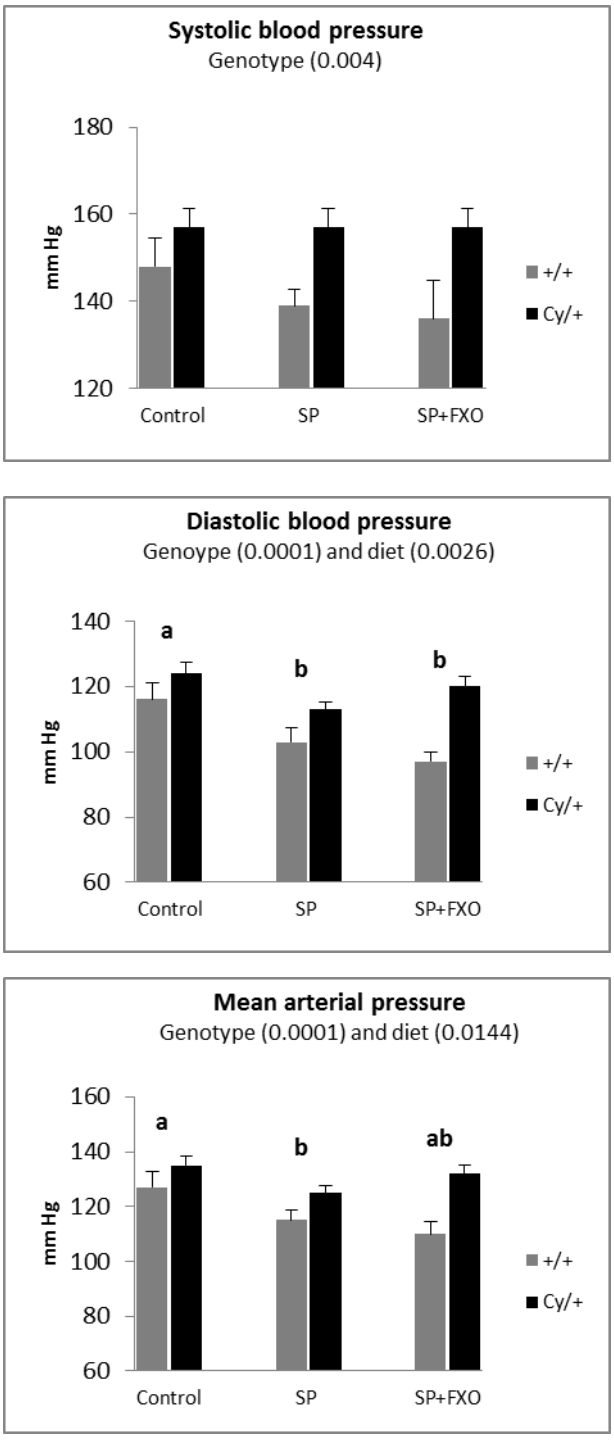


Figure 4.2. Blood pressure in normal and diseased Han:SPRD-*Cy* rats given control, SP, or SP+FXO diets. Systolic and diastolic blood pressures were measured by the tail-cuff plethysmography method. Values are means ± SE (n=6 for +/+ and 12 for *Cy*/+ groups). Values with different letters indicate significant differences in diet effects ($p<0.05$).

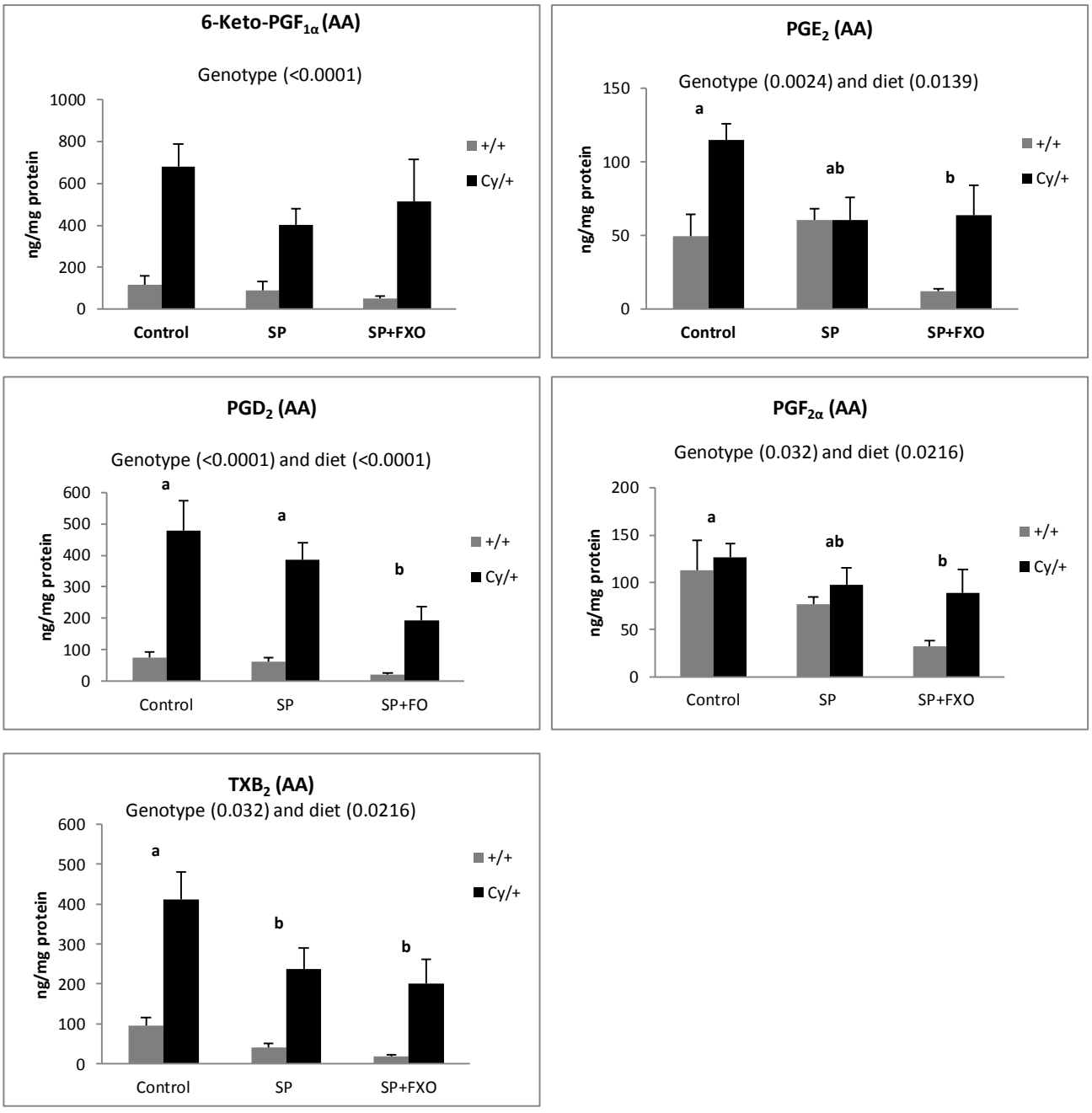


Figure 4.3. Cortical COX oxylipin levels (ng/mg protein) in normal and diseased Han:SPRD-*Cy* rats given control, SP, or SP+FXO diets. Cortical oxylipin levels were analyzed by HPLC-tandem mass spectrometry. The fatty acid precursor for each oxylipin is denoted in parentheses. Values are means ±SE (n=6 for +/+ and 12 for *Cy*/+ groups). Values with different superscript letters indicate significant differences in diet effects ($p<0.05$).

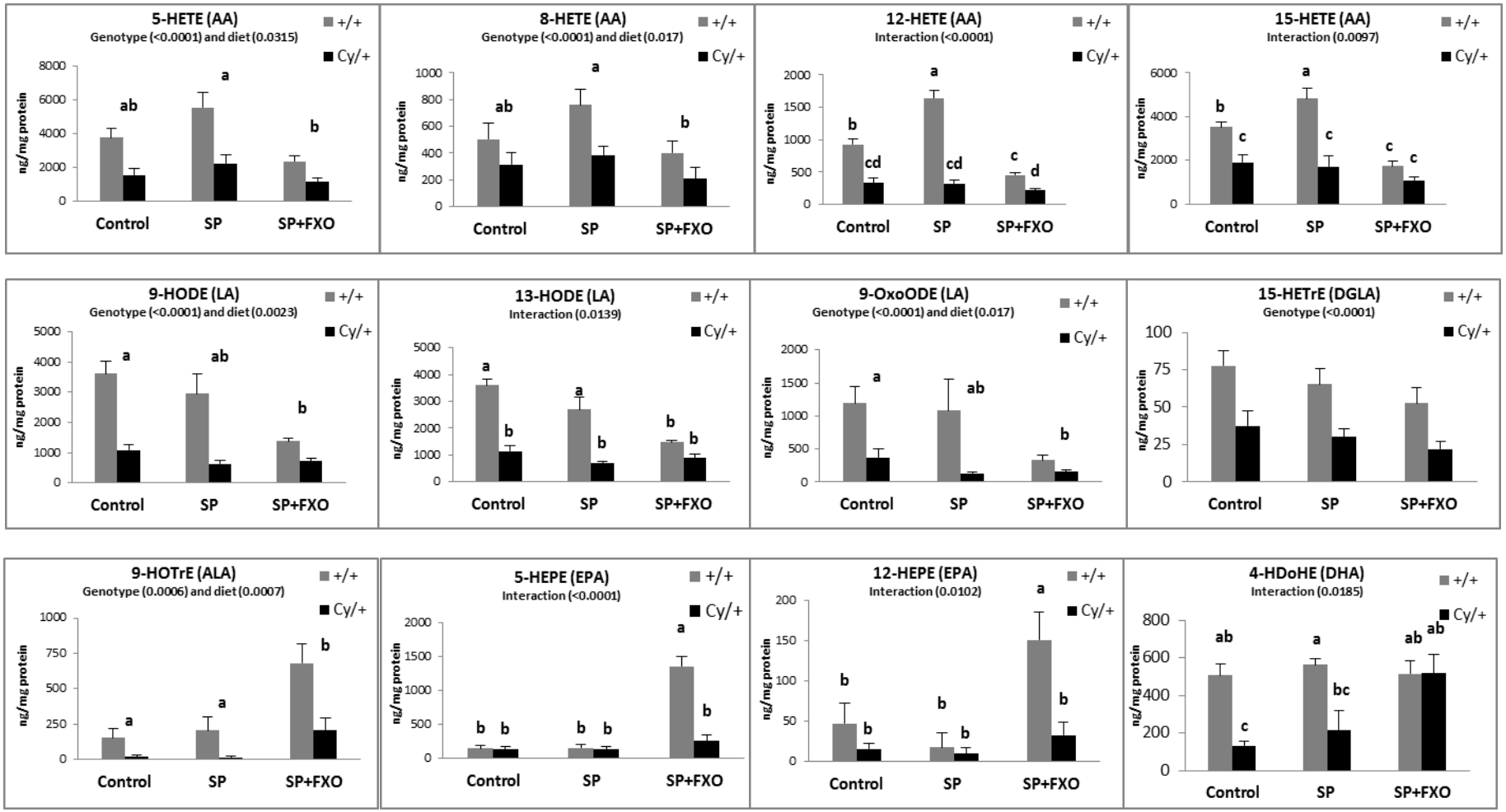


Figure 4.4. Cortical LOX oxylipin levels (ng/mg protein) in normal and diseased Han:SPRD-*Cy* rats given control, SP, or SP+FXO diets. Cortical oxylipin levels were analyzed by HPLC-tandem mass spectrometry. The fatty acid precursor for each oxylipin is denoted in parentheses. Values are means ±SE (n=6 for +/+ and 12 for *Cy*/+ groups). Values with different superscript letters indicate significant differences in diet effects ($p<0.05$).

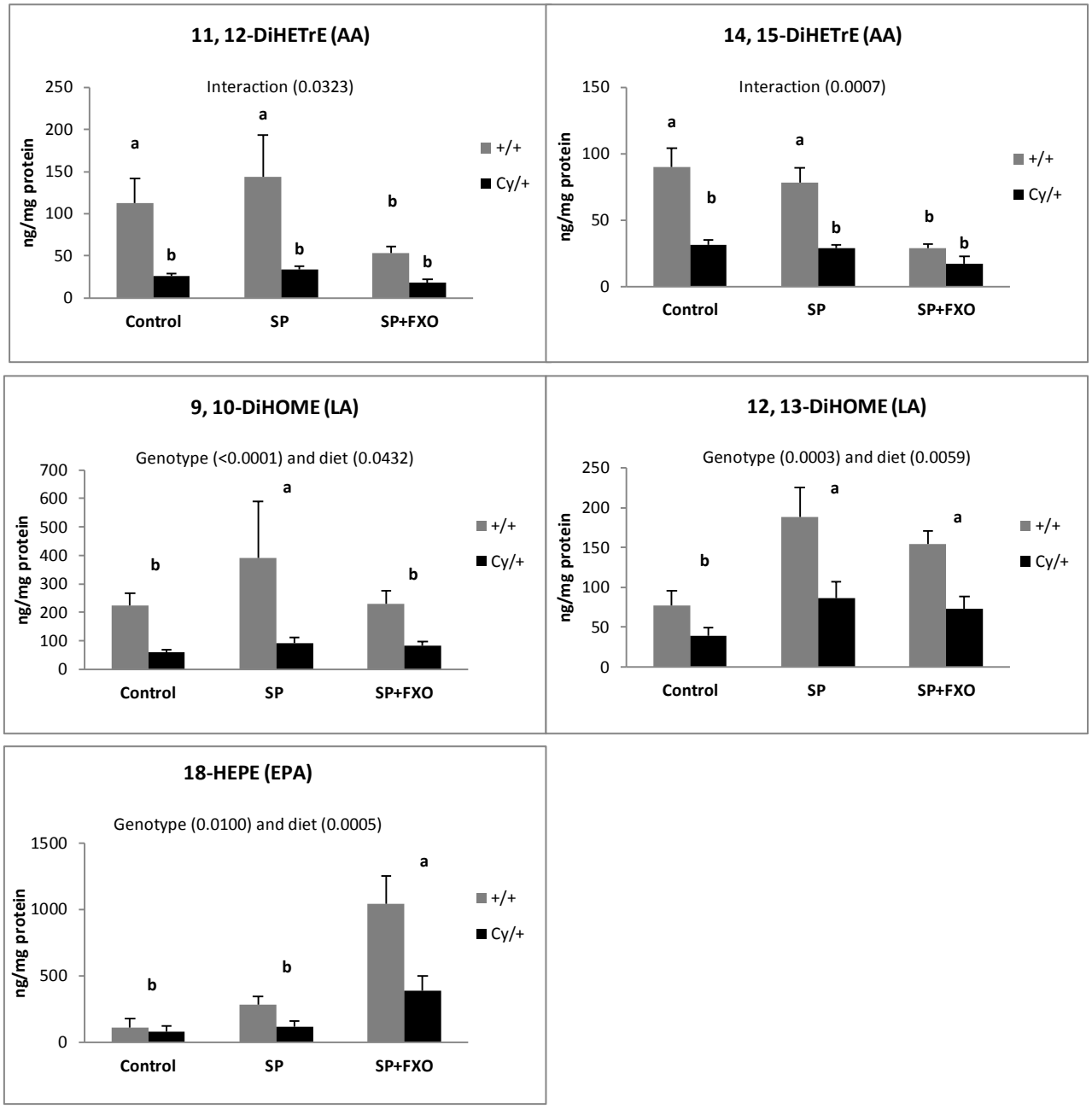


Figure 4.5. Cortical CYP oxylipin levels (ng/mg protein) in normal and diseased Han:SPRD-*Cy* rats given control, SP, or SP+FXO diets. Cortical oxylipin levels were analyzed by HPLC-tandem mass spectrometry. The fatty acid precursor for each oxylipin is denoted in parentheses. Values are means ±SE. Values with different superscript letters indicate significant differences in diet effects ($p<0.05$).

4.5 Discussion

The current study confirms and extends previous findings of altered renal oxylipins in the Han:SPRD-*Cy* rat. Previously we have reported that AA derived COX oxylipins are elevated and AA and LA derived LOX oxylipins are reduced in diseased kidneys [11,12]. The current study confirmed these findings and further demonstrates that DGLA (15-HETrE) and n-3 fatty acid derived LOX oxylipins, including those from ALA and DHA (9-HOTrE and 4-HDoHE), are also lower in diseased kidneys. Furthermore, CYP derived oxylipins from both n-6 and n-3 fatty acids (11,12- and 14,15-DiHETrE, 9,10- and 12-13-DiHOME, 18-HEPE) are lower in cystic compared to normal kidneys in this model. Dietary SP ameliorated some of these alterations (TXB_2, 9,10- and 12,13-DiHOME), while dietary FXO improved some PGD_2, PGE_2, $PGF_{2\alpha}$, TXB_2, 9-HOTrE, 4-HDOHE, and 18-HEPE) and worsened others (13-HODE, 11,12- and 14,15-DiHETrE).

The precise roles of these oxylipin alterations in cystic kidney disease remain to be elucidated, but may include several mechanisms. Elevated COX oxylipins such as PGE_2 and TXA_2 are associated with increased epithelial cell proliferation, cAMP levels and cystogenesis [40-43], and in the current study were correlated with serum cystatin C. Inhibiting the production of these oxylipins using selective or non-selective COX inhibitors markedly reduces disease progression in the Han:SPRD-*Cy* rat [12-14]. The implications of the reduction in LOX oxylipins with disease and the negative correlation with serum creatinine is less clear, as further LOX inhibition does not appear to worsen the disease [12]. LOX oxylipins can have both beneficial and detrimental effects in disease [44,45], with the n-3 oxylipins generally being more protective [46]. The reduced levels of LOX oxylipins also may simply be as a result of less substrate fatty

acid availability due to the increased demand by the COX enzymes and the overall lower levels of fatty acid in diseased compared to normal kidneys, although correlations between COX and LOX oxylipins were not significant (data not shown).

This may also be the case for the CYP oxylipins, which have many protective cardiovascular and renal properties, including anti-hypertensive, anti-inflammatory, anti-fibrotic, natriuretic and anti-apoptotic effects [22,23,47]. The reduction in these oxylipins may be related to disease progression and the elevated blood pressure in these rats, since the CYP oxylipin diols were not only lower in diseased compared to normal rats, but also correlated negatively with serum creatinine and cystatin C and with blood pressure. Similar to the reduced renal DiHETrE in the rats in the current study, plasma levels of epoxy-eicosatrienoic acid, a CYP oxylipin that is metabolized to DiHETrE, are lower in patients with cystic kidney disease [25]. We also scanned for the CYP oxylipin, 20-HETE, since it is elevated in diseased kidneys of the Balb/C polycystic kidney (BPK) mouse, and inhibition of this oxylipin reduces disease progression [48,49]. However, 20-HETE was below the limit of detection (1 ng/mg dry tissue) in the Han:SPRD-*Cy* rat kidney, as previously reported [11].

Providing SP reduced TXB_2, while adding FXO to the SP reduced PGE_2, PGD_2, TXB_2 and $PGF_{2\alpha}$. Consistent with previous studies with COX inhibitors [12-14], this reduction in COX oxylipins by SP and FXO was associated with slower disease progression. In the current study, this reduction in COX oxylipins by SP and FXO also was associated with a reduction in blood pressure in diseased rats. Providing SP did not significantly alter the levels of LOX oxylipins in diseased kidneys. However, levels of 12- and 15-HETE were increased with SP feeding in normal kidneys, consistent with our previous study that showed a modest or no increase in LOX

oxylipin levels with SP [11]. SP also increased the lowered levels of two of four diols produced via the CYP pathway, specifically those derived from LA. While CYP diols derived from the longer chain fatty acids such as AA are potent vasodilators involved in the regulation of blood pressure [22-24], less is known regarding those derived from LA. The current correlation results reveal a negative association with not only the AA diols, but also with the LA diols suggesting that LA derived CYP oxylipins also may be important hypotensive agents. If this is the case, the increased levels with SP feeding may help explain the beneficial effects of SP on blood pressure in this disease. The role of the LA oxylipins in this disease, however, remains to be explored.

The exact mechanism by which SP alters oxylipins is unknown. SP feeding did not alter kidney fatty acid levels in the current study, consistent with previous findings [11], so it does not appear to alter oxylipins by altering their substrate fatty acids. SP may mediate its effects by affecting COX, LOX or CYP activity. In a prior study we demonstrated that dietary SP reduced overall COX activity and COX1, but not COX2 protein levels, suggesting that it may specifically inhibit COX1 activity [11]. Based on the similar effects of SP on the different HODE and HETE isomers in the current study, on the other hand, it does not appear to have specific LOX activity. The current study may suggest that SP has greater effects on the LA compared to the AA derived CYP oxylipins, but further studies examining the effects of SP on specific oxylipin biosynthetic enzymes and substrates are warranted.

The benefits and effects of SP on oxylipins also may be related to the bioactive peptides produced via digestion of dietary SP in the gastrointestinal tract. Several of these have been reported to inhibit angiotensin converting enzyme (ACE) activity, which mediates its effects via changes in COX oxylipins, as well as itself being regulated by COX products [50-52]. ACE inhibition in PKD is effective in lowering blood pressure and there is evidence in humans and in

rat models that this treatment is associated with slowing of disease progression [53,54]. It also has been speculated that the unique amino acid composition of SP such as its higher levels of arginine could impart renoprotective effects [55]. However, examination of amino acid profiles of several protein sources that affect disease progression in this model do not support this [56]. Isoflavones also have been identified as the potential beneficial ingredient in SP, as they have multiple effects on the kidney, such as effects on vasodilation, ACE inhibition, inhibition of cell proliferation, inhibition of the Na-K-Cl cotransporter of the thick ascending limb of Henle's loop, and its antioxidant effect, as reviewed in [57]. However, depletion of isoflavones from SP does not block the protective effect of SP on disease progression in this model [17].

In contrast to dietary proteins, dietary oils do alter the fatty acid compositions of tissues, and therefore can potentially alter oxylipin production in this way. Dietary fish oil was therefore examined as a complement to dietary SP in a previous study, but adding dietary fish oil to SP did not provide additional protection, possibly because it reduced LOX levels even further [11]. On the other hand, in the CD1-*pcy/pcy* mouse model of cystic kidney disease, dietary FXO was protective towards both disease progression and oxylipin alterations [21]. Since dietary FXO alone reduces disease progression in the Han:SPRD-*Cy* rat, and because FXO may not alter fatty acid compositions to as great an extent as fish oil, we hypothesized that combining dietary FXO with SP may be advantageous. However, adding FXO to SP also had no apparent additive effect on slowing disease progression. This may be due to the fact that despite lowering the elevated COX levels, it also further lowered LOX and CYP oxylipins derived from n-6 fatty acids, similar to the effect of dietary fish oil [11]. Even though dietary FXO increased the lowered levels of ALA and DHA derived oxylipins, the detrimental effects on the n-6 oxylipins may have counteracted these positive effects. As such, prior to clinical studies in humans, further study on

the specific role of renal oxylipins in cystic kidney disease are warranted, including examination of the potential differences in males and females, as only males have been used in these studies to date [11-15].

One of the reported differences between dietary ALA compared to DHA is that the former does not alter tissue DHA levels, while the latter does, due to the apparent slow conversion of ALA to DHA once incorporated into tissues [58]. Our fatty acid analysis of the kidneys in this study corroborates this finding. However, interestingly, the ALA provided by dietary FXO was sufficiently converted to DHA and to 4-HDoHE to restore the levels of this DHA oxylipin to normal levels. This also was observed in the CD1-*pcy/pcy* mouse [21] and suggests that ALA conversion to DHA is sufficient to provide substrate for adequate oxylipin analysis. Thus the conversion of ALA to DHA may not be slow, but highly regulated to prevent overproduction of oxylipins. Further support for this concept comes from a study in CD1-*pcy/pcy* mice in which dietary DHA increased DHA levels (oxylipins were not measured) and worsened disease progression [34].

4.6 Conclusions

The current study confirms that COX oxylipins are elevated and n-6 derived LOX metabolites are reduced by disease in this model of cystic kidney disease and extends these findings to n-3 fatty acid derived LOX oxylipins. Further, it also demonstrates that CYP derived oxylipins are lowered by disease in this renal disorder. The beneficial effects of SP on disease were associated with amelioration of several oxylipin alterations (TXB_2, 9,10- and 12,13-DiHOME); combining FXO with SP improved some (PGD_2, PGE_2, $PGF_{2\alpha}$, TXB_2, 9-HOTrE, 4-HDOHE, and 18-HEPE) but worsened other oxylipin alterations (13-HODE, 11,12- and 14,15-DiHETrE) and did not provide further disease protection.

4.7 Acknowledgements

This work was supported by grants from the Manitoba Agri-Food Research and Development Initiative and the Natural Sciences and Engineering Research Council of Canada (**NSERC**). Support to N.I. was provided by the NSERC Food Advancement through Science and Training (FAST) program, by the Libyan-North American scholarship program and a University of Manitoba Graduate Fellowship. The authors thank the Richardson Centre for Functional Foods and Nutraceuticals for providing access to equipment, and Joy Gauthier, Dennis Labossiere, Tanja Winter, and Melanie Gregoire for providing technical assistance.

Conflict of Interest

All the authors declare no competing interests.

4.8 References

[1] Hao C-, Breyer MD. Physiological regulation of prostaglandins in the kidney. Annual Review of Physiology 2008;70:357-77.

[2] Hodeify RF, Kreydiyyeh SI. PGE2 reduces net water and chloride absorption from the rat colon by targeting the Na+/H+ exchanger and the Na+ K+ 2Cl- cotransporter. Prostaglandins Leukot Essent Fatty Acids 2007;76:285-92.

[3] Mori A, Saito M, Sakamoto K, Narita M, Nakahara T, Ishii K. Stimulation of prostanoid IP and EP(2) receptors dilates retinal arterioles and increases retinal and choroidal blood flow in rats. Eur J Pharmacol 2007;570:135-41.

[4] Dolegowska B, Pikula E, Safranow K, Olszewska M, Jakubowska K, Chlubek D et al. Metabolism of eicosanoids and their action on renal function during ischaemia and reperfusion: the effect of alprostadil. Prostaglandins Leukot Essent Fatty Acids 2006;75:403-11.

[5] Kitahara M, Eitner F, Ostendorf T, Kunter U, Janssen U, Westenfeld R et al. Selective cyclooxygenase-2 inhibition impairs glomerular capillary healing in experimental glomerulonephritis. J Am Soc Nephrol 2002;13:1261-70.

[6] Vukicevic S, Simic P, Borovecki F, Grgurevic L, Rogic D, Orlic I et al. Role of EP2 and EP4 receptor-selective agonists of prostaglandin E(2) in acute and chronic kidney failure. Kidney Int 2006;70:1099-106.

[7] Stitt-Cavanagh EM, Faour WH, Takami K, Carter A, Vanderhyden B, Guan Y et al. A maladaptive role for EP4 receptors in podocytes. J Am Soc Nephrol 2010;21:1678-90.

[8] Darlametsos IE, Varonos DD. Role of prostanoids and endothelins in the prevention of cyclosporine-induced nephrotoxicity. Prostaglandins Leukot Essent Fatty Acids 2001;64:231-9.

[9] Xu ZG, Miao LN, Cui YC, Jia Y, Yuan H, Wu M. Angiotensin II type 1 receptor expression is increased via 12-lipoxygenase in high glucose-stimulated glomerular cells and type 2 diabetic glomeruli. Nephrol Dial Transplant 2009;24:1744-52.

[10] Xie C, Wang DH. Inhibition of renin release by arachidonic acid metabolites, 12(s)-HPETE and 12-HETE: role of TRPV1 channels. Endocrinology 2011;152:3811-9.

[11] Ibrahim NH, Jia Y, Devassy JG, Yamaguchi T, Aukema HM. Renal cyclooxygenase and lipoxygenase products are altered in polycystic kidneys and by dietary soy protein and fish oil treatment in the Han:SPRD-Cy rat. Mol Nutr Food Res 2014; 58:768-81.

[12] Ibrahim NH, Gregoire M, Devassy JG, Wu Y, Yoshihara D, Yamaguchi T et al. Cyclooxygenase product inhibition with acetylsalicylic acid slows disease progression in the Han:SPRD-Cy rat model of polycystic kidney disease. Prostaglandins Other Lipid Mediat 2014;116-117C:19-25.

[13] Sankaran D, Bankovic-Calic N, Ogborn MR, Crow G, Aukema HM. Selective COX-2 inhibition markedly slows disease progression and attenuates altered prostanoid production in Han:SPRD-cy rats with inherited kidney disease. Am J Physiol Renal Physiol 2007;293:F821-30.

[14] Xu T, Wang N, Fu L, Ye C, Yu S, Mei C. Celecoxib inhibits growth of human autosomal dominant polycystic kidney cyst-lining epithelial cells through the VEGF/Raf/MAPK/ERK signaling pathway. Mol Biol Rep 2012;39:7743-53.

[15] Peng CY, Sankaran D, Ogborn MR, Aukema HM. Dietary Soy Protein Selectively Reduces Renal Prostanoids and Cyclooxygenases in Polycystic Kidney Disease. Exp Biol Med 2009;234:737-43.

[16] Cahill LE, Peng CY, Bankovic-Calic N, Sankaran D, Ogborn MR, Aukema HM. Dietary soya protein during pregnancy and lactation in rats with hereditary kidney disease attenuates disease progression in offspring. Br J Nutr 2007;97:77-84.

[17] Ogborn MR, Nitschmann E, Bankovic-Calic N, Weiler HA, Aukema HM. Dietary soy protein benefit in experimental kidney disease is preserved after isoflavone depletion of diet. Exp Biol Med 2010;235:1315-20.

[18] Hwang SY, Taylor CG, Zahradka P, Bankovic-Calic N, Ogborn MR, Aukema HM. Dietary soy protein reduces early renal disease progression and alters prostanoid production in obese fa/fa Zucker rats. J Nutr Biochem 2008;19:255-62.

[19] Lu J, Bankovic-Calic N, Ogborn M, Saboorian MH, Aukema HM. Detrimental effects of a high fat diet in early renal injury are ameliorated by fish oil in Han:SPRD-cy rats. J Nutr 2003;133:180-6.

[20] Ogborn MR, Nitschmann E, Bankovic-Calic N, Weiler HA, Aukema H. Dietary flax oil reduces renal injury, oxidized LDL content, and tissue n-6/n-3 FA ratio in experimental polycystic kidney disease. Lipids 2002;37:1059-65.

[21] Yamaguchi T, Devassy JG, Gabbs M, Ravandi A, Nagao S, Aukema HM. Dietary flax oil rich in alpha-linolenic acid reduces renal disease and oxylipin abnormalities, including formation

of docosahexaenoic acid derived oxylipins in the CD1-pcy/pcy mouse model of nephronophthisis. Prostaglandins Leukot Essent Fatty Acids 2015;94:83-9.

[22] Makita K, Takahashi K, Karara A, Jacobson HR, Falck JR, Capdevila JH. Experimental and/or genetically controlled alterations of the renal microsomal cytochrome P450 epoxygenase induce hypertension in rats fed a high salt diet. J Clin Invest 1994;94:2414-20.

[23] Imig JD, Zhao X, Zaharis CZ, Olearczyk JJ, Pollock DM, Newman JW et al. An orally active epoxide hydrolase inhibitor lowers blood pressure and provides renal protection in salt-sensitive hypertension. Hypertension 2005;46:975-81.

[24] Imig JD, Zhao X, Capdevila JH, Morisseau C, Hammock BD. Soluble epoxide hydrolase inhibition lowers arterial blood pressure in angiotensin II hypertension. Hypertension 2002;39:690-4.

[25] Lorthioir A, Joannides R, Remy-Jouet I, Freguin-Bouilland C, Iacob M, Roche C et al. Polycystin deficiency induces dopamine-reversible alterations in flow-mediated dilatation and vascular nitric oxide release in humans. Kidney Int 2015;87:465-72.

[26] Brown JH, Bihoreau MT, Hoffmann S, Kranzlin B, Tychinskaya I, Obermuller N et al. Missense mutation in sterile alpha motif of novel protein SamCystin is associated with polycystic kidney disease in (cy/+) rat. J Am Soc Nephrol 2005;16:3517-26.

[27] Sankaran D, Bankovic-Calic N, Cahill L, Yu-Chen Peng C, Ogborn MR, Aukema HM. Late dietary intervention limits benefits of soy protein or flax oil in experimental polycystic kidney disease. Nephron - Experimental Nephrology 2007;106:e122-8.

[28] Aukema HM, Housini I. Dietary soy protein effects on disease and IGF-I in male and female Han:SPRD-cy rats. Kidney Int 2001;59:52-61.

[29] Kubota Y, Umegaki K, Kagota S, Tanaka N, Nakamura K, Kunitomo M et al. Evaluation of blood pressure measured by tail-cuff methods (without heating) in spontaneously hypertensive rats. Biol Pharm Bull 2006;29:1756-8.

[30] Yoshihara D, Kugita M, Sasaki M, Horie S, Nakanishi K, Abe T et al. Telmisartan ameliorates fibrocystic liver disease in an orthologous rat model of human autosomal recessive polycystic kidney disease. PLoS One 2013;8:e81480.

[31] Ogborn MR, Nitschmann E, Weiler HA, Bankovic-Calic N. Modification of polycystic kidney disease and fatty acid status by soy protein diet. Kidney Int 2000;57:159-66.

[32] Ogborn MR, Nitschmann E, Weiler HA, Bankovic-Calic N. Modification of polycystic kidney disease and fatty acid status by soy protein diet. Kidney Int 2000;57:159-66.

[33] Hervey GR. Determination of creatinine by the Jaffe reaction. Nature 1953;171:1125.

[34] Sankaran D, Lu J, Bankovic-Calic N, Ogborn MR, Aukema HM. Modulation of renal injury in pcy mice by dietary fat containing n-3 fatty acids depends on the level and type of fat. Lipids 2004;39:207-14.

[35] Schafer K, Gretz N, Bader M, Oberbaumer I, Eckardt KU, Kriz W et al. Characterization of the Han:SPRD rat model for hereditary polycystic kidney disease. Kidney Int 1994;46:134-52.

[36] Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248-54.

[37] Aukema HM, Lu J, Borthwick F, Proctor SD. Dietary fish oil reduces glomerular injury and elevated renal hydroxyeicosatetraenoic acid levels in the JCR:LA-cp rat, a model of the metabolic syndrome. Br J Nutr 2012:1-9.

[38] Deems R, Buczynski MW, Bowers-Gentry R, Harkewicz R, Dennis EA. Detection and quantitation of eicosanoids via high performance liquid chromatography-electrospray ionization-mass spectrometry. Methods Enzymol 2007;432:59-82.

[39] Hall LM, Murphy RC. Electrospray mass spectrometric analysis of 5-hydroperoxy and 5-hydroxyeicosatetraenoic acids generated by lipid peroxidation of red blood cell ghost phospholipids. J Am Soc Mass Spectrom 1998;9:527-32.

[40] Elberg D, Turman MA, Pullen N, Elberg G. Prostaglandin E 2 stimulates cystogenesis through EP4 receptor in IMCD-3 cells. Prostaglandins and Other Lipid Mediators 2012;98:11-6.

[41] Elberg G, Elberg D, Lewis TV, Guruswamy S, Chen L, Logan CJ et al. EP 2 receptor mediates PGE 2-induced cystogenesis of human renal epithelial cells. American Journal of Physiology - Renal Physiology 2007;293:F1622-32.

[42] Mangoo-Karim R, Ye M, Wallace DP, Grantham JJ, Sullivan LP. Anion secretion drives fluid secretion by monolayers of cultured human polycystic cells. American Journal of Physiology - Renal Fluid and Electrolyte Physiology 1995;269:F381-8.

[43] Belibi FA, Reif G, Wallace DP, Yamaguchi T, Olsen L, Li H et al. Cyclic AMP promotes growth and secretion in human polycystic kidney epithelial cells. Kidney Int 2004;66:964-73.

[44] Camara NO, Martins JO, Landgraf RG, Jancar S. Emerging roles for eicosanoids in renal diseases. Curr Opin Nephrol Hypertens 2009;18:21-7.

[45] Hao CM, Breyer MD. Physiologic and pathophysiologic roles of lipid mediators in the kidney. Kidney Int 2007;71:1105-15.

[46] Calder PC. Polyunsaturated fatty acids and inflammatory processes: New twists in an old tale. Biochimie 2009;91:791-5.

[47] Chaudhary KR, Batchu SN, Das D, Suresh MR, Falck JR, Graves JP et al. Role of B-type natriuretic peptide in epoxyeicosatrienoic acid-mediated improved post-ischaemic recovery of heart contractile function. Cardiovasc Res 2009;83:362-70.

[48] Park F, Sweeney WE,Jr, Jia G, Akbulut T, Mueller B, Falck JR et al. Chronic blockade of 20-HETE synthesis reduces polycystic kidney disease in an orthologous rat model of ARPKD. Am J Physiol Renal Physiol 2009;296:F575-82.

[49] Park F, Sweeney WE, Jia G, Roman RJ, Avner ED. 20-HETE mediates proliferation of renal epithelial cells in polycystic kidney disease. J Am Soc Nephrol 2008;19:1929-39.

[50] Jensen BL, Schmid C, Kurtz A. Prostaglandins stimulate renin secretion and renin mRNA in mouse renal juxtaglomerular cells. Am J Physiol 1996;271:F659-69.

[51] Stichtenoth DO, Wagner B, Frolich JC. Effect of selective inhibition of the inducible cyclooxygenase on renin release in healthy volunteers. J Investig Med 1998;46:290-6.

[52] Farzamirad V, Aluko RE. Angiotensin-converting enzyme inhibition and free-radical scavenging properties of cationic peptides derived from soybean protein hydrolysates. Int J Food Sci Nutr 2008;59:428-37.

[53] Jia G, Kwon M, Liang HL, Mortensen J, Nilakantan V, Sweeney WE et al. Chronic treatment with lisinopril decreases proliferative and apoptotic pathways in autosomal recessive polycystic kidney disease. Pediatr Nephrol 2010;25:1139-46.

[54] Goto M, Hoxha N, Osman R, Dell KM. The renin-angiotensin system and hypertension in autosomal recessive polycystic kidney disease. Pediatr Nephrol 2010;25:2449-57.

[55] Watanabe G, Tomiyama H, Doba N. Effects of oral administration of L-arginine on renal function in patients with heart failure. J Hypertens 2000;18:229-34.

[56] Aukema HM, Gauthier J, Roy M, Jia Y, Li H, Aluko RE. Distinctive effects of plant protein sources on renal disease progression and associated cardiac hypertrophy in experimental kidney disease. Molecular Nutrition and Food Research 2011;55:1044-51.

[57] Anderson JW. Beneficial effects of soy protein consumption for renal function. Asia Pac J Clin Nutr 2008;17 Suppl 1:324-8.

[58] Talahalli RR, Vallikannan B, Sambaiah K, Lokesh BR. Lower efficacy in the utilization of dietary ALA as compared to preformed EPA + DHA on long chain n-3 PUFA levels in rats. Lipids 2010;45:799-808.

Chapter 5

5. Cyclooxygenase product inhibition with acetylsalicylic acid slows disease progression in the Han:SPRD-Cy rat model of cystic kidney disease

This manuscript has been published in the Prostaglandins and Other Lipid Mediators, 2014 Nov 4;116-117C:19-25. doi: 10.1016/j.

Naser H.M. Ibrahim[1,2,4], Melanie Gregoire[1,2], Jessay G. Devassy[1,2], Yinhong Wu[1,2], Daisuke Yoshihara[5], Tamio Yamaguchi[1,2], Shizuko Nagao[5], and Harold M. Aukema[1,2,3,4]

[1]Department of Human Nutritional Sciences, University of Manitoba, Winnipeg, Manitoba, Canada

[2]Canadian Centre for Agri-Food Research in Health and Medicine, St. Boniface Hospital Research Centre, Winnipeg, Manitoba, Canada

[3] Manitoba Institute of Child Health, Manitoba, Winnipeg, Manitoba, Canada

[4]the Richardson Center for Functional Foods and Nutraceuticals

[5] Education and Research Center of Animal Models for Human Diseases, Fujita Health University, Toyoake, Aichi, Japan

5.1 Abstract

Renal cyclooxygenase (COX) derived oxylipins are elevated and lipoxygenase (LOX) products are reduced in the Han:SPRD-*Cy* rat model of cystic kidney disease. Selective COX2 inhibition reduces kidney disease progression, but COX1 levels also are elevated in this model. Since the effect of reducing the products of both COX isoforms and the role of LOX products is not known, weanling normal and diseased Han:SPRD-*Cy* littermates were given either low dose acetylsalicylic acid (ASA), nordihydroguaiaretic (NDGA) or no treatment for eight weeks. Renal oxylipins were altered in the diseased compared to normal cortex, with COX products being higher and LOX products being lower. ASA reduced COX products, cyst growth and kidney water content, while NDGA reduced LOX products without altering disease progression or kidney function. Hence, a human equivalent ASA dose equal to less than one regular strength aspirin per day slowed disease progression, while further reduction of LOX products did not worsen disease progression.

Keywords:

Cystic kidney disease; acetylsalicylic acid; NDGA; oxylipin.

5.2 Introduction

Cystic kidney disease is one of the most frequently inherited renal diseases, and encompasses a group of disorders characterized by numerous renal cysts. The continuous growth of these cysts over time results in massive kidney enlargement, renal damage, and reduction in function, which eventually leads to end stage renal disease (ESRD) and death [1]. Cystic kidney disease occurs in approximately 1:500 to 1:1000 individuals in the general population, is the fourth leading cause of renal failure, and is responsible for 10-15% of ESRD [1]. There is no cure for cystic kidney disease and there is no therapy that is clinically effective in slowing the progression of this disease [2,3].

Oxylipins are important regulators of normal renal function as mediators of vascular tone, salt and water balance, renin release, as well as being important in acute and chronic renal diseases by mediating inflammatory and fibrotic processes in response to kidney injury [4-7]. With respect to cystic kidney disease, cyclooxygenase (COX) derived oxylipins such as prostaglandin (PG) E_2, and PGI_2, stimulate cell proliferation, fluid secretion, and cyst formation in primary cultured PKD cells and in inner medullary collecting duct cells in *vitro* [8-10]. We have shown that renal COX derived oxylipins are elevated and lipoxygenase (LOX) derived products are lower in the Han:SPRD-*Cy* rat model of cystic kidney disease [11-14], as well as in another model of cystic kidney disease which displays renal cyst development, the *pcy* mouse model of cystic kidney disease [15].

Studies in the Han:SPRD-*Cy* rat and other models of renal disease have demonstrated that selective COX2 inhibition can reduce kidney disease progression [16-19]. However, we have shown that both COX1 and COX2 activities are elevated in diseased kidneys in the Han:SPRD-*Cy* rat [13]. Considering the concerns surrounding the use of COX2 inhibitors [20],

the lack of effective therapies to date [2,3], and the fact that both COX1 and COX2 are elevated, it was of interest to examine the potential benefits of a commonly used dual COX isoform inhibitor such as ASA [21].

Further rationale for also examining the potential benefit of COX1 inhibition in this disorder comes from nutritional studies with this model. Dietary soy protein (SP) slows disease progression and reduces the elevated COX activities and prostanoid levels in diseased kidneys in the Han:SPRD-*Cy* rat [11]. The effect of the SP was primarily in the cortex, the region in which most cysts are found in Cy/+ kidneys [11,22], and was associated with a reduction in the elevated levels of COX1 protein levels and activity in this region [11].

In addition to reducing the increased COX products in diseased kidneys, dietary SP also was associated with amelioration of the reduced LOX products in diseased compared to normal kidneys in the Han:SPRD-*Cy* rat [11,15]. However, as with the effects on COX products, whether the change in the LOX products is a cause or an effect of the change in disease progression due to dietary SP is not known. Intervention with dietary fish oil (FO) suggests that reducing LOX products in this disease may be detrimental, since dietary FO reduced both COX and LOX products, but was not as effective in slowing disease progression as dietary SP which reduced COX and elevated LOX products [11]. Previous studies have shown that high levels of the LOX inhibitor NDGA cause cyst formation in kidneys [23,24], but these may be non-specific toxic effects, as other chemicals with no apparent LOX inhibitory activity at high levels also cause renal cyst formation [25]. Therefore, to directly explore the role of LOX products in this disorder, LOX was inhibited with a low dose of NDGA to determine whether further reduction of LOX products in this cystic kidney disease model would worsen disease progression.

5.3 Materials and methods

5.3.1 Animals and treatments

All animal procedures were approved by the University of Manitoba Committee on Animal Care (protocol F09-012) and were in accordance with the guidelines of the Canadian Council on Animal Care. Han:SPRD-*Cy* rats were derived from our breeding colony, which originated from breeders provided to us by Dr. Benjamin Cowley (at the University of Kansas Medical Center). Male weanling Han:SPRD-*Cy* rats were genotyped and at 3 wk of age 12 *Cy/+* and 6 +/+ rats (18/group) were randomly assigned into each of three different treatments. The 1st group received the standard (AIN-93G) diet [26] with no drug added and served as the control group; the 2nd group received the standard diet with 0.05% NDGA (LOX inhibitor, Cayman Chemical) added, a dose that that has been shown to inhibit LOX products in animal studies [27,28]; the 3rd group received the standard diet with 0.04% ASA (COX inhibitor, Cayman Chemical) added, a human equivalent dose of 290 mg/day or less than one regular strength aspirin per day (1 ASA= 325 mg) [29]. All diet ingredients were purchased from Dyets (Bethlehem, PA) and stored at 4 ^{0}C in sealed containers as described [11]. Diet details in supplementary Table S3.

The study continued for 8 wk, during which the rats were housed singly in a temperature and humidity controlled environment with a 12 hour day/night cycle and free access to water and diet. 2 days prior to termination, rats were lightly anesthetized with isoflurane gas and blood pressure was determined by tail-cuff plethysmography. At the end of the study, rats were anesthetized with isoflurane and terminated by cardiac puncture to obtain blood for serum. Tissues were removed and weighed. Left kidneys were cut longitudinally across the hilum and one half was fixed in 10% buffered formalin for morphological and histological analysis, while

the right kidneys were separated into cortex and medulla, snap frozen in liquid nitrogen and stored in -80^{0}C for further analysis [11,16].

5.3.2 Histologic and morphometric assessment

Formalin fixed kidneys were embedded in paraffin, sectioned at 5 µm and processed using our previously described methods [11,30]. Cystic and fibrosis areas were captured from 20 random fields (×40 magnification) of Masson's trichrome or Hematoxylin and Eosin (H&E) stained kidney sections and indices (% of total field) were measured and calculated by a naive observer using LUZEX AP software (NIRECO CO.LTD, Tokyo, Japan) [31]. Proportional cyst and fibrosis areas were multiplied by kidney weight to estimate volume as described in [32]. To avoid underestimation of fibrosis due to empty cystic areas in the sections, fibrosis measurements were corrected to solid tissue areas of these sections.

5.3.3 Serum biochemistry

Biochemical measurements were performed by a single observer who was blinded to treatment and disease status. Serum cystatin C was determined using a commercial ELISA kit (Bio Vendor Research and Diagnostic Products, Candler, NC, USA). Serum creatinine concentrations were measured calorimetrically using a modified Jaffe reaction [33]. Intra-assay CVs were calculated for all assays; if the CV for any sample or standard was ≥ 10 the sample was repeated.

5.3.4 Oxylipin and fatty acid analyses

Renal eicosanoid, fatty acid and total protein levels were analyzed as previously described [11]. Briefly, 45 mg of tissue lyophilates were homogenized on ice in 30 volumes of Tyrode's. After homogenization, Triton X-100 was added and mixed to achieve a final concentration of 0.01%. Protein concentrations of the homogenates were determined using the Bradford method [34]. The

endogenous renal oxylipin levels in all samples were analyzed by HPLC-tandem mass spectrometry as described [11] based on the method of Deems *et al* [35]. Quantification of oxylipins was determined using the stable isotope dilution method [35]. Intra-assay variability was determined by processing replicates of two samples and the inter-assay was determined by processing 4 replicates in 3 batches. Excellent linearity with r^2 >0.999 was obtained for an accuracy of 99%. The internal standards, *m/z* transitions, retention times and detector response factors used to measure the targeted oxylipins are described in Table S7 [11].

For analysis of renal phospholipid content, total lipids were extracted as described [11]. Renal phospholipid fractions were purified by TLC plates using heptane/isopropyl ether/acetic acid 60/40/3 (v/v) as the mobile phase. Phospholipid was identified by comigration with authentic standards purchased from Nu-Chek Prep (MN). Fatty acids in renal phospholipid were transmethylated using methanolic HCl and analyzed by gas-liquid chromatography as described [11].

5.3.5 Statistical analysis

Data were expressed as mean ± standard error (SE). All data were analyzed using 2-way ANOVA using the GLM procedure of SAS (SAS, version 9.2, Cary, NC). Normality of the data was assessed using the Shapiro-Wilk's test (W>0.05 for normally distributed data) and the data were normalized by logarithmic transformation if necessary. When the overall main or interaction effect was significant, Tukey's test was used for simple effect comparisons. Statistical significance for main and interaction effects was set at P<0.05. Pearson's correlation analysis was used to test the relationship between renal pathology and oxylipin levels.*5.4 Results*

5.4.1 Renal pathology and function

Diseased rats had significantly larger kidneys and higher water content compared to normal rats (Table 5.1). Neither drug treatment resulted in significant differences in renal size, but histological analysis revealed that cortical cyst volume was 20% lower in ASA treated rats compared to control, but not different in the medulla or in NDGA treated rats (Figure 5. 1A). Representative histology images are shown in Figure 5.1B. Consistent with cyst volume changes, water content was 21% lower in ASA, but not NDGA treated rats. Renal fibrosis was lower with drug treatment, but the differences were not significantly different from control. Renal function was impaired in diseased rats, as indicated by elevated serum creatinine and cystatin C, but drug treatment did not alter these parameters. Diseased compared to normal rats also were slightly smaller at the end of the study, but drug treatment did not alter body weight (Table 5.1). Liver weight was affected by disease and treatments, as diseased rats had smaller livers compared to normal rats and rats given NDGA had larger livers compared to the control and the ASA groups (Table 5.1). Systolic blood pressure (SBP) and mean arterial blood pressure (MAP) in diseased rats was significantly higher compared to normal rats and ASA and NDGA did not modify either blood pressure measure.

5.4.2 Oxylipin levels

Since cyst formation and oxylipin changes in the Han:SPRD-Cy rat occur primarily in the cortex [11], oxylipin and octadecanoid levels were examined in this part of the kidney. As previously observed, diseased compared to normal rats given the control treatment had higher levels of cortical COX products, 6-keto-$PGF_{1\alpha}$, PGE_2, TXB_2, as well as total COX products, but not $PGF_{2\alpha}$ or PGD_2 (Figure 5.2). Conversely, diseased kidneys also had lower levels of cortical LOX products 5-, 8-, 12-, and 15-HETE and 9-hydroxy-octadecadienoic acid (9-HODE), 9-oxoODE and 13-HODE, as well as total HETEs (Figure 5.3A and 5.3B).

Giving rats ASA reduced the disease related elevation in COX products. As expected, ASA did not have any significant effects on the LOX products except for 5-HETE, where ASA resulted in lower levels of cortical 5-HETE compared to the control treatment, consistent with its reported dual COX and 5-LOX inhibitory activity [36]. NDGA treatment resulted in lower cortical levels of 5-, 8-, 15-, and total HETEs but did not alter the levels of 12-HETE, 9- and 13-HODE, and 9-OxoODE. NDGA also did not affect COX product levels, with the exception of a lowering of $PGF_{2\alpha}$.

Interestingly, the changes in oxylipins were not reflected in precursor fatty acid levels, as the levels of almost all fatty acids were lower in diseased kidneys (Table 5.2). When expressed as a proportion (mol%, data not shown) of total fatty acids, arachidonic acid was lower in diseased compared to normal kidneys. Thus the increase in COX products in diseased kidneys occurred despite the absolute and relatively lower levels of this precursor fatty acid. The importance of the COX product alterations in relation to disease was further demonstrated by correlation analysis across experimental groups, which showed that kidney weight (r=0.47), and

cortical cyst (r=0.50) and fibrosis volumes (r=0.60) positively correlated ($P<0.05$) with total COX products, but not with total HETE.

Table 5.1. Physiologic parameters in normal and diseased Han:SPRD-*Cy* rats given no drug, NDGA, or ASA.

Drug	Control		NDGA		ASA		
Genotype	**+/+**	**Cy/+**	**+/+**	**Cy/+**	**+/+**	**Cy/+**	**Effects (P-values)**
Body weight (g)	401 (10.3)	376 (5.3)	404 (8.4)	382 (5.4)	400 (7.4)	370 (4.6)	Genotype (<0.005)
Kidney weight (g)	2.68 (0.09)	9.49 (0.43)	2.71 (0.06)	9.41 (0.34)	2.69 (0.38)	8.26 (0.38)	Genotype (<0.0001)
Kidney/body weight (g/100 g)	0.67 (0.01)	2.50(0.09)	0.67 (0.01)	2.46 (0.07)	0.68 (0.02)	2.23 (0.08)	Genotype (<0.0001)
Kidney water (g)	1.07 (0.06)^{c}	3.69 (0.19)^{a}	1.13 (0.05)^{c}	3.74 (0.14)^{a}	1.07 (0.03)^{c}	2.91 (0.18)^{b}	Interaction (0.0397)
Liver weight (g)	16.01 (0.51)	13.51 (0.27)^{b}	18.03 (0.76)	15.01 (0.31)^{a}	15.45 (0.29)	12.53 (0.29)^{b}	Genotype and drug (<0.0001)
Liver/body wt (g/100 g)	3.99 (0.07)	3.56 (0.04)^{b}	4.46 (0.13)	3.93 (0.05)^{a}	3.87 (0.10)	3.39 (0.06)^{b}	Genotype and drug (<0.0001)
Heart weight (g)	1.55 (0.12)	1.44 (0.04)	1.51 (0.09)	1.48 (0.08)	1.44 (0.07)	1.50 (0.06)	No effect
Heart/body wt (g/100 g)	0.41 (0.04)	0.38 (0.01)	0.37 (0.02)	0.39 (0.02)	0.36 (0.02)	0.41 (0.02)	No effect
Serum creatinine (µg/dl)	5.41 (0.14)	7.14 (0.22)	5.48 (0.17)	6.90 (0.19)	5.36 (0.12)	7.66 (0.15)	Genotype (<0.0001)
Serum cystatin C (ng/ml)	1.54 (0.16)	2.38 (0.16)	1.45 (0.07)	2.20 (0.19)	1.36 (0.13)	2.68 (0.23)	Genotype (<0.0001)
Systolic blood pressure (BP) (mmHg)	148 (6.50)	157 (4.30)	140 (5.20)	156 (1.90)	149 (5.20)	157 (3.30)	Genotype (0.0051)
Mean Arterial Pressure (MAP) (mmHg)	127 (5.59)	135 (4.06)	121 (4.03)	128 (2.47)	128 (4.59)	134 (4.06)	Genotype (0.0374)

Values are means (SE), (n=6 for +/+ and 12 for *Cy*/+ groups). Average CV for serum creatinine = 1.5 and for serum cystatin C = 2.7.
Values in the same row with different superscript letters indicate significant differences in drug effects (P<0.05).

Table 5.2. Renal cortex phospholipid fatty acid compositions in normal and diseased Han:SPRD-*Cy* rats given no drug, NDGA, or ASA

Drug	Control diet		NDGA		ASA		Effects (P-values)
Genotype	**+/+**	**Cy/+**	**+/+**	**Cy/+**	**+/+**	**Cy/+**	
C18:0	8809 (2280)	2246 (295)	10334 (2953)	2335 (390)	11870 (334)	2415 (466)	Genotype (<0.0001)
C18:1	2256 (600)	673 (90)	3323 (549)	535 (128)	3052 (84)	658 (70)	Genotype (<0.0001)
C18:2n6	4477 (1374)	979 (155)	5523 (1375)	935 (87)	6290 (46)	722 (98)	Genotype (<0.0001)
C18:3n3	56 (15)	13 (6)	83 (21)	21 (2)	76 (20)	15 (2)	Genotype (<0.0001)
C18:3n6	34 (8)	4 (1)	40 (11)	9 (3)	46 (2)	9 (3)	Genotype (<0.0001)
C20:3n3	65 (1)	3 (2)	50 (12)	4 (3)	67 (2)	4 (4)	Genotype (0.023)
C20:3n6	259 (75)	54 (10)	365 (84)	517 (464)	359 (7)	41 (3)	Genotype (0.0004)
C20:4n6	9508 (2635)	2156 (318)	11964 (2958)	1652 (578)	13625 (499)	1701 (185)	Genotype (<0.0001)
C20:5n3	66 (19)	4 (4)	62 (16)	11 (4)	90 (3)	15 (2)	Genotype (<0.0001)
C22:0	219 (65)	74 (13)	301 (4)	64 (3)	277 (14)	61 (2)	Genotype (<0.0001)
C22:4n6	42 (23)	37 (6)	37 (13)	54 (3)	57 (5)	53 (4)	No effect
C22:5n3	162 (250)	78 (24)	172 (44)	48 (1)	366 (202)	43 (4)	Genotype (0.0003)
C22:6n3	503 (189)	76 (28)	635 (164)	93 (8)	610 (211)	93 (11)	Genotype (<0.0001)
C24:0	1460 (431)	331 (92)	1900 (514)	245 (31)	2112 (114)	275 (61)	Genotype (0.007)
C24:1	404 (118)	57 (9)	442 (117)	57 (5)	540 (20)	46 (5)	Genotype (<0.0001)

Values are means (SE), expressed in ng/mg protein (n=8/group).

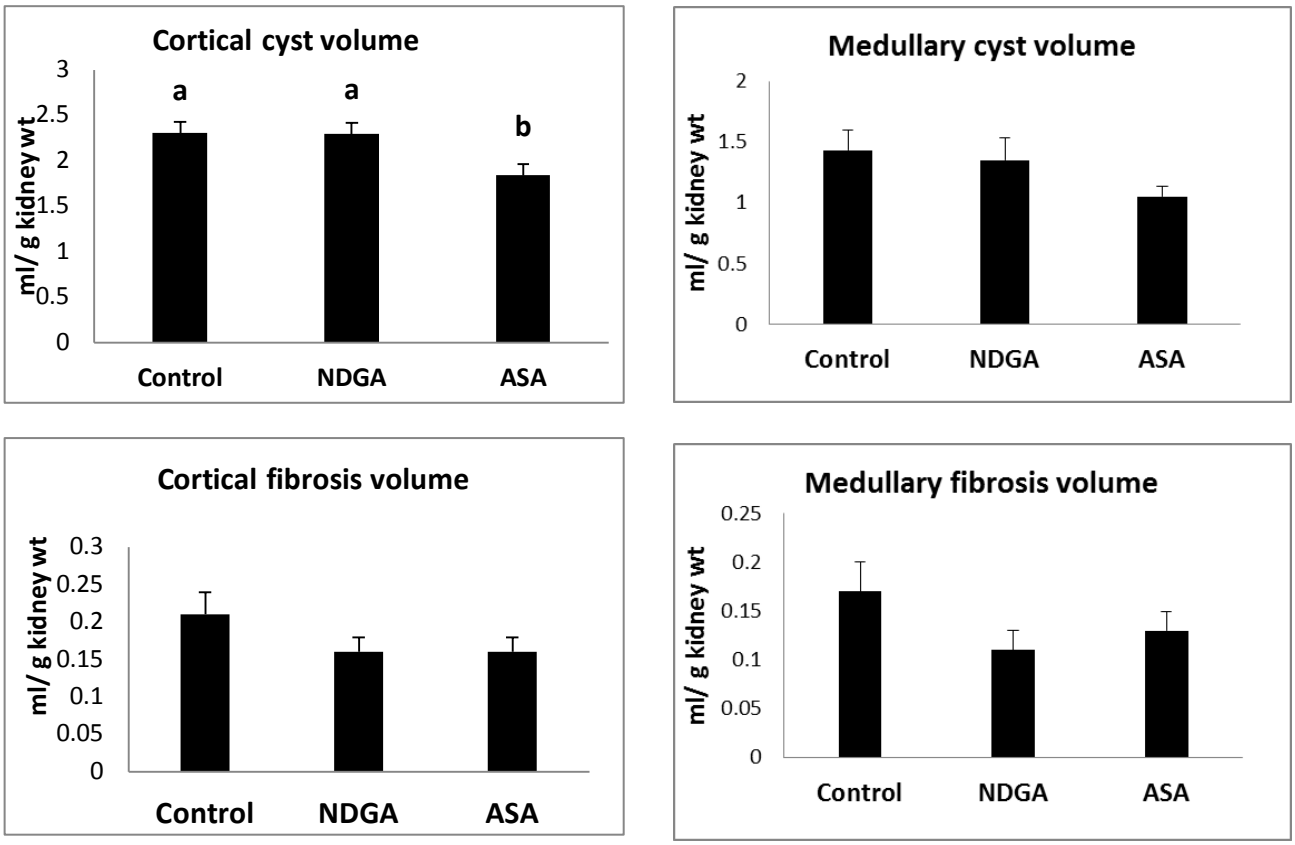


Figure 5.1A. Cortical and medullary cyst and fibrosis volumes in diseased Han:SPRD-*Cy* rats given no drug, 0.05% NDGA, or 0.04% ASA in the diet. Cyst areas were captured from 20 random fields (×40 magnification) of H&E stained kidney sections and indices were measured and calculated by a naive observer using LUZEX AP software (NIRECO CO.LTD, Tokyo, Japan). The product of proportional cyst area and kidney weight was used to estimate cyst volume. Fibrosis volume was calculated in the same way using Masson's trichrome stained sections. Values are means ± SE (n=12/treatment group). Values on the same graph with different letters are significantly different ($P<0.05$). Representative images are shown in Figure 5.1B.

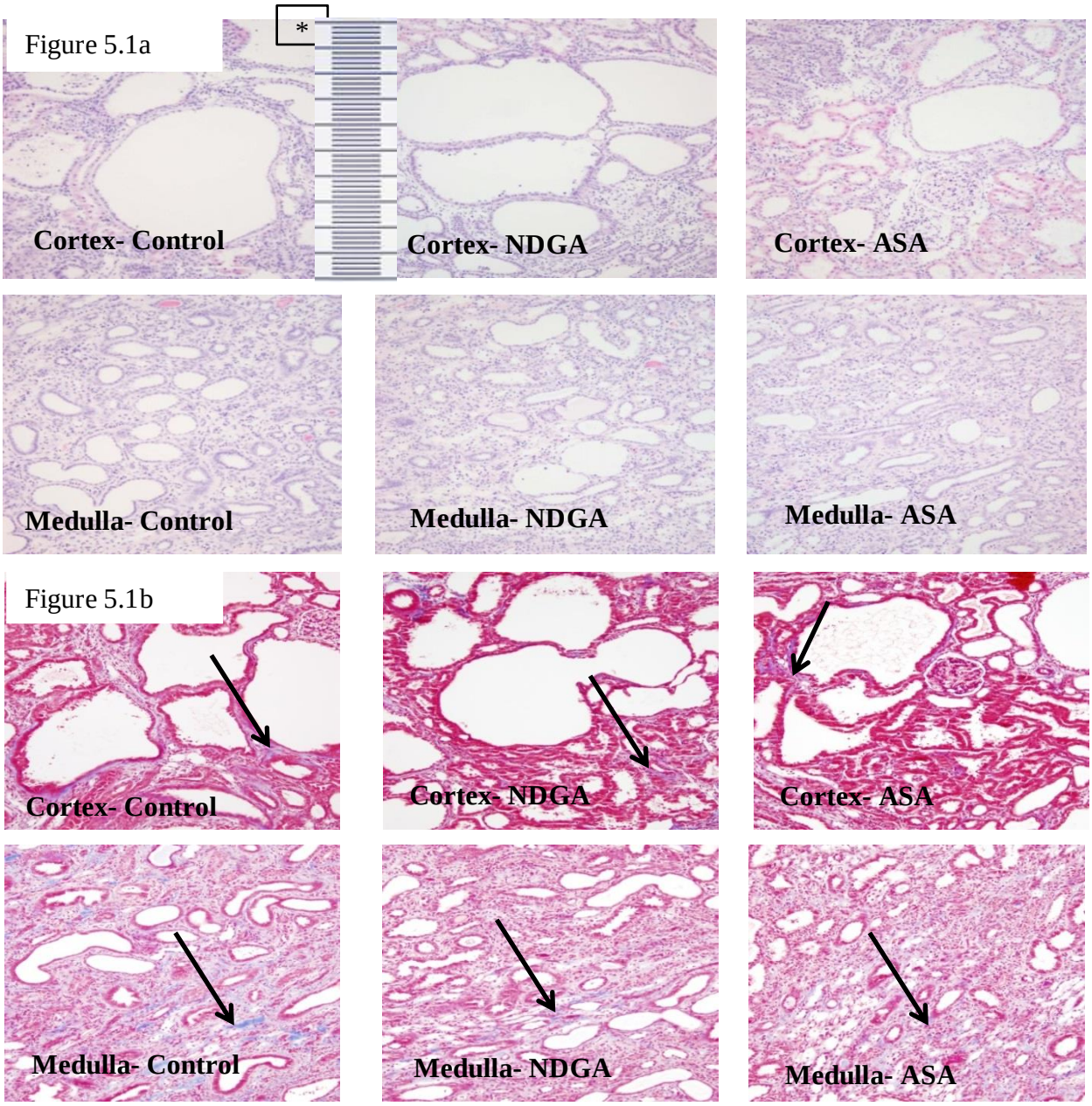


Figure 5.1B. Cortical and medullary (A) cyst and (B) fibrosis volumes in diseased Han:SPRD-*Cy* rats given no drug, 0.05% NDGA, or 0.04% ASA in the diet. Cyst areas were captured from 20 random fields (×40 magnification) of H&E stained kidney sections and indices were measured and calculated by a naive observer using LUZEX AP software (NIRECO CO.LTD, Tokyo, Japan). The product of proportional cyst area and kidney weight was used to estimate cyst volume. Fibrosis volume was calculated in the same way using Masson's trichrome stained sections. *1 space of a stage micrometer = 10 µm.

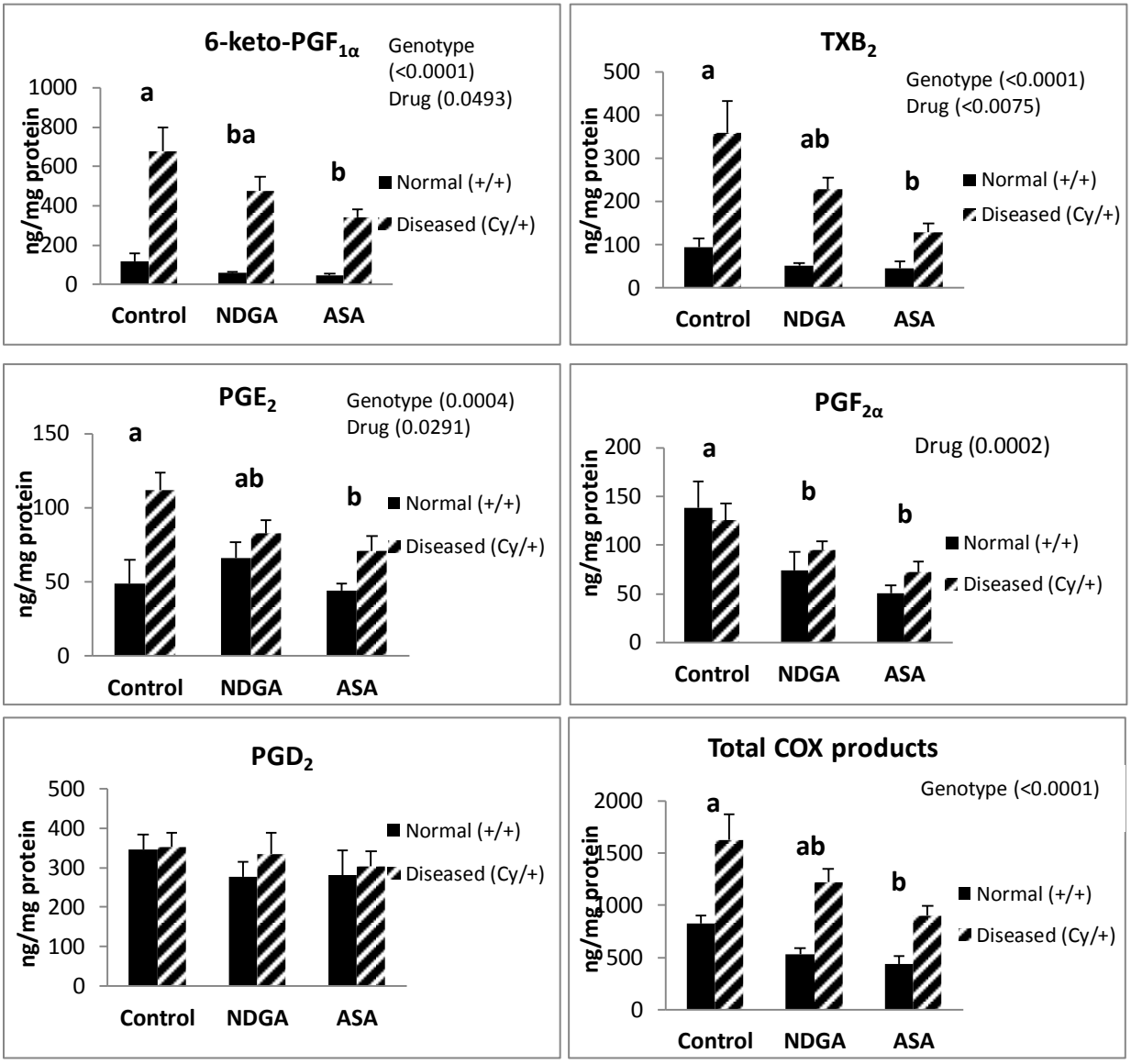


Figure 5.2. COX products in the cortex of Han:SPRD-*Cy* rats given no drug, 0.05% NDGA, or 0.04% ASA in the diet. Renal oxylipin levels were analyzed by HPLC-tandem mass spectrometry. Values are means ± SE (n=6 for +/+ and 10 for *Cy*/+ groups). Main effects (P value) are indicated on individual graphs; where drug effects (P<0.05) were present, letters indicate significant differences. PG, prostaglandin; TX, thromboxane.

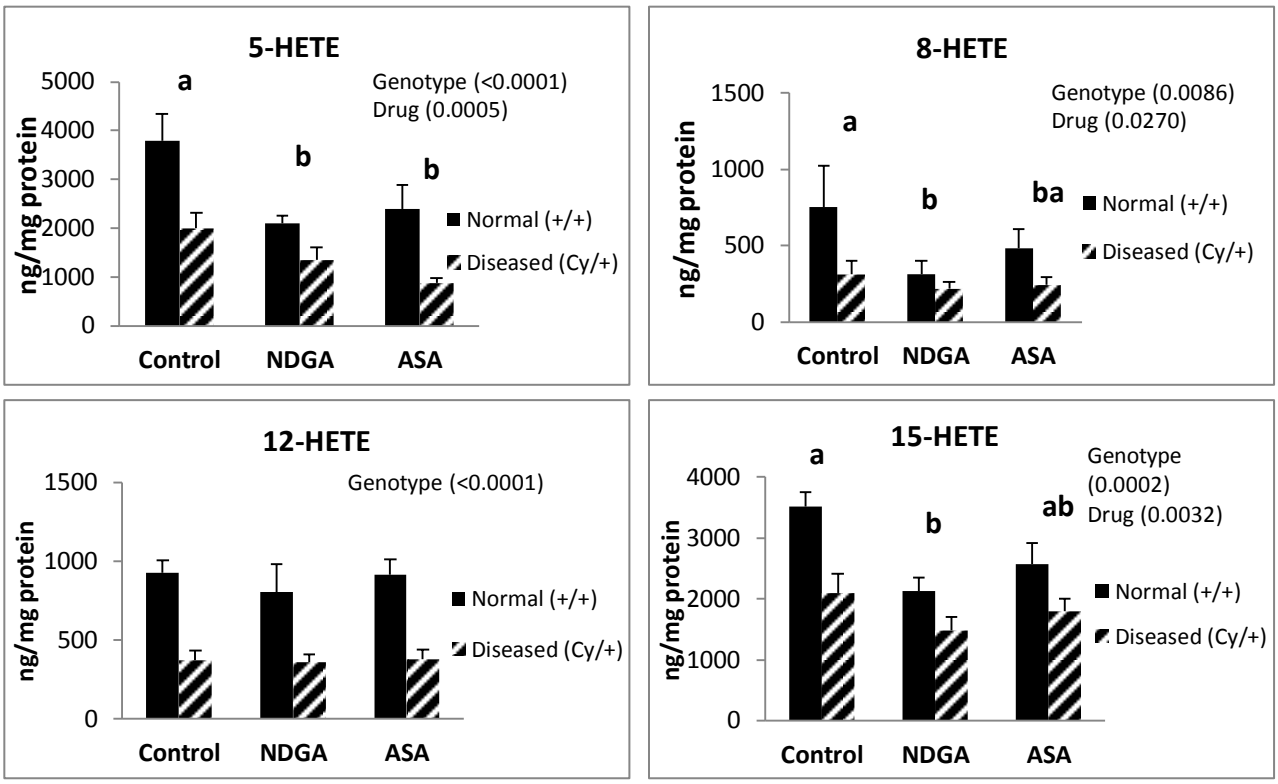


Figure 5.3A. LOX products in the cortex of Han:SPRD-*Cy* rats given no drug, 0.05% NDGA, or 0.04% ASA in the diet. Renal oxylipin levels were analyzed by HPLC-tandem mass spectrometry. Values are means ± SE (n=6 for +/+ and 10 for *Cy*/+ groups). Main effects (P value) are indicated on individual graphs; where drug effects (P<0.05) were present, letters indicate significant differences. HETE, hydroxy-eicosatetraenoic acid; HODE, hydroxy-octadecadienoic acid.

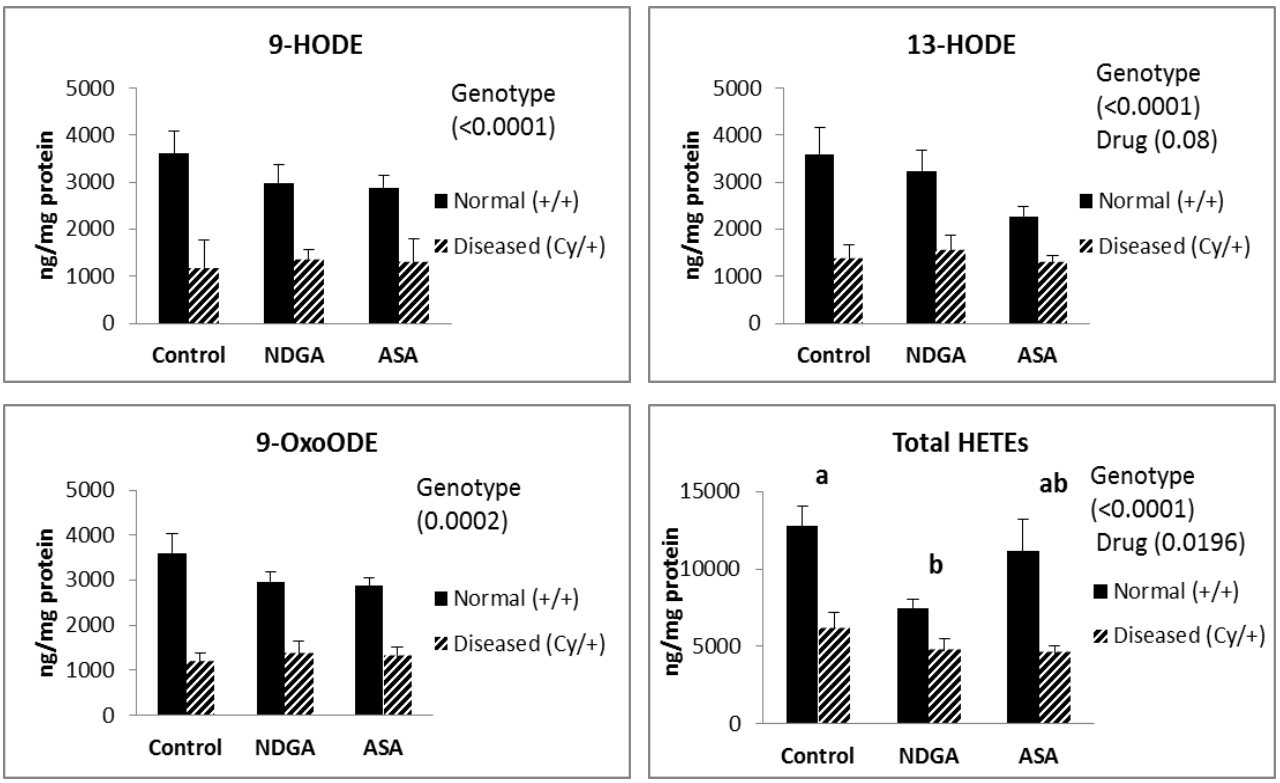


Figure 5.3B. LOX products in the cortex of Han:SPRD-*Cy* rats given no drug, 0.05% NDGA, or 0.04% ASA in the diet. Renal oxylipin levels were analyzed by HPLC-tandem mass spectrometry. Values are means ± SE (n=6 for +/+ and 10 for *Cy*/+ groups). Main effects (P value) are indicated on individual graphs; where drug effects (P<0.05) were present, letters indicate significant differences. HETE, hydroxy-eicosatetraenoic acid; HODE, hydroxy-octadecadienoic acid.

5.5 Discussion

This study demonstrates that inhibition of both COX isoforms slows cyst formation and disease progression in the Han:SPRD-*Cy* rat model of cystic kidney disease. Previously, we have demonstrated that renal prostanoid levels and total COX activity are higher in diseased compared to normal Han:SPRD-*Cy* rats [11,13,16], and that selective COX2 inhibition reduces cyst growth and fibrosis in diseased kidneys [16,17]. Since renal COX1 levels also are elevated in this model [11,13], and considering the complications of using selective COX2 inhibitors [20], the therapeutic effects of a commonly used dual COX isoform inhibitor was examined. ASA given at a human adult equivalent dose of less than one tablet of aspirin per day reduced cyst growth, as indicated by reduced water content and cyst volume. Cyst development and time-dependent structural disruption of renal architecture plays a central role in the genesis of cystic kidney disease and cyst growth is a key predictor of deterioration of renal function [37-40]. Furthermore, changes in cyst volume occur prior to changes in fibrosis in the early stages of cystic kidney disease [37,39,40], indicating that this reduction in early cyst growth would have a long term beneficial effect on disease progression. Such studies are needed to determine whether these positive effects on disease progression will ultimately result in improved renal function.

Increased levels of renal COX products in this disorder may contribute to disease progression through multiple mechanisms [8,9,41], including stimulation of fluid secretion and cell proliferation, important components of cyst formation in cystic kidney disease [42,43] that are mediated in part via activation of 3' 5'-cyclic adenosine-monophosphate (cAMP) [41,44]. cAMP formation is regulated in part by prostaglandins [41], and TXB_2 and PGE_2 stimulate renal cell proliferation [8,45,46]. In renal epithelial cells from cystic kidney disease patients and mice with cystic kidney disease, PGE_2 stimulates cAMP production, cyst formation, and proliferation

[8-10]. In the current study, these COX products were higher in diseased compared to normal kidneys and ASA reduced these elevated levels in parallel with a reduction in cyst volume. This reduction in the levels of COX products by ASA may therefore decrease levels of cAMP, prevent fluid secretion, and cyst proliferation, thereby possibly retarding cyst growth and disease progression in this rat model of cystic kidney disease.

To date there is no cure for cystic kidney disease and there is no available therapy that can prevent or slow the cyst expansion in this disorder [2,3]. ASA is among the most prescribed NSAIDs and one of the most frequently used over-the counter drugs worldwide. Its therapeutic effect has been demonstrated in a number of disease conditions [47-50]. A concern raised regarding the use of NSAIDs is their potential to cause renal damage in patients with renal insufficiency [51-53]. However, not all NSAIDs have the same effects on the kidney: for example, ibuprofen may be more nephrotoxic than Sulindac or other NSAIDs [51,54]. Furthermore, case control and cohort studies showed no adverse renal effects for ASA use; a population based case control study that targeted mainly people with ESRD demonstrated that there is no increased risk of ESRD with ASA use [55]. This observation is also supported by clinical trials, such as the first UK Heart and Renal Protection Study which reported that low dose of ASA (100 mg/d) did not significantly impair kidney function or increase the risk for major bleeding [56]. This indicates that low dose ASA has the potential to be safely used as a therapeutic intervention to reduce kidney disease progression in cystic kidney disease. However, whether higher doses of ASA would be detrimental in this model or in human cystic kidney disease still needs to be investigated.

In previous studies we have demonstrated that dietary soy protein, FXO, or conjugated linoleic acid reduce kidney disease progression when initiated at early stages of the disease, but

that late intervention limits these beneficial effect in the Han:SPRD-*Cy* rat model [57,58]. Similarly, in the current study, ASA also was initiated in the early stages of the disease, so whether late intervention with ASA will have similar effects should be investigated. However, it does confirm the importance of early intervention in the disease process.

In contrast to COX, products of the LOX pathway are reduced in diseased compared to normal Han:SPRD-*Cy* rats. Inhibition of this pathway with low levels of NDGA would have been expected to worsen the disease, but this was not observed in the current study. This may suggest that either the decreased levels of LOX products in this model of cystic kidney disease are a result of and not a cause of the disease, or that some of the other important biological activities of NDGA, including its antioxidant effect, anti-proliferative effect, and inhibitory effect on transforming growth factor beta activities [59], may have mitigated the potential deleterious effects of NDGA on the kidney. Also, it is important to note that in addition to inhibiting LOX products, NDGA also reduced the renal levels of $PGF_{2\alpha}$, an effect that has been reported previously [60]. In addition, the levels of several other COX products were in between the levels found in kidneys from rats given the control and ASA treatments, but not different from either. Therefore, it is possible that a concomitant lowering of COX products in rats given the NDGA treatment may have counteracted any harmful effects of inhibiting LOX products in diseased kidneys. Selective LOX inhibitors that do not affect COX products will need to be investigated in this model in order to address the question as to whether lower LOX products are directly associated with disease progression.

At high levels NDGA can induce cystic nephropathy [23,24] as well as liver toxicity in rodents [61]. The levels of NDGA used in the current study were much lower than those that cause renal toxicity, but a small enlargement of the livers was observed in the NDGA treated

group. This is consistent with a possible liver toxicity effect, which has been observed with higher levels of this drug [61]

Renal phospholipid composition was examined because their fatty acids are precursors to oxylipin formation. Although both the AA and LA precursors to the products examined herein were lower in diseased kidneys, the COX products tended to be elevated, while the LOX products were lower in disease. We have previously reported such discrepancies between fatty acid and oxylipin levels in this model [11], as well as in a model of obesity-associated nephropathy [62]. This provides further evidence of the importance of the COX enzyme activity (and its inhibition) in the progression of this disease.

5.6 Conclusion

This study demonstrates that the renal COX products are elevated and LOX products are reduced in diseased compared to normal Han:SPRD-*Cy* rats. A low dose of ASA mitigated the elevated COX products in diseased kidneys and reduced renal cyst growth in this model of cystic kidney disease. Hence, a human equivalent dose equal to less than one regular strength ASA per day slowed disease progression, while further reduction of LOX products did not worsen disease progression in this rat model of cystic kidney disease.

5.7 Acknowledgements

This work was supported by a grant from The Natural Sciences and Engineering Research Council of Canada (NSERC). Support to N.I was provided by the NSERC Food Advancement through Science and Training (FAST) program and by the Libyan-North American scholarship program. The authors thank the Richardson Centre for Functional Foods and Nutraceuticals for providing access to equipment, and Joy Gauthier, Tanja Winter, and Dennis Labossiere for providing technical assistance with the oxylipin and fatty acid analyses.

5.8 References

[1] Niemczyk M, Niemczyk S, Paczek L. Autosomal dominant polycystic kidney disease and transplantation. Ann Transplant 2009;14:86-90.

[2] Wuthrich RP, Mei C. Pharmacological management of polycystic kidney disease. Expert Opin Pharmacother 2014;15:1085-95.

[3] Spithoven EM, Kramer A, Meijer E, Orskov B, Wanner C, Caskey F et al. Analysis of data from the ERA-EDTA Registry indicates that conventional treatments for chronic kidney disease do not reduce the need for renal replacement therapy in autosomal dominant polycystic kidney disease. Kidney Int 2014; 86:1244-52.

[4] Hao C-, Breyer MD. Physiological regulation of prostaglandins in the kidney. Annual Review of Physiology 2008;70:357-77.

[5] Francois H, Facemire C, Kumar A, Audoly L, Koller B, Coffman T. Role of microsomal prostaglandin E synthase 1 in the kidney. Journal of the American Society of Nephrology 2007;18:1466-75.

[6] Jensen BL, Schmid C, Kurtz A. Prostaglandins stimulate renin secretion and renin mRNA in mouse renal juxtaglomerular cells. Am J Physiol 1996;271:F659-69.

[7] Matzdorf C, Kurtz A, Höcherl K. COX-2 activity determines the level of renin expression but is dispensable for acute upregulation of renin expression in rat kidneys. American Journal of Physiology - Renal Physiology 2007;292:F1782-90.

[8] Elberg D, Turman MA, Pullen N, Elberg G. Prostaglandin E 2 stimulates cystogenesis through EP4 receptor in IMCD-3 cells. Prostaglandins and Other Lipid Mediators 2012;98:11-6.

[9] Elberg G, Elberg D, Lewis TV, Guruswamy S, Chen L, Logan CJ et al. EP 2 receptor mediates PGE 2-induced cystogenesis of human renal epithelial cells. American Journal of Physiology - Renal Physiology 2007;293:F1622-32.

[10] Mangoo-Karim R, Ye M, Wallace DP, Grantham JJ, Sullivan LP. Anion secretion drives fluid secretion by monolayers of cultured human polycystic cells. American Journal of Physiology - Renal Fluid and Electrolyte Physiology 1995;269:F381-8.

[11] Ibrahim NH, Jia Y, Devassy JG, Yamaguchi T, Aukema HM. Renal cyclooxygenase and lipoxygenase products are altered in polycystic kidneys and by dietary soy protein and fish oil treatment in the Han:SPRD-Cy rat. Mol Nutr Food Res 2014;58:768-81.

[12] Peng CY, Sankaran D, Ogborn MR, Aukema HM. Dietary Soy Protein Selectively Reduces Renal Prostanoids and Cyclooxygenases in Polycystic Kidney Disease. Exp Biol Med 2009;234:737-43.

[13] Warford-Woolgar L, Peng CY, Shuhyta J, Wakefield A, Sankaran D, Ogborn M et al. Selectivity of cyclooxygenase isoform activity and prostanoid production in normal and diseased Han:SPRD-cy rat kidneys. Am J Physiol Renal Physiol 2006;290:F897-904.

[14] Wakefield AP, Ogborn MR, Ibrahim N, Aukema HM. A dietary conjugated linoleic acid treatment that slows renal disease progression alters renal cyclooxygenase-2-derived prostanoids in the Han: SPRD-cy rat. J Nutr Biochem 2012;23:908-14.

[15] Yamaguchi T, Lysecki C, Reid A, Nagao S, Aukema HM. Renal Cyclooxygenase Products are Higher and Lipoxygenase Products are Lower in Early Disease in the pcy Mouse Model of Adolescent Nephronophthisis. Lipids 2014;49 (1):39-47.

[16] Sankaran D, Bankovic-Calic N, Ogborn MR, Crow G, Aukema HM. Selective COX-2 inhibition markedly slows disease progression and attenuates altered prostanoid production in Han:SPRD-cy rats with inherited kidney disease. Am J Physiol Renal Physiol 2007;293:F821-30.

[17] Xu T, Wang N, Fu L, Ye C, Yu S, Mei C. Celecoxib inhibits growth of human autosomal dominant polycystic kidney cyst-lining epithelial cells through the VEGF/Raf/MAPK/ERK signaling pathway. Mol Biol Rep 2012;39:7743-53.

[18] Cheng HF, Wang CJ, Moeckel GW, Zhang MZ, McKanna JA, Harris RC. Cyclooxygenase-2 inhibitor blocks expression of mediators of renal injury in a model of diabetes and hypertension. Kidney Int 2002;62:929-39.

[19] Wang JL, Cheng HF, Shappell S, Harris RC. A selective cyclooxygenase-2 inhibitor decreases proteinuria and retards progressive renal injury in rats. Kidney Int 2000;57:2334-42.

[20] Morales E, Mucksavage JJ. Cyclooxygenase-2 inhibitor-associated acute renal failure: case report with rofecoxib and review of the literature. Pharmacotherapy 2002;22:1317-21.

[21] Wu KK. Aspirin and salicylate: An old remedy with a new twist. Circulation 2000;102:2022-3.

[22] Schafer K, Gretz N, Bader M, Oberbaumer I, Eckardt KU, Kriz W et al. Characterization of the Han:SPRD rat model for hereditary polycystic kidney disease. Kidney Int 1994;46:134-52.

[23] Evan AP, Gardner KD,Jr. Nephron obstruction in nordihydroguaiaretic acid-induced renal cystic disease. Kidney Int 1979;15:7-19.

[24] Goodman T, Grice HC, Becking GC, Salem FA. A cystic nephropathy induced by nordihydroguaiaretic acid in the rat. Light and electron microscopic investigations. Lab Invest 1970;23:93-107.

[25] Yamaguchi T, Wallace DP, Magenheimer BS, Hempson SJ, Grantham JJ, Calvet JP. Calcium restriction allows cAMP activation of the B-Raf/ERK pathway, switching cells to a cAMP-dependent growth-stimulated phenotype. J Biol Chem 2004;279:40419-30.

[26] Reeves PG, Nielsen FH, Fahey GC,Jr. AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. J Nutr 1993;123:1939-51.

[27] Mikuni M, Yoshida M, Hellberg P, Peterson CA, Edwin SS, Brannstrom M et al. The lipoxygenase inhibitor, nordihydroguaiaretic acid, inhibits ovulation and reduces leukotriene and prostaglandin levels in the rat ovary. Biol Reprod 1998;58:1211-6.

[28] Downey BR, Mootoo JE, Doyle SE. A role for lipoxygenase metabolites of arachidonic acid in porcine ovulation. Anim Reprod Sci 1998;49:269-79.

[29] Health USDo, Services H, Food, Administration D, Evaluation CfD, CDER R. Guidance for Industry: Estimating the Maximum Safe Starting Dose in Initial Clinical Trials for Therapeutics in Adult Healthy Volunteers. 2005.

[30] Aukema HM, Gauthier J, Roy M, Jia Y, Li H, Aluko RE. Distinctive effects of plant protein sources on renal disease progression and associated cardiac hypertrophy in experimental kidney disease. Molecular Nutrition and Food Research 2011;55:1044-51.

[31] Yoshihara D, Kugita M, Sasaki M, Horie S, Nakanishi K, Abe T et al. Telmisartan ameliorates fibrocystic liver disease in an orthologous rat model of human autosomal recessive polycystic kidney disease. PLoS One 2013;8:e81480.

[32] Ogborn MR, Nitschmann E, Weiler HA, Bankovic-Calic N. Modification of polycystic kidney disease and fatty acid status by soy protein diet. Kidney Int 2000;57:159-66.

[33] Hervey GR. Determination of creatinine by the Jaffe reaction. Nature 1953;171:1125.

[34] Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248-54.

[35] Deems R, Buczynski MW, Bowers-Gentry R, Harkewicz R, Dennis EA. Detection and quantitation of eicosanoids via high performance liquid chromatography-electrospray ionization-mass spectrometry. Methods Enzymol 2007;432:59-82.

[36] Kaur J, Bhardwaj A, Huang Z, Knaus EE. Aspirin analogues as dual cyclooxygenase-2/5-lipoxygenase inhibitors: Synthesis, nitric oxide release, molecular modeling, and biological evaluation as anti-inflammatory agents. ChemMedChem 2012;7:144-50.

[37] Fonseca JM, Bastos AP, Amaral AG, Sousa MF, Souza LE, Malheiros DM et al. Renal cyst growth is the main determinant for hypertension and concentrating deficit in Pkd1-deficient mice. Kidney Int 2014.

[38] Chen D, Ma Y, Wang X, Yu S, Li L, Dai B et al. Clinical characteristics and disease predictors of a large Chinese cohort of patients with autosomal dominant polycystic kidney disease. PLoS One 2014;9:e92232.

[39] Grantham JJ, Torres VE, Chapman AB, Guay-Woodford LM, Bae KT, King BF,Jr et al. Volume progression in polycystic kidney disease. N Engl J Med 2006;354:2122-30.

[40] Torres VE, King BF, Chapman AB, Brummer ME, Bae KT, Glockner JF et al. Magnetic resonance measurements of renal blood flow and disease progression in autosomal dominant polycystic kidney disease. Clin J Am Soc Nephrol 2007;2:112-20.

[41] Lone AM, Taskén K. Proinflammatory and immunoregulatory roles of eicosanoids in T cells. Frontiers in Immunology 2013;4.

[42] Mochizuki T, Tsuchiya K, Nitta K. Autosomal dominant polycystic kidney disease: Recent advances in pathogenesis and potential therapies. Clinical and Experimental Nephrology 2013;17:317-26.

[43] Patel V, Chowdhury R, Igarashi P. Advances in the pathogenesis and treatment of polycystic kidney disease. Curr Opin Nephrol Hypertens 2009;18:99-106.

[44] Belibi FA, Reif G, Wallace DP, Yamaguchi T, Olsen L, Li H et al. Cyclic AMP promotes growth and secretion in human polycystic kidney epithelial cells. Kidney Int 2004;66:964-73.

[45] Breyer MD, Breyer RM. G protein - Coupled prostanoid receptors and the kidney. Annual Review of Physiology 2001;63:579-605.

[46] Schmitz PG, Zhang K, Dalal R. Eicosapentaenoic acid suppresses PDGF-induced DNA synthesis in rat mesangial cells: involvement of thromboxane A2. Kidney Int 2000;57:1041-51.

[47] Tougeron D, Sha D, Manthravadi S, Sinicrope FA. Aspirin and colorectal cancer: Back to the Future. Clin Cancer Res 2013.

[48] Goren A, Liu X, Gupta S, Simon TA, Phatak H. Warfarin and Aspirin Use for Stroke Prevention Among Patients With Atrial Fibrillation: The US National Health and Wellness Survey. Am J Ther 2013.

[49] Bartolucci AA, Tendera M, Howard G. Meta-analysis of multiple primary prevention trials of cardiovascular events using aspirin. Am J Cardiol 2011;107:1796-801.

[50] Veitonmäki T, Tammela TLJ, Auvinen A, Murtola TJ. Use of aspirin, but not other non-steroidal anti-inflammatory drugs is associated with decreased prostate cancer risk at the population level. Eur J Cancer 2013;49:938-45.

[51] Ciabattoni G, Cinotti GA, Pierucci A, Simonetti BM, Manzi M, Pugliese F et al. Effects of sulindac and ibuprofen in patients with chronic glomerular disease. Evidence for the dependence of renal function on prostacyclin. N Engl J Med 1984;310:279-83.

[52] Whelton A, Scott H, Stout RL. Effects of Misoprostol on Nonsteroidal Anti-Inflammatory Drug-Induced Renal Insufficiency in Patients with Stable Chronic Renal Failure: A Double-Blind, Crossover Study. Am J Ther 1995;2:858-63.

[53] Plantinga L, Grubbs V, Sarkar U, Hsu CY, Hedgeman E, Robinson B et al. Nonsteroidal anti-inflammatory drug use among persons with chronic kidney disease in the United States. Ann Fam Med 2011;9:423-30.

[54] Whelton A, Stout RL, Spilman PS, Klassen DK. Renal effects of ibuprofen, piroxicam, and sulindac in patients with asymptomatic renal failure. A prospective, randomized, crossover comparison. Ann Intern Med 1990;112:568-76.

[55] Perneger TV, Whelton PK, Klag MJ. Risk of kidney failure associated with the use of acetaminophen, aspirin, and nonsteroidal antiinflammatory drugs. N Engl J Med 1994;331:1675-9.

[56] Baigent C, Landray M, Leaper C, Altmann P, Armitage J, Baxter A et al. First United Kingdom Heart and Renal Protection (UK-HARP-I) study: biochemical efficacy and safety of simvastatin and safety of low-dose aspirin in chronic kidney disease. Am J Kidney Dis 2005;45:473-84.

[57] Sankaran D, Bankovic-Calic N, Cahill L, Yu-Chen Peng C, Ogborn MR, Aukema HM. Late dietary intervention limits benefits of soy protein or flax oil in experimental polycystic kidney disease. Nephron - Experimental Nephrology 2007;106:e122-8.

[58] Scatliff CE, Bankovic-Calic N, Ogborn MR, Aukema HM. Effects of dietary conjugated linoleic acid in advanced experimental polycystic kidney disease. Nephron Exp Nephrol 2008;110:e44-8.

[59] Lee CH, Jang YS, Her SJ, Moon YM, Baek SJ, Eling T. Nordihydroguaiaretic acid, an antioxidant, inhibits transforming growth factor-beta activity through the inhibition of Smad signaling pathway. Exp Cell Res 2003;289:335-41.

[60] Cooke RG, Ahmad N. Suppression of oxytocin-induced prostaglandin F2alpha release after intra-uterine nordihydroguariaretic acid administration in ewes. Prostaglandins Other Lipid Mediat 2000;60:27-33.

[61] Lambert JD, Zhao D, Meyers RO, Kuester RK, Timmermann BN, Dorr RT. Nordihydroguaiaretic acid: hepatotoxicity and detoxification in the mouse. Toxicon 2002;40:1701-8.

[62] Caligiuri SP, Love K, Winter T, Gauthier J, Taylor CG, Blydt-Hansen T et al. Dietary linoleic acid and alpha-linolenic acid differentially affect renal oxylipins and phospholipid fatty acids in diet-induced obese rats. J Nutr 2013;143:1421-31.

Chapter 6

6. Overall conclusions

6.1 Overall discussion and conclusion

Cystic kidney disease is the most common severe hereditary disease, found in approximately 1:500 to 1:1000 individuals in the general population, is the fourth leading cause of renal failure, and it is responsible for 10-15% of the cases of ESRD [1-3]. There is no cure for cystic kidney disease and there is no therapy that is clinically effective in slowing the progression of this disease. Nevertheless, animal models of cystic kidney disease play a very important role in understanding the molecular genetics and cellular pathophysiologic mechanisms responsible for the development and progression of the disease [4,5]. Animal models have also allowed for testing the effects of different types of drugs and dietary interventions on disease progression and laid the foundation for the development of potential therapies for the disease.

The current thesis provides a comprehensive analysis of the effects of disease and diet [soy protein (SP), fish oil (FO) and flax oil (FXO)] on renal oxylipins levels (produced form the three pathways in both the kidney cortex and medulla) and related enzyme activities (Chapter 3) and how these alterations correlate to disease progression and blood pressure in the Han:SPRD-*Cy* rat model of cystic kidney disease. This thesis also investigated for the first time the effect of COX (both isoforms) and LOX inhibitors on oxylipin levels and disease progression in the same rat model.

This thesis (chapter 3) demonstrates that renal COX products and enzyme activities are elevated, but LOX products from both AA and LA were lowered in disease in both the cortex

and medulla in the Han:SPRD-*Cy* rat model of cystic kidney disease. In previous studies, our lab demonstrated that COX levels and phospholipase A_2 are altered in the *pcy* mouse and Han:SPRD-*Cy* rat models of cystic kidney disease [6,7] and select COX products (TXB_2 , PGE_2, and 6-keto $PGF_{1\alpha}$) are higher in diseased compared to normal kidneys in the Han:SPRD-*Cy* rat [6-9]. Furthermore, pharmacologic inhibition of COX2 reduces kidney disease progression in the same model [7,10], and suppresses proliferation of human PKD epithelial cells in cell culture [10]. This thesis confirms the important role of these oxylipins in disease progression and demonstrates that other COX, LOX, and CYP metabolites are also altered by disease in the Han:SPRD-*Cy* rat**.** Furthermore, This thesis demonstrates (chapter 3) that oxylipin alterations occur predominantly in the cortical region of the diseased kidney [11], the part in which most cysts are found in diseased kidneys [12].

This alteration in oxylipins levels in the kidney can affect disease progression via different mechanisms. Previous studies have demonstrated that increased levels of renal COX oxylipins such as TXA_2 and PGE_2 promote epithelial cell proliferation, cystogenesis, and increase cAMP levels [13-16]. The current thesis demonstrated that inhibiting the production of these metabolites and other n-6 oxylipins produced from the COX pathway using dietary interventions and aspirin markedly reduces disease progression in the Han:SPRD-*Cy* rat [11,17].

Since dietary SP and FO slow renal disease progression in the Han:SPRD-*Cy* rat model of cystic kidney disease and affect renal oxylipins through different mechanisms, it was of interest in the current thesis to examine whether they would have additive and/or synergistic effects on disease progression. Consistent with previous studies [8,18-20], chapter 3 shows that both SP and FO individually reduced kidney disease progression. Nonetheless, the current thesis demonstrated that dietary SP is more effective than FO in reducing cyst growth and fibrosis

volume in the cortex and the medulla, and adding FO to the SP diet did not improve its beneficial effects. Both SP and FO feeding were associated with a reduction in renal COX products produced from n-6 fatty acids and feeding rats with FO resulted in increased levels of EPA derived anti-inflammatory oxylipins [11].

It is not known how SP alters kidney disease progression and prostanoid production, since it does not alter the fatty acid composition of the kidney. As discussed in the literature reviewer section (chapter 2), SP has a very unique amino acid composition and contains numerous biologically active compounds such as isoflavones, saponins, and peptides that may contribute to its protective effects [21,22]. It has been demonstrated that the beneficial effects of SP were preserved in experimental kidney disease even after isoflavone depletion of the diet [23], indicating that SP may not reduce disease progression through its isoflavone content. Another proposed mechanism is that SP has more arginine and less methionine (sulphur amino acid) compared to casein protein. Indeed, it has been demonstrated that renal L-arginine and NO synthesis are significantly reduced in patients with chronic kidney disease or ESRD [24] and infusion of L-arginine has been shown to increase RBF and GFR, and decrease proteinuria in experimental animals [25]. However, the administration of L-arginine has been shown to have only a very modest beneficial effect on the development of renal cystic disease in the Han:SPRD-*Cy* rat [26]. In another study, hemp and pea protein had the same levels of methionine and higher levels of L-arginine compared to the SP diet but had much different effects on disease progression and blood pressure in the Han:SPRD-*Cy* rat model [27]. While hemp and SP were beneficial, pea protein was actually detrimental. However, these three plant proteins have different mineral composition and have different biologically active compounds that may have played a role in the different effects observed as well.

A plausible mechanism is that the beneficial effect of SP might be related to the bioactive peptides produced via digestion of the proteins. SP contains peptides and proteins such as Bowman-Birk inhibitor (BBI), Kunit inhibitor, and lunasin. These proteins have been reported to reduce total cholesterol levels in the blood, prevent lung tumorigenesis [28], reduce high blood pressure [29], stimulate the immune system [30], inhibit inflammation and PGE_2 production, and reduce reactive oxygen species (ROS), which might contribute to its anti-inflammatory activity and ability to reduce disease progression [31]. Bioactive peptides have also been shown to inhibit angiotensin I converting enzyme activity (ACE), which mediates its effects via changes in oxylipin levels as well as being itself regulated by COX products [32-34]. Future studies to investigate the effect of different SP derived peptides on kidney disease progression and oxylipin levels in the kidney warrant further investigation.

Feeding rats with FO alters the phospholipid fatty acid composition of the kidney and renal oxylipin levels, resulting in a reduction in proinflammatory oxylipins derived from AA [11]. However, in addition to reducing n-6 COX metabolites, FO also further reduced the already lowered n-6 LOX products in diseased kidneys, which might have reduced its beneficial effect on disease [11]. Dietary FO contains both EPA and DHA, whereas FXO contains ALA, so FXO may not have as strong a LOX oxylipin lowering effect as FO did. Also dietary FXO reduces kidney disease progression in the Han:SPRD-*Cy* rat and in *pcy* mouse models of cystic kidney disease [35,36]. In the latter model, the beneficial effect of FXO on disease was associated with a reduction in the elevated n-6 COX oxylipins, but it did not alter the n-6 LOX oxylipins and increased the lowered levels of LOX oxylipins produced from n-3 fatty acids, suggesting that FXO may be more effective than fish oil in this regard [11,36]. Therefore, it was of interest in

the current thesis to examine the effects of combining FXO with SP on disease progression and renal oxylipin levels produced from all three pathways in the Han:SPRD-*Cy* model.

The results of this study (chapter 4) shows that dietary SP reduced the loss in renal function and lowered the elevated blood pressure in diseased rats and FXO did not provide further disease protection. Adding FXO to the SP diet altered the phospholipid fatty acid composition of the kidney and resulted in higher levels of ALA, EPA, and DPA. While FXO did not alter the renal LA and DHA levels, it increased the levels of ALA- and DHA-derived LOX oxylipins, and increased the LA- and EPA-derived CYP oxylipins, in diseased kidneys. What is fascinating is the increased levels of DHA-derived LOX oxylipins with feeding FO despite the fact the DHA levels in the kidney were not affected by FXO feeding, indicating that the tissue fatty acid composition does not always correlate to oxylipin levels [36,37]. Similar findings were reported in the *pcy* mouse model of cystic kidney disease [36]. This shows that ALA was converted to DHA in amounts that are enough to restore the levels of DHA oxylipins and suggests that the conversion of ALA to DHA may be regulated by the need for oxylipin production [36].

As for LOX products, consistent with chapter 3 [11,17], this study demonstrates that renal LOX oxylipins, including DHA-derived LOX oxylipins, were reduced by disease. It has been suggested that the activities of the COX and LOX are regulated by the availability of the substrate [38]. However, in the current study the levels of all fatty acids were lower in diseased compared to normal rats but AA-derived COX oxylipins were higher and n-6 and n-3- PUFAs derived LOX and CYP oxylipins were lower in diseased kidneys. It could be speculated that the high levels of COX products resulted in reduced levels of LOX products in the diseased kidneys where both COX and LOX are competing for the same substrate. This increased demand by the

COX pathway also could explain the reduction in the n-3 and n-6 PUFAs LOX metabolites, as all n-3 and n-6 PUFAs were reduced in the cortex of diseased compared to normal kidneys. However, the presence of potential cross-talk between the COX and the LOX pathways or another independent disease effect on LOX, also may explain the reduction in LOX products in diseased kidneys. It has been shown that the LOX product (12-HETE) activates COX2 protein expression in pancreatic β cells [39] and in mesangial cells in a time and dose dependent manner [38]. In diabetic nephropathy, it is has been demonstrated that COX and the 12/15-LOX products cross- activate the corresponding enzymes and thereby greatly augment the process involved in kidney disease progression [38].

Among the important finding of this research was that diseased compared to normal kidneys have lower n-3 and n-6 PUFA-derived CYP oxylipins. Dietary SP increased the levels of LA-derived CYP oxylipins (9,10 and 12,13-DiHOME), while FXO increased the levels of LA- (12,13-DiHOME) and EPA-derived (18-HEPE) CYP oxylipins in diseased kidneys. Some studies have reported that some of these oxylipins have anti-inflammatory, anti-fibrotic, natriuretic, anti-apoptotic, and antihypertensive properties, and contribute to maintenance of renal hemodynamic function [40-42]. Therefore, the reduction observed in the oxylipins produced from the CYP pathways may be related to the elevated blood pressure and disease progression in these rats. Similar to the reduced renal DiHETrE in the rats in the current study, plasma levels of epoxy-eicosatrienoic acid, a CYP oxylipin that is metabolized to DiHETrE, are lower in patients with cystic kidney disease [43].

The effect of SP in the first study was stronger than the 2nd study in terms of reducing cyst and fibrosis volume [11]. This discrepancy in SP effects between studies suggests that the beneficial effects of SP may be mediated by multiple components within it [21,22], [32-34].

Some studies have demonstrated that the beneficial effect of SP could be mediated by its isoflavone content [31,44]. Isoflavones have many properties through which it could affect kidney disease progression, including its vasodilator effects, inhibition of protein tyrosine kinase, ACE inhibition, inhibition of cell proliferation and nitric oxide production, inhibition of Na^+-K^+-Cl^- cotransporter of the thick ascending limb of the Henle's loop, and its antioxidant effect [45]. Studies have used different types and doses of isoflavones to test these effects. However, the optimal dose still needs to be determined.

Rats that were given genistein at 5, 100, and 500 µg/g feed had a significant dose-dependent increase in total genistein levels in different organs including brain, liver and kidney [46]. Similar results were reported for daidzein in another study in which daidzein was found to be three- to fivefold higher in the kidney than in plasma [47]. This data points to the high levels of uptake and storage of isoflavones and metabolites in the kidney and other tissues and indicate a possible toxic effect if these isoflavones were taken at higher doses [47]. Thus, the effect of high isoflavones intake could be as harmful on the kidney as a high protein diet [48].

Based on these observations, we analyzed the levels of isoflavones in the SP diets in the 1st and 2nd studies and found that the levels of genestin and daidzin (the glycosylated forms of genistein and daidzein) consumed by rats in the 2nd study were higher compared to the 1st study. The average of genestin consumed in the 1st study per day was 27 mg/kg/day (5.4 mg/day) compared to 47 mg/kg/day (9.3 mg/day) in the 2nd study. For daidzin, it was 15 mg/kg/day (2.9 mg/day) in the 1st study compared to 27 mg/kg/day (5.4 mg/day) in the 2nd study.

The effect of high genestin and daidzin on the kidney is not well investigated, where most of the studies were conducted using genistein and daidzein. It is generally accepted that the

aglycone form of isoflavones has the highest estrogenic activity. However, glucosides are quickly hydrolyzed to produce the aglycone form, so administration of either the glucoside or aglycone leads to absorption of the biologically active aglycone [49]. It has been shown that oral exposure of neonatal mice to genestin (25mg/kg) results in elevated estrogenic activity similar to the response to a subcutaneous dose (20 mg/kg) of genistein [49]. In the *pcy* mouse model of cystic kidney disease, it has been reported that genistein supplementation of a casein-based diet did not reduce kidney disease progression and that the beneficial effect of SP may be unrelated to its genistein content.[44]. However, doses between 25-75 mg/kg of genestin results in adverse consequences on the female reproductive system in mice [49]. Based on the previous evidence, it could be speculated that the high levels of these isoflavones in the 2nd study might have reduced the beneficial effects of the SP diet.

We have previously demonstrated that the benefit of SP in cystic kidney disease is preserved after isoflavone depletion of diet [23]. However, none of the previous studies investigated the effect of high levels of isoflavones (such as genestin and daidzin) on the kidney. In SP, genistein is mainly present as a glycoside, genistin; in spite of this, most studies have focused on genistein. More dose-dependent studies are needed to investigate the effects of these isoflavones (genestin and daidzin) on kidney disease progression in this renal disorder.

To directly investigate the role of COX and LOX products in this disorder, the COX and LOX pathways were inhibited with a low dose of aspirin or NDGA, respectively. The results of this study are presented in chapter 5 and it demonstrates that inhibition of both COX isoforms using a low dose of aspirin suppresses the elevated levels of oxylipins produced from the COX pathway in diseased kidneys and results in a reduction in cyst volume and water content in diseased kidneys, while further reduction of LOX products with NDGA does not worsen disease

progression in this renal disorder (chapter 5) [17]. There is no cure for cystic kidney disease and there is no therapy that is clinically effective in slowing its progression [2,50]. However, our research demonstrates that a human equivalent dose equal to less than one regular strength aspirin per day slows disease progression in this rat model of cystic kidney disease, an important finding that will open the door for more research and clinical trials to look for treatment options that target oxylipin production in the kidney.

In conclusion, our research demonstrates that COX oxylipins are elevated and n-6 derived LOX metabolites are reduced by disease in this model of CKD, and demonstrates that n-3 PUFA derived LOX oxylipins, including those derived from ALA and DHA are also lower in diseased kidneys. Furthermore, the current study also reveals that CYP derived oxylipins are also lowered by disease in this renal disorder. The beneficial effect of SP on disease was associated with amelioration of several oxylipin alterations in parallel with a reduction in kidney disease production, improvement in kidney function and blood pressure. However, combining dietary SP with FO or FXO improved some but worsened other oxylipin alterations and did not provide further disease protection. Furthermore, both COX1 and COX2 activities are elevated in diseased kidneys and blocking this pathway using aspirin reduced disease progression in the Han:SPRD-*Cy* rat. Thus, oxylipins play a very important role in kidney disease progression and may be a good target for future treatment of this renal disorder.

The main (novel) findings of the this thesis are outlined below:

- The current thesis provided evidence that renal oxylipins produced from the COX, LOX, and CYP are altered in cystic kidney disease. While n-6 COX products were elevated, both n-6 and n-3 LOX and CYP metabolites were lowered in diseased

compared to normal Han:SPRD-*Cy* rats. This is very important since we showed in the current study that inhibiting the COX enzyme using aspirin reduced disease progression in this model. The same effect was observed in rats fed the SP diet. These finding are very important for the development of a potential treatment for this renal disorder that targets these alterations in oxylipin metabolism within diseased kidneys.

- Both COX1 and COX2 levels are elevated in the diseased kidneys of the Han:SPRD-*Cy* rat. Given the concerns surrounding the cardiovascular safety of selective COX2 inhibitors, aspirin (dual inhibitor) may provide a safer potential treatment option for cystic kidney disease. However, before we can come up with any recommendations the findings of the current study still need to be replicated in humans.
- The beneficial effect of the SP was primarily in the cortex, the region in which most cysts are found in Cy/+ kidneys [11,12], and was associated with a reduction in the elevated levels of COX1 protein levels and activity in this region. This shows that oxylipin production is not homogenous within the kidney. Despite the fact that the medulla had more oxylipins than the cortex, the alteration in oxylipin production was more noticeable in the cortex than the medulla. Similarly, the effect of SP on disease progression and oxylipins was stronger in the cortex than in the medulla.
- In addition to reducing the increased COX products in diseased kidneys, dietary SP also was associated with amelioration of the reduced LOX products in diseased compared to normal kidneys in the Han:SPRD-*Cy* rat.

- Dietary FO reduced both COX and LOX products produced from n-6 fatty acids and increased COX metabolites produced from n-3 fatty acids, but was not as effective in slowing disease progression as dietary SP which reduced COX and elevated LOX products in diseased kidneys. This is very important to note because it shows that dietary interventions that reduce COX product but not LOX oxylipins may be more beneficial and more effective in reducing disease progression in the Han:SPRD-*Cy* rat model of cystic kidney disease.
- As expected, inhibition of both COX isoforms using a low dose of aspirin mitigated the elevated COX products in diseased kidneys and reduced renal cyst growth in this model of cystic kidney disease. Investigating the effects of different doses of aspirin (dose dependent study) in an orthologous model of cystic kidney disease will provide more information about the efficacy and safety of aspirin in treating this renal disorder.
- Further reduction of LOX products did not worsen disease progression in this renal disorder. Therefore, we reject the hypothesis that NDGA will accelerate kidney disease progression in this model.
- CYP derived oxylipins from both n-6 and n-3 fatty acids are lower in cystic compared to normal kidneys. Dietary SP ameliorated some of these alterations, while dietary FXO improved some and worsened others. But the reduction in COX oxylipins by SP and FXO was associated with improved kidney function and a reduction in diastolic and mean arterial blood pressure in diseased rats.

6.2 Summary of findings (re Figures 1.1-1.3)

Study-1

	COX products	LOX products
Diseased Kidney	**Higher**	**Lower**
SP	**Reduced by SP**	**Increased by SP**
----------Reduction in kidney disease progression----------		
FO	**N6 products reduced by FO**	**Reduced by FO**
Adding FO to SP	**Does not improve the beneficial effect of SP**	

Study-2

	COX products	LOX products	CYP products
Diseased Kidney	**Higher**	**Lower**	**Lower**
SP	**Reduced by SP**	**Increased by SP**	**Increased by SP**
----------Reduction in kidney disease progression----------			
FXO	**Reduced n6 products by FXO**	**Improved some and worsened others**	**Improved some and worsened others**

Study-3

	COX products	LOX products
Diseased Kidney	**Higher**	**Lower**
Aspirin	**Reduce COX products and disease progression**	
NDGA	**Reduced some COX products**	**Reduce LOX products, no effect on disease progression**

6.3 Significance of the research

We believe that the use of dietary and pharmacological approaches to investigate the effect of diet and disease on oxylipin levels and oxylipin related enzymes (chapter 3) in normal and diseased kidney is a key to the development of therapeutic approaches to slow kidney disease progression, improve kidney function, and blood pressure in this renal disorder. This project will add to the knowledge of the effect of diet and disease on renal oxylipins.

This thesis is the first to investigate the effects of diet and disease on oxylipins produced from all three pathways in the kidney. Also, these studies are the first to run separate analysis on the cortex and the medulla in this disorder.

The study also demonstrates the potential use of a common over-the-counter drug (Aspirin) in the treatment of this disorder, a disorder for which there is currently no cure. It also provides mechanistic information of the oxylipins affected by aspirin treatment, demonstrating that it reduces the elevated COX products in diseased kidneys. Furthermore, it shows that lowering the already lower LOX products does not worsen the disease, providing further mechanistic evidence for the importance of COX products in this disease.

Food and pharmaceutical industries will benefit from the scientific knowledge presented in this thesis and the published papers. Additionally, the findings of the current thesis will have the potential to contribute to improving quality of life of patients with cystic kidney disease via using a natural health product such as SP.

6.4 Limitations and future directions

6.4.1 Limitations

There are several limitations to the work described in this thesis. Phenotypically the Han:SPRD-*Cy* rat is a model of human ADPKD but genetically, recent studies show that it is an orthologous model of nephronophthisis (NPHP). The Han:SPRD-*Cy* rat is a very well established spontaneous hereditary model of cystic kidney disease in which the macroscopic and histological appearance of kidneys in diseased heterozygous rats closely resembles that of human ADPKD. This model has played a very important role in understanding the pathophysiological mechanisms of the disease. Furthermore, dietary fish FO and SP have been shown to reduce disease progression in this model and we wanted to investigate how these dietary interventions affect disease and renal oxylipin alterations in the same model. Hence, the model was appropriate for achieving the objective of the current study since we were referring to it as a model of cystic kidney disease.

Another limitation of the current study is the amount of SP, FO and FXO used in this study are not the same amount that would be normally consumed by humans. A large amount of SP will have to be consumed by humans to see the beneficial effects observed in our animal studies. Replacing all sources of protein with SP might not be practical.

Another potential limitation of the current study is that we investigated the effect of only one dose of aspirin on disease progression and oxylipin levels in the kidney. However, it is not clear whether a higher dose will have the same beneficial effects on disease or would be detrimental in a long-term study.

Another limitation of the current study was that the study was not designed to determine the main compound(s) in the SP diet that is responsible for its beneficial effects.

6.4.2 Future directions

We have scanned for and detected a large number of oxylipins, but there are more oxylipins that may contribute to disease progression in this renal disorder. Examination of a greater array of oxylipins will be very important to complete the picture of oxylipin profile in diseased rats.

It is not clear whether a higher dose of aspirin than the one used in our study will have the same beneficial effects on disease or would be detrimental in a long-term study. A dose-dependent study is required to establish the maximum safe dose to reduce disease progression using different models of cystic kidney disease. Investigating the effect of aspirin on kidney disease progression and renal oxylipin levels in an orthologous model of ADPKD or NPHP also warrants further investigation.

In the current study we used aspirin to inhibit the production of all COX metabolites; a potential future study is to investigate the effects of inhibiting specific COX derived oxylipins instead of all of them. For example, inhibiting TXB_2 or PGE_2, since they are altered the most by disease. This will give us more information about the role played by these metabolites in disease progression. At the same time, testing the effect of administrating exogenous COX oxylipins (such as TXB_2 and PGE_2) or LOX metabolites (such as HETEs and HDoHE) to animals with cystic kidney disease will also provide more information about the role played by these oxylipin in disease progression.

In addition to its unique amino acid composition, SP has many biologically active compounds that can contribute to its reno-protective effects; investigating the impact of specific

peptides and proteins derived from SP as well as amino acids (such as arginine and glycine) on kidney disease progression and renal oxylipin levels warrants further investigation.

The type of oxylipin produced depends mainly on the PUFA being oxidized and the enzyme metabolizing the PUFA. The most well-known precursor of oxylipins is the 20 carbon, omega-6 PUFA, AA. However, oxylipins can also be produced from the omega-3 PUFA, EPA or ALA. It is not known how different levels of n-3 PUFA alter oxylipin profiles in cystic kidney disease. The amount of oil used in the current study is high in EPA and ALA and might not reflect normal human consumption. Therefore, studying the effects of lower levels of EPA, DHA, and ALA in FO and FXO on renal oxylipin levels and disease progression in an orthologous model of cystic kidney disease will be another excellent future step. The lower levels of dietary EPA, DHA, and ALA will provide novel information about how different levels of dietary EPA and ALA can influence the renal oxylipin profile. Thus, such studies will allow for potential diagnostic and treatment measures in this renal disorder.

To advance the research in this area to human PKD, the first step would be to study the serum oxylipin levels in humans with and without PKD. If this study confirms similar patterns that have been identified in this thesis, this will help in establishing treatment agents that target these alterations in PKD, reducing disease progression and improving quality of life.

6.4 References

[1] Niemczyk M, Niemczyk S, Paczek L. Autosomal dominant polycystic kidney disease and transplantation. Ann Transplant 2009;14:86-90.

[2] Wuthrich RP, Mei C. Pharmacological management of polycystic kidney disease. Expert Opin Pharmacother 2014;15:1085-95.

[3] Chen D, Ma Y, Wang X, Yu S, Li L, Dai B et al. Clinical characteristics and disease predictors of a large Chinese cohort of patients with autosomal dominant polycystic kidney disease. PLoS One 2014;9:e92232.

[4] Nagao S, Kugita M, Yoshihara D, Yamaguchi T. Animal models for human polycystic kidney disease. Exp Anim 2012;61:477-88.

[5] Torres VE, Harris PC. Polycystic kidney disease: genes, proteins, animal models, disease mechanisms and therapeutic opportunities. J Intern Med 2007;261:17-31.

[6] Aukema HM, Adolphe J, Mishra S, Jiang J, Cuozzo FP, Ogborn MR. Alterations in renal cytosolic phospholipase A2 and cyclooxygenases in polycystic kidney disease. FASEB J 2003;17:298-300.

[7] Sankaran D, Bankovic-Calic N, Ogborn MR, Crow G, Aukema HM. Selective COX-2 inhibition markedly slows disease progression and attenuates altered prostanoid production in Han:SPRD-cy rats with inherited kidney disease. Am J Physiol Renal Physiol 2007;293:F821-30.

[8] Peng CY, Sankaran D, Ogborn MR, Aukema HM. Dietary Soy Protein Selectively Reduces Renal Prostanoids and Cyclooxygenases in Polycystic Kidney Disease. Exp Biol Med 2009;234:737-43.

[9] Warford-Woolgar L, Peng CY, Shuhyta J, Wakefield A, Sankaran D, Ogborn M et al. Selectivity of cyclooxygenase isoform activity and prostanoid production in normal and diseased Han:SPRD-cy rat kidneys. Am J Physiol Renal Physiol 2006;290:F897-904.

[10] Xu T, Wang N-, Fu L-, Ye C-, Yu S-, Mei C-. Celecoxib inhibits growth of human autosomal dominant polycystic kidney cyst-lining epithelial cells through the VEGF/Raf/MAPK/ERK signaling pathway. Mol Biol Rep 2012;39:7743-53.

[11] Ibrahim NH, Jia Y, Devassy JG, Yamaguchi T, Aukema HM. Renal cyclooxygenase and lipoxygenase products are altered in polycystic kidneys and by dietary soy protein and fish oil treatment in the Han:SPRD-Cy rat. Mol Nutr Food Res 2014;58:768-81

[12] Schafer K, Gretz N, Bader M, Oberbaumer I, Eckardt KU, Kriz W et al. Characterization of the Han:SPRD rat model for hereditary polycystic kidney disease. Kidney Int 1994;46:134-52.

[13] Elberg D, Turman MA, Pullen N, Elberg G. Prostaglandin E 2 stimulates cystogenesis through EP4 receptor in IMCD-3 cells. Prostaglandins and Other Lipid Mediators 2012;98:11-6.

[14] Elberg G, Elberg D, Lewis TV, Guruswamy S, Chen L, Logan CJ et al. EP 2 receptor mediates PGE 2-induced cystogenesis of human renal epithelial cells. American Journal of Physiology - Renal Physiology 2007;293:F1622-32.

[15] Mangoo-Karim R, Ye M, Wallace DP, Grantham JJ, Sullivan LP. Anion secretion drives fluid secretion by monolayers of cultured human polycystic cells. American Journal of Physiology - Renal Fluid and Electrolyte Physiology 1995;269:F381-8.

[16] Belibi FA, Reif G, Wallace DP, Yamaguchi T, Olsen L, Li H et al. Cyclic AMP promotes growth and secretion in human polycystic kidney epithelial cells. Kidney Int 2004;66:964-73.

[17] Ibrahim NH, Gregoire M, Devassy JG, Wu Y, Yoshihara D, Yamaguchi T et al. Cyclooxygenase product inhibition with acetylsalicylic acid slows disease progression in the Han:SPRD-Cy rat model of polycystic kidney disease. Prostaglandins Other Lipid Mediat 2014;116-117C:19-25.

[18] Cahill LE, Peng CY, Bankovic-Calic N, Sankaran D, Ogborn MR, Aukema HM. Dietary soya protein during pregnancy and lactation in rats with hereditary kidney disease attenuates disease progression in offspring. Br J Nutr 2007;97:77-84.

[19] Ogborn MR, Nitschmann E, Weiler HA, Bankovic-Calic N. Modification of polycystic kidney disease and fatty acid status by soy protein diet. Kidney Int 2000;57:159-66.

[20] Aukema HM, Housini I. Dietary soy protein effects on disease and IGF-I in male and female Han:SPRD-cy rats. Kidney Int 2001;59:52-61.

[21] Watanabe G, Tomiyama H, Doba N. Effects of oral administration of L-arginine on renal function in patients with heart failure. J Hypertens 2000;18:229-34.

[22] Gabardi S, Munz K, Ulbricht C. A review of dietary supplement-induced renal dysfunction. Clin J Am Soc Nephrol 2007;2:757-65.

[23] Ogborn MR, Nitschmann E, Bankovic-Calic N, Weiler HA, Aukema HM. Dietary soy protein benefit in experimental kidney disease is preserved after isoflavone depletion of diet. Exp Biol Med 2010;235:1315-20.

[24] Schmidt RJ, Yokota S, Tracy TS, Sorkin MI, Baylis C. Nitric oxide production is low in end-stage renal disease patients on peritoneal dialysis. Am J Physiol 1999;276:F794-7.

[25] Klahr S, Morrissey J. L-arginine as a therapeutic tool in kidney disease. Semin Nephrol 2004;24:389-94.

[26] Yoshida I, Bengal R, Torres VE. Gender-dependent effect of L-NAME on polycystic kidney disease in Han:SPRD rats. Am J Kidney Dis 2000;35:930-6.

[27] Aukema HM, Gauthier J, Roy M, Jia Y, Li H, Aluko RE. Distinctive effects of plant protein sources on renal disease progression and associated cardiac hypertrophy in experimental kidney disease. Molecular Nutrition and Food Research 2011;55:1044-51.

[28] Kennedy AR, Kritchevsky D, Shen WC. Effects of spermine-conjugated Bowman-Birk inhibitor (spermine-BBI) on carcinogenesis and cholesterol biosynthesis in mice. Pharm Res 2003;20:1908-10.

[29] Mallikarjun Gouda KG, Gowda LR, Rao AG, Prakash V. Angiotensin I-converting enzyme inhibitory peptide derived from glycinin, the 11S globulin of soybean (Glycine max). J Agric Food Chem 2006;54:4568-73.

[30] Tsuruki T, Kishi K, Takahashi M, Tanaka M, Matsukawa T, Yoshikawa M. Soymetide, an immunostimulating peptide derived from soybean beta-conglycinin, is an fMLP agonist. FEBS Lett 2003;540:206-10.

[31] Dia VP, Berhow MA, Gonzalez De Mejia E. Bowman-Birk inhibitor and genistein among soy compounds that synergistically inhibit nitric oxide and prostaglandin E2 pathways in lipopolysaccharide-induced macrophages. J Agric Food Chem 2008;56:11707-17.

[32] Jensen BL, Schmid C, Kurtz A. Prostaglandins stimulate renin secretion and renin mRNA in mouse renal juxtaglomerular cells. Am J Physiol 1996;271:F659-69.

[33] Stichtenoth DO, Wagner B, Frolich JC. Effect of selective inhibition of the inducible cyclooxygenase on renin release in healthy volunteers. J Investig Med 1998;46:290-6.

[34] Farzamirad V, Aluko RE. Angiotensin-converting enzyme inhibition and free-radical scavenging properties of cationic peptides derived from soybean protein hydrolysates. Int J Food Sci Nutr 2008;59:428-37.

[35] Ogborn MR, Nitschmann E, Bankovic-Calic N, Weiler HA, Aukema H. Dietary flax oil reduces renal injury, oxidized LDL content, and tissue n-6/n-3 FA ratio in experimental polycystic kidney disease. Lipids 2002;37:1059-65.

[36] Yamaguchi T, Devassy JG, Gabbs M, Ravandi A, Nagao S, Aukema HM. Dietary flax oil rich in alpha-linolenic acid reduces renal disease and oxylipin abnormalities, including formation of docosahexaenoic acid derived oxylipins in the CD1-pcy/pcy mouse model of nephronophthisis. Prostaglandins Leukot Essent Fatty Acids 2015;94:83-9.

[37] Caligiuri SP, Love K, Winter T, Gauthier J, Taylor CG, Blydt-Hansen T et al. Dietary linoleic acid and alpha-linolenic acid differentially affect renal oxylipins and phospholipid fatty acids in diet-induced obese rats. J Nutr 2013;143:1421-31.

[38] Xu ZG, Li SL, Lanting L, Kim YS, Shanmugam N, Reddy MA et al. Relationship between 12/15-lipoxygenase and COX-2 in mesangial cells: potential role in diabetic nephropathy. Kidney Int 2006;69:512-9.

[39] Han X, Chen S, Sun Y, Nadler JL, Bleich D. Induction of cyclooxygenase-2 gene in pancreatic beta-cells by 12-lipoxygenase pathway product 12-hydroxyeicosatetraenoic acid. Mol Endocrinol 2002;16:2145-54.

[40] Makita K, Takahashi K, Karara A, Jacobson HR, Falck JR, Capdevila JH. Experimental and/or genetically controlled alterations of the renal microsomal cytochrome P450 epoxygenase induce hypertension in rats fed a high salt diet. J Clin Invest 1994;94:2414-20.

[41] Imig JD, Zhao X, Zaharis CZ, Olearczyk JJ, Pollock DM, Newman JW et al. An orally active epoxide hydrolase inhibitor lowers blood pressure and provides renal protection in salt-sensitive hypertension. Hypertension 2005;46:975-81.

[42] Imig JD, Zhao X, Capdevila JH, Morisseau C, Hammock BD. Soluble epoxide hydrolase inhibition lowers arterial blood pressure in angiotensin II hypertension. Hypertension 2002;39:690-4.

[43] Lorthioir A, Joannides R, Remy-Jouet I, Freguin-Bouilland C, Iacob M, Roche C et al. Polycystin deficiency induces dopamine-reversible alterations in flow-mediated dilatation and vascular nitric oxide release in humans. Kidney Int 2015;87:465-72.

[44] Tomobe K, Philbrick DJ, Ogborn MR, Takahashi H, Holub BJ. Effect of dietary soy protein and genistein on disease progression in mice with polycystic kidney disease. American Journal of Kidney Diseases 1998;31:55-61.

[45] Anderson JW. Beneficial effects of soy protein consumption for renal function. Asia Pac J Clin Nutr 2008;17 Suppl 1:324-8.

[46] Chang HC, Churchwell MI, Delclos KB, Newbold RR, Doerge DR. Mass spectrometric determination of Genistein tissue distribution in diet-exposed Sprague-Dawley rats. J Nutr 2000;130:1963-70.

[47] Janning P, Schuhmacher US, Upmeier A, Diel P, Michna H, Degen GH et al. Toxicokinetics of the phytoestrogen daidzein in female DA/Han rats. Arch Toxicol 2000;74:421-30.

[48] Jia Y, Hwang SY, House JD, Ogborn MR, Weiler HA, O K et al. Long-term high intake of whole proteins results in renal damage in pigs. J Nutr 2010;140:1646-52.

[49] Jefferson WN, Doerge D, Padilla-Banks E, Woodling KA, Kissling GE, Newbold R. Oral exposure to genistin, the glycosylated form of genistein, during neonatal life adversely affects the female reproductive system. Environ Health Perspect 2009;117:1883-9.

[50] Spithoven EM, Kramer A, Meijer E, Orskov B, Wanner C, Caskey F et al. Analysis of data from the ERA-EDTA Registry indicates that conventional treatments for chronic kidney disease do not reduce the need for renal replacement therapy in autosomal dominant polycystic kidney disease. Kidney Int 2014;86:1244-52.

[51] Yamaguchi T, Lysecki C, Reid A, Nagao S, Aukema HM. Renal cyclooxygenase products are higher and lipoxygenase products are lower in early disease in the *pcy* mouse model of adolescent nephronophthisis. Lipids 2014 49:39-47.

[52] Cowley BD, Gudapaty S, Kraybill AL, Barash BD, Harding MA, Calvet JP et al. Autosomal-Dominant Polycystic Kidney-Disease in the Rat. Kidney Int 1993;43:522-34.

[53] J. Kaspareit-Rittinghausen FD. Animal model of human disease hereditary polycystic kidney disease. 1991;139.

7. Appendices

Appendix 1: Copyright license for previously published materials

This Agreement between Naser H.M. Ibrahim ("You") and John Wiley and Sons ("John Wiley and Sons") consists of your license details and the terms and conditions provided by John Wiley and Sons and Copyright Clearance Center.

Your confirmation email will contain your order number for future reference.

Get the printable license.

License Number	3600841149953
License date	Apr 02, 2015
Licensed Content Publisher	John Wiley and Sons
Licensed Content Publication	Molecular Nutrition & Food Research
Licensed Content Title	Renal cyclooxygenase and lipoxygenase products are altered in polycystic kidneys and by dietary soy protein and fish oil treatment in the Han:SPRD-Cy rat
Licensed Content Author	Naser H. M. Ibrahim and *et al.*
Licensed Content Date	Oct 30, 2013
Licensed Content Pages	14
Type of use	Dissertation/Thesis
Requestor type	Author of this Wiley article
Format	Print and electronic
Portion	Full article
Will you be translating?	No
Title of your thesis / dissertation	Effects of Diet and Disease on Renal Oxylipins and Related Enzymes in the Han:SPRD-Cy rat Model of Cystic Kidney Disease
Expected completion date	May 2015
Expected size (number of pages)	220
Requestor Location	
Billing Type	Invoice
Billing address	
Total	0.00 CAD

This is a License Agreement between Naser H.M. Ibrahim ("You") and Elsevier ("Elsevier"). The license consists of your order details, the terms and conditions provided by Elsevier, and the payment terms and conditions.

Get the printable license.

License Number	3603161441331
License date	Apr 06, 2015
Licensed content publisher	Elsevier
Licensed content publication	Prostaglandins and Other Lipid Mediators
Licensed content title	Cyclooxygenase product inhibition with acetylsalicylic acid slows disease progression in the Han:SPRD-Cy rat model of polycystic kidney disease
Licensed content author	None
Licensed content date	January–March 2015
Licensed content volume number	116
Licensed content issue number	n/a
Number of pages	7
Type of Use	reuse in a thesis/dissertation
Portion	full article
Format	both print and electronic
Are you the author of this Elsevier article?	Yes
Will you be translating?	No
Title of your thesis/dissertation	Effects of Diet and Disease on Renal Oxylipins and Related Enzymes in the Han:SPRD-Cy rat Model of Cystic Kidney Disease
Expected completion date	May 2015
Estimated size (number of pages)	220
Elsevier VAT number	GB 494 6272 12
Permissions price	0.00 USD
VAT/Local Sales Tax	0.00 USD / 0.00 GBP
Total	0.00 USD

April 28, 2015

Co-authors permission request for articles to be used in PhD thesis.

Dear Co-authors,

I am requesting your permission to include in my PhD thesis the following manuscripts, of which you are co-authors:

1) Ibrahim NH, Jia Y, Devassy JG, Yamaguchi T, Aukema HM. Renal cyclooxygenase and lipoxygenase products are altered in polycystic kidneys and by dietary soy protein and fish oil treatment in the Han:SPRD-Cy rat. Mol Nutr Food Res, 2013.

2) Ibrahim NH, Gregoire M, Devassy JG, Wu Y, Yoshihara D, Yamaguchi T, Nagao S, and Aukema HM. Cyclooxygenase product inhibition with acetylsalicylic acid slows disease progression in the Han:SPRD-Cy rat model of polycystic kidney disease. Prostaglandins Other Lipid Mediators, 2014.

3) Ibrahim NH, Yoshihara D, Nagao S, Yamaguchi T, and Aukema HM. Dietary soy protein and flax oil effects on cystic kidney disease and oxylipin alterations in the Han:SPRD-*Cy* rat. Submitted for publication to Prostaglandins and Other Lipid Mediators; March, 2015.

Thesis title: Effects of Diet and Disease on Renal Oxylipins and Related Enzymes in the Han:SPRD-*Cy* rat Model of Cystic Kidney Disease.

My thesis will be posted electronically and will be accessible for free to a worldwide audience from the University of Manitoba's digital repository called MSpace located at https://mspace.lib.umanitoba.ca/index.jsp. I do not expect any commercial profits from my thesis.

Your name will appear within the designated chapter of my thesis in recognition of your contribution to this work.

According to the University of Manitoba regulation, I need your permission to include this/these manuscripts/manuscripts as part of my thesis.

Thank you,

Naser Ibrahim

Your Name: [redacted] Signature: [redacted]

I am a co-author on manuscript number: (1) ✓ (2) ✓ or (3) ✓

April 28, 2015

Co-authors permission request for articles to be used in PhD thesis.

Dear Co-authors,

I am requesting your permission to include in my PhD thesis the following manuscripts, of which you are co-authors:

1) Ibrahim NH, Jia Y, Devassy JG, Yamaguchi T, Aukema HM. Renal cyclooxygenase and lipoxygenase products are altered in polycystic kidneys and by dietary soy protein and fish oil treatment in the Han:SPRD-Cy rat. Mol Nutr Food Res, 2013.

2) Ibrahim NH, Gregoire M, Devassy JG, Wu Y, Yoshihara D, Yamaguchi T, Nagao S, and Aukema HM. Cyclooxygenase product inhibition with acetylsalicylic acid slows disease progression in the Han:SPRD-Cy rat model of polycystic kidney disease. Prostaglandins Other Lipid Mediators, 2014.

3) Ibrahim NH, Yoshihara D, Nagao S, Yamaguchi T, and Aukema HM. Dietary soy protein and flax oil effects on cystic kidney disease and oxylipin alterations in the Han:SPRD-*Cy* rat. Submitted for publication to Prostaglandins and Other Lipid Mediators; March, 2015.

Thesis title: Effects of Diet and Disease on Renal Oxylipins and Related Enzymes in the Han:SPRD-*Cy* rat Model of Cystic Kidney Disease.

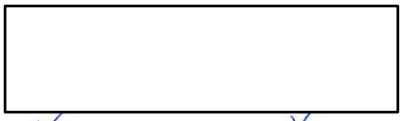
My thesis will be posted electronically and will be accessible for free to a worldwide audience from the University of Manitoba's digital repository called MSpace located at https://mspace.lib.umanitoba.ca/index.jsp. I do not expect any commercial profits from my thesis.

Your name will appear within the designated chapter of my thesis in recognition of your contribution to this work.

According to the University of Manitoba regulation, I need your permission to include this/these manuscripts/manuscripts as part of my thesis.

Thank you,

Naser Ibrahim

Your Name: Daisuke Yoshihara, Ph.D. Signature: 

I am a co-author on manuscript number: (1)_____(2)_____ or (3)_____

April 28, 2015

Co-authors permission request for articles to be used in PhD thesis.

Dear Co-authors,

I am requesting your permission to include in my PhD thesis the following manuscripts, of which you are co-authors:

1) Ibrahim NH, Jia Y, Devassy JG, Yamaguchi T, Aukema HM. Renal cyclooxygenase and lipoxygenase products are altered in polycystic kidneys and by dietary soy protein and fish oil treatment in the Han:SPRD-Cy rat. Mol Nutr Food Res, 2013.

2) Ibrahim NH, Gregoire M, Devassy JG, Wu Y, Yoshihara D, Yamaguchi T, Nagao S, and Aukema HM. Cyclooxygenase product inhibition with acetylsalicylic acid slows disease progression in the Han:SPRD-Cy rat model of polycystic kidney disease. Prostaglandins Other Lipid Mediators, 2014.

3) Ibrahim NH, Yoshihara D, Nagao S, Yamaguchi T, and Aukema HM. Dietary soy protein and flax oil effects on cystic kidney disease and oxylipin alterations in the Han:SPRD-*Cy* rat. Submitted for publication to Prostaglandins and Other Lipid Mediators; March, 2015.

Thesis title: Effects of Diet and Disease on Renal Oxylipins and Related Enzymes in the Han:SPRD-*Cy* rat Model of Cystic Kidney Disease.

My thesis will be posted electronically and will be accessible for free to a worldwide audience from the University of Manitoba's digital repository called MSpace located at https://mspace.lib.umanitoba.ca/index.jsp. I do not expect any commercial profits from my thesis.

Your name will appear within the designated chapter of my thesis in recognition of your contribution to this work.

According to the University of Manitoba regulation, I need your permission to include this/these manuscripts/manuscripts as part of my thesis.

Thank you,

Naser Ibrahim

Your Name: Jessay Devassy Signature:--  ----

I am a co-author on manuscript number: (1)__✓_____(2)___✓_____ or (3)_____

April 28, 2015

Co-authors permission request for articles to be used in PhD thesis.

Dear Co-authors,

I am requesting your permission to include in my PhD thesis the following manuscripts, of which you are co-authors:

1) Ibrahim NH, Jia Y, Devassy JG, Yamaguchi T, Aukema HM. Renal cyclooxygenase and lipoxygenase products are altered in polycystic kidneys and by dietary soy protein and fish oil treatment in the Han:SPRD-Cy rat. Mol Nutr Food Res, 2013.

2) Ibrahim NH, Gregoire M, Devassy JG, Wu Y, Yoshihara D, Yamaguchi T, Nagao S, and Aukema HM. Cyclooxygenase product inhibition with acetylsalicylic acid slows disease progression in the Han:SPRD-Cy rat model of polycystic kidney disease. Prostaglandins Other Lipid Mediators, 2014.

3) Ibrahim NH, Yoshihara D, Nagao S, Yamaguchi T, and Aukema HM. Dietary soy protein and flax oil effects on cystic kidney disease and oxylipin alterations in the Han:SPRD-*Cy* rat. Submitted for publication to Prostaglandins and Other Lipid Mediators; March, 2015.

Thesis title: Effects of Diet and Disease on Renal Oxylipins and Related Enzymes in the Han:SPRD-*Cy* rat Model of Cystic Kidney Disease.

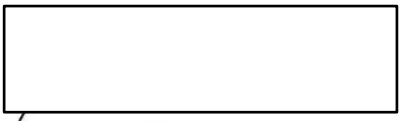
My thesis will be posted electronically and will be accessible for free to a worldwide audience from the University of Manitoba's digital repository called MSpace located at https://mspace.lib.umanitoba.ca/index.jsp. I do not expect any commercial profits from my thesis.

Your name will appear within the designated chapter of my thesis in recognition of your contribution to this work.

According to the University of Manitoba regulation, I need your permission to include this/these manuscripts/manuscripts as part of my thesis.

Thank you,

Naser Ibrahim

Your Name: Melanie Gregoire Signature: 

I am a co-author on manuscript number: (1)_____(2)__✓__ or (3)_____

April 28, 2015

Co-authors permission request for articles to be used in PhD thesis.

Dear Co-authors,

I am requesting your permission to include in my PhD thesis the following manuscripts, of which you are co-authors:

1) Ibrahim NH, Jia Y, Devassy JG, Yamaguchi T, Aukema HM. Renal cyclooxygenase and lipoxygenase products are altered in polycystic kidneys and by dietary soy protein and fish oil treatment in the Han:SPRD-Cy rat. Mol Nutr Food Res, 2013.

2) Ibrahim NH, Gregoire M, Devassy JG, Wu Y, Yoshihara D, Yamaguchi T, Nagao S, and Aukema HM. Cyclooxygenase product inhibition with acetylsalicylic acid slows disease progression in the Han:SPRD-Cy rat model of polycystic kidney disease. Prostaglandins Other Lipid Mediators, 2014.

3) Ibrahim NH, Yoshihara D, Nagao S, Yamaguchi T, and Aukema HM. Dietary soy protein and flax oil effects on cystic kidney disease and oxylipin alterations in the Han:SPRD-*Cy* rat. Submitted for publication to Prostaglandins and Other Lipid Mediators; March, 2015.

Thesis title: Effects of Diet and Disease on Renal Oxylipins and Related Enzymes in the Han:SPRD-*Cy* rat Model of Cystic Kidney Disease.

My thesis will be posted electronically and will be accessible for free to a worldwide audience from the University of Manitoba's digital repository called MSpace located at https://mspace.lib.umanitoba.ca/index.jsp. I do not expect any commercial profits from my thesis.

Your name will appear within the designated chapter of my thesis in recognition of your contribution to this work.

According to the University of Manitoba regulation, I need your permission to include this/these manuscripts/manuscripts as part of my thesis.

Thank you,

Naser Ibrahim

Your Name: Shizuko Nagao, Ph.D. Signature:

I am a co-author on manuscript number: (1)_____ (2) _____ or (3)_____

April 28, 2015

Co-authors permission request for articles to be used in PhD thesis.

Dear Co-authors,

I am requesting your permission to include in my PhD thesis the following manuscripts, of which you are co-authors:

1) Ibrahim NH, Jia Y, Devassy JG, Yamaguchi T, Aukema HM. Renal cyclooxygenase and lipoxygenase products are altered in polycystic kidneys and by dietary soy protein and fish oil treatment in the Han:SPRD-Cy rat. Mol Nutr Food Res, 2013.

2) Ibrahim NH, Gregoire M, Devassy JG, Wu Y, Yoshihara D, Yamaguchi T, Nagao S, and Aukema HM. Cyclooxygenase product inhibition with acetylsalicylic acid slows disease progression in the Han:SPRD-Cy rat model of polycystic kidney disease. Prostaglandins Other Lipid Mediators, 2014.

3) Ibrahim NH, Yoshihara D, Nagao S, Yamaguchi T, and Aukema HM. Dietary soy protein and flax oil effects on cystic kidney disease and oxylipin alterations in the Han:SPRD-*Cy* rat. Submitted for publication to Prostaglandins and Other Lipid Mediators; March, 2015.

Thesis title: Effects of Diet and Disease on Renal Oxylipins and Related Enzymes in the Han:SPRD-*Cy* rat Model of Cystic Kidney Disease.

My thesis will be posted electronically and will be accessible for free to a worldwide audience from the University of Manitoba's digital repository called MSpace located at https://mspace.lib.umanitoba.ca/index.jsp. I do not expect any commercial profits from my thesis.

Your name will appear within the designated chapter of my thesis in recognition of your contribution to this work.

According to the University of Manitoba regulation, I need your permission to include this/these manuscripts/manuscripts as part of my thesis.

Thank you,

Naser Ibrahim

Your Name: Tamio Yamaguchi Signature: 

I am a co-author on manuscript number: (1) ✓ (2) ✓ or (3) ✓

Appendix 2: Method details

- Blood pressure measurements (IITC Model 29 SSP)
- Histology protocol
- Serum creatinine assay lab protocol
- Serum cystatin C determination
- Homogenization of lyophilized kidney tissue (lab protocol)
- Oxylipin analysis via LC-MS/MS fraction
- Western immunoblotting (lab protocol)
- Total protein determination (lab protocol)
- Kidney fatty acid analysis (lab protocol)

Blood pressure measurements (IITC Model 29 SSP)

Before using the blood pressure machine, make sure that you have everything you need for the process.

What do you need?

1- Anaesthesia machine with induction chamber, charcoal canister and Isoflurane (make sure that you have the small loading tool for the Isoflurane)
2- Oxygen tank
3- Computer with the Acqknowledge-3 software downloaded on it
4- Fume hood (make sure it is working) or a proper scavenger system

Setting up the anaesthesia machine:

1- Connect the anaesthesia machine to the oxygen tank and make sure that you have enough oxygen pressure in the cylinder (more than 500 litres on the oxygen regulator)
2- Connect the anaesthesia machine to the induction chamber
3- Connect the charcoal filter to the induction chamber (passive scavenger system). Please remember to weigh the filter before using it; the filter should be changed if it has been used for more than 12 hours or its weight increased by 50 gm.
4- Put the induction chamber in the fume hood (active scavenger system)
5- Check if you have enough Isoflurane level, if not add some

The blood pressure machine has 4 parts (please see the attached pictures):

1- The control box (main box) (Figure A.1)
2- The channel box (2 parts) ((Figure A.2)
3- The sphygmomanometer (the cuff, the air bumper and the pressure meter, gauge) (Figure A.3)
4- The heating chamber ((Figure A.4)

Putting the blood pressure machine together

1- Connect the two parts of the channel box to each other and then connect the control box to the channel box (channel 3 wire goes to the pressure and channel 5 goes to pulse in the control box)
2- Attach the sphygmomanometer to its respective position-input in the control box (input), BNC plug in and plastic tubing. (you can always change the cuff if you think it is too old or there is a hole in it)
3- Connect the blood pressure machine to the computer (USB)

4- There is no connection between the blood pressure machine and the heating chamber (just plug it to the power par)

Blood pressure machine calibration

Pressure channel:

1- Calibration should be made with the filter switch in the OFF position but once calibrated the filter should be ON
2- Open the MP100 drop menu and select " show input value" and keep this window active during the calibration
3- Pressure channel (3): top graph, click on the vertical axis number and change the volt to 0.5 volts
4- When the pressure is zero mmHg on the pressure meter then it should be very small on the input value window (you can test that by pressing on the rubber air bulb (inflating ball) and releasing the air valve in the sphygmomanometer and looking at the gauge and the input value window at the same time)
5- Increase pressure to 300 mmHg and adjust input value to about 5 Volt (it should be about 5 on its own)
6- Decrease pressure to 120 mmHg and with the pressure adjustment knob (in the control box) adjust such that the input value is 2 Volt

Pulse channel:

1- Select the bottom graph (lower part of the screen), which is your pulse recording
2- Switch to Band Pass (control box) and adjust the recording to zero
3- Switch back to low pass and using offset knob mover line back to zero. Offset knob should end up at about 5.
4- Switch back to the Band Pass
5- Pulse gain knob should be between 8-9 (control box)
6- Filter back on (control box)

Measuring blood pressure (Acqknowledge-3 **program)**

1- Select the Acqknowledge-3 program from the desktop
2- Setup 2 channels by clicking on analog channels under the MP100 drop menu use channel 3 for pressure and channel 5 for pulse (please note that channel 1 does not work). Every time you select your channels you have to close the input channels dialog box and open it again to make sure that the channels you have selected still activated and they did not change
3- From the MP100 drop menu select set up a acquisition and select 200 samples/ second and increase the total length of measurement time to 60 seconds.
4- Click on the start icon (lower right end of the screen), the screen will be divided into 2 parts (upper and lower). The upper part is the pressure part (green chart), the lower part is the pulse part (pink chart).

5- Click on the volts area of the vertical axis of the upper part and change the volts to 2 in the dialog box
6- Click on the volts area of the vertical axis of the lower part and change the volts to 0.5 in the dialog box
7- Turn on the oxygen regulator and make sure that the oxygen flow meter is between 2 and 3
8- Put the rat in the induction chamber and turn on the Isoflurane valve to 5
9- Wait for 3 minutes. Turn the Isoflurane valve off (make sure you do)
10- Take the rat out to the heating chamber; pull the tail out of one of the holes of the heating chamber side. (the rat will be unconscious for only 2 minutes; so you have to be quick)
11- Place the blood pressure cuff into the tail and make sure it is at the base of the rat's tail
12- Press the start icon in the software (lower right end of the screen). You should start seeing two lines, green and pink, running across the screen from left to right
13- Inflate the rubber air bulb until the meter on the gauge is between 120 and 220 (one smooth push without stopping)
14- Very slowly release the air valve attached to the bulb
15- You should see a nice pink wave across the lower part of the screen like the one in the figure 4
16- You can take more than one reading
17- When you are satisfied with your graphs click end (lower right end of the screen)
18- Save your diagram under the same ID name of your rat by clicking on file icon and save as
19- You can either read your graph immediately or read it latter when you finish all of your rats (I would recommend reading it immediately)
20- Wait for your rat to fully recover while on the heating chamber. Once awake take him back to his cage
21- Tip, the heating chamber might get really hot, therefore, make sure that the temperature in the heating chamber is below 40 C^0.

.

Figure A.1: The main box

Figure A.2: The channels box

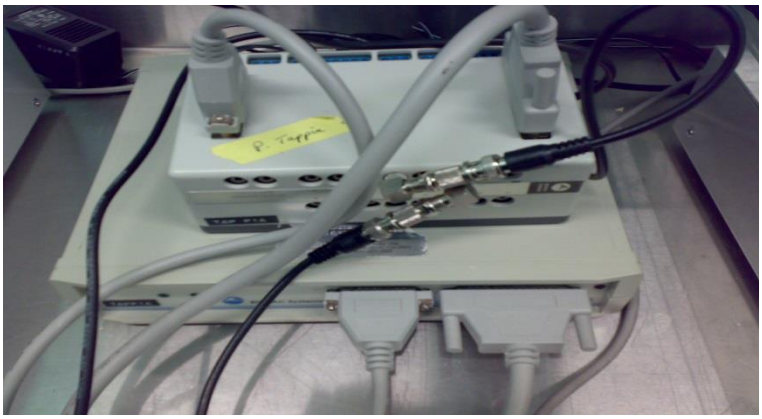


Figure A.3: The sphygmomanometer

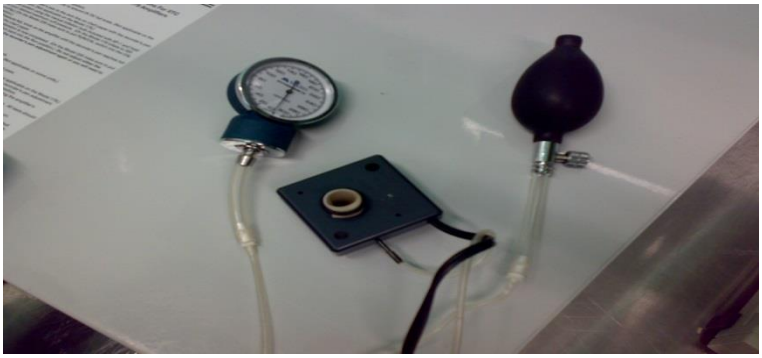
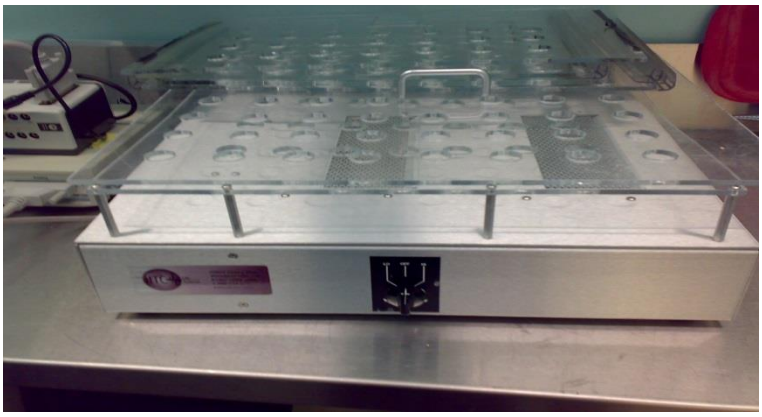


Figure A.4: The heating chamber



Histology Protocol

Use of Microscope

- Take photos of your slides using the Olympus BX60 Microscope
- Open SPOT advanced software.
- Turn on microscope
- Turn on very left black box labelled SPOT
- Place a slide under the microscope
- Make sure you have the same light settings every time you take photographs
- Take representative photos of your slide
- If taking photographs for cyst and fibrosis areas: take 13 photos of the cortex and 13 of the medulla
- Focus the photo by clicking the focus button on the right hand side of the computer
- Then move your slide to take a new photo, focus if you need to, and then click the 2nd button on the right of the computer screen to continually take photos.

Fibrosis

- Open Image-Pro Plus Software 6.0
- Select Complete
- Select Done
- Select magnification
- Create a colour standard
- Now put the mouse over your kidney photograph and select the truest colours of blue you see that represent fibrosis. Look at the colour representation cube on the count/size window to better tell the difference between colours.
- Once you're happy with your colour selection, click File.
- Click Save File and save your colour standard under a recognizable name.
- You must load this standard for every picture you analyze for fibrosis.
- Now open your cortex photos for one rat ID.
- Under the measure tab, click the data collector.
    - Under the layout tab, select "image" in the first drop down box. Then select name and the click the right arrow to add it to the right.
    - Where it says "image" in the drop down box, select "count/size" then select "area" from the list below.
    - The dropdown box below select sum, then click the arrow to add it to the list to the right.
    - Click the top tab labelled "Data list". This is where your data will be placed.
    - Now, close the "segmentation – image x" window. In the count/size window click "select colours"
    - Click "File"
    - Click "Load File"
    - Select the colour standard that you just created
    - Click "yes" to replace the ranges.
    - Close the segmentation window

- One the count/size window, select “Count”
- In the data collector window, select “collect now”.
- The area sum of the fibrosis from your photo should appear in the data list.
- Once you have finished analyzing your cortex photos for that one rat, click the “export” tab in the data collector window.
- Select export target
- Select export now. Your data will be exported to the active excel sheet. To change the export options, click “export options”.
- You can then proceed with your medulla pictures for the rat in the same way.

If analyzing cystic area in cystic kidneys, you can do it in a similar way. Instead of selecting the blue colours, you can select the white colours that represent the cysts.

Serum Creatinine Assay Protocol

Reference: Heinegard D, Tiderstrom G. 1973 Determination of serum creatinine by a direct colorimetric method. Clinica Chimica Acta 43: 305-310.

SOLUTIONS

Prepare in Advance & Keep as Stock:

(a) 0.05 M Sodium Phosphate and 0.05 M Sodium Borate (*keep refrigerated*)

- Add 13.40 g of $Na_2HPO_4{\cdot}7H_20$ (sodium phosphate) and 6.9 g of $BNaO_2{\cdot}4H_20$ (sodium borate) to 900 mL of distilled water
- Add 10 M aqueous sodium hydroxide (drop by drop) to reach a pH of 12.7
- Top up solution with distilled water to 1 L

(sodium phosphate 268.07 g = 1 mole
13.4035 g = 0.05 mole)

(sodium borate 137.9g = 1 mole
6.895g = 0.05 mole)

**Na_2HPO_4 can be used if $Na_2HPO_4{\cdot}7H_20$ is not available.

(Na_2HPO_4 142 g = 1 mole
7.1 g = 0.05 mole)

To make 10 M Sodium Hydroxide:

(Sodium Hydroxide 40g = 1 mole
400 g = 10 mole)

For example: to make 100 ml 10 M sodium hydroxide: dissolve 40g sodium hydroxide in small amount of distilled water (i.e. 80 ml) in a beaker, and then transfer sodium hydroxide solution into a 100 ml volumetric flask and add distilled water to reach the line.

(b) 4% aqueous SDS (room temperature)

- Add 40 g SDS to 1 L distilled water

* SDS is a detergent and therefore can become bubbly very easily; swirl to mix

(c) 60% Acetic Acid solution (room temperature)

- 60 mL concentrated Acetic Acid + 40 mL distilled water

(c) Picric Acid 1.3% (room temperature) **(**Sigma, cat. # P6744-1GA)

Make Fresh Daily:

(**a) Picric solution** (Combine in proportions of 2:2:1)

- 2 volumes 0.05 M Sodium Phosphate and 0.05 M Sodium Borate solution (a)
- 2 volumes of 4% aqueous SDS (b)
- 1 volume Picric Acid (d)

For example: To make 25 mL (enough for one 96 well plate)
10 ml 0.05 M Sodium Phosphate and 0.05 M Sodium Borate solution (a)
10 ml 4% aqueous SDS (b)
5 ml Picric Acid (c)
*Swirl to mix

(b) 15% Acetic Acid solution (prepare using a 1:4 dilution)

- 1 mL 60% Acetic Acid
- 3 mL Picric solution

PROCEDURE

Step 1: Prepare standards, samples, and reagents

Standards: (make new for each plate)
Use standard set from Sigma (cat. # C3613) *See note on bottom of last page.
Set contains stock concentrations of: 0.01mg/ml, 0.03mg/ml, & 0.10mg/ml

For Serum – use standard concentrations of 0.0025mg/ml, 0.050mg/ml, 0.01mg/ml, 0.03mg/ml, 0.05mg/ml, and 0.06mg/ml (0.08mg/ml and 0.10mg/ml can also be prepared to increase accuracy of standard curve).

Samples:

Serum – No dilution required

Reagents:
Prepare picric solution and 15% acetic acid solution as directed above

Step 2: Plate 20 µl of blank (deionized water), standards, and samples to wells of 96 well-plate (Fisher, cat # 07-200-641)**.** Make duplicates or triplicates of each.
Step 3: Add 200 µl of Picric Solution to each well using a multi-tip pipet with yellow tips (and mix by filling and expelling tips once.
(There may be a few bubbles present due to SDS, this is not a problem during the incubation because the bubbles are popped before reading plate)

Step 4: Cover plate with well cover sheet (Cayman, cat # 400012) and incubate at room temperature on orbital shaker at 140rpm or Vortex-Genie 2 at lowest speed for 40 minutes.

After 40 minutes, remove well cover sheet (this will cause the bubbles to disappear) and place well-plate on the microplate reader and shake every 15-20 seconds for 5 seconds for the last 3-4 minutes.

Step 5: Read 96-well plate at 500 nm on microplate reader. Print and save results.

Step 6: Add 20 µl of 15% Acetic Acid solution using a multi-tip pipet (make sure pipet tips touch the bottom of each well) and mix by filling and expelling the tip once.

Step 7: Incubate at room temperature for 6 minutes (begin timing as soon as 15% Acetic Acid solution is added to the last row of wells). To ensure adequate mixing, place well-plate on the microplate reader and shake every 15-20 seconds for 5 seconds for the last 3-4 minutes.

Step 8: Read 96-well plate at 500 nm on microplate reader. Print and save results

Step 9: Calculations (Use Microsoft Excel spread sheet)

a) To find the final absorbance of standards and samples:

Final absorbance = pre-acid absorbance values – post-acid absorbance values

b) Take average of results (any CV less than 10% is acceptable)

c) Construct a standard curve with absorbance on the x-axis and concentration on the y-axis. Using the equation given from this curve, calculate the concentration of creatinine in serum samples. (If samples were diluted, multiply concentration by the dilution factor.)

* When receiving new standards from Sigma, aliquot solutions into labeled 0.5ml micro centrifuge tubes and store in fridge. This helps reduce the change in standard concentration over time. Each time a stock standard is needed; take from a new 0.5ml tube.

Serum Cystatin C Determination

ELISA kit (BioVendor Cat. No.: RD391009200R)

STORAGE
Storage of the kit: Store the complete kit at 2-8°C. Under these conditions, the kit is stable until the expiration date (see label on the box).

PRECAUTIONS
- Wear gloves and laboratory coats when handling immunodiagnostic materials
- This kit contains components of animal origin. These materials should be handled as potentially infectious
- Avoid contact with the acidic Stop Solution and Substrate Solution, which contains hydrogen peroxide and tetramethylbenzidine (TMB). Wear gloves and eye and clothing protection when handling these reagents.

- **MATERIAL REQUIRED BUT NOT SUPPLIED**
- Deionized (distilled) water
- Test tubes for diluting samples
- Glassware (graduated cylinder and bottle) for Wash Solution (Dilution Buffer)
- Precision pipettes to deliver 5-1000 □l with disposable tips
- Multichannel pipette to deliver 100 □l with disposable tips
- Absorbent material (e.g. paper towels) for blotting the microtitrate plate after washing
- Vortex mixer
- Orbital microplate shaker capable of approximately 300 rpm

REAGENT SUPPLIED
(All reagents need to be brought to room temperature prior to use)

Kit Components	*State*	*Quantity*
Antibody Coated Microtiter Strips	ready to use	96 wells
Biotin Labelled Antibody	ready to use	13 ml
Streptavidin-HRP Conjugate	ready to use	13 ml
Master Standard	lyophilized	2 vials
Quality Control HIGH	lyophilized	2 vials
Quality Control LOW	lyophilized	2 vials
Dilution Buffer	ready to use	2 x 13 ml
Wash Solution Conc. (10x)	concentrated	100 ml
Substrate Solution	ready to use	13 ml
Stop Solution	ready to use	13 ml
Product Data Sheet (White Booklet)		1 pc
Certificate of Analysis (Yellow Sheet)		1 pc

PREPARATION OF REAGENTS

1. To make **Master Standard Stock**, refer to Certificate of Analysis (Yellow Sheet) for current volume of Dilution Buffer needed for reconstitution of standard.
Let it dissolve at least 15 minutes with occasional gentle shaking (not to foam).

Prepare set of standards using Dilution Buffer as follows (**preparation for duplicates**):

Volume of Standard	*Dilution Buffer*	*Concentration*
Master standard stock	-	25 ng/ml
250 µl of stock	250 µl	12.5 ng/ml
250 µl of 12.5 ng/ml	250 µl	6.25 ng/ml
250 µl of 6.25 ng/ml	250 µl	3.13 ng/ml
250 µl of 3.13 ng/ml	250 µl	1.56 ng/ml
250 µl of 1.56 ng/ml	250 µl	0.78 ng/ml

Do not store the diluted Standard solutions.

2. To make **Quality Controls HIGH, LOW,** refer to the Certificate of Analysis (Yellow Sheet) for current volume of Dilution Buffer needed for reconstitution and for current Quality Control concentration**.** Let it dissolve at least 15 minutes with occasional gentle shaking (not to foam).
Expected concentrations are written in Certificate of Analysis (Yellow Sheet).

Do not store the reconstituted Quality Controls.

3. Wash Solution Conc. (10x)
Dilute Wash Solution Concentrate (10x) ten-fold in 900 ml of distilled water to prepare a 1x working solution. Example: 100 ml of Wash Solution Concentrate (10x) + 900 ml of distilled water for use of all 96-wells.

PREPARATION OF SAMPLES

Dilute **rat serum samples** 500x with Dilution Buffer just prior to the assay in two steps as follows:
Dilution A (20x):
Add 5 µl of sample into 95 µl of Dilution Buffer. **Mix well** (not to foam). Vortexing is recommended.
Dilution B (25x):
Add 10 µl of Dilution A into 240 µl of Dilution Buffer for duplicates to prepare final dilution (500x). **Mix well** (not to foam). Vortex is recommended.
Do not store the diluted samples.

ASSAY PROCEDURE

1. Pipet **100 µl** of **diluted Standards, Quality Controls, Dilution Buffer (=Blank)** and

samples, preferably in triplicates, into the appropriate wells.

2. Incubate the plate at room temperature (ca. 25°C) for **1 hour**, shaking at ca. 300 rpm on an orbital microplate shaker.

3. Wash the wells 3-times with Wash Solution (0.35 ml per well). After final wash, invert and tap the plate strongly against paper towel.

4. Add **100 µl** of **Biotin Labelled Antibody** into each well.

5. Incubate the plate at room temperature (ca. 25°C) for **1 hour**, shaking at ca. 300 rpm on an orbital microplate shaker.

6. Wash the wells 3-times with Wash Solution (0.35 ml per well). After final wash, invert and tap the plate strongly against paper towel.

7. Add **100 µl** of **Streptavidin-HRP Conjugate** into each well.

8. Incubate the plate at room temperature (ca. 25°C) for **30 minutes**, shaking at ca. 300 rpm on an orbital microplate shaker.

9. Wash the wells 3-times with Wash Solution (0.35 ml per well). After final wash, invert and tap the plate strongly against paper towel.

10. Add **100 µl** of **Substrate Solution** into each well. Avoid exposing the microtiter plate to direct sunlight. **Covering the plate with e.g. aluminium foil** is recommended.

11. Incubate the plate for **10 minutes** at room temperature. **Do not shake the plate** during the incubation. The incubation time may be extended [up to 20 minutes] if the reaction temperature is below than 20°C.

12. Stop the colour development by adding **100 µl** of **Stop Solution**.

13. Determine the absorbance of each well using a microplate reader set to 450 nm, preferably with the reference wavelength set to 630 nm (acceptable range: 550 - 650 nm).
Subtract readings at 630 nm (550 - 650 nm) from the readings at 450 nm. **The absorbance should be read within 5 minutes following step 12.**

HOMOGENIZATION OF LYOPHILIZED KIDNEY TISSUE

Created by Tanja Winter – November 23/09

Step 1: Prepare List of Conditions

Condition	Treatment	Incubation time (37°C water bath)
1	200uL homogenate	0 minutes
4	200uL homogenate	5 minutes

Step 2: Calculate amount of Kidney Homogenate needed

2 conditions X 200uL homogenate per condition
= 400uL homogenate required + 20uL for protein assay
= 420uL total X 1.1 (allow for losses)
= 462uL total

Make 500uL total to ensure enough homogenate available. Make 50% more for Western Analysis (ie. 500ul). Therefore at least you need 1000uL total.

Use Tyrode's (pH 7.6) salt solution to homogenize dried kidney tissue. For every 70 mg of dried kidney tissue 2000uL of Tyrode's (ph7.6) is required.
Therefore, if 45 mg of dried kidney tissue and you need 1285.7 uL of Tyrode's (7.6).

$$\frac{70\text{mg}}{2000\text{uL}} = \frac{45\text{ mg}}{X\text{ uL}}$$

The smallest volume of fresh Tyrode's (7.6) that can be made without having major difficulty with pH adjustments is 100mL.

Step 3: Homogenizing

1. Ensure there is enough prepared of:
    - Tyrode's salt solution (pH 7.6) (Check for deterioration. See Solutions Preparation below)
    - ETYA Aliquots
    - 1.4xwhole cell buffer

2. Prepare 1% Triton Solution as per instructions below (see Solutions Preparation).
3. Prepare a wash bucket of 10% bleach in water to soak tools contaminated with biological hazards.
4. Turn on water bath to 37C
5. Turn on the Accuspin 3R centrifuge to 4C and insure microtube rotor is in place
6. Turn on the ultracentrifuge to 4C and insure ultracentrifuge tube rotor is in place (if completely preparing westerns fraction today)
7. Label 16 x 125mm disposable glass test tubes with sample ID's

8. Obtain a large container of ice
9. Prepare and label three disposable glass tubes (16 x 125mm) with 100% ethanol and three disposable glass tubes (16 x 125mm) with ultrapure water for cleaning the homogenizer
10. Move the homogenizer to the fume hood
11. Remove the required lyophilized kidney samples from the -80C freezer and keep on ice.
12. Weigh and record (actual mass) 45 mg lyophilized kidney sample into labeled tubes (prepared in step 5), cover with parafilm and immediately place on ice.
13. Return the remaining lyophilized kidney samples to the -80 freezer
14. Calculate, record, and add required amount of Tyrode's (pH7.6) to each massed kidney sample.

Amount Tyrode's Required (uL) = (2000ul / 70mg)(X mg lyophilized tissue ie 45mg)

= 1285.71ul

15. Clean homogenizer before use, after use, and in between each sample by:
    - 3 tubes ethanol x 30 seconds each at speed 13
    - Dab with kimwipe to dry
    - 3 tubes ultrapure water x 30 seconds each at speed 13
    - Dab with kimwipe to dry
16. Place test tube containing lyophilized kidney tissue in a small plastic container containing ice.
17. Insert rotor into test tube and homogenize at speed 13 for 30 seconds. Avoid generating bubbles. Stop and check that all kidney tissue is at the bottom of the tube. If not, use rotor tip to push everything to the bottom of the test tube.
18. Homogenize again for another 30 seconds (speed 13).
19. Repeat steps 15 – 17 for each sample. Remember to keep tubes covered with parafilm and on ice as much as possible.
20. Remove 500 ul for Westerns fraction from the LC-MS/MS fraction.
21. If Westerns fraction is being stored for later preparation, move westerns fraction into labeled 1.5ml microtube and store at -80C. If westerns fraction is being prepared now, move westerns fraction into ultracentrifuge tube.
22. Continue below for:
    - Oxylipin Analysis via LC-MS/MS Fraction
    - Enzyme Analysis via Western Blot Fraction

Oxylipin ANALYSIS VIA LC-MS/MS FRACTION

1. Calculate amount of 1% Triton solution to add to the remaining LC-MS/MS fraction in the 16x125mm tubes (should be around 1ml). Final concentration of Triton in homogenate should be 0.01%.

   Amount 1% Triton solution to add (uL) = (0.01)(X ul homogenate ie 1285.7ul)

   = 13ul

2. Vortex for 10 seconds each
3. Incubate, covered, on ice for 10 minutes
4. While waiting, reconstitute ETYA aliquot(s) as instructed below (ETYA Prep)
5. Vortex again for 10 seconds each
6. Incubate, covered, on ice for 10 minutes
7. Vortex again for 10 seconds each
8. Incubate, covered, on ice for 10 minutes
9. Vortex and aliquot 200ul of LC-MS/MS homogenate into labeled 1.5mL microtubes for each appropriate condition.
10. Vortex and aliquot 20ul of LC-MS/MS homogenate into labeled 0.5mL microtubes for protein assay. Store in -80C freezer.
11. Vortex each microtube for 10 seconds.
12. Working quickly, add 800ul of chilled ETYA solution to the time 0 samples and vortex for 5 seconds.
13. Incubate the time 0 samples on ice for 20 minutes.
14. Incubate the time 5 minute samples in the 37C water bath for 10 minutes. Step 12, 13 and 14 should be done at the same time (as you can).
15. After 5 minute incubation in 37C water bath (time 10 minute samples), quickly add 800ul of chilled ETYA solution to stop the reaction.
16. Incubate time 5 minute samples on ice for 20 minutes.
17. After 20 minutes are up, centrifuge the time 0 minute samples at 12,000g (11400rpm) at 4C for 5 minutes.
18. After 5 minute centrifuge (time 0 samples), draw off the supernatant and place in prepared 1.5mL microtubes. Keep on ice.
19. After 20 minutes are up, centrifuge the time 5 minute samples at 12,000g (11400rpm) at 4C for 5 minutes
20. After 5 minute centrifuge (time 5 minute samples), draw off the supernatant and place in prepared 1.5mL microtubes.
21. Store samples (time 0 and time 5 minutes) in -80C freezer for future oxylipin extraction.

Western immunoblotting (Lab protocol)

Remove western fractions from the -80C freezer and thaw on ice then move thawed and vortexed homogenate from microtubes into ultracentrifuge tubes

Sample Preparation - A dose response gel must be performed first to optimize the amount of protein being loaded. For this purpose, we are using an example loading amount of 14ug.

1. Set at least 400ml of H2O to boil in 1L beaker on a hotplate.
2. Calculate the volumes of samples to be used based on the protein concentration as follows:

Example: **For one gel loading of 14 µg of protein using 2x sample buffer:**

14 (µg) / Protein concentration (µg/µl) = volume of sample
7 – Volume of sample = volume of water

To prepare the sample as calculated:
(Volume of sample + volume of water) + 7µl sample buffer (since 14/2 = 7) = 14µl total

Using a protein concentration of 6.32 µg/µl as an example:
14µg /6.32µg/µl = 2.22µl of sample 7µl - 2.22µl = 4.78µl of water 2.22 + 4.78 + 7µl of buffer = 14µl total

If the protein concentration is less then 2.0, you must use 6x sample buffer. The calculation is as follows for loading 14µg of protein: 14ug/6x = 2.33ul of sample buffer

E.g. Protein concentration = 1.4µg/µl
14/1.4 = 10.00 (sample volume)
14 – 2.33 =11.67 11.67 – 10.00 = 1.67 (water volume)
Then add 2.33µl of 6x sample buffer (since 14/6 = 2.33) to obtain a total volume of 14µl.

Note: Prepare an extra 10% of all volumes (sample, water, sample buffer) to ensure that you have prepared enough sample to load onto the gel.

3. To prepare the sample use the 1.5ml microcentrifuge tubes. Label the tubes with permanent marker on the top and side. **Add the ddH_2O first**, then sample and finally buffer. Make sure to keep samples on ice at all times. Add sample buffer to samples in the fume hood. Seal samples with parafilm around the tops, the heat from the water bath will cause the tops to pop open and you will get water in your sample if you don't.

4. Heat samples in boiling water (100°C) for 5 minutes, this will denature the proteins.

5. Remove samples from water and cool in ice for about a minute. Centrifuge at 5000g for 1min and place back on ice. The samples are now ready to be loaded on the gel.

Note: If you plan on storing the samples overnight or over the weekend, you do not need to boil, just place in -20°C. When you are ready to use the samples you will need to continue on starting with Step 5.

Running the Gel

1. Fill the outer chamber with cold 1x used running buffer between the min and max line (running buffer may be re-used approximately 8-10 times for filling the large chamber). **Unclip bottom of gel sandwich** and place in chamber, make sure to get rid of all bubbles at the bottom of the glass sandwich by shaking the gel sandwich in the chamber containing running buffer. Fill upper compartment of apparatus with new 1x running buffer. Gently remove stacking gel comb by pulling straight up. Wash wells with approximately 100µl of new running buffer.

2. Moving from left to right, apply 14µl of sample to wells (for samples prepared as above so that 14µg of protein is loaded into each well) using gel loading pipette tips (Fisherbrand Cat No. 02-707-86) in blue box for a max of 10µl and white box for 14µl. Also apply standards and markers to the wells. For cPLA2 standard (Genetics Institute) apply 3µl and for BenchMark Prestained Ladder (Invitrogen, Cat No. 10748-010) apply 10µl (ladder is stored in the -20^{o}C freezer). Fill outer running chamber to max fill line with used 1x running buffer.

Note: For gels that we prepare the maximum well amount that can be easily loaded is 21µL before samples spill into the other wells.

3. Run precast gels at 125V (set mA to max of 400mA) for 1.5 hours or until blue band is .5cm from the bottom of the glass sandwich. If you are using gels you have made, run at 200V. Check for bubbles when you start running the gel behind the sandwich in the inner chamber to make sure it is running.
4. Note: Gels may be run in the 4°C fridge or on the counter.
   *gels in fridge tend to run slower (set time for 2 hours but check progress at 1.5 hours).

Removing the Gel

1. When the running time on the electrophoresis apparatus is just past 1 hour, cut the PVDF membrane you will be using to transfer the proteins onto (GE Healthcare Amersham Hybond-P Cat No. RPN2020F) to the desired size (You can use the template inside the membrane roll). Remove the paper cover on both sides using forceps.
2. Using a pencil, label the membrane with the date and gel number at the bottom of the membrane. Cut the top left hand corner of the membrane in order to indicate the first lane. Make sure to always wear gloves and use forceps when handling the membrane.
3. Place the membrane in plastic container and pour a small amount of Methanol on the membrane, approximately 5ml. Gently swirl for 10 seconds and then fill the container half full with ddH_2O. Place container on rocker for 7-8 minutes. Pour methanol/water into methanol waste container and add new 1x transfer buffer to the membrane container for equilibration. Place it back on shaker until the gel is ready for protein transfer.

4. When the blue band reaches 0.5cm from the bottom of the gel, turn off the power unit and remove the gel apparatus from the chamber. Depending on the percentage of the gel, the running time is approximately 1 hour and 30 minutes. Pour the inner chamber new running buffer into the used running buffer in the large chamber. Unscrew gel apparatus screws and place gel sandwich on paper towels. Separate the two glass plates by prying the plates apart using the spacers. Using a knife cut off the stacking gel from separating gel and the blue band on the gel (if it has not run off). Cut the top left corner of the gel to indicate which is lane 1. Put the cut off parts of the gel into the gel waste box which is in the fume hood. Pour the used 1x running buffer back into its bottle and mark that it was used again.
5. Carefully place gel into container with a sufficient amount of 1x transfer buffer to cover gel. Place the container with gel on rocker and allow the gel to equilibrate for 5 – 10 minutes.

Electroblotting

1. Using Western blot filter paper (Thermo scientific Cat No. 88600); soak two in new 1X transfer buffer. Place negative metal cathode in the bottom of the transfer apparatus. Next place the plastic grid, smooth side face up. Soak 2 sponges in new 1x transfer buffer in a separate plastic container and place on plastic grid, then one piece of filter paper that had been soaking. Using clean gloves, place the gel on the filter paper. Place the membrane on the gel with the cut corners lining up and the date facing up using forceps. Once the membrane has touched the gel, try not to move the membrane. Place a piece of transfer buffer soaked filter paper on top of the membrane. To remove bubbles use a glass test tube, cleaned with ethanol, and roll it across the filter paper. Place two or three more transfer buffer soaked sponges on top of the filter paper (use as many sponges as needed so that everything is quite tight in the transfer apparatus).

Note: In order to know which side of the membrane the protein is transferred, keep the cut corners on the left. When you remove the membrane, the reverse side will have the proteins on it, making the cut corner on the right.

*You can load 2 gels in one transfer apparatus using this method – put a couple sponges in between the two gels.

2. Place second plastic grid, smooth side down, on sponges. Next place positive anode on top of grid and cover with clear plastic piece. Slide into transfer chamber. Fill apparatus with new 1x transfer buffer until the top of sponges are just covered.
3. **Black (-ve) anode is connected to the left, attached to bottom plate and red (+ve) anode is connected on the right, attached to top plate**. Connect to power unit and transfer for 1:35 hours at 24V and 400mA. Check for bubbles running up the side along the back metal plate to know it is transferring. **If you accidently reverse the current by connecting the cords to the wrong plates, your proteins will run backwards onto your filter paper!**

Note: Membranes may be transferred in 4°C or on counter.

Note: For each transfer approximately 300ml of new 1x transfer buffer is required.

Blocking

1. Prepare 5% skim milk solution (2.5g skim milk in 50 ml 1xTBS/Tween). Shake well to ensure all the skim milk powder has dissolved. The blocking solution can be reused until it separates with a max of 2 weeks. Store in the 4^{o}C fridge.
2. Turn off the power unit and remove membrane from transfer apparatus and place in dish using forceps. Flip the membrane so that the side facing the gel is now facing up.
3. Place the membrane in PS for 5 minutes and then wash it with ddH_2O then image it on the Fluorochem Q Imager for protein detection
4. Wash the membrane with water 2 times
5. Pour the 5% skim milk solution into the dish slowly covering membrane. Cover and place on rocker (or shaker) for 1 hour. Gel can be dried in the gel waste box or checked for any remaining protein as described in the staining and de-staining procedure at the end of the protocol. Cut the membrane before or after the blocking step if doing more than one primary antibody.

Adding Antibodies

1. Using a 15ml centrifuge tube, prepare primary antibody in a 2% skim milk/1x TBS-T solution, 0.3g skim milk in 15ml TBS/T. Pour off blocking solution and slowly pour on primary antibody. Cover and place in chromatography refrigerator **on rocker** overnight or at room temperature for 2- 3 hours on rocker. Put the blocking solution back in the 4^{o}C fridge until two-weeks from preparation date.

Note: See table at end of protocol for the different treatments used depending on which antibody used.

2. Remove 2% skim milk/1x TBS-T solution with primary antibody from membrane and save in -20°C freezer if using Cayman antibodies. If using Santa Cruz antibodies, solution must be stored in 4°C fridge. (Antibody can be reused up to five times).
3. Pour 1x TBS-T on membrane and place on shaker or on rocker for length of time stated in column titled First Washing in the table at the end of protocol.
4. Using a 15ml centrifuge tube, prepare secondary antibody (see table to determine type and concentration depending on primary antibody used) in **1x TBS-T** and add to membrane after pouring off the 1x TBS-T from washings. Cover and put on shaker or put on rocker for 1 hour.
5. Pour off secondary antibody (secondary antibody solution is not usually saved since it is used in such low concentrations) **Secondary can be poured down the sink**. Add 1x TBS-T and place on shaker or on rocker for length of time stated in column titled Second Washing in the table at the end of protocol.

Developing the Membrane using the Fluorochem Q Imager

1. Make an approx. 1:2 dilution using the Chemiluminescent Peroxidase Substrates (Sigma, CPS160-1KT) (4^{o}C storage), 350µl of reagent and 700µl of buffer. The substrates should

be at room temperature when used and mixed 4-5 minutes ahead of time (store in a dark e.g. drawer).

2. Pour the 1x TBS-T off the membrane. Dab off excess using a kimwipe to the edge of the membrane. Don't touch the surface of the membrane with the kimwipe. Place membrane on a clear plastic container with a flat bottom using forceps. Evenly distribute the solution over the surface of the membrane. Cover the plastic container with Saran wrap. **Make sure there are no creases in the Saran wrap over the membrane area or the photo will have black lines in it.**
3. Open the program by double clicking on the icon.
4. Click [**Acquire]** and click [**Live].** Ensure the aperture is set at 0.95.
5. Open the door to the cabinet and place membrane on shelf. Holding door slightly ajar so you can see the image on the screen, center the membrane and focus the lens to get a clear image. Close door tightly.
6. From the drop down menu under **Load Protocol**, select **Chemi high-med.**
7. Click on **[Acquire]**.
8. When the image is complete, save the image in your file. Click on **[Reverse]** to create a white background. Adjust the **[gamma]**, **[white**], and **[black]** slider settings until you get an optimal image: visible bands with low background.
9. Click on **[Analysis Tools]** tab. Select **[Band Analysis]**. Click on the square under **Single Region Tools**. Place your cursor in the corner of the darkest (largest) band and create a box around the band, keeping out as much background as possible.
10. Select the box you just created and click on **[multi-region copy].** Center your arrow on another band and left click. Another box exactly the same size as the first one will be created. Do this for every band. Some boxes may have to be moved to encompass the entire band.
11. Click **on [background]** tab. Click on **[Multi-regional background**]. Create a box that is approximately the same size as the band box, is representative of the lane background, and has no other visible bands in it.
12. Highlight together the band box and the background box.
13. Click on link background. Do these two steps for all bands.
14. Click [**print screen]** and open "**Paint**" from **Accessories**. Under **Edit,** select **Paste**. Your band analysis result along with imaged membrane will appear. Save for your lab book.
15. When closing, a message will appear, "Do you want to save current analysis?" Select **NO**.
16. To take an image of the ladder click on "Acquire" and turn on the reflective white light. Change the diaphragm so that the light is enough to see the ladder on the computer. Change the time to 8 milliseconds and at Medium/Medium. Click on Acquire image. Save image as you did for the ones above.

SOLUTIONS PREPARATION

To Reconstitute Tyrode's Salts Without Sodium Bicarbonate
Product# T2145 (Sigma)

Tyrode's salts powder comes prepackaged from Sigma-Aldrich and is kept in the fridge. Please refer to the product insert for full product information. Powdered salts are hygroscopic and should be protected from moisture. The entire contents of each package should be used immediately after opening. Preparing a concentrated salt solution is not recommended as precipitates may form. Tyrode's Salts are meant to maintain cellular pH and osmotic balance.

1. Measure out 1L of ultrapure water in a graduated cylinder. Water temperature should be 15-20C
2. Transfer about 800ml of measured water into a 2000ml beaker. Add a large stir bar (careful not to splash) and place on stir plate. Begin gentle stirring.
3. Add powdered Tyrode's salts and continue stirring until dissolved. Do NOT heat.
4. Rinse original Tyrode's salts packaging with some (NOT all) of the remaining 200ml measured water to remove all traces of powder. Add to solution in step 3.
5. Transfer solution to a 1L volumetric flask and bring to volume using some of the remaining 200ml measured water in graduated cylinder from step 1.
6. Insert volumetric stopper and invert 10x to mix.
7. Transfer to a 1L glass bottle covered with tin foil to protect from light and clearly label as Tyrode's salts WITHOUT NaHCO3. Store in the refrigerator (2-8C).

To Make 100mL of Tyrode's (pH 7.6)

1. Measure 100ml of reconstituted Tyrode's salt solution into a graduated cylinder.
2. Weigh 100mg of powdered sodium bicarbonate (Sigma, S5761) into a 125ml Erlenmeyer flask.
3. Cover flask with tin foil to protect from light. Add a stir bar.
4. Transfer about 80ml of measured Tyrode's in graduated cylinder from step 1 to the Erlenmeyer flask.
5. Completely dissolve powder into solution by placing on stir plate and stirring (apprx 15 min). Powder must be completely dissolved before adjusting pH.
6. While continuing to stir, adjust the pH of the solution to pH 7.6 using 1N HCl or 1N NaOH. Normally to achieve pH 7.6, a couple of drops of 1N HCl are required.
7. Transfer solution to a 100mL volumetric flask and bring to volume using some of the remaining 20ml measured Tyrode's in graduated cylinder from step 1.
8. Insert volumetric stopper and invert 10x to mix.
9. Transfer to a 100mL glass bottle covered with tin foil to protect from light and clearly label as Tyrode's salts (pH 7.6). Store in the refrigerator (2-8C).

NOTE: Tyrode's that has sodium bicarbonate added and has been pHed can deteriorate. Deterioration can be recognized by:

- pH change
- precipitate or particulates
- cloudy appearance
- colour change

Check all these signs before using.

Use Tyrode's (pH 7.6) to make 1% Triton.

To Make 1% Triton

This solution mixes best if Tyrode's (pH 7.6) is at room temperature.

1. Weigh out 0.02 g of Triton solution in a 20ml scintillation vial
2. Add 2.0 ml of room temperature Tyrode's (pH 7.6) using a 1.0ml eppendorf pipette.
3. Cover with cap and vortex well.
4. Cover with tin foil to protect from light and chill on ice or in fridge. Store remains in refrigerator.

A final concentration of 0.01% Triton is required in the homogenate to disrupt lipids and release proteins. This will ONLY be added to the LC-MS/MS fraction.

Therefore add 10uL of 1% Triton (pH 7.6) to 1000uL of kidney homogenate for the LC-MS/MS fraction only to make a 1% Triton final solution (Step 1 under oxylipin Analysis via LC-MS/MS Fraction).

i.e. Volume 1% Triton to add (ul) = (0.01 final concentration)(1000uL kidney homog)

To Make ETYA

ETYA (5,8,11,14 - eicosatetraynoic acid) inhibits arachidonic acid uptake. Nonspecifically inhibits cyclooxygenase and all lipoxygenases in whole cells. Inhibits PLA_2 and cytochrome P-450. To inhibit oxylipin enzymes in the sample solution a 50uM concentration is required.

Solutions of ETYA should be made fresh daily. Compound is subject to oxidation. Lasts up to 12 months stored as solid.

To Make ETYA Aliquots:

1. Chill 3ml of ethanol in 4C fridge.
2. Purge chilled ethanol with inert gas (nitrogen).
3. On ice, transfer 2.5ml (use 1000ul eppendorf pipette) of chilled, purged ethanol directly into the 20mg ETYA vial (Enzo Life Sciences, Cat# ET-004).
4. Replace air over ETYA in ethanol solution with inert gas (nitrogen).
5. Vortex. Insure ETYA has dissolved completely.
6. Aliquot 32.6ul (use 100ul eppendorf pipette) of ETYA solution into 2.0mL minicentrifuge tubes. Should take about 76 tubes to completely aliquot solution.
7. Dry down under gentle stream of nitrogen.
8. Replace air over remaining ETYA solid with inert gas (nitrogen).
9. Label tubes as "0.8796 umol ETYA, [date], [name]".
10. Store in 4C fridge.

To Reconstitute on Day of Use:

1. Keep on ice.
2. Add 180ul (use 200ul eppendorf pipette) of 100% ethanol to the 0.8796 umol ETYA aliquot and vortex to dissolve ETYA.
3. Transfer 13900ul (use 5000ul pipette –> 5000ul x 2 + 3900 x1) of chilled Tyrode's (pH 7.6) into a 20ml glass scintillation vial.
4. Transfer the 180ul of reconstituted ETYA in ethanol from step 2 into the 13900ul chilled Tyrode's prepared in step 3. Total volume is 13900ul + 180ul = 14080ul.
5. Vortex well to mix.
6. Each vial contains enough ETYA solution for four SAMPLES (SAMPLES = 4 conditions, therefore 4 samples x 4 conditions = 12 x 800uL aliquots)
7. If processing more than four samples, reconstitute appropriate number of vials into appropriate container.

Calculations:

ETYA stock concentration = 20mg ETYA / 2.5 ml ethanol = 8 mg/ml
Convert to mg/L = (8 mg/ml)(1000ml/1L) = **8000 mg/L**

ETYA MW = 296.5 g/mol
Convert to mg/umol = (296.5 g/mol)(1000mg/1g)(1mol/1x10^{6}umol) = **0.2965 mg/umol**

ETYA stock concentration in uM = (8000 mg/L)(1umol/0.2965mg) = **26981.45025 uM**

Concentration sub-stock ETYA solution required for final 50uM ETYA in sample solution = ((50uM)(1000uL final volume ie. 200ul sample + 800ul ETYA sub-stock) / 800ul sub-stock) = **62.5uM**

Amt 62.5uM ETYA solution required per sample = 800ul x 4 conditions = 3200uL/sample
Account for losses = (3200uL)(1.1) = 3520 uL/sample
Aliquot for 4 samples = (3520ul/sample)(4 samples) = **14080 ul/4 samples**

Amount ETYA stock required to make 14080ul of 62.5uM ETYA solution = ((62.5uM)(14080ul) / 26981.45025uM) = **32.615uL**

Western Blotting Buffers

All ingredients should be mixed first, then add 1N or 10N NaOH (depending on initial pH) to the solution to result in an end pH of 7.2-7.4. Usually, a couple of drops of 10N NaOH suffices.

Component	**1.4xwhole cell Buffer 20mL**	**Final Concentration**
500 mM Tris-HCl Gibco, xxxx (6.1 g/ 100ml)	2 ml	50 mM
0.5 M sucrose (17.2 g/ 100ml) Sigma, S9378	10 ml	250 mM
200 mM EDTA (pH = 7.6) Sigma, ED4SS, (0.76 g/10 ml)	200 µl	2 mM
100 mM EGTA (pH = 7.5) Sigma, E4378 (0.38 g/10 ml) Add NaOH to get it to dissolve and bring pH to 7.5	200 µl	1 mM
0.4 M NaF (0.168 g/10 ml) Sigma, S6521	2.5 µl	50 µM
ddH_2O Water	6.104 ml	
10 % Triton X-100 Sigma T8787	1 ml	1 %
10 mM Na orthovanadate Sigma, S6508 (1.839 mg/ml)	200 µl	100 µM
2.5 mg/ml aprotinin Sigma, A1153	200 µl	25 µg/ml
1 mg/ml leupeptin Sigma, L2884	500 µl	25 µg/ml
2.5 mg/ml pepstatin (in 90:10 methanol: glacial acetic acid) Sigma, P5318	200 µl	25 µg/ml
1 mg/ml STI, Sigma, T9003	20 µl	1 µg/ml
50mM ABSF (12 mg/ml) 4-(2-aminoethyl) benzene-sulfonyl fluoride, Sigma, A8456	57.6 µl	144 µM
b-mercaptoethanol	14 µl	10 mM

Total Protein Determination

1. Take samples out of the freezer to thaw but keep on ice.
2. Take the following out of 4C fridge to come to room temperature for pipetting:
    - 2mg/ml BSA stock solution (Sigma P0834-10X1ML).
    - Tyrode's buffer or whichever solution tissue is suspended in for dilutions
    - Bradford reagent
3. Keep original samples on ice. Standards and diluted samples are better to be left at room temperature for pipetting.
4. Gather 1.5 ml centrifuge tubes, as many needed for 7 standards and all samples.
5. Label tubes with standard concentrations or sample numbers (with dilution factor). Use as a guide for sample dilutions: serum 200X, urine 10X, kidney homogenates 20X. Dilutions may not be this high. (see attached instructions for serial dilution).
6. Make standard dilutions using BSA stock solution. The BSA is in a small glass vial. Transfer with a pipette into a 2.0mL microcentrifuge tube. Standards should range in concentration from 1mg/ml to 0.1mg/ml. If you are doing serial dilution, remember to make enough needed for next dilution and triplicate samples. The concentrations will be 1mg/mL, 0.75mg/mL, 0.56mg/mL, 0.42mg/mL, 0.32mg/mL, 0.24mg/mL, & 0.12mg/mL. Do not make standards lower than 0.12mg/ml; the curve will no longer be linear. Always vortex solutions after mixing and before pipetting out for next serial dilution. You can refer to the attached protocol *Instructions for Serial Dilution* for an appropriate serial dilution.
7. Prepare diluted samples according to step #4. I.e.: 10µl of kidney homogenate sample with 190µl of Tyrode's (pH 7.6)/ultrapure H2O = 20x dilution. Vortex samples after diluting.
8. Label a template guide with the position of the blank, standards and samples.
9. Vortex standards and diluted samples directly before pipetting onto plate. Working in triplicate, pipette 10µl of water/Tyrode's buffer in wells A1, A2, and A3 of plate (Costar, 3368). These will be your blanks. Pipette standards and samples in the same way. When pipetting into the wells, use reverse pipetting technique. This will give more accurate results.
10. Swirl bottle of Bradford dye (Bradford Reagent, Sigma B6916) gently and fill reagent reservoir, pouring out only enough you will need. Never pour unused Bradford back into the bottle. Using a 300µl multichannel pipette, pipette 200µl of Bradford into each well. Mix contents with the pipette. The time in which you add Bradford from the first well to the last should only be 10 minutes. Cover plate with plate cover and incubate plate on rocker or orbital shaker for 15 minutes at room temperature. Shake or rotate gently. The protein-dye complex is stable up to 60 minutes.
11. The wavelength for reading the plate is 595nm. Set up plate reader (in Dr. Aluko's lab) using the GENLAB software to the correct plate template while plate is incubating. Time is **IMPORTANT**. **Do not** let your samples incubate longer than 15 minutes. The results will not be representative of the sample. This is a colorimetric assay with the color being directly proportional to the protein concentration. If the plate is incubated longer, the color will be darker, therefore skewing protein concentrations. Bring plate to plate reader before 15 minutes are up so you can read plate at exactly 15 min.

Kidney Fatty Acid Analysis

Extraction:

*Always use volatile solvents in fume hood (most especially chloroform and any of the ethers)! Volatile (flammable) solvents are stored in the yellow flammable cabinets.
**Glass Tubes must always be soaked in Contrad overnight and rinsed thoroughly with distilled water, so prepare clean tubes the day before you run samples. To speed up the drying time they may be placed in the oven at 100^0 until completely dry.

***Keep samples on ice
****Do not wash chromatography tanks with soap

1. Take your prepared glass 12ml tube with 250ul of tissue homogenate (sample + Tyrode's) out of the -20^0C freezer and put on ice to let thaw.

2. Turn on the Accuspin 3R centrifuge to 4°C.

3. Put the TLC plate in a 110°C oven for 1 hour. Doing so completely dries out the plate. After an hour, place the TLC plate into a dessicator. Turn oven down to 80°C.

4. Turn on the Nitrogen bath to 37°C. Make sure the bath is filled with distilled water until the water reaches the bottom of the sample holder.

5. Prepare a blank tube with 250uL of Tyrodes with every set of samples you do.

5. Add **2.5mL (2500ul)** 2:1 chloroform:methanol with 0.01% BHT to your homogenate and blank. To prepare solution: 0.03g butylated hydroxytoluene, 200mL chloroform, 100mL methanol = 0.06g BHT, 400mL chloroform, 200mL methanol. Vortex

6. Add **10uL** of each standard using a 10uL pipette.
Standard: C15:0 – 10mg/ml – Phospholipid (PL)

All standards are made up in 2:1 chloroform:methanol
This amount is only for the test sample. You will need to optimize the volume of standard to add to the samples according to the peak heights in the test sample and adjust accordingly

7. Add **2.25mL (2250ul)** of 2:1 chloroform:methanol to the homogenate.

8. Cap and vortex for 15 seconds.

9. Add 1.2**mL (950ul)** 0.73% NaCl. *To prepare solution: 0.73g NaCl in 100mL de-ionized water = 3.65g NaCl in 500mL de-ionized water.*

10. Cap and vortex for 30 seconds, put on ice. (Takes approx. 45min to get to this step.)

11. Centrifuge for 10 minutes at 800g or RCF. It is crucial to place and balance tubes properly or breakage may occur**.** If you have an odd number of tubes, add an extra tube with similar solvents and water volumes to balance the load.

12. Get 4mL glass vials with Teflon lids and label with sample ID's using tape.

13. Take the tubes out of the centrifuge when completed. You should see 2 layers. Using a glass Pasteur pipette with small yellow bulb, carefully extract the lower phase without taking the upper phase. Slide the tip of the Pasteur pipette down the side while pushing out bubbles, and slowly draw up the lower phase and release it into your newly labeled 4ml vials.
*Be careful not to extract the upper phase. If the 2 layers were accidently disturbed and mixed, re-centrifuge the tube (make sure to balance in the centrifuge). (This step takes approx.30 - 45 min, depending on how many samples you have.)

14. Before placing your samples in the nitrogen bath, turn on the nitrogen tank so that a gentle stream comes out the open the needles that you will be using. Wash the needles with chloroform (place the needle in a scintillation vial with 100% chloroform for 5 seconds) and then let any remaining chloroform volatilize.

15. Place your samples (in the 4mL vials) in the nitrogen bath with the lids off. Carefully lower the needles close to the sample and turn the nitrogen gas on until you see a gentle ripple in the sample. Keep in the nitrogen bath until the vial is completely dry. (Samples take approx. 45 min to dry.)

16. Remove samples from nitrogen one at a time adding 100uL of 2:1 chloroform:methanol , screwing on lid and vortexing. Samples are vulnerable to oxidation between being removed from nitrogen and adding solvent.

Thin Layer Chromatography (TLC):

1. Turn on oven to around 88℃ (or lower heat if still on from heating TLC plate). The temperature desired for methylation is 80℃, but when the oven is opened, the temperature rapidly drops. 8-10℃ above desired temperature gives some leeway.

2. Make up the solvent tank and let it equilibrate for at least half an hour.

- Cut a piece of chromatography paper to fit in tank so that it covers the back and sides. You should only need to fold the paper in half lengthwise and tear.
- To make solvent for tank, get a glass graduated cylinder (100ml or 250ml), in fume hood fill graduated cylinder with 60ml of Heptane, 40ml of Isopropyl ether, and 3ml of acetic acid. You can pour Heptane and isopropyl ether into graduate cylinder so it reaches 100ml then pipette 3ml of acetic acid in. Pour solvent mixture into tank and close, fastening on lid. Do this in the fume hood and leave tank in the fume hood for solvent to volatilize.
- Depending on which solvent method is appropriate, you may be using 85.0ml petroleum ether, 15.0ml diethyl ether and 0.5ml acetic acid in the chromatography tank.

3. Using the TLC template with 6 or 8 lanes (depending on how many samples you have), create equal and parallel vertical lanes on your silica plate (that was previously heated) using the dull side of a razor blade. Be careful not to make any marks on any other part of the plate or to touch the plate (you could accidentally transfer lipids onto the plate from your gloves). Leave at least 1cm on each side of plate. Only use pencil on the plate, **no ink**.

4. Turn on nitrogen tank with small drying hose attached. Using a 100ul pipette, draw up 50uL of your sample and slowly spot it along the horizontal line drop by drop in the center of the lane. After each drop, dry with nitrogen hose.

5. Place the plate in the tank until the solvent line reaches 1 cm from the top. Keep plate as vertical as possible. This will take approximately 20-30 minutes. Make sure you check the solvent front at 20min. While plate is running, label your 12ml tubes with sample ID and either PL, TG and get your weigh paper ready for scraping plate (fold creases)

6. Let solvent volatilize off TLC plate in the fume hood. You'll know it has when the plate is removed from the fume hood and you don't smell a strong odour of ***solvent*** (a slight odour of acetic acid, ie vinegar, is fine). Dry the plate with a gentle stream of nitrogen gas. Samples are again susceptible to oxidation, so don't dry for any longer than 10 minutes. It should only take a minute or two using nitrogen gas to dry.

7. Protect the back of the fume hood and other samples in the fume hood by placing a large piece of cardboard along the back of the fume hood. Stand up TLC plate in the center of the cardboard.

8. Attach hose to the nitrogen gas in fume hood and to bottle with 0.1 % ANS solution in water and spray a fine layer onto the TLC plate. 0.1% ANS can last about a week. Check the color of the solution, if it is a dark yellowish/brown, it is no longer good to use. To prepare 0.1%

ANS, weigh out 100 mg ANS (8-Anilino-1-Naphthalene-Sulfonic Acid) and add 100 mL of milli-Q water. Protect from light using tinfoil.

9. In a dark room wearing UV safety glasses, use the hand held UV light to mark the PL, FFA, Chol and TG lines using a pencil, indicating where to scrape. R_f (retention factor) for PL = 0, R_f for TG =0.61 (PL is at the origin and TG is about 60% of the way to the solvent front).

10. Since static is a factor, wipe your gloves with a dryer sheet and keep it handy for further use if needed. Be careful not to contaminate your samples with the dryer sheet.

11. **IMPORTANT Wear a mask when scraping as silica dust is extremely hazardous. Also, work in an area of the lab away from others **** Using a razor blade, scrape the indicated portions onto a creased weigh paper and carefully transfer to the corresponding, labeled 12mL screw top test tube.

12. If you're stopping at this point and completing acid methylation the following day, add only 1.5mL (1500ul) toluene, use Teflon tape, **cap tightly** and vortex. Store overnight in -20C. Then add 1.2mL (1200ul) of methanolic HCl, kept in 4^0 fridge, to the tubes the following day when you begin acid methylation.

Acid Methylation

Turn on oven (about 88C) and allow it to reach temperature, if it is not already on

1 Add 1.5mL (1500ul) toluene and add 1.2mL (1200ul) of methanolic HCl, kept in 4^0 fridge, to the tubes, use Teflon tape, **cap tightly** (very important!) and vortex for 30 seconds. With a marker, mark the liquid level of each tube.

(You can stop at this point if you don't put methanolic HCL in the sample. Just store the samples in 1.5mL of toluene in the -20^0freezer. Continue on the next day by adding the methanolic acid and going on to step 2.)

2. Place tubes in a metal rack in a preheated 80^0C oven for 1.5 hours. After 30 minutes if the volume decreases, remove the tubes from the oven, let cool, add more methanolic HCl to achieve the original volume and place back in oven.

3. After 1.5 hours**,** remove rack from oven and cool for 10-15 minutes.

4. In the fume hood, add 1mL of de-ionized water to the tubes, cap and vortex for 15 seconds. Centrifuge for 5 minutes at 800g or RCF, brake on low.

5. Using glass Pasteur pipette tip with yellow rubber bulb, transfer the top layer to a clean 2mL GC vial (**glass**), being careful not to transfer the bottom layer and cap. Place in the -20°C freezer.

Later you can thaw the sample, dry under nitrogen using a dry bath (you can use the water bath, just be careful). It is very important not to get any water in your sample at this point.

6. Once the solvent has evaporated, immediately add 200uL of hexane to the tubes *(adjust volume according to concentration on GC after samples have been run)* and cap. Work with one sample at a time. Again, samples are susceptible to oxidation between removal from nitrogen gas and addition of solvent. Store the samples in the -20^0C freezer in a labeled box.
When they are ready to be run on the GC, label GC vials with sample number, date and your initials and transfer 100uL into a **glass** insert. Cap and store in -20^0C freezer (top shelf, right in front) in a sample box clearing stating that they are ready to be run on GC and marked "Attn: Dennis". to place an analytical service request in with Dennis.

Appendix 3: Supplementary data

- **Table S1**. Diet composition for study 1 (Chapter 3)
- **Table S2.** Diet composition for study 2 (Chapter 4)
- **Table S3. AIN-93G mineral mix details** (Chapter 4)
- **Table S4**. Cortical and medullary cyst and fibrosis volumes in diseased Han:SPRD-*Cy* rats given control, SP, or SP+FXO diets. (n=12/ group) (Chapter 4).
- **Table S5.** Correlation analysis of kidney function parameters and total COX and LOX oxylipins (Chapter 4).
- **Table S6.** Diet composition for study 3 (Chapter 5).
- **Table S7.** HPLC-tandem mass spectrometry parameters for oxylipins scanned for in this thesis.
- **Table S8** Fatty acid composition of the diet

Table S1. Diet composition for study 1 (Chapter 3)

Ingredients (g/kg)	CP SO	CP FO	SP SO	SP FO
Cornstarch	397.486	397.486	407.2556	405.756
Casein protein (87%)	200	200	0	0
Soy protein (92%)	0	0	189	189
fish oil	0	52.5	0	52.5
Dextrinized cornstarch	132	132	132	132
Sucrose	100	100	100	100
Soybean oil	70	17.5	70	17.5
Fiber	50	50	50	50
Mineral mix (G)	35	35	35	35
Vitamin mix	10	10	10	10
Threonine	0	0	0.2	0.2
Methionine	0	0	2.4	2.4
L-Cystine	3	3	1.5	3
Choline bitartrate (41.1% choline)	2.5	2.5	2.5	2.5
Tert-butylhydroquinone (TBHQ)	0.014	0.014	0.014	0.014
total	1000	1000	1000	1000

All diet ingratiates were purchased from Dyets (Bethlehem, PA)

Table S2. Diet composition for study 2 (Chapter 4)

Ingredients (g/kg)	Control	SP	SP+FXO
Cornstarch	397.5	397.5	397.5
Casein protein (87%)	200	0	0
Soy protein (92%)	0	189	189
Dextrinized cornstarch	132	132	132
Sucrose	100	100	100
Soybean oil	70	70	17.5
Flax oil	0	0	52.5
Fiber	50	50	50
Mineral mix (G)	35	35	35
Vitamin mix	10	10	10
Threonine	0	0.2	0.2
Methionine	0	2.4	2.4
L-Cystine	3	1.5	1.5
Choline bitartrate (41.1% choline)	2.5	2.5	2.5
Tert-butylhydroquinone (TBHQ)	0.014	0.014	0.014

All diet ingratiates were purchased from Dyets (Bethlehem, PA)

Table S3. AIN-93G mineral mix details. Values are the amount when the mineral mix is added to the diet in Supplementary Table S2 (Chapter 4).

Mineral	mg/kg diet
Calcium	5200.0
Phosphorus	1561.0
Potassium	3600.0
Sulfur	300.0
Sodium	1019.0
Chloride	1571.0
Magnesium	507.0
Iron	35.0
Zinc	30.0
Manganese	10.0
Copper	6.0
Iodine	0.2
Molybdenum	0.15
Selenium	0.15
Silicon	5.0
Chromium	1.0
Fluoride	1.0
Nickel	0.5
Boron	0.5
Lithium	0.1
Vanadium	0.1

Table S4. Cortical and medullary cyst and fibrosis volumes in diseased Han:SPRD-*Cy* rats given control, SP, or SP+FO diets. (n=12/ group) (Chapter 4).

ml/ gm kidney wt.	**Control**	**SP**	**SP+FXO**	**Effects (P-value)**
Cortical cyst volume	2.14 (0.14)	2.08 (0.21)	2.33 (0.21)	No effect
Cortical fibrosis volume	0.21 (0.03)	0.24 (0.04)	0.21 (0.04)	No effect
Medullary cyst volume	1.43 (0.17)	1.43 (0.19)	1.22 (0.19)	No effect
Medullary fibrosis volume	0.16 (0.03)	0.12 (0.02)	0.14 (0.02)	No effect

Cyst areas were captured from 20 random fields (×40 magnification) of H&E stained kidney sections and indices were measured and calculated by a naive observer using LUZEX AP software (NIRECO CO.LTD, Tokyo, Japan). The product of proportional cyst area and kidney weight was used to estimate cyst volume. Fibrosis volume was calculated in the same way using Masson's trichrome stained sections. Values are means ± SE, (n = 12 /group).

Table S5. Correlation analysis of kidney function parameters and total COX and LOX oxylipins (Chapter 4).

Oxylipins	**Serum Creatinine r (P value)**	**Serum cystatin C r (P value)**
COX oxylipins	0.16 (0.3963)	0.50 (0.0135)
LOX oxylipins	-0.49 (0.0040)	-0.24 (0.3011)
CYP oxylipins	-0.54 (0.0010)	-0.41 (0.0321)

P<0.05 is significant

Table S6. Diet composition for study 3 (Chapter 5)

Ingredients (g/kg)	Control	Aspirin (ASA)	NDGA
Cornstarch	397.486	397.486	397.486
Casein protein (87%)	200	200	200
Dextrinized cornstarch	132	132	132
Sucrose	100	100	100
Soybean oil	70	70	70
Fiber	50	50	50
Mineral mix (G)	35	35	35
Vitamin mix	10	10	10
L-Cystine	3	3	3
Choline bitartrate (41.1% choline)	2.5	2.5	2.5
Tert-butylhydroquinone (TBHQ)	0.014	0.014	0.014
Aspirin (ASA)	0	0.4	0
NDGA	0	0	0.5
Total	1000	1000	1000

All diet ingratiates were purchased from Dyets (Bethlehem, PA)

Table S7. HPLC-tandem mass spectrometry parameters for oxylipins scanned for in this thesis. Highlighted are oxylipins scanned for, but not present at detectable levels.

Metabolite	Internal standard	Precursor Ion	Fragment Ion	Detector Response Factor	r^2	Retention Time
12,13-DiHOME	*12,13-DiHOME-d4*	313.00	183.00	2.9909	0.9995	14.85
14,15-DiHETrE	*14,15*-DiHETrE-*d11*	337.00	207.00	1.3602	0.9995	15.08
18-HEPE	*15-HETE-d8*	317.00	215.00	0.4272	0.9953	15.65
9-HEPE	*5-HETE-d8*	317.00	123.00	22.6352	0.9962	16.35
9-HOTrE	*9-HODE-d4*	293.00	171.00	0.6674	0.9996	15.55
9-HODE	*9-HODE-d4*	295.00	171.00	1.7306	0.9999	16.76
19-HETE	*15-HETE-d8*	319.00	275.00	0.5246	0.9988	15.83
12-HETE	*15-HETE-d8*	319.00	179.00	2.5826	0.9999	17.21
9,10-DiHOME	*9,10-DiHOME-d4*	313.00	201.00	3.9356	0.9909	15.15
13-HOTrE	*13-HODE-d4*	293.00	195.00	0.3884	0.9896	15.64
11,12-DiHETrE	*11,12*-DiHETrE *-d11*	337.00	167.00	2.1154	0.9907	15.75
20-HETE	*20-HETE-d6*	319.00	289.00	1.8863	0.9498	15.96
8-HETE	*5-HETE-d8*	319.00	155.00	1.1603	0.9921	17.3
13-HODE	*13-HODE-d4*	295.00	195.00	1.6181	0.9993	16.58
15-HEPE	*5-HETE-d8*	319.00	155.00	1.1603	0.9921	17.3
TXB_2	*TxB_2-d4*	369.00	169.00	1.3754	1	7.85
$PGF_{2\alpha}$	*$PGF_{2\alpha}$-d4*	353.00	193.00	3.3709	0.9879	8.82
PGE_2	*PGE_2-d4*	351.00	271.00	3.7736	0.9997	9.17
9-HETE	*5-HETE-d8*	319.00	151.00	0.4478	0.9997	17.46
12-HEPE	*15-HETE-d8*	317.00	179.00	0.9932	0.9955	15.25
8,9-DiHETrE	*8,9*-DiHETrE-*d11*	337.00	155.00	0.286	0.9998	16.09
16-HETE	*15-HETE-d8*	319.00	232.00	0.9875	0.9902	16.27
PGD_2	*PGD_2-d4*	351.00	189.00	1.0347	0.999	9.64
8-HEPE	*5-HETE-d8*	317.00	155.00	0.4955	0.9893	16.25
15-HETE	*15-HETE-d8*	319.00	219.00	1.1664	0.9998	16.77
5-HETE	*5-HETE-d8*	319.00	115.00	1.4348	0.9972	17.71
5,6-DiHETrE	*11,12*-DiHETrE *-d11*	337.00	145.00	0.8458	0.9955	16.46
9-OxoODE	*5-OxoETE-d7*	293.00	185.00	2.9681	0.9964	17.05
6-keto-$PGF_{1\alpha}$	*6-keto-$PGF_{1\alpha}$-d4*	369.00	163.00	1.6375	0.9991	6.07
LtB4	*LtB4-d4*	335.00	195.00	1.7742	0.9952	14.05
11-HEPE	*5-HETE-d8*	317.00	195.00	0.405	0.9997	16.08
11-HETE	*5-HETE-d8*	319.00	167.00	5.17816	0.9995	17.05
13-OxoODE	*5-OxoETE-d7*	293.00	113.00	7.5212	0.9393	16.79
5-HEPE	*5-HETE-d8*	317.00	115.00	0.8847	0.9993	16.56
14-HDoHE	*15-HETE-d8*	343.23	161.00	1.2743	0.9832	17.1
15-HETrE	*13-HODE-d4*	321.24	221.00	3.7356	0.9992	17.28

4-HDoHE	*5-HETE-d8*	343.23	101.00	3.8587	0.997	17.75
TxB_3	*TxB_2-d4*	367.00	169.00	0.7926	0.9985	8.25
$PGF_{3\alpha}$	*TxB_2-d4*	351.00	193.00	0.5944	0.9998	9.32
PGE_3	*PGE_2-d4*	349.00	269.00	0.5990	0.9831	10.3
PGD_3	*PGE_2-d4*	349.00	269.00	0.3058	0.9924	10.6

Table S8 Fatty acid composition of the diet

Protein	Casein		Soy	
Lipid	Soy	Fish	Soy	Fish
C18:0	29 (1)	17 (0.3)	31 (1)	16 (0.4)
C18:1	144 (3)	52 (1)	159 (4)	49 (1)
C18:2n6	371 (9)	74 (1)	439 (9)	83 (1)
C18:3n3	53 (1)	14 (0.2)	62 (1)	15 (0.3)
C18:3n6	1.5 (0.1)	2.1 (0.1)	1.8 (0.1)	2 (0.1)
C20:3n3	0.2 (0.01)	0.8 (0.01)	0.9 (0.03)	0.9 (0.02)
C20:3n6	0 (0)	1 (0.01)	0 (0)	1 (0.01)
C20:4n6	0.1 (0)	3 (0.04)	0 (0)	3 (0.1)
C20:5n3	0.1 (0.01)	32 (0.4)	0.2 (0.01)	32 (0.8)
C22:0	2.2 (0.1)	0.9 (0.02)	0.8 (0.1)	2 (0.01)
C22:4n6	0.2 (0.1)	0.6 (0.01)	0.4 (0.1)	0.5 (0.01)
C22:5n3	0.2 (0.01)	5 (0.1)	0.2 (0.02)	5 (0.1)
C22:6n3	0.1 (0.01)	22 (0.3)	0.1 (0.01)	22 (0.6)
C24:0	0.7 (0.03)	0.4 (0.01)	0.7 (0.02)	0.3(0.01)