Effect of dietary oils and protein and cyclooxygenase-2 inhibition on disease progression and oxylipin alterations in cystic kidney disease

By

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ABSTRACT

Autosomal dominant polycystic kidney disease (ADPKD) is the most common inherited kidney disease in humans. In animal models that are non-orthologous to ADPKD, dietary soy protein and flax oil slow disease progression, while fish oil feeding has inconsistent effects and has not been compared directly with flax oil. Therefore, weanling Pkd2$^{WS25/-}$(Pkd2) mice orthologous for ADPKD were provided diets containing soy protein or casein, and soy, flax or fish oil for 13 weeks. Renal disease was assessed by histomorphometric analysis of cysts and fibrosis, and measurement of serum urea nitrogen and creatinine levels. In contrast to findings in non-orthologous models, these dietary interventions did not alter disease progression.

Oxylipins are bioactive lipid metabolites that are altered in the kidneys of models that are non-orthologous to ADPKD. In particular, oxylipins derived via the cyclooxygenase (COX) pathway are elevated. Therefore, targeted lipidomic analysis of renal oxylipins of diverse models of PKD (including the Pkd2 mouse) was performed by HPLC-MS/MS. There were differing patterns of oxylipin alterations amongst models, but COX oxylipins were consistently elevated across all types of PKD. The dietary interventions had unique effects on the kidney oxylipin profile despite not altering disease. Kidney oxylipins were higher in females for oxylipins derived via the COX pathway, and higher in males for other pathways.

Dietary high protein (HP) causes normal renal hypertrophy, worsens renal disease and reportedly increases COX oxylipins (prostanoids). However, we found that dietary HP did not alter renal prostanoids and other oxylipins in *pcy* mice (non-orthologous model) provided a HP diet for 13 weeks, despite having the expected physiological renal effects. On the other hand, 13 weeks of feeding a selective COX2 inhibitor (celecoxib) reduced disease in the Pkd2 mouse.

In conclusion, dietary soy protein, flax or fish oil do not alter disease in the Pkd2 mouse model of ADPKD. Kidneys from diverse PKD models display elevated prostanoids, and prostanoids also were higher in females, but diet and sex effects (or lack thereof) on oxylipins did not correlate with disease. However, the slowing of disease with celecoxib suggests that COX2 inhibitors may have therapeutic value for the treatment of ADPKD.

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DEDICATION

I dedicate this thesis to

My parents, *Md Abdul Wohab* and *Most Nurunnahar*

My son, *Ayman Jaman*

and

My wife, *Afroza Ferdouse*

THESIS OUTLINE

This thesis is prepared following a manuscript format, and it is composed of five manuscripts. The thesis begins with a literature review (chapter 1). The literature review consists of brief information on cystic kidney diseases, oxylipins, and the effects of disease, diet and sex on renal oxylipins. In chapter 2, the rationale, hypothesis and objectives are presented. Manuscripts 1, 2, 3, 4 and 5 appear as chapters 3, 4, 5, 6 and 7, respectively. Manuscript 1, 2, 3 and 5 were published in PLoS One, Biochim Biophys Acta-Molecular and Cell Biology of Lipids, Prostaglandins, Leukotrienes and Essential Fatty Acids, and the Journal of Nephrology, respectively. Manuscript 4 is being prepared for submission to the Journal of Nutritional Biochemistry. The thesis is concluded with a general discussion, conclusions and future directions (chapters 8 and 9).

AUTHOR CONTRIBUTIONS

This thesis was primarily written by M Monirujjaman, with the exception of manuscripts 1 and 3 (chapters 3 and 5). For these two chapters, M Monirujjaman conducted the animal feeding, tissue collection, statistical analysis and prepared the results tables for the Pkd2 mouse studies. The first authors of these chapters (3 and 5) were the primary authors of these manuscripts, with contributions by M Monirujjaman to the editing of these manuscripts. Detailed author contributions to the manuscript chapters are detailed below.

Manuscript 1 (chapter 3):

Yamaguchi T, Devassy JG, **Monirujjaman M**, Gabbs M, Aukema HM.

Lack of Benefit of Early Intervention with Dietary Flax and Fish Oil and Soy Protein in Orthologous Rodent Models of Human Hereditary Polycystic Kidney Disease

M Monirujjaman conducted the animal feeding, tissue collection and statistical analysis for the Pkd2 mouse study. JG Devassy and T Yamaguchi conducted animal feeding, tissue collection, and urine sample analysis of the PCK rats. JG Devassy conducted the histology and statistical analysis in the PCK rat study and captured the histology pictures of Pkd2 mice. JG Devassy also prepared the figures used in the paper. M Gabbs helped with the animal feeding and sample collection. HM Aukema, JG Devassy, M Monirujjaman and T Yamaguchi developed the concept and designed experiments and edited the manuscript. HM Aukema was primarily responsible for writing the manuscript and responding to reviewer comments.

Manuscript 2 (chapter 4):

Md Monirujjaman, Jessay G. Devassy, Tamio Yamaguchi, Nikhil Sidhu, Masanori Kugita, Melissa Gabbs, Shizuko Nagao, Jing Zhou, Amir Ravandi, Harold M. Aukema.

Distinct Bioactive Lipids Alterations in Diverse Models of Cystic Kidney Diseases

M Monirujjaman conducted the animal feeding, tissue collection, oxylipin extraction and quantification of the Pkd2 mouse study. M Monirujjaman performed statistical analysis and prepared results tables for the Pkd2 and *jck* mouse studies. M Monirujjaman was also primarily responsible for the writing of the manuscript and responding to reviewer comments.
JG Devassy and T Yamaguchi conducted the breeding and colony expansion and feeding phase of the PCK rats and the two Pkd1 mouse studies. JG Devassy conducted procedures for lipid extraction and oxylipin analysis, statistical analysis and prepared results tables for these three studies. M Gabbs helped with the animal feeding and sample preparation for oxylipin analysis for these three studies. Masanori Kugita and Shizuko Nagao conducted the breeding and colony expansion, feeding phase and tissue collection of the *jck* mouse study. Nikhil Sidhu conducted procedures for lipid extraction and oxylipin analysis of the *jck* mouse study. J Zhou provided Pkd1 mice and provided input for the manuscript, and A Ravandi provided the mass-spectrometric facilities for the oxylipin analysis. HM Aukema, T Yamaguchi, JG Devassy and M Monirujjaman developed the concept, designed experiments and edited the manuscript for final submission.

Manuscript 3 (chapter 5):

Jessay G. Devassy, Tamio Yamaguchi, **Md Monirujjaman**, Melissa Gabbs, Amir Ravandi, Jing Zhou, Harold M. Aukema.

Distinct effects of dietary flax compared to fish oil, soy protein compared to casein, and sex on the renal oxylipin profile in models of polycystic kidney disease

M Monirujjaman conducted the animal feeding, tissue collection, oxylipin extraction and quantification, and statistical analysis of oxylipins for the Pkd2 mouse study. M Monirujjaman prepared the results tables for the Pkd2 study. Devassy and T Yamaguchi conducted animal feeding of the PCK rats and the two Pkd1 mouse studies. JG Devassy carried out lipid extraction and oxylipin analysis, and statistical analysis of these three studies. JG Devassy was primarily responsible for writing the manuscript and responded to reviewer comments. J Zhou provided Pkd1 mice, and A Ravandi provided the mass-spectrometric facilities for the oxylipin analysis. HM Aukema, T Yamaguchi, JG Devassy and M Monirujjaman developed the concept, designed experiments and edited the manuscript for final submission.

Manuscript 4 (chapter 6):

Md Monirujjaman, Harold M. Aukema.

Dietary high protein does not alter renal prostanoids and other oxylipins in normal mice and mice with inherited renal disease

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Manuscript 5 (chapter 7):

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Cyclooxygenase 2 inhibition slows disease progression and improves the altered renal lipid mediator profile in the Pkd2$^{WS25/-}$ mouse model of autosomal dominant polycystic kidney disease

M Monirujjaman conducted the animal feeding, tissue collection, oxylipin extraction and quantification, image capture and analysis and statistical analysis for the study. M Monirujjaman was primarily responsible for the writing of the manuscript and responding to reviewer comments. HM Aukema and M Monirujjaman developed the concept, designed experiments and edited the manuscript for final submission.

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LIST OF ABBREVIATIONS

AA	Arachidonic acid
AdA	Adrenic acid
ADPKD	Autosomal dominant polycystic kidney disease
AIN	American Institute of Nutrition
ALA	α-linolenic acid
ANOVA	Analysis of variance
ARPKD	Autosomal recessive polycystic kidney disease
ASA	Acetylsalicylic acid
cAMP	Cyclic adenosine monophosphate
COX	Cyclooxygenase
cPLA2	Cytosolic phospholipase A2
CREB	cAMP response element binding protein
CFTR	Cystic fibrosis transmembrane conductance regulator
CYP	Cytochrome P450
DGLA	Dihomo-γ-linolenic acid
DHA	Docosahexaenoic acid
DiHDoHE	Dihydroxy-docosahexaenoic acid
DiHDPE	Dihydroxy-docosapentaenoic acid
DiHEDE	Dihydroxy-eicosadienoic acid
DiHEPE	Dihydroxy-eicosapentaenoic acid
DiHETE	Dihydroxy-eicosatetraenoic acid
DiHETrE	Dihydroxy-eicosatrienoic acid

DiHODE	Dihydroxy-octadecadienoic acid
DiHOME	Dihydroxy-octadecenoic acid
DiHOTrE	Dihydroxy-octadecatrienoic acid
%E	% Energy
ELISA	Enzyme-linked immunosorbent assay
EpDPE	Epoxy-docosapentaenoic acid
EpETE	Epoxy-eicosatetraenoic acid
EpETrE	Epoxy-eicosatrienoic acid
EpODE	Epoxy-octadecadienoic acid
EpOME	Epoxy-octadecenoic acid
EPA	Eicosapentaenoic acid
ERK	Extracellular signal-regulated kinase
ESRD	End-stage renal disease
Ex	Eoxin
GLA	γ-linolenic acid
GLM	General linear model
GFR	Glomerular filtration rate
HDoHE	Hydroxy-docosahexaenoic acid
HEPE	Hydroxy-eicosapentaenoic acid
HETE	Hydroxy-eicosatetraenoic acid
HETrE	Hydroxy-eicosatrienoic acid
HHTrE	hydroxy-heptadecatrienoic acid
HODE	Hydroxy-octadecadienoic acid

HOTrE	Hydroxy-octadecatrienoic acid
HP	High protein
HpDoHE	Hydroperoxy-docosahexaenoic acid
HpETE	Hydroperoxy-eicosatetraenoic acid
HpETrE	Hydroperoxy-eicosatrienoic acid
HpEPE	Hydroperoxy-eicosapentaenoic acid
HPLC/MS/MS	High performance liquid chromatography tandem mass spectrometry
HpODE	Hydroperoxy-octadecadienoic acid
HpOTrE	Hydroperoxy-octadecatrienoic acid
KLF15	Kruppel-like factor-15
LA	Linoleic acid
LLE	Liquid liquid extraction
LOX	Lipoxygenase
LP	Low protein
Lt	Leukotriene
MAPK	Mitogen-activated protein kinase
MIF	Macrophage migration inhibitory factor
MiR	Micro-RNA
mTOR	Mechanistic target of rapamycin
NP	Normal protein
NPHP	Nephronophthisis
NSAID	Nonsteroidal anti-inflammatory drug
oxo-DoHE	Oxo-docosahexaenoic acid

oxo-EPE	Oxo-eicosapentaenoic acid
oxo-ETE	Oxo-eicosatetraenoic acid
oxo-ODE	Oxo-octadecadienoic acid
oxo-OTrE	Oxo-octadecatrienoic acid
PBS	Phosphate buffered saline
PC	Polycystin
PD	Protectin
PDCD4	Programmed cell death 4
PG	Prostaglandin
PGEM	Prostaglandin E metabolite
pI:pC	Polyinosinic polycytidylic acid
PKA	Protein kinase A
PKD	Polycystic kidney disease
RAS	Renin-angiotensin system
Rv	Resolvin
SE	Standard error
SPE	Solid phase extraction
sEH	Soluble epoxide hydrolase
TriHOME	Trihydroxy-octadecenoic acid
Tx	Thromboxane

Chapter 1: Literature Review

Autosomal dominant polycystic kidney disease (ADPKD), autosomal recessive polycystic kidney disease (ARPKD) and nephronophthisis (NPHP) are major genetic forms of cystic kidney diseases. However, there is a scarcity of effective treatments to slow disease progression in these disorders. Some evidence in NPHP models shows that dietary treatment with soy protein, flax and/or fish oil may provide an effective treatment option in these disorders. This needs to be examined in orthologous models of ADPKD, the most common genetic form of cystic kidney disease, and is examined more extensively in this thesis. Secondly, alterations in a limited number of lipid mediators called oxylipins have been documented in NPHP. So the second main thrust of this thesis is to examine the renal oxylipin profile in a model of ADPKD and to examine the effects of several diets, a drug, and sex differences on these oxylipins, and their effects on disease progression.

In the following literature review, the major genetic forms of cystic kidney diseases ADPKD, ARPKD and NPHP will be discussed briefly. We will then describe the oxylipin pathways and provide some evidence of oxylipin alterations in NPHP models. We will also discuss effects of inhibiting a specific group of oxylipins on NPHP progression. This will be followed by a discussion of the effects of dietary soy protein, flax oil and fish oil in the treatment of PKD and their effects on oxylipin alterations. We will then discuss the renal effects of dietary high protein (HP) in renal disease and its effects on renal oxylipins. This will be followed by a section on sex difference in renal oxylipins. Finally, there will be a brief description of the orthologous Pkd2 mouse model of ADPKD and *pcy* mouse model of NPHP, used in this thesis.

1.1 Cystic kidney diseases

Cystic kidney diseases encompass a group of hereditary, nonhereditary, and acquired disorders characterized by growth and proliferation of numerous renal cysts, and development of a variety of external manifestations that can ultimately lead to end stage renal disease (ESRD) (1-3). Disease development can occur any stage of life i.e., in infancy, childhood, or adulthood (3). Cystic kidney diseases also are known as ciliopathies, since almost all genes mutated in these diseases encode protein products that are linked to the biology and function of primary cilia (1).

The most common genetic form of cystic kidney diseases is polycystic kidney disease (PKD), which is caused by mutations in one or more genes that encode proteins that primarily function in cilia (4, 5). Two major types of PKD in humans are ADPKD and ARPKD. ADPKD is more prevalent and is characterized by the slow bilateral development of large fluid-filled renal cysts (4, 5). Due to the development of large cysts, the kidneys become enlarged and their functional integrity becomes diminished, ultimately resulted in progressive renal failure (6). The cysts may be up to 10 to 20 cm in diameter with hundreds to thousands of them within a single kidney; clinically significant cysts are also common in the pancreas, intestine, and in the liver (especially in women). Common symptoms in ADPKD patients are the presence of hypertension, flank pain, recurrent urinary tract infections, polyuria, hematuria and kidney stone formation. Patients with ADPKD have an increased risk of heart-valve defects and aortic aneurysms (7), and some patients have five times the risk of sudden death from intracerebral aneurysms compared to general population (8). In ADPKD, clinically significant impairments of renal function occur usually after age of forty, and by 70 years of age 50% of patients experience ESRD, which requires renal replacement therapy. In the fourth decade of life renal function

begins to decline and glomerular filtration rate (GFR) also begins to decrease by 4.4–5.9 mL/min/year (9). ADPKD is discussed further in section 1.1.1.

ARPKD is much rarer than ADPKD, with a prevalence of 1 in 20,000 live births (10). Tremendous bilateral enlargement of the kidneys, pulmonary hypoplasia, and impaired lung formation are characteristic features of ARPKD which often cause fetal or neonatal death (10). The majority of babies who survive the perinatal period develop hepatic fibrosis and renal failure. ARPKD is caused by a mutation in the polycystic kidney and hepatic disease-l (PKHD1) gene found in chromosome 6p (11). ARPKD is discussed further in section 1.1.2.

Another form of cystic kidney diseases is known as nephronophthisis (NPHP), one of the most common genetic disorders causing ESRD in children (12). NPHP will be discussed further in section 1.1.3.

1.1.1 Autosomal dominant polycystic kidney disease (ADPKD)

ADPKD occurs worldwide, in all races with a prevalence of 1:400 to 1:1000, affecting approximately 12.5 million people worldwide (13). It is the fourth leading cause of ESRD and affects about 600,000 individuals (5000 to 6000 new cases each year) in the United States (14, 15). In 2016, a total of 5,597 new patients were diagnosed with ESRD in Canada, of them 3.8% were primarily diagnosed with ADPKD (16). ADPKD patients develop numerous fluid filled cysts in utero that continue to grow and expand throughout life until they damage the surrounding tissues and impair normal renal function (17). Although a small proportion of tubules develop cysts, their continuous expansion over decades causes significant damage to the kidney structure, and accompanied by inflammation, fibrosis, apoptosis and oxidative stress, ultimately results in renal failure (18-20). Men with ADPKD progress to renal failure

approximately 5 years earlier than women, but women with more than 3 pregnancies also have increased risk of ESRD. However, there is significant variability in disease progression between individuals, ranging from renal failure occurring in the neonatal period to patients in their nineties with kidneys that are still functioning (21).

ADPKD is heterogeneous and two responsible genes have been identified: i) *PKD1* is found in the chromosome region 16p13.3 and accounts for ~85% cases, ii) *PKD2* is found in chromosome region 4q21 and accounts for ~15% cases (22, 23). Some patients shows similar symptoms and characteristics of ADPKD without having mutations in *PKD1* or *PKD2* genes, suggesting the presence of a third responsible gene (24). However, the proposed gene, *PKD3*, has not been identified yet. Moreover, re-sampling and re-analysis of clinical information, and mutation screening for *PKD1/PKD2* genes in those patients and families does not provide any evidence to support the existence of a third PKD gene (25). Homozygous or compound heterozygous mutations of PKD are embryonically lethal (26). More severe renal disease is observed in individuals who are heterozygous for both *PKD1* and *PKD2* mutations and survive to adulthood (27). Although ADPKD1 and ADPKD2 result from mutations in different proteins, most studies until very recently have not specified the form of the disease. Indeed, prior to the discovery of specific gene defects, these two forms were referred to as Adult PKD.

The *PKD1* gene encodes polycystin-1 (PC1), a large integral trans-membrane glycoprotein with a molecular mass of ~460 kDa and composed of 4303 amino acids (AA). On the other hand, the *PKD2* gene encodes polycystin-2 (PC2), which is an integral membrane glycoprotein formed by 968 AA residues with a molecular mass ~110 kDa. PC2 is primarily found in the endoplasmic reticulum (ER); however, it can also be found in the primary apical cilium, mitotic spindles, centrosome and plasma membrane. On the other hand, PC1 shares

localization with PC2 in the plasma membrane, primary cilium and potentially in the ER (28). It is thought that PC1 might function as a mechanosensor, whereas, PC2 is a nonselective calcium channel protein. PC1-PC2 protein complexes located in the primary cilium have important roles disease progression. The cilium which projects into the lumen is thought to have sensory functions in the tubular epithelial cells (29). Lack of functional PC1 in transgenic mice does not affect cilia formation; however, increased Ca^{2+} influx does not occur in response to physiological fluid flow in isolated cells from these transgenic mice. Similarly, blocking the activity of PC2 by blocking antibody also abolishes the flow response in the wild-type cells (30). These findings provide evidence for the mechanosensor roles of the polycystin complex in the primary cilia, which detects flow changes and thus leads to Ca^{2+} influx through the polycystin complex. The higher Ca^{2+} level inside cells in turn induces Ca^{2+} release from intracellular stores (29). The *PKD1* and *PKD2* gene product work together in a receptor-channel complex which regulates calcium based intra-cellular signaling, and mutations in either PC1 or PC2 protein disrupt this signaling pathway. Therefore, mutations in PC1 or PC2 result in a similar disease outcome and indistinguishable clinical phenotypes (31). However, the functions of the individual polycystins as well as functions of the PC1-PC2 receptor-channel complex are poorly understood. The immediate downstream pathways of PC1-PC2 complex have not been identified yet (31).

It has been shown that ADPKD renal tubular cells have elevated cAMP levels, impaired calcium signaling, low calcium stores in the endoplasmic reticulum and low intracellular calcium levels (32, 33). Polycystin proteins are involved in development of tubules and the vasculature in the kidney as well as the liver, heart, brain and other tissues, but the effects are most significant in the kidney. Defects in one or both of these proteins results in loss of proper cell orientation

and secretion, increased cell proliferation and apoptosis, extracellular matrix remodeling and cyst formation (34).

Interestingly, cystogenesis may derive from gain-of-function of PKD1 or PKD2 as well (35, 36). Thus, too much or too little PKD1/PKD2 is cystogenic, and their expression and function have to be strictly regulated. A very small proportion of tubules develop cysts despite the fact that all cells in an individual with ADPKD have one copy of a mutated PKD gene and one copy of a normal allele. The most prevalent explanation of this is currently the "two-hit" theory which proposes that cysts begin to form only when the normal allele sustains a "hit" resulting in both alleles being mutated. Evidence for a "third hit" resulting from renal injury which accelerates cyst formation and disease progression has been reported (37). Altering the environmental influences that affect the rates of second or third "hits" or mutations is a potential means of modifying the development of disease; the potential role of diet in this remains to be investigated.

1.1.2 Autosomal recessive polycystic kidney disease (ARPKD)

ARPKD is a much less common but a more severe form of PKD than the ADPKD form. It results from a defect in the *PKHD1* gene which codes for fibrocystin (also called polyductin) (38, 39). Fibrocystin appears to function as part of the polycystin complex and is involved in calcium homeostasis and in the maintenance of the three-dimensional structure of the tubules. Enlarged kidneys filled with cysts are the main symptoms, but liver cysts and fibrosis, portal and systemic hypertension, and deformities in the face, spine and limbs also occur (38, 39). Renal cysts in ARPKD are primarily found in the collecting ducts, in contrast to ADPKD, where they can also originate from other parts of the nephron. This form of PKD typically presents early in

life with 30-50% of patients dying shortly after birth. For those who survive past the first month, however, the long-term prognosis improves, with a five-year survival rate of 75-90% (40, 41). More than half of these patients require renal replacement therapy by 10-20 years of age (42, 43).

1.1.3 Nephronophthisis (NPHP)

NPHP is characterized by inflammation, fibrosis and development of corticomedullary cysts (usually in the latter stage of disease) (44, 45), which ultimately lead to polyuria, polydipsia, anemia, and growth retardation (12). It is the most common cause of renal failure in the first three decades of life with a prevalence of 1 in 50,000 to 80,000 worldwide. In North America, NPHP is the reason for 5–10% cases of ESRD in children (45-47). Clinically NPHP can be divided into infantile, juvenile, and adolescent/adult types (44). Mutations in more than 25 genes underlying this disorder have been identified, encoding protein products that are mostly related to cilia (12, 48). *NPHP1* gene mutation is most common, and accounts for approximately 20% of cases (44).

Since the phenotypic expression in NPHP animal models are similar to the phenotypic expression of human PKD, many of the previous studies (e.g. dietary studies, oxylipin studies – see sections following) with PKD were carried using NPHP models, particularly before the genetic defects in these models had been established. However, since the underlying genetic causes of NPHP are different from human PKD, findings in NPHP models need to be examined and confirmed in orthologous models of ADPKD prior to moving these pre-clinical studies to clinical studies. This is one of the objectives of this thesis.

1.2 Oxylipins

Oxylipins are a group of biologically active compounds present in tissues, blood and urine that are formed by oxidative metabolism of polyunsaturated fatty acids (PUFA) released from membrane phospholipids by the action of cytosolic phospholipase A_2 ($cPLA_2$) (49). They are thought to be short-lived and not stored; however, the presence of steady-state levels of both free and esterified oxylipins in tissues such as the liver, adipose, kidney, ileum, etc. provide evidence that they can be stored (50-52). Oxylipins are important regulators of tissue homeostasis, inflammation and signaling, and they have been documented to be involved in many diseases including renal disease, cardiovascular disease, and cancer (53-57). Arachidonic acid (AA), which is a n-6 fatty acid, is the most well-known precursor of oxylipins in the body that gives rise to eicosanoids (58). However, oxylipins can also be produced from other PUFA precursors including linoleic acid (LA), dihomo-γ-linolenic acid (DGLA), α-linolenic acid (ALA), eicosapentaenoic acid (EPA), adrenic acid (AdA) and docosahexaenoic acid (DHA). Different oxylipins vary in their carbon chain length, and presence and position of double bonds. The type of oxylipins produced depends on PUFA oxidizing enzymes and type of PUFA being oxidized (59). These PUFA precursors can be obtained directly from the diet or they can be synthesized *de novo* in the body from LA and ALA into longer chain PUFA. In general, a higher level of n-3 PUFA derived oxylipins are produced with consumption of an n-3 rich diet, whereas higher levels of n-6 derived oxylipins are produced with intake of an n-6 rich diet (60). However, the types of oxylipins produced in the body also depends on the type and levels of PUFA in the tissue phospholipids, enzyme preferences for each PUFA as well as the enzyme present for metabolizing these PUFA into oxylipins in each tissue. Therefore, it is important to

measure tissue oxylipin profile directly in order to understand the effects of PUFA that are mediated via oxylipins (60).

Distinct classes of oxylipins are formed by the action of cyclooxygenase (COX), lipooxygenase (LOX) and cytochrome P_{450} (CYP) enzymes upon different physiological and pathological stimuli, such as hormones, growth factors and cytokines (60-62). The COX enzymatic pathway results in the formation of prostaglandins (PG) and thromboxanes (Tx), collectively known as prostanoids; the LOX enzymatic pathway produces leukotrienes (LTs) and hydroxy-fatty acids; the CYP enzymatic pathway results in formation of hydroxyl fatty acids via ω-hydroxylase activity and epoxy fatty acids via epoxygenase activity (53, 60-62). Figure 1.1 shows simplified pathways of oxylipin production from various PUFAs. For detailed information about oxylipins, their source fatty acids, and the enzymes responsible for their production please refer to our recent review (60).

1.2.1 The cyclooxygenase (COX) pathway

The COX pathway converts PUFA into their respective prostanoids– i.e. PGs and TXs (63-65). Prostanoids contain one or more double bonds and a characteristic five-carbon ring structure (60). Generation of prostanoids involves a series of reactions that begins with prostaglandin H synthase (PGHS) that oxidizes PUFA into an unstable intermediate hydroperoxy endoperoxide PGH (oxidation reaction). This is further metabolized by prostanoid synthases, including prostaglandin D synthase (PGDS), prostaglandin E synthase (PGES), prostaglandin F synthase (PGFS), prostaglandin I synthase (PGIS) and thromboxane synthase (T_XS), to produce subsequent prostanoids (53, 58, 60, 66). The COX pathway converts DGLA, AA, EPA and AdA into 1-, 2-, 3- and dihomo-2-series prostanoids, respectively (60, 67, 68).

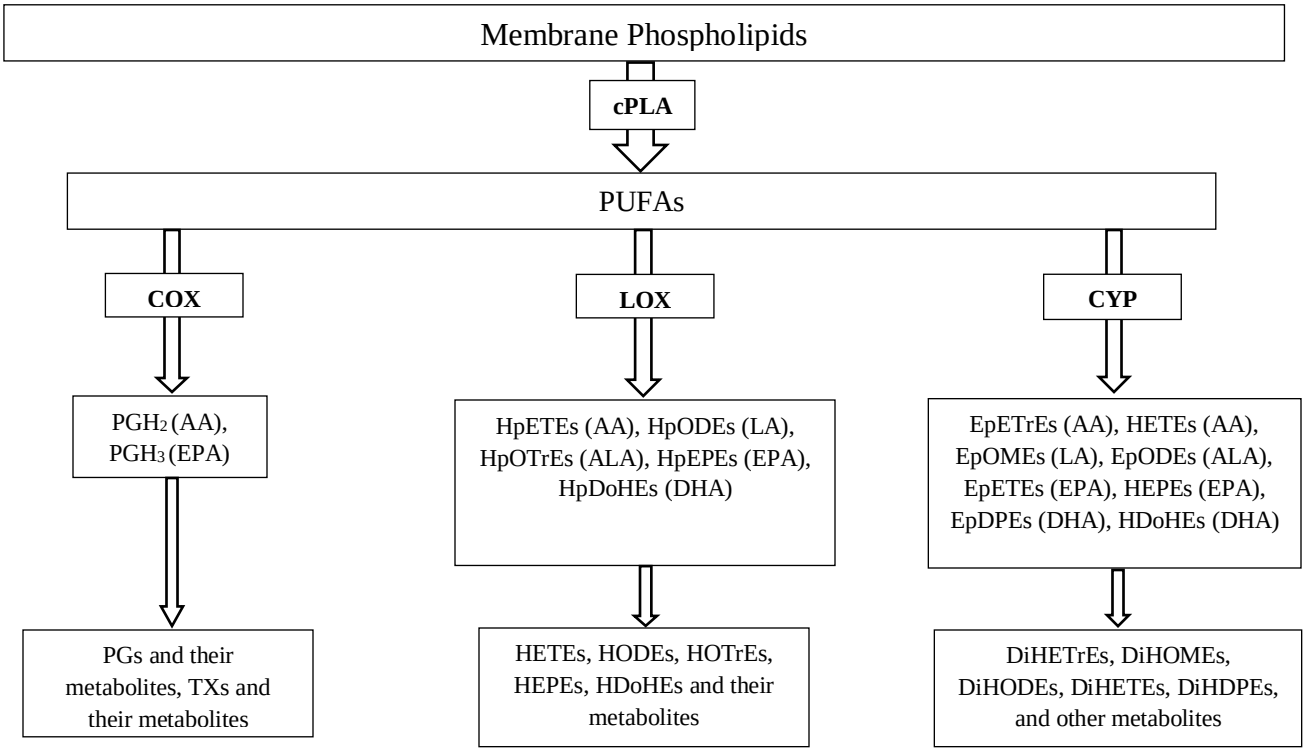

Figure 1.1 Simplified pathways of oxylipin production from various PUFAs. PUFA precursors are indicated in parentheses in the first level of boxes.

Abbreviations: AA, Arachidonic acid; ALA, α- linolenic acid , COX, Cyclooxygenase; $cPLA_2$, Cytosolic phospholipase A_2; CYP, Cytochrome P_{450}; DHA, Docosahexaenoic acid; DiHETrE, Dihydroxy-eicosatetraenoic acid; DiHETE, Dihydroxy-eicosatetraenoic acid; DiHODE, Dihydroxy-octadecadienoic acid; DiHOME, Dihydroxy-octadecenoic acid, DiHOTrE, Dihydroxy-octadecatrienoic acid; EPA, Eicosapentaenoic acid, EpDPE, Epoxy-docosapentaenoic acid; EpETE, Epoxy-eicosatetraenoic acid; EpETrE, Epoxy-eicosatrienoic acid; EpODE, Epoxy-octadecadienoic acid; EpOME, Epoxy-octadecenoic acid; HDoHE, Hydroxy-docosahexaenoic acid; HEPE, Hydroxy-eicosapentaenoic acid; HETE, Hydroxy-eicosatetraenoic acid; HODE, Hydroxy-octadecadienoic acid; HOTrE, Hydroxy-octadecatrienoic acid; HpDoHE, Hydroperoxy-docosahexaenoic acid; HpETE, Hydroperoxy-eicosatetraenoic acid; HpEPE, Hydroperoxy-eicosapentaenoic acid; HpODE, Hydroperoxy-octadecadienoic acid; HpOTrE, Hydroperoxy-octadecatrienoic acid; PG, Prostaglandin; LA, Linoleic acid; LOX, Lipoxygenase; PUFA, Polyunsaturated fatty acid; TX, thromboxane.

Two isoforms of the COX enzyme, COX1 and COX2, have been found in humans. A new member of the COX family, COX3 has been identified (69, 70). This isoform is encoded by a gene similar to COX1; however, it contains a different intronic sequence and is expressed mainly in the hypothalamus and pituitary gland of rats (70, 71). To the best of our knowledge, COX3 is not expressed in humans and there is no information about the expression and role of this isoform of COX in the kidneys. COX1 was first isolated from sheep seminal vesicles (72), and is constitutively expressed in most adult tissues, whereas, COX2 was first isolated from mouse and chicken fibroblast cell cultures, and is inducible and is upregulated in response to various physiological stimuli (73, 74). Both COX1 and COX2 proteins are composed of about 600 amino acids and share 60%-65% homology within and 85%-90% homology between species at the amino acid level (74, 75). Although evidence shows that COX1 is expressed constitutively and COX2 is inducible, both COX1 and COX2 enzyme activity are higher in a number of renal disorders and nephritic models (76, 77). Prostanoids derived from COX1 generally carry out homeostatic functions, whereas higher levels of prostanoids derived from COX2 induction mediate the inflammatory responses and are the target for many anti-inflammatory drugs. A new class of nonsteroidal anti-inflammatory drugs (NSAIDs) known as selective COX2 inhibitors selectively blocks COX2 activity and prevent higher prostanoid production without affecting the COX1 enzyme activity (76, 78). This class of drugs (selective COX2 inhibitors) are different from traditional NSAIDs, as traditional NSAIDs block both COX1 and COX2 activity, whereas, selective COX2 inhibitors selectively block COX2 enzyme activity and COX1 remains functional.

1.2.2 The lypooxygenase (LOX) pathway

The second pathway of oxylipin formation is the LOX pathway that converts PUFA into hydroxy fatty acids and their subsequent metabolites, including leukotrienes, lipoxins, resolvins, protectins, maresins, hepoxilins and eoxins (60). There are seven murine functional LOX genes that have been identified; of them, six are on chromosome 11 and one gene (5-LOX) is on chromosome 6. In contrast, six human functional LOX genes have been identified, five of which are on chromosome 17 and only the 5-LOX gene is on chromosome 10 (79). The conventional classification of LOX enzymes is based on positional specificity of AA oxegenation to form hydroperoxy and hydroxy fatty acid substrates. However, this classification has several limitations (60, 79). In humans and rodents, 5-,12- and 15-LOX are primarily attributed to oxylipin formation and are the most studied (60, 80). The first step of the LOX pathway is to form hydroperoxy fatty acids, which is followed by formation of hydroxy fatty acids. Hydroxy fatty acids can be further metabolized to their keto, di- or tri- hydroxy derivatives (60).

1.2.3 The cytochrome P_{450} (CYP) pathway

The third pathway of oxylipin formation is the CYP pathway that converts PUFA into epoxy- or hydroxy fatty acids by epoxygenase or ω-hydroxylase activity (60). CYP enzymes are so named because in the reduced state they bind to carbon monoxide and have their highest absorbance at 450 nm. The CYP enzymes constitute a diverse superfamily of more than 8,700s protein that share a common tertiary folding structure with overall < 25% amino acid sequence similarity (81). They are divided into multiple families and subfamilies, and in most mammalian tissues CYP2C and CYP2J enzymes metabolize PUFA by epoxygenase activity, whereas the CYP4A and CYP4F subfamilies metabolize PUFA by hydroxylase activity (81). Oxylipins

derived from AA by CYP enzyme activity (such as epoxyeicosatrienoic acids and hydroxy-eicosatetraenoic acids) have been shown to regulate cell proliferation and differentiation, ion transport mechanisms, vascular tone, inflammation, renal hemodynamics, and salt and water reabsorption and secretion (54, 82).

1.3 Oxylipins in cystic kidney diseases

Oxylipins have diverse functions in the body, and most functions of oxylipins are still being elucidated (59). One way they can mediate their effects is by activating a group of nuclear receptor proteins knows as peroxisome proliferator-activated receptors (PPARs). PPARs function as transcription factors that regulate expression of different genes. They can also act in a paracrine or autocrine manner by diffusing through the plasma membrane and signal through G protein-coupled receptors (83, 84). Many oxylipins directly interact with receptors that mediate their effects via stimulatory or inhibitory G proteins (85-87). Because of variation in type and location of receptors, oxylipin effects are diverse. For example, PGE_2 has both vasodilatory and vasoconstrictor effects, while PGI_2 has primarily vasodilatory effects. Prostanoids also can influence inflammation (PGE_2), cell growth and death (PGI_2), and tubular transport processes (PGI_2), in addition to the hemodynamic effects (56, 87, 88). Other oxylipins such as 12-HETE modulate the activity of signaling proteins that affect renal cell proliferations (89, 90). Since the effects of oxylipins are often different and sometimes opposing, it is important to assess the role of multiple oxylipins in tissues.

In normal unchallenged kidneys, oxylipin levels are usually low; however, they can be generated rapidly in response to physiological stimuli. In normal kidneys, oxylipins play a critical role in maintaining GFR by regulating hemodynamics and salt/water homeostasis;

however, in diseased kidneys, oxylipins also enhance disease progression by inflammatory and proliferative processes in response to renal injury (53, 56). It has been shown that protein and mRNA level of enzymes involved in AA release ($cPLA_2$) and prostanoid formation (COX1 and COX2) are altered in Han:SPRD-Cy rat and *pcy* mouse models of NPHP (77, 91) (PKD animal models referred in this thesis including the Han:SPRD-Cy rat and the *pcy* mouse are included in the Table 1.1). In a recent lipidomic analysis of kidneys from *pcy* mice, it has been shown that COX oxylipin levels are overall increased as disease worsens, and levels of specific HETEs derived from the lipoxygenase and/or cytochrome P450 pathways are reduced (92). It also has been shown that treatment with the COX2 selective inhibitor NS398 reduced prostanoid production and reduced cyst growth, interstitial fibrosis, proliferation, inflammation and oxidant injury in the kidneys of Han:SPRD-Cy rat (76). These findings are consistent with the previous observations where COX2 inhibitors were reno-protective in other forms of chronic renal injury (93-95).

An inhibitor of the cytochrome P450 pathway also reduces disease in the Balb/c polycystic kidney (non-orthologous) mouse model of ARPKD. Administration of this inhibitor for 14 days resulted in reduced kidney size and serum urea and creatinine (96).

1.3.1 COX derived oxylipins in renal cystic disease progression

COX enzymes are expressed in both the cortex and medulla of kidneys (97), and their expression is altered in diseased kidneys of Han:SPRD-Cy rats and *pcy* mice models of NPHP (91). COX1 and COX2 enzyme activities are higher in diseased compared to normal kidneys in Han:SPRD-Cy rats (77, 98). Following pharmacological inhibition of COX by non-selective or selective inhibitors, disease progression markedly slows down as evidenced by reduced renal

cyst proliferation and renal fibrosis in this model (76, 99-101). Is has also been shown that COX derived oxylipins, in particular those derived from AA, such as $PGF_{2\alpha}$, 6-keto-$PGF_{1\alpha}$, PGE_2, and TxB_2 were higher in Han:SPRD-Cy rat kidneys (76, 77). AA derived PGD_2, PGE_2, 6-keto-$PGF_{1\alpha}$, $PGF_{2\alpha}$, 15-d-PGJ_2, TxB_2 and 12-HHTrE were also higher in diseased compared to normal kidneys in the *pcy* mice model (92). Moreover, AA derived PGE_2, TxB_2 and 6-keto-$PGF_{1\alpha}$ were elevated as early as 30 days of age in diseased kidneys of *pcy* mice (102). Results from these studies clearly signify the importance of COX, and COX oxylipins in renal cystic disease progression in NPHP.

The actual mechanism by which these altered oxylipins lead to disease progression are not clearly known; however, several possible mechanisms including stimulation of epithelial cell proliferation and transepithelial fluid secretion have been described (57, 103, 104). These two processes are important for expansion of renal cysts (105, 106), and both are stimulated by elevated cAMP (57, 107). In relation to COX oxylipins, PGE_2 causes cyst formation, cell proliferation and intracellular cAMP production in primary cultured ADPKD cells (87, 104, 108). In several cystic kidney disease models, renal cAMP is elevated (109-111), and cAMP can stimulates fluid secretion and epithelial cell proliferation in human kidney cyst cells (112). COX oxylipins such as PGE_2 mediate their effects via G protein-coupled receptors resulting in the stimulation of protein kinase A (PKA) signaling (87, 104, 108, 113). Activated PKA in turn can stimulate B-Raf and the mitogen-activated protein kinase (MAPK) cascade in cyst epithelial cells, resulting in the phosphorylation of extracellular signal-regulated kinase (ERK) (113). ERK and MAPK in turn activate cAMP response element binding protein (CREB) and thus promote transcriptional activity related to renal cell proliferation (87, 108, 114).

In several orthologous mouse models of ADPKD as well as in human ADPKD kidney miR-21 is upregulated (108, 115). cAMP signaling can transactivates miR-21 in renal cells (108). miR-21 is a target for the programmed cell death 4 (PDCD4) tumor suppressor gene and suppress its expression (116), while in cyst epithelial cells inactivation of miR-21 resulted in increased PDCD4 expression (108). PDCD4 is a pro-apoptotic gene and a subset of PDCD4 null (*Pdcd4*$^{-/-}$) mice spontaneously develop kidney cysts (117). It has been shown that *in vitro* treatment with PGE_2 up-regulates miR-21 expression and down-regulates PDCD4 proteins in colonic adenocarcinoma cells, while selective COX2 inhibition reverses this (116). COX oxylipins can increase cAMP level by inhibiting cAMP phosphodiesterase enzymes, which selectively break-down the phosphodiester bond in cAMP and reduce its cellular level (118). Overall these studies provide strong evidence regarding COX oxylipin roles, in particular those derived from AA, in cystic kidney disease progression in different models.

1.3.2 Effects of COX inhibition in the kidney

NSAIDs are used to reduce inflammation and pain and are the most commonly prescribed and used drugs worldwide (119, 120). NSAIDs are used to treat a number of inflammatory diseases including arthritis, headache, dysmenorrhea, dental pain, gout and ankylosing spondylitis (121-123). NSAIDs can be classified into two types based on their mechanism of action: traditional nonselective NSAIDs (tNSAIDs) and selective COX inhibitors (119). The tNSAIDs inhibit both COX1 and COX2 enzyme activity, and thus inhibit overall COX metabolite production (123, 124). On the other hand, selective COX inhibitors such as rofecoxib, valdecoxib and celecoxib, selectively inhibit COX2, and are commonly used for the treatment of inflammatory diseases such as arthritis (125).

It has been found that tNSAIDs are associated with an increased risk of gastrointestinal ulcers which include gastrointestinal hemorrhage, obstruction and perforation (126-129). About 15-30% patients who regularly take tNSAIDs develop gastric or duodenal ulcers (130). The ulcerogenic effects of tNSAIDs are thought to relate to its capacity to inhibit COX1 in the gastric mucosa (119). The use of selective COX2 inhibitors is an alternative option to reduce the risk of these gastrointestinal events (119).

In diseased kidneys, the majority of COX activity is due to the COX2 isoform (77), and inhibition of the formation of the COX oxylipins with selective COX2 inhibitors reduces disease progression and attenuates altered oxylipin production in the Han:SPRD-Cy rat model of NPHP (76, 131), as well as in models of other kidney diseases (99, 132, 133). Selective COX2 inhibition in human ADPKD cyst-lining epithelial cells suppresses cell cycle progression, inhibits proliferation, and induces apoptosis (131). Whether similar effects are observed in ADPKD is still unknown and are explored in this thesis.

Selective COX2 inhibitors such as coxibs can cause peripheral edema, hypertension, sodium retention, hyperkalemia, papillary necrosis and renal insufficiency (134). A large meta-analysis of 114 studies including 116,094 patients showed that an increased risk of hypertension, renal dysfunction and peripheral edema was associated with selective COX2 inhibitor rofecoxib, whereas, lower risk of hypertension and renal dysfunction was associated with celecoxib (135). Despite the fact that COX2 inhibitors have been associated with acute renal dysfunction and myocardial infarction (136-138), there is a scarcity of effective treatment options for PKD to date. The fact that COX2 inhibitors effectively reduced disease progression in the Han:SPRD-Cy rat, as well as other models of renal disease (99, 101, 132, 133), provides rationale for the use of selective COX2 inhibitor in ADPKD as a potential therapeutic approach for this disorder.

1.3.3 Effects of LOX and CYP oxylipins in cystic kidneys

Although previous studies focused on COX derived oxylipins, more specifically AA metabolites, oxylipins derived from LOX or CYP pathway or other PUFA derived COX oxylipins might have important functions in PKD progression. Studies show that LOX and CYP derived oxylipins as well as other PUFA (other than AA) derived COX oxylipins are also altered in models of NPHP (92, 139). In general, oxylipins derived from n-6 PUFA are considered to be pro-inflammatory; on the other hand oxylipins derived from n-3 PUFA are considered to be less inflammatory or anti-inflammatory (140-142). In the Han:SPRD-Cy rat, LOX derived 9-HODE (139), and in *pcy* mice several LOX derived oxylipins (both from n-3 and n-6 PUFA) were lower in diseased kidneys (92). In kidneys, LOX derived oxylipins play important role in the regulation of renal blood flow, vasoconstriction, renal blood pressure and GFR (143). However, the function of most LOX oxylipins in kidneys is still unknown.

Studies examining the level and effect of CYP derived oxylipins in PKD are rare; however, in one study with the *pcy* mouse model of NPHP, the CYP pathway was also altered in disease, and CYP derived n-3 and n-6 oxylipins were lower in diseased compared to normal *pcy* mouse kidneys (92). Plasma epoxyeicosatetraenoic acid (EpETE), a CYP derived oxylipin, was lower in plasma of patients with ADPKD (144). CYP derived oxylipins have been proven to have anti-inflammatory, vasodilatory, anti-apoptopic and anti-fibrotic effects which provide cardiovascular and renal benefits (82, 145, 146). For example, epoxyeicosatrienoic acid (EpETrE) is a vasodilator, and it produces vascular relaxation and maintains vascular homeostasis through the production of the vasodilator nitric oxide by upregulating endothelial nitric oxide activity (143, 147). Therefore, it is reasonable to speculate that LOX and CYP

derived oxylipins play important roles in PKD progression and role of these oxylipins need to be explored further.

In sum, several studies have shown that oxylipins are altered in the Han:SPRD-Cy rat and in the *pcy* mouse models of NPHP, but there are no data on models of ADPKD. Further, only a limited number of oxylipins were analyzed in these studies (76, 77, 92, 102). Serum oxylipins from human ADPKD patients also display alterations, but renal changes are unknown. Therefore, another objective of the present thesis was to analyze the renal oxylipin profile in orthologous models of ADPKD.

1.4 Challenges in oxylipin analysis and analytical methods

The main challenge with oxylipin analysis is that they are present in very lower concentrations in the biological samples and are very unstable. Tissue degradation and auto-oxidation can occur within few seconds of collection; moreover, non-enzymatic lipid peroxidation can occur even at -20C (148, 149). Therefore, tissue samples should be frozen in liquid nitrogen as soon as possible and stored at -80C. Blood samples should be collected and stored on ice and processed as quickly as possible. Short storage of blood at room temperature before further processing has a huge impact on the concentration of several oxylipins (150). Delaying serum collection and freezing after centrifugation can also decrease the levels of some oxylipins (150).

The method of euthanasia also may have great effects on several oxylipins. For example: rats euthanized by decapitation had 10–40 times higher PGs in brain tissue, when compared to euthanasia by focused microwave radiation (151). Similar effect also have been observed for

mouse kidney tissue, in which renal PGs increases up to 140-fold, when analyzed without microwave fixation (152).

In order to monitor oxylipin extraction efficiency and to quantify oxylipins, stable internal standards (IS) are used. However, not every oxylipin has a corresponding IS, in which case a common IS for a group of oxylipins is used. In a recent study, Wang et al. used 26 deuterated ISs for the analysis of 184 oxylipins (153). In the present thesis, we used 20-22 deuterated ISs to detect and quantify oxylipins from kidney tissue.

One of the critical steps in oxylipin analysis is the extraction process from biological samples. Liquid liquid extraction (LLE) methods have been used for a long time to extract target compounds from aqueous samples; however, this method also extracts other impurities which makes the separation and quantification process difficult. Moreover, LLE is time consuming and use of organic chemicals makes this technique less favourable. The most popular solid phase extraction (SPE) technique overcomes these difficulties. However, different major labs use different SPE methods. An extensive comparison of these techniques is studied in (154).

In this thesis we have used the StrataX SPE technique. StrataX SPE technique effectively removes matrix contaminants and increases reproducibility (148). This technique saves processing time since multiple samples can be processed simultaneously. Also, sample variations are reduced by deconditioning resistant sorbent, which is also stable at pH 1-14 (148). However, the StrataX SPE technique has some limitations. For example, the extraction efficiency of the StrataX protocol was low when compared to other SPE techniques. Also, low recovery rates were observed for several deuterated ISs with this technique (154).

Oxylipins derived from the same precursor PUFA contain very similar structures. Therefore, a rapid, sensitive and highly accurate analytical method is required for oxylipin

analysis from biological samples. Several methods are used to detect and quantify oxylipins. For example: enzyme-linked immunosorbent assay (ELISA), thin-layer chromatography, high-performance liquid chromatography (HPLC) coupled with ultraviolet detection (HPLC-UV), HPLC coupled with fluorescent detectors (HPLC-FLD), gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-mass spectrometry (LC-MS) (148).

The immunoassay ELISA was used for quantitation of oxylipins for a long time. However, this method requires a specific antibody for each oxylipin to be detected, which makes this assay expensive and inefficient for quantitation of a large number of oxylipins. Moreover, only limited numbers of antibodies are commercially available.

Other methods such as HPLC-UV, HPLC-FLD and GC-MS provide opportunity to analyze multiple oxylipins with greater efficiency. However, these methods require oxylipins to be derivatized chemically. Different derivatization methods are available; however, none of these derivatization methods are suitable for all oxylipins. Also, the derivatization process is labour intensive and time consuming; moreover, there are risks of thermal decomposition of oxylipins and undesirable side reactions with reagents (148).

LC-MS has become the most powerful technique for oxylipin detection and quantitation. LC-MS provides better separation and quantitation of oxylipins at a very low level. Addition of an electrospray ionization multi-reaction monitoring (ESI-MRM) mode on the MS provides greater resolving power with high sensitivity for oxylipin detection and quantitation from biological samples. In ESI a high voltage is applied to the oxylipin carboxylate moiety to become ionized. The MRM technique uses a triple quadrupole MS (Q1, q2 and Q3). In Q1, parent ions are separated based on their mass/charge (m/z) ratio; in q2 the parent ion is fragmented to form daughter ions, and in Q3 one or more specific daughter ion(s) is detected based on its m/z ratio.

By using LC-ESI-MS/MS hundreds of oxylipins can be analyzed within a short period of time (153).

1.5 Dietary treatment of PKD

There is scarcity of treatment options to slow disease in PKD, and existing therapies for patients are designed to counteract and reduce symptoms of the disorder, such as hypertension (155, 156). Pharmacological treatment such as the use of the vasopressin receptor antagonist tolvaptan provides a therapeutic option, however, with considerable side effects (157).

Therefore a potential treatment that is of great interest to patients with PKD involves the use of dietary modification to not only attenuate disease progression but also to manage complications such as hypertension, cardiovascular disease, protein energy malnutrition, dyslipidemia, anemia, and bone loss, associated with cystic kidney diseases (158, 160). Several studies with NPHP and ARPKD models shows that dietary management might be a potential treatment option in PKD (92, 160-162); most of these studies investigated modification of the type and quantity of dietary protein and fat content (92, 159-167). Dietary soy protein and flax oil rich in ALA reduced disease progression in Han:SPRD-Cy rats and *pcy* mice, as evident from reduced kidney size, water content, cyst growth and fibrosis (92, 139, 161, 163, 168-174). On the other hand, diet containing fish oils, rich in EPA and DHA, have showed inconsistent results (139, 163, 175, 176). Other dietary interventions that have demonstrated benefits include conjugated linoleic acid treatment in Han:SPRD-Cy model but not in *pcy* mice (177-179), water in PCK rats (180), citrate in Han:SPRD-Cy rats but not *pcy* mice (181-183), and fat or protein reduction in Han:SPRD-Cy rats and *pcy* mice (163, 173, 175, 176, 184, 185). These other dietary interventions also demonstrate contrasting effects of diet in NPHP and ARPKD models,

implicating the necessity for dietary studies in orthologous models of ADPKD. Importantly, several dietary treatments in NPHP models carried out by our lab [e.g. soy protein, low protein (LP), fish oil, flax oil (161, 163, 173-175, 184)] showed beneficial effects on cyst growth, histological changes, renal function and survival. However, acceptance of the efficacy of these dietary interventions in PKD is hampered by the lack of studies in orthologous animal models. Until studies in these models demonstrate similar efficacy, human clinical trials will not occur.

Although dietary treatments may not cure the disease, improvement in symptoms and slowing of disease development will improve the quality of life of patients in addition to delaying onset of renal failure. The importance of delaying disease is reflected in the significantly increased healthcare costs in patients with PKD with low GFR (~5-fold higher costs in patients with GFR<15 mL/min compared to those with GFR>90 mL/min) (186).

1.5.1 Effect of dietary soy protein on cystic kidney disease and renal oxylipins

Generally, plant proteins are considered to be lower quality proteins compared to animal proteins. However, soy protein, a high-quality plant protein, is exceptional and comparable with standard animal protein such as casein (187). The use of dietary soy protein in the treatment of kidney disease in general is gaining interest, since several human and animal studies are showing beneficial effects of this dietary intervention in slowing disease progression of other renal diseases (139, 160, 188, 189). Studies have demonstrated that dietary soy protein is effective in slowing disease, as evident by reduced cyst growth, inflammation and fibrosis, serum creatinine and urea nitrogen and improved creatinine clearance in Han:SPRD-Cy rat and the *pcy* mouse model (161, 171-173, 190). In the obese fa/fa Zucker rats soy protein feeding improved renal function and ameliorated kidney disease progression by restoring renal nitric oxide synthase

alterations and improving renal NO production (191). In another study with the same model soy protein feeding showed similar results with attenuation of the altered level of renal prostanoid 6-keto-$PGF_{1\alpha}$ (160). The effect of soy protein on oxylipin levels are not clear; however, several lines of evidence indicate that renal oxylipin alterations are associated with the beneficial effects of soy protein (98, 139). In the Han:SPRD-Cy rat, soy protein feeding reduced disease progression and ameliorated renal prostanoids (6-keto-$PGF_{1\alpha}$ and TxB_2), $cPLA_2$ and COX enzyme alterations in disease (98). In a recent study with the same model, soy protein feeding was effective in reducing disease progression and this effect was parallel to the reversal of disease associated alterations of renal oxylipins (139). Soy protein feeding mitigated the altered levels of renal AA derived 6-keto-$PGF_{1\alpha}$, PGE_2, and TxB_2 in this model. A study with healthy human volunteers showed that consumption of a soy based diet for 3 weeks effectively lowered GFR in association with reduced urinary 6-keto-$PGF_{1\alpha}$ excretion (192), illustrating the ability of soy protein to alter these prostanoids in humans as well.

Results from these studies suggest that soy protein has effects on oxylipins derived from n-6 PUFA, more particularly, AA derived oxylipins. Some other studies have shown that soy protein can alter the level and activity of Δ^6-desaturase, an enzyme of the AA synthesis pathway (193, 194).

Dietary soy protein reduces disease progression in the Han:SPRD-Cy rat and the *pcy* mouse model of NPHP (161, 171-173, 190). Whether similar effects occur in orthologous models of ADPKD needs to be tested before making any recommendation for dietary soy protein to PKD patients. Moreover, the effect of soy protein on renal oxylipins needs to be further investigated, since only a limited number of oxylipins were analyzed previously (98, 139, 192).

Therefore, another objective of the present thesis was to examine the effect of dietary soy protein on disease progression and renal oxylipin alterations in an orthologous mouse model of ADPKD.

1.5.2 Effect of dietary flax and fish oil on cystic kidney disease and renal oxylipins

Diets containing n-3 fatty acids generally are regarded as beneficial with respect to health and disease, and they have been shown to be reno-protective in several kidney disease models (195-197). N-3 fatty acids such as ALA primarily found in flax oil, and longer chain EPA and DHA in fish oils, are thought to exert their renoprotective effect by shifting the oxylipin production from n-6 (AA) to n-3 (ALA and EPA/DHA) fatty acids. This reduces the excess inflammatory oxylipin production from AA and enhances the less inflammatory or anti-inflammatory oxylipin production from ALA and EPA/DHA (198). Diets with higher n-3 containing PUFA enhance production of protective oxylipins such as 3-series prostanoids, lipoxins and hydroxy-docosahexaenoic acids (HDoHEs), hydroxy-eicosatetraenoic acids (HEPEs) (199, 200); therefore, flax oil or fish oil feeding might reduce kidney disease progression by reducing inflammation in kidneys.

1.5.2.1 Effect of dietary flax oil on cystic kidney disease and renal oxylipins

Several studies have been carried out to investigate the beneficial effects of flax oil in NPHP. Flax oil feeding reduces renal injury in the Han:SPRD-Cy rat, as measured by cystic damage, fibrosis, macrophage infiltration, epithelial proliferation and oxidized LDL content in the kidneys, with concomitant increase of tissue n-3/n-6 ratio (174). In another study, Sankaran *et al.* showed that diet containing flax oil slowed early fibrosis progression in young *pcy* mouse (163). However, none of abovementioned studies have measured oxylipins in those models. Recently, Yamaguchi *et al.* demonstrated that dietary flax oil reduces disease progression and

mitigated oxylipin abnormalities in the *pcy* mice model (92). They showed that total COX derived oxylipins are higher, whereas LOX and CYP derived oxylipins are lower in diseased compared to normal kidneys. And, flax oil feeding mitigated the elevated COX derived oxylipins formed from AA, and increased LOX and CYP derived oxylipins formed from ALA and DHA (92). Whether the disease lowering benefits of flax oil are mediated via reduction of AA derived COX oxylipins or elevation of ALA and DHA oxylipins, or both, is not clear. In obesity associated nephropathy, dietary flax oil reduced AA and LA derived proliferatory and pro-inflammatory oxylipins, and elevated ALA and EPA derived oxylipins in rat kidneys (49). In humans, consumption of 6 g ALA/day for four weeks lowered selected AA and LA derived oxylipins in their plasma (200). A limited number of studies has shown that ALA derived hydroxy-octadecatrienoic acids (HOTrEs) have anti-inflammatory properties *in-vitro* (201), and EPA derived oxylipins can oppose the detrimental effects of AA derived oxylipins (202). It can be speculated from the abovementioned studies that flax oil is effective in reduction of NPHP, and its effect may be mediated in part by mitigation of altered oxylipins in diseased kidneys. Therefore, the effect of flax oil in modulating oxylipin level and in reduction of ADPKD was tested in the present thesis.

1.5.2.2 Effect of dietary fish oil on cystic kidney disease and renal oxylipins

Fish oil is another source of n-3 fatty acids, rich in EPA and DHA, that appears to show beneficial effects in the Han:SPRD-Cy rat (175) and other renal disease models (203, 204), but not always in the *pcy* mouse (163, 176, 205). Intraperitoneal EPA injection for 8 weeks reduced disease progression in the KKA(y)/Ta mouse model of type 2 diabetic nephropathy (204). In another study with renal nephrectomy in dogs, fish oil provided protective effects against early

renal insufficiency (203). In this model there was a lower serum cholesterol concentration and a tendency to lower urinary PGE_2 and TxA_2 excretion (203).

In the case of cystic kidney diseases, a high fat diet (20 g/100 g diet) worsened renal disease progression in the Han:SPRD-Cy rat, as indicated by larger kidney size, greater renal fibrosis and lower creatinine clearance. A diet containing fish oil ameliorated the detrimental effects of the high fat diet as reflected in lower kidney weights, kidney water content, cyst volumes and serum cholesterol and triglyceride concentrations (175). In a recent study, it has been shown that fish oil feeding reduced both COX and LOX oxylipins produced from n-6 fatty acids and increased 3-series prostanoids in both normal and diseased kidneys; however these differences did not parallel disease changes (139). In an earlier study with *pcy* mice, Yamaguchi *et al.* showed that treatment with n-3 oil rich in EPA markedly reduced mean kidney weights and tubular dilatation in male and female *pcy* mice, and lowered total cyst area in male *pcy* mice (205). However, Sankaran *et al.* found that DHA rich algal oil treatment lead to higher kidney weights, total cyst area and serum urea nitrogen (163). Results of no beneficial effects of fish oil have been documents in other studies with NPHP model as well (176, 206). None of these studies however measured tissue oxylipin levels.

Although a recent study with Han:SPRD-Cy rat model demonstrates that fish oil modulates some disease-associated oxylipins in kidneys (139), the effect of fish oil in PKD remains unclear. Therefore, another objective of the current thesis was to investigate the effects of fish oil on disease progression and oxylipin alterations using an orthologous ADPKD animal model.

1.6 HP diet in renal diseases

HP diets are popular for the management of overweight and obesity. However, there are controversial data regarding the safety of long-term HP diet consumption. Although HP diets have been shown to improve body composition, satiety and aid weight loss (207-209), there is evidence that HP intake increases GFR, renal blood flow, kidney volume and kidney weight in normal kidneys (192, 210-215).

Several animal studies have shown that HP feeding results in enlarged kidneys with increased GFR, renal hypertrophy, glomerulosclerosis and renal fibrosis (216-220). Feeding a HP diet [35% energy (%E) from protein] for 17 months in healthy Sprague-Dawley rats led to larger kidneys, increased proteinuria and creatinine clearance, larger glomeruli and enhanced glomerulosclerosis (220). In a similar study, HP feeding for 4 month and 8 months resulted in enlarged kidneys with higher renal and glomerular volumes, and increased fibrosis and glomerulosclerosis in pigs (207). Renal monocyte chemoattractant protein-1 and plasma homocysteine levels were also higher (216). Results from these studies indicate that long-term intakes of protein even within the upper limit of the acceptable macronutrient distribution range (AMDR) might be detrimental for renal health.

HP feeding also increases renal protein excretion and proteinuria. In a study in which healthy Sprague-Dawley rats were provided diets containing 6, 20 and 35%E) from protein for 20 months, the HP diet significantly worsened proteinuria, tubulo-interstitial changes, focal glomerular sclerosis and was associated with deteriorating overall renal function (221). There was a strong correlation between dietary protein content and renal lesions and proteinuria in this study.

Results from human subjects show conflicting results with HP diet consumption. Most studies with human subjects have been short term, based on a single meal of HP, and focused on mostly renal hemodynamic effects (210, 211, 213-215). Frank *et al.* showed that a HP diet alters renal functions as well as renal hemodynamics (222). In this study young healthy human subjects were provided a HP (2.4 g/kg/d) or NP (1.2 g/kg/d) diet for seven days in a crossover design manner. This short-term HP feeding significantly increased GFR, BUN and renal excretion of uric acid, sodium, and albumin (222). In contrast, the 11-year prospective cohort Nurses' Health Study has shown that long term protein intake (63 to 90 g/d) is not associated with a decline in renal function in women (42 to 68 y older) with normal renal function. However, this study also shows that high total protein intake was associated with renal function decline in women with mild renal insufficiency (223). In another study with healthy obese volunteers, it has been shown that a low-carbohydrate high-protein diet over 2 years was not associated with increased GFR, urinary albumin, or fluid and electrolyte imbalance compared to a low-fat diet (224). In a recent study with pre-diabetic overweight and obese adults, a HP diet (35-40%E from protein) for one year was not associated with changes in creatinine clearance, GFR or serum creatinine (225). Two meta-analyses also indicated no noticeable adverse effects of HP diet on kidney health (226, 227). However, another meta-analysis showed that HP diets are associated with increased GFR, serum concentrations of uric acid and urea, and urinary calcium excretion (228).

Compared to healthy individuals, HP diets are detrimental to kidney function in persons with pre-existing kidney disease or who are susceptible to kidney disease such as those with diabetes (229-231). Several studies showed that the risk of kidney disease increases by 40 to 83% in individuals with overweight, obesity or the metabolic syndrome (232, 233). Introducing HP diet to this population with increased risk of kidney dysfunction will add another detrimental

factor (228). To support this, the Nurses' Health Study showed that higher intake of HP may enhance kidney disease progression in women with an already established mild renal insufficiency (223).

1.6.1 HP diet in cystic kidney diseases

Very few studies have investigated the effect of dietary protein on cystic kidney diseases and none of them have tested effects of HP diet. Tomobe *et al.* investigated effects of LP (6 g/100 g diet) compared to normal protein (NP) diets (25 g/100 g diet) in young male *pcy* mice. They showed that LP diet feeding for 3.5 months reduced kidney weight by 50% and cyst area by 40% (184). In another study male Han:SPRD-Cy rats were fed a LP (8 g/100 g diet) or a NP (20 g/100 g diet) diet. Feeding LP diet for 4 months reduced renal volume and total cyst volume compared to a NP diet in rats. Feeding LP compared HP also improved renal function, as indicated by lower serum creatinine and urea concentrations (185). Moreover, LP feeding reduced proliferating cell nuclear antigen-positive cells, apoptotic cells, inflammation and fibrosis in this model (234). In a recent study, supplementation of branched-chain amino acids (BCAAs) in a $Pkd1^{flox/flox}$:Mx1-Cre orthologous mouse model of ADPKD led to a greater kidney/body weight ratio and higher cystic index in both the kidney and liver. This study also showed that BCAAs enhance proliferation of cyst-lining cells and upregulate mTOR and MAPK/ERK pathways (235). These pathways are important in regulating cyst growth in ADPKD. Protein restriction delayed ESRD in human PKD patients as indicated by slower increases in serum creatinine concentration (236). However, in a larger human trial there was no difference in follow-up compared to baseline disease progression in patients with either

advanced PKD or significantly impaired renal function who consumed 0.7−1.2 g/kg/day of protein (237).

1.6.2 HP diet and renal oxylipins

HP intake increases GFR, renal blood flow, kidney volume and kidney weight in normal kidneys and these changes were associated with increased glomerular production of PGE_2, PGI_2, and TxA_2 in Wister rat kidneys (238), and PGE_2 and TxB_2 in Sprague-Dawley rat kidneys (239). Studies carried out by Yanagisawa *et al.* showed that a HP diet containing 40%E fromcasein enhances the activity of membrane associated PLA_2 and COX enzymes with concomitant increased production of PGE_2, 6-keto $PGF_{1\alpha}$ and TxB_2 in rat glomeruli (240, 241). Two other studies also showed higher urinary excretion of PGE_2 in normal rats fed HP diets (242, 243). Study with normal and three distinct rat models of experimental renal disease including diabetes mellitus, heymann nephritis, and partial renal ablation, showed that feeding a HP diet (40%E from protein) led to52-257% increased production of glomerular PGE_2, $PGF_{2\alpha}$, and TxB_2 in all study animals. Glomerular production of PGE_2 and TxB_2 was also higher in the presence of AA, which indicates augmentation of glomerular COX activity by HP diet feeding (239). In another study rats with reduced renal mass, HP feeding for 2 weeks significantly increased proteinuria, GFR and urinary PGE_2 excretion. Prostanoid (PGE_2, 6-keto PGF1α, and TxB_2) production in isolated glomeruli also increased several folds in the HP group. Moreover, a COX inhibitor(indomethacin) reduced GFR in rats provided HP diets (238). It appears that renal prostanoids have profound effects on renal function and can be modulated by HP feeding. However, the abovementioned studies showing higher level of select COX derived prostanoids

with HP diet were carried out *ex vivo*, and a recent *in vivo* study showed that a short term HP diet had little effect on COX derived prostanoid alteration in normal rat kidneys (244).

Thus, while it is clear that HP diets have detrimental effects in kidneys with already established disease, no study has investigated the effect of a HP diet on renal cystic disease progression. Therefore, another objective of the thesis was to examine the effect of dietary HP on diseased cystic kidneys, using the *pcy* mouse model of NPHP. Further, since the effect of HP diets on renal prostanoids is not clear, another objective in this thesis was to examine the effect of longer-term dietary HP on renal prostanoids and other oxylipins in normal and cystic kidneys. The *pcy* mouse model was used due to the lack of availability of an orthologous ADPKD model. An advantage of using the *pcy* mouse was that increased renal prostanoids had been demonstrated with disease in this model, so it was reasoned that they may be susceptible to increase renal prostanoids due to dietary HP.

1.7 Sex differences in kidneys and renal oxylipins

It has been found that men with ADPKD start hemodialysis therapy 1.3 years earlier than women (245), while progression to renal failure is approximately 5 years earlier than women (246). Although there is a slight gender difference in cystic kidney disease progression, most animal studies with dietary interventions are carried out in male animals (247-251). Male rats with PKD display lower GFR and plasma flow with related abnormalities of other renal factors, compared to females. Administration of 17β-estradiol showed a protective effect in ovariectomized female rats on disease progression by regulating gene expression (252). Dietary soy protein attenuates kidney disease progression by reducing cyst volume and improvement of renal functional parameters only in male Han:SPRD-Cy rats but not in females (170). In another

study with female PCK rats, dietary intervention with soy protein or fish oil showed no beneficial effects (247). Gender differences in disease are clear in rat models such as in Han:SPRD-Cy and PCK rat models, however, data in mouse models are less clear; moreover, no data are available for PKD animals. Therefore, the studies in this thesis included both male and female animals.

Sex also is an important regulator of oxylipin metabolism. There are sex specific differences in enzymes that synthesize and degrade AA derived oxylipins (253, 254). Female rats secrete higher levels of urinary PGE_2 and PGI_2 compared to diseased male rats with type II Bartter syndrome (255). Moreover, plasma PGE_2 and PGI_2 levels were higher in female compared to hypertensive male rats (256). Renal PGE_2 synthase and COX2 protein expression are higher in females compared to males (253). Female hormones might affect activity of enzymes involved in oxylipin metabolism (254, 257, 258). For example, activity of renal prostaglandin 9-ketoreductase, enzyme that converts PGE_2 into $PGF_{2\alpha}$, was about 50% higher in female compared to male rats. The enzyme activity was decreased by gonadectomy in females, but not in males. Estradiol (a female hormone) treatment elevated prostaglandin 9-ketoreductase activity both in males and females, while testosterone (male hormone) treatment had no effect (258). Recently it has been shown that sex hormones influence expression of specific transporters responsible for PG clearance and expression of these transporters are lower in female rat kidneys (259). These findings indicate that there might be sex specific differences in the effects of dietary intervention on renal cystic disease and oxylipin production. Therefore, both male and female animals were included in the studies in this thesis, to determine whether there sex specific difference in disease progression and oxylipin alterations in renal cystic diseases.

1.8 PKD animal models

Experimental animal models are important to understand molecular mechanisms of disease onset and progression; they are also important for the investigatin of potential therapeutic treatments (260). In this thesis orthologous Pkd2 and *pcy* mouse models were used and discussed in the section 1.8.1 and 1.8.2, respectively. Other PKD animal models referred in this thesis are briefly discussed in the Table 1.1.

1.8.1 Orthologous Pkd2 mice

Most nutritional intervention studies were carried out using Han:SPRD-Cy rat and the *pcy* mouse models of NPHP, which are phenotypically similar to human ADPKD but genetically different. Disease development in Han:SPRD-Cy rat is caused by a mutation in the *Anks6* gene (formally known as *Pkdr1*) (260), and disease in the *pcy* mouse is caused by a *Nphp3* gene mutation (261). A recent human study with PKD patients failed to show beneficial effects of n-3 PUFA which has been found beneficial in these models (262). Moreover, in a recent study Maditz *et al*. found no beneficial effects of soy protein and fish oil feeding in the PCK rat model of ARPKD (247). This indicates the necessity of using orthologous models in intervention studies to make any dietary recommendations for humans. Until recently, a model that is orthologous to human ADPKD2 was not practical for intervention studies since complete homozygous knockouts are embryonic lethal and heterozygous knockouts develop the disease very slowly and with much variability (263). The recent development of a conditional knockout, however, now provides the opportunity to test nutritional interventions in this model of ADPKD.

The Pkd2$^{WS25/-}$ mouse was the first conditional ADPKD2 knockout reported and is still a suitable conditional *Pkd2* knockout available for intervention studies (263, 264). To generate the

Pkd2$^{WS25/-}$ model, Pkd2$^{WS25/WS25}$ and Pkd2$^{+/-}$ mice were crossed. This resulted in a control (Pkd2$^{WS25/+}$) that does not develop cysts and a diseased mouse (Pkd2$^{WS25/-}$) in which one of the *Pkd2* alleles is mutated and the other contains the *WS25* mutation inserted into the first intron. This insertion results in an increased rate of somatic mutations ("second hits") in the *Pkd2* gene and reliably results in a slowly progressive renal cystic disease that models human ADPKD2. By 16 wk of age these mice consistently develop renal cysts (265), so our interventions were for 13 wk, starting at 3 wk of age. In this model, development of cystic disease is similar to human ADPKD, with progressive and relatively constant cyst growth. As in the human form, renal function is affected only once significant enlargement of the kidneys occurs (19, 263, 266). Dr. Somlo (Yale University) collaborated with us and assisted in establishing a colony of these mice for our studies. This was the main model used in the current thesis, as it is orthologous to ADPKD.

1.8.2 *Pcy* mice model of nephronophthisis

The *pcy* mouse is an orthologous animal model to human NPHP containing a mutation in *NPHP3* gene (261). In this model renal tubules are enlarged as early as embryonic day 15, and cyst development occurs at the cortico-medullary junction by weaning. Starting from the distal tubules, numerous cysts develop throughout the whole nephron by week 30, which ultimately leads to ESRD after the age of 35 weeks (260). Due to lack of availability of Pkd2 mice for all studies, the *pcy* mouse was used in the dietary HP study in the current thesis. This model was chosen because we had previously shown that it displayed alterations of renal oxylipins (92, 102), and that LP diets slowed disease progression in this model (175, 176).

Table 1.1 PKD animal models referred to in the thesis.

Animal	Brief description
Han:SPRD-Cy rat (267)	The Han:SPRD-Cy rat is a model of NPHP. In this model *Pkdr1* (also called *Cy, Samd6, or Anks6*) gene is mutated. The *Pkdr1* gene product SamCystin is expressed in the proximal tubules. Renal cysts develope both in homozygous (*Cy/Cy*) and heterozygous (*Cy/+)* animals. However, in homozygous rats disease progression is rapid with a short 3-week life span. Slow disease progression is observed in heterozygous rats with the average life span is approximately 1.5 years in females and 1 year in males.
jck mouse (268)	The *jck* mouse is a model of NPHP. In this model the *Nphp9* gene (also called *Nek8*) gene is mutated. The *Nphp9* gene product is NIMA (never in mitosis A-related kinase 8), a serine/threonine kinase expressed in primary cilia which affects normal expression of the PKD1 and PKD2 gene products. At the age of 4 weeks initial renal cysts are observed, and at the age of 10 weeks cysts are developed fully.
PCK rat (269)	The PCK rat is model of ARPKD. In this model the *Pkhd1* gene is mutated. The *Pkhd1* gene product fibrocystein is expressed in the primary cilia. Numerous cysts are clearly visible on the surface of the kidney and liver at the age of 1 year. PCK rat survive approximately 1.5 years.
Bpk (BALB/c polycystic kidney) mouse (270)	The *Bpk* mouse is a model of ARPKD. This mouse displays renal cysts derived from collecting ducts, and liver cysts derived from bile ducts, which are similar to those found in human ARPKD. The *bicc1* gene responsible for disease in this mouse and the *bicc1* gene product can also be found in the primary cilia.
Mx1Cre^{+}*Pkd1*$^{flox/flox}$ (Pkd1) mouse (271)	This is an orthologous mouse model of human ADPKD1. This model

	originated by conditional knockout of the *Pkd1* gene using the Pkd1-Cre/loxp system. Cysts are derived from either collecting tubules or distal tubules but not from proximal tubules. Disease development in this model depends on timing of *Pkd1* inactivation. For example: *Pkd1* inactivation in 5-week old mice results in slow disease progression after 6 to 9 weeks of age; however, a severe polycystic phenotype is observed after 1 year. On the other hand, *Pkd1* inactivation in 1-week old mice results in massive cyst development after 6 weeks of age.

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Chapter 2

Rationale, Hypothesis and Objectives

2.1 Overall hypothesis: PKD progression will be reduced by dietary soy protein, flax oil, fish oil and a selective COX2 inhibitor, and increased by dietary HP. These effects will be accompanied by parallel effects on the altered renal oxylipins in PKD.

2.2 Overall objectives: The objectives of the present research were to investigate the effect of dietary soy protein, oils containing n-3 fatty acids, a HP diet, and a selective COX2 inhibitor on PKD progression, to determine whether renal oxylipins are altered in orthologous and non-orthologous models of PKD, and to determine the effects of these interventions on altered renal oxylipins. Secondary objectives were to examine sex difference in renal oxylipins.

2.3 Specific hypotheses and objectives

Rationale 1: Dietary soy protein and flax oil reduces disease progression in the Han:SPRD-Cy rat and *pcy* mouse models of NPHP (1-5), while fish oils show beneficial effects in the Han:SPRD-Cy rat (6), but not always in the *pcy* mouse (7, 8). The studies with soy protein and fish oil used male (4) animals as disease progression is greater in males; however, recent studies with the female PCK rat model of ARPKD showed conflicting results (9). Moreover, the effects of dietary soy protein or n-3 oils in orthologous models of ADPKD have not yet been determined in either males or females.

Hypothesis 1: Dietary soy protein and flax or fish oil will reduce disease progression in male and female Pkd2 mice with ADPKD.

Objective 1: Examine whether dietary intervention using soy protein, flax oil or fish oil improves renal disease in the Pkd2 orthologous mouse model of ADPKD.

Rationale 2: In the Han:SPRD-Cy rat and *pcy* mouse models of NPHP, renal oxylipins are altered in disease (10-14). Whether oxylipins are altered in ADPKD is not clear. Serum oxylipins from human ADPKD patients display alterations (15), but renal changes are unknown. Moreover, previous studies with NPHP models used only male animals, but sex also may be an important regulator of oxylipin metabolism.

Hypothesis 2: Disease and sex alter the renal oxylipin profile (specifically, COX oxylipins will be higher and LOX and CYP oxylipins will be lower) in the Pkd2 mouse model of ADPKD.

Objective 2: Compare normal and diseased animals to determine if there are disease and sex specific alterations in renal oxylipins in the Pkd2 mouse model of ADPKD.

Rationale 3: Dietary soy protein, flax and fish oil influence the production of oxylipins in the kidneys; however, only a few oxylipins have been studied (5, 10, 11). Also, oxylipin alterations by dietary flax oil were associated with reduced renal disease (5, 11), but not always with fish oil (8). Moreover, no studies have compared the effects of soy protein compared to casein, and fish compared to flax oil on the oxylipin profile in an orthologous ADPKD model, side-by-side, in males and females.

Hypothesis 3: Sex difference and dietary interventions with soy protein, fish or flax oil will alter the oxylipin profile (specifically, flax oil will increase ALA derived oxylipins, while fish oil will increase EPA and DHA derived oxylipins) in the Pkd2 mouse model of ADPKD.

Objective 3: Examine the effect of dietary soy protein, oils containing n-3 fatty acids and sex on renal oxylipins in the Pkd2 mouse model of ADPKD.

Rationale 4: *Ex vivo* studies have indicated that HP feeding increases renal prostanoid production (16-18); however, a recent short-term *in vivo* study failed to show this (19). Moreover, no study has examined renal oxylipins in male and female mice, with and without kidney disease, provided dietary HP for a long-term period.

Hypothesis 4: A HP diet will enhance disease progression and exacerbate disease specific alterations in renal oxylipins (specifically, HP will increase COX derived prostanoids) in the *pcy* mouse model of NPHP.

Objective 4: Examine the effects of a HP diet and sex on disease progression and renal oxylipins in male and female *pcy* mice with NPHP.

Rationale 5: Studies with non-orthologous models of PKD showed a reduction of disease progression with selective COX2 inhibition (20-22). However, no study has been carried out with orthologous ADPKD animals.

Hypothesis 5: Selective COX2 inhibition will attenuate disease progression and disease specific alterations in renal COX oxylipins in the Pkd2 mouse model of ADPKD.

Objective 5: Examine the effect of a selective COX2 inhibitor (celecoxib) on disease progression and renal oxylipins in the Pkd2 orthologous mouse model of ADPKD.

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2.5 Transition to next chapter

As discussed in chapter 1, dietary soy protein and flax oil consistently reduce renal injury in the Han:SPRD-Cy rat and *pcy* mouse models of NPHP (1-5), whereas, fish oils appear to have inconsistent effects in these models (6-8). These dietary oils have not been tested in orthologous ADPKD animals, nor have they been tested side by side. Therefore, the next chapter will examine (hypothesis 1) whether dietary intervention using soy protein, flax oil or fish oil improves renal disease progression in the Pkd2 mouse, an orthologous model of ADPKD, and in the PCK rat model of ARPKD.

My role in this collaborative study was as follows:

I conducted the animal feeding, tissue collection, oxylipin extraction and quantification, and statistical analysis for the Pkd2 mouse study, and I prepared the results table for the Pkd2 portion of the study. I have also edited the manuscript draft. For contributions of other authors please refer to Author Contributions section (p.vii-p.x).

Chapter 3

Lack of benefit of early intervention with dietary flax and fish oil and soy protein in orthologous rodent models of human hereditary polycystic kidney disease

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3.1 Abstract

Rationale for dietary advice in polycystic kidney disease (PKD) is based in part on animal studies that have examined non-orthologous models with progressive development of cystic disease. Since no model completely mimics human PKD, the purpose of the current studies was to examine the effects of dietary soy protein (compared to casein) or oils enriched in n-3 fatty acids (fish or flax oil compared to soy oil) on early disease progression in two orthologous models of PKD. The models studied were Pkd2$^{WS25/-}$ mice as a model of autosomal dominant PKD, and PCK rats as a model of autosomal recessive PKD. After 13 weeks of feeding, dietary fish (but not flax) oil resulted in larger kidneys and greater kidney water content in female Pkd2$^{WS25/-}$ compared to control mice. After 12 weeks of feeding male PCK compared to control rats, both fish and flax compared to soy oil resulted in enlarged kidneys and livers, greater kidney water content and higher kidney cyst area in diseased rats. Dietary soy protein compared to casein had no effects in Pkd2$^{WS25/-}$ compared to control mice. In PCK rats, kidney and liver histology were not improved, but lower proteinuria and higher urine pH suggest that soy protein could be beneficial in the long term. Therefore, in contrast to studies in non-orthologous models during the progressive development phase, these studies in orthologous PKD models do not support dietary advice to increase soy protein or oils enriched in n-3 oils in early PKD.

3.2 Introduction

Hereditary polycystic kidney disease (PKD) is characterized by countless renal cysts and often also displays significant liver cysts. The two major types of PKD are autosomal dominant PKD (ADPKD) and autosomal recessive PKD (ARPKD). Approximately 80-85% of ADPKD is caused by mutations in *PKD1,* the gene for polycystin 1, and the remaining 15-20% of cases are caused by mutations in *PKD2,* which codes for polycystin 2 (1). ARPKD is much less common but is a more severe form of disease that primarily affects the pediatric population. ARPKD is caused by a mutation in the polycystic kidney and hepatic disease-1 (*PKHD1*) gene, which codes for polyductin/fibrocystin (2).

Despite the promise of pharmacological treatments such as vasopressin receptor antagonists, the presence of undesirable side effects (3) and the lack of efficacy of current treatments in delaying the need for renal replacement therapy (4), has led to much interest in alternative treatments such as dietary therapy. This is evidenced by ADPKD diet studies currently in progress (5,6), as well as nutritional advice on several national PKD association web pages addressing nutrition questions (7-9). In their dietary advice, PKD Foundations in both Canada and the US cite animal studies as evidence of the potential effectiveness of dietary soy and plant proteins. Dietary soy protein has been particularly effective in several spontaneous rodent models of renal cystic diseases such as the Han:SPRD-*Cy* rat with the mutated *Anks6* (formally called *Pkdr1*) gene and the *pcy*/*pcy* (*pcy*) mouse that harbors the *pcy* mutation (10-16). In these models, renal cyst disease develops progressively and soy protein feeding resulted in lower kidney size and water content, along with reduced cyst growth and fibrosis, when replacing casein as the protein source in the standard AIN93 laboratory rodent diet (17).

The PKD Foundations in Canada and the US also advise patients to consume n-3 fatty acids, again based on animal model data. Indeed, dietary flax oil (enriched in α-linolenic acid) reduces kidney size, water content, cyst growth and fibrosis in both the Han:SPRD-*Cy* rat and the *pcy* mouse (18-22). However, fish oil (enriched in EPA and DHA) appears to have beneficial effects in the Han:SPRD-*cy* rat (23,24), but not always in the *pcy* mouse (21,25-27).

In both dietary soy protein and n-3 oil interventions, male animals have been used, as they typically display greater disease progression. However, recent studies in the female PCK rat model of ARPKD have suggested that dietary soy protein or fish oil may not be effective in this orthologous model of ARPKD (28). Further, the effects of dietary soy protein or n-3 oils in orthologous models of ADPKD have not yet been determined in either males or females, nor have male PCK rats been tested with these dietary treatments. Therefore, the effects of soy protein compared to casein and flax or fish oil compared to soy oil were examined in the early stages of disease in males and females of an orthologous mouse model of ADPKD ($Pkd2^{WS25/-}$ mice) and in the male PCK rat model of ARPKD.

3.3 Materials and Methods

All animal procedures were approved by the University of Manitoba Animal Care Committee and adhered to the guidelines of the Canadian Council on Animal Care.

3.3.1 $Pkd2^{WS25/-}$ mice

$Pkd2^{WS25/WS25}$ and $Pkd2^{+/-}$ breeders were obtained from Dr. Stefen Somlo at Yale University (New Haven, CT, U.S.A.) (29). These genotypes were crossed to produce mice with diseased ($Pkd2^{WS25/-}$) or normal ($Pkd2^{WS25/+}$) phenotypes. All mice were given diets based on the American Institute of Nutrition (AIN) 93G standard diet for laboratory rodents (17), which has

casein as the standard protein source and soy oil as the standard oil. The experimental diets contained either an equivalent amount of soy protein that replaced the casein, or either flax oil or fish oil that replaced 80% of the soy oil, as shown in Table 3.1 and detailed in previous studies of non-orthologous models of PKD (11,21). All oils and diet ingredients were purchased from Dyets Inc. (Bethlehem, PA, USA). The oils contained 0.02% tBHQ to prevent oxidation and diet ingredients were stored at 4°C. Diet was freshly prepared twice per month and stored in sealed containers at -20°C until feeding. Routine examination of texture, odor, and color indicated that the oils were not oxidized. Mice were housed singly in cages with plastic enrichment domes in a temperature- and humidity-controlled environment with a 12 hour day/night cycle and were given free access to water and diet.

The feeding period was for 13 weeks, from 3 to 16 weeks of age, and feed and water disappearance were determined during week 6 of feeding to estimate feed and water intakes, respectively. Mice were monitored daily and no mice became ill or died. Mice were anesthetized to surgical plane using isofluorane and euthanized via exsanguination. Normal and diseased mice were identified by the absence or presence of renal cysts. Body, kidney and liver weights were recorded before placing the left kidney and a portion of the liver in 10% formalin for 24h, followed by transfer to PBS at 4°C until further processing. The right kidney and another portion of the liver were snap frozen in liquid nitrogen, and lyophilized to determine tissue water content.

Formalin fixed kidneys and livers were embedded in paraffin, sectioned at 5 µm and tissue sections were stained with Masson's trichrome to measure cyst and fibrosis area as previously described (21,24). A Nikon D600 FX DSLR camera equipped with a 60mm F2.8 Macro lens (Nikon Corporation, Mississauga, Canada) was used to capture images of backlit

whole kidney sections. Macro rings were used between the camera body and lens to achieve 2.5X magnification. This allowed clear identification of open spaces from complete coverage of the kidney or liver sections in each picture.

Table 3.1 Details of experimental diets based on the AIN-93G diet for laboratory rodents [17].

Protein source	Casein			Soy Protein		
Oil source	Soy oil	Flax oil	Fish oil	Soy oil	Flax oil	Fish oil
Ingredient	*g/kg diet*					
Casein (87% protein)	200	200	200	-	-	-
Soy protein (92% protein)	-	-	-	*189*	*189*	*189*
Soybean oil	70	*14*	*14*	70	*14*	*14*
Fish oil	-		*56*	-	-	*56*
Flax oil	-	*56*	-	-	*56*	-
Cornstarch	397.5	397.5	397.5	*408.5*	*408.5*	*408.5*
Dextrinized cornstarch	132	132	132	132	132	132
Sucrose	100	100	100	100	100	100
Fibre (cellulose)	50	50	50	50	50	50
Mineral mix (AIN93G)[1]	35	35	35	35	35	35
Vitamin mix (AIN93G)[1]	10	10	10	10	10	10
L-cystine	3	3	3	3	3	3
Choline bitartrate	2.5	2.5	2.5	2.5	2.5	2.5
Tert-butylhydroquinone[2]	0.014	0.014	0.014	0.014	0.014	0.014

[1]Details of the mineral and vitamin mix are found in [17].

[2]Antioxidant added to the oils.

The italicized numbers are those that are modified from the control diet (casein protein, soy oil) to create the experimental diets. Diet ingredients were purchased from Dyets Inc. (Bethlehem, PA) and Harlan Teklad (Madison, WI).

Quantitative analysis of cyst area of the whole kidney section and the sample liver section was performed using Image Pro software (Media Cybernetics, Silverspring, MO), after coloring in the white areas of tubular lumen spaces to eliminate these from measurement. In addition to cyst area, the blue areas in stained sections were used to examine fibrosis by densitometry as previously described (21,24).

3.3.2 PCK rats

Weanling male PCK rats were purchased from a commercial breeder (Charles River, QC, Canada) and normal and diseased rats were provided the same diets and housing conditions as described for study 1. PCK rats were fed these diets for 12 weeks, from 4 to 16 weeks of age, and feed and water disappearance determined during week 10 of the feeding period using metabolic cages. Rats were monitored daily and one rat in the casein fish oil group was euthanized in week 2 due to poor overall condition and was found to have an enlarged heart upon autopsy. A second rat in the casein fish oil group was terminated in week 12 due to excessive weight loss and poor condition, and was found to have enlarged kidneys, liver and spleen upon autopsy. Rats were anesthetized as described for mice. At termination, tissues were processed as described above for Pkd2$^{WS25/-}$ tissues, with the exception of cyst area measurements, for which a Nikon D90 DX DSLR camera was used. Tubular lumen spaces could not be accurately differentiated from cysts in these sections, so white spaces from both the cysts and tubular lumen spaces in these sections were quantified together. Serum and urinary creatinine were measured using the Jaffe reaction as modified by Heinegard & Tiderstrom and adapted for micro assay (21). Creatinine clearance was calculated using urine volume and urine creatinine from week 10 of the feeding period and serum creatinine from termination. Urine pH was measured

immediately after 24 hour urine collection and urine protein was determined using the Bradford protein assay method with bovine serum albumin as a standard (30). Blood pressure was measured in conscious rats at week 12 of feeding period using a multichannel blood pressure system with a tail-cuff sphygmomanometer (Coda 6 System, Kent Scientific, Torrington, Conn), as described (31).

3.3.3 Statistical Analyses

To first determine effects of disease and sex, only those provided soy oil (control oil) were compared. In Pkd2$^{WS25/-}$ and Pkd2$^{WS25/+}$ mice, 3-way ANOVA (sex x disease x protein) revealed that there were no protein effects on disease, so mice given soy protein and casein were combined for analyses by 2-way (disease x sex) ANOVA. For PCK rats, only males were used, so disease effects in the soy oil fed rats were tested using t-tests.

Dietary effects were tested in Pkd2$^{WS25/-}$ animals only, and 3-way ANOVA (sex x oil x protein) again revealed that protein had no effect on any parameters. Therefore, mice given soy protein and casein were combined and a 2-way ANOVA (oil x sex) was performed. For PCK rats 2-way ANOVA (oil x protein) was used, as only males were used for this study.

All ANOVA were performed using the GLM procedure of SAS (SAS, version 9.3, Cary, NC) followed by Duncan's Multiple Range test to delineate significant oil or interaction effects. Normality of the data was assessed using the Shapiro–Wilk's test, and non-normal data was normalized by log transformation where possible. If normality was not achieved, data were analyzed using the Kruskal–Wallis test. Statistical significance for main and interaction effects was set at $P < 0.05$. All data are presented as mean±SE.

3.4 Results

3.4.1 Pkd2$^{WS25/-}$ mice

At the end of the feeding period, the area comprising both cysts and tubular lumen made up ~15-20% of the kidney section areas in Pkd2$^{WS25/-}$ mice (Fig 3.1A-D). On the other hand, livers displayed fewer and smaller cysts, with 42% of mice exhibiting no cysts at all, and none displaying significant fibrosis (Fig 3.2A-D). As well, 3-way (sex x disease x protein) ANOVA of the soy oil fed mice revealed that there was no protein effect on any of the parameters measured, indicating no benefit of soy protein compared to the casein protein. The protein groups were therefore combined, and in mice provided the soy oil diets, cyst development resulted in higher kidney weights and water content in diseased compared to normal mice (Table 3.2). Consistent with the small and sporadic liver cysts observed, liver weights were not elevated in diseased compared to normal mice. Body and tissue weights were higher in males compared to females, but there were no sex differences in any other parameters (Table 3.2).

With respect to dietary oil effects, fish oil effects on renal disease were only observed in female mice: females given fish oil compared to either soy or flax oil had higher kidney weights and kidney water content. However, there were neither significant dietary oil effects on renal cyst area, nor on any liver parameters. All parameters in flax oil fed mice were similar to soy oil fed mice, with the exception of higher kidney weights in flax oil fed male mice. There were no dietary effects on feed intake, water intake or body weight (Table 3.3 and Figs 3.3 and 3.4).

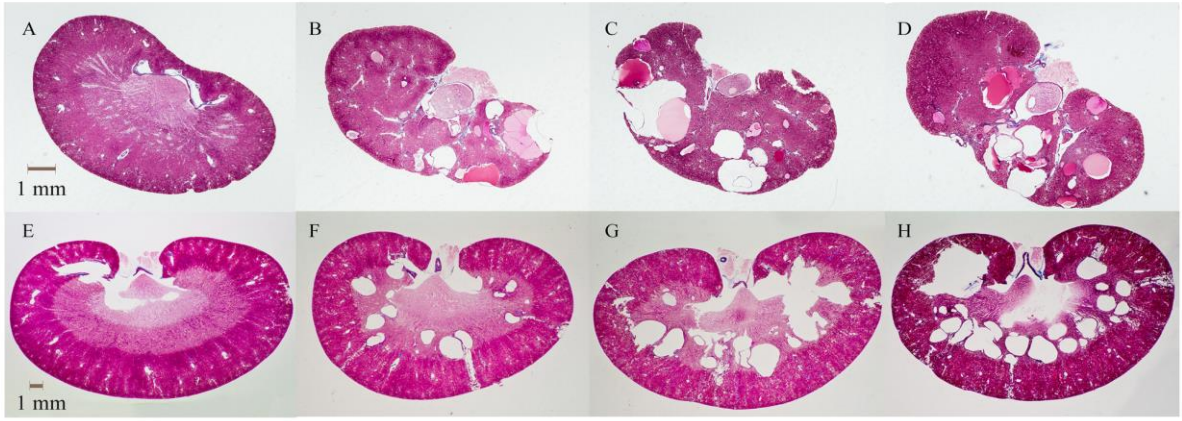

Fig 3.1 Kidney sections. Sections A-D are from Pkd2 mice and E-F are from PCK rats as follows: (A) Pkd2$^{WS25/+}$ (normal) and (B) Pkd2$^{WS25/-}$ (diseased) mice provided soy oil, Pkd2$^{WS25/-}$ (diseased) mice provided (C) flax oil or (D) fish oil, (E) normal and (F) PCK rats provided soy oil, PCK rats provided (G) flax oil or (H) fish oil. Scale bar = 1 mm.

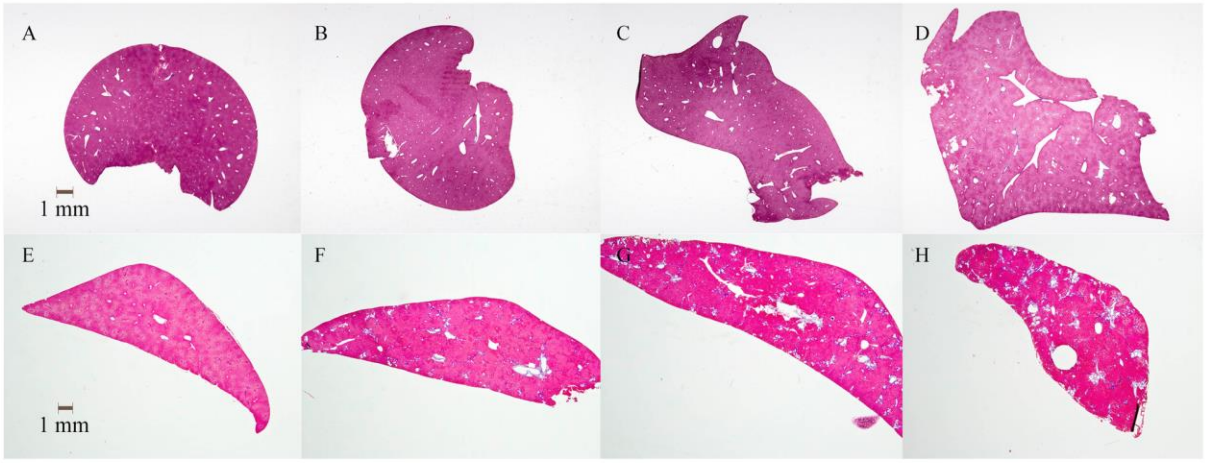

Fig 3.2 Liver sections. Sections A-D are from Pkd2 mice and E-F are from PCK rats as follows: (A) Pkd2$^{WS25/+}$ (normal) and (B) Pkd2$^{WS25/-}$ (diseased) mice provided soy oil, Pkd2$^{WS25/-}$ (diseased) mice provided (C) flax oil or (D) fish oil, (E) normal and (F) PCK rats provided soy oil, PCK rats provided (G) flax oil or (H) fish oil. Scale bar = 1 mm.

Table 3.2 Disease and sex effects in Pkd2$^{WS25/-}$ and Pkd2$^{WS25/+}$ mice

	Pkd2$^{WS25/+}$		Pkd2$^{WS25/-}$			
	Male	Female	Male	Female	P<0.05	
Body weight (g)	26.2±2.2	19.5±0.6	27.1±1.7	19.3±1.0		S
Kidney						
weight (g)	0.28±0.02	0.21±0.01	0.37±0.04	0.27±0.03	D	S
weight / body weight (g/100g)	1.05±0.04	1.11±0.04	1.37±0.01	1.40±0.12	D	
water content (%)	72.2±0.6	71.5±1.0	75.4±1.8	74.8±1.1	D	
cyst area / section (pixels x10^3)			468±94	419±99		
cyst area / kidney area (%)	-	-	16.6±5.5	20.9±8.3		
Liver						
weight (g)	1.10±0.12	0.77±0.03	0.98±0.15	0.77±0.06		S
weight / body weight (g/100g)	4.06±0.13	3.98±0.06	4.25±0.09	3.95±0.13		
water content (%)	66.9±0.5	66.7±0.7	66.7±0.6	67.2±2.0		
cyst area / liver area (%)	-	-	0.24±0.20	0.07±0.03		
Feed intake (g/24h)	3.3±0.3	3.6±0.5	3.1±0.4	3.1±0.3		
Water intake (mL/24h)	3.5±0.5	4.1±0.4	3.2±0.3	3.9±0.8		
N	7	12	7	5		

Data from mice provided soy oil diets only. Values are mean±SE. D, disease; S, sex.

Table 3.3 Dietary oil and sex effects in Pkd2$^{WS25/-}$ (diseased) mice

	Soy Oil		Flax Oil		Fish Oil		
	Male	Female	Male	Female	Male	Female	P<0.05
Body weight (g)	27.1±1.73	19.3±1.03	27.9±0.93	20.8±0.60	27.6±1.30	20.6±1.42	S
Kidney							
weight (g)	0.37±0.04^{b}	0.27±0.03^{b}	0.53±0.09^{a}	0.34±0.03^{b}	0.41±0.06^{ab}	0.57±0.10^{a}	I
weight / body weight (g/100g)	1.37±0.11^{b}	1.40±0.12^{b}	1.89±0.33^{b}	1.63±0.15^{b}	1.44±0.14^{b}	2.75±0.37^{a}	I
water content (%)	75.4±1.8^{b}	74.8±1.1^{b}	78.4±2.2^{ab}	75.9±2.3^{b}	75.5±0.6^{b}	83.0±1.1^{a}	I
cyst area / section (pixels x10^{3})	468±94	419±99	774±292	566±131	599±145	998±230	
cyst area / kidney area (%)	16.6±5.5	20.9±8.3	23.0±5.9	24.4±5.5	22.6±5.1	32.3±7.2	
Liver							
weight (g)	0.98±0.15	0.77±0.06	1.18±0.06	0.89±0.02	1.18±0.06	0.93±0.06	S
weight / body weight (g/100g)	4.25±0.09	3.95±0.13	4.22±0.10	4.30±0.14	4.29±0.09	4.50±0.15	
water content (%)	66.7±0.6	67.2±2.0	65.9±1.4	66.8±1.1	65.6±0.4	66.1±1.0	
cyst area / liver area (%)	0.24±0.20	0.07±0.03	0.07±0.03	0.21±0.12	0.09±0.03	0.69±0.33	
Feed intake (g/24h)	3.1±0.4	3.1±0.3	2.6±0.1	3.2±0.4	2.3±0.2	4.2±0.6	
Water intake (mL/24h)	3.2±0.3	3.9±0.8	5.2±0.5	4.8±0.4	3.7±0.5	3.4±0.3	
N	7	5	5	7	8	4	

Values are mean±SE. With significant interaction effects, differing lower case superscript letters indicate significant simple effect differences between values. I, interaction; S, sex.

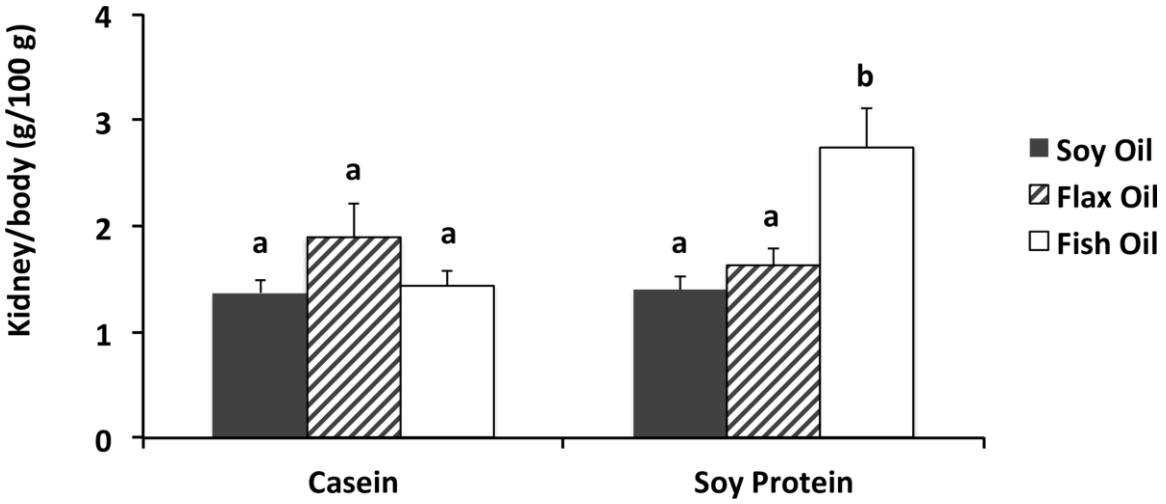

Fig. 3.3 Dietary oil and sex effects on kidney size in Pkd2$^{WS25/-}$ (diseased) mice. There was a diet x sex interaction and differing lower case superscript letters indicate significant simple effect differences between values. Data from Table 3.3.

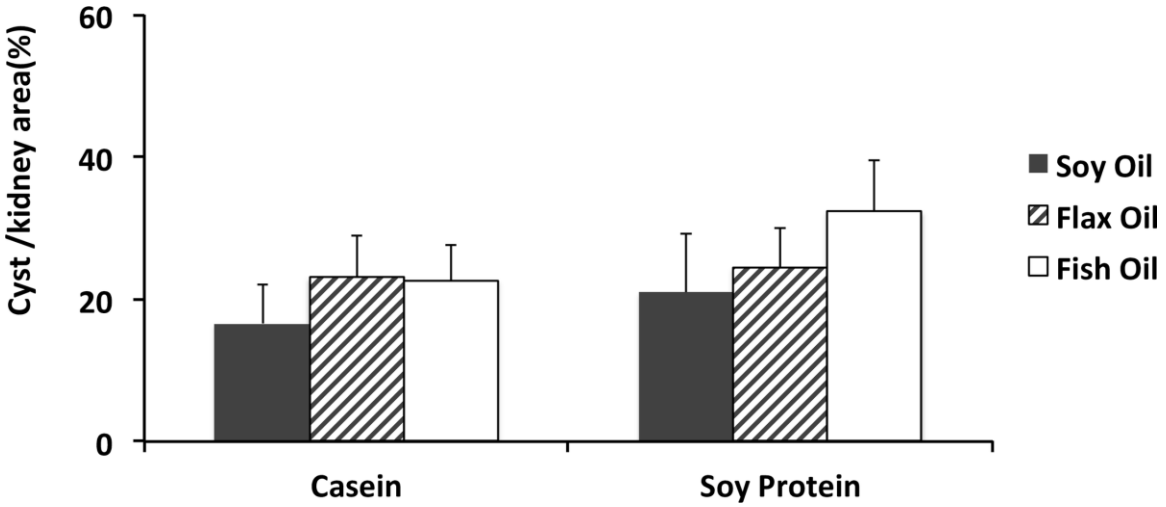

Fig. 3.4 Dietary oil and sex effects on renal cyst area in Pkd2$^{WS25/-}$ (diseased) mice. There were no significant diet or sex effects. Data from Table 3.3.

3.4.2 PCK rats

PCK rats developed both kidney (Fig 3.1E-H) and liver cysts (Fig 3.2E-H) by 16 weeks of age. Renal cysts developed to a greater extent than hepatic cysts, with the cyst and lumen areas being ~ 4 times higher in kidneys compared to livers (Table 3.4). In contrast to $Pkd2^{WS25/-}$ mice, fibrosis was detected in the livers but very little fibrosis was observed in the kidneys of PCK rats. The presence of cysts was reflected in higher kidney and liver weights and higher lumen and cyst area, as shown in normal and PCK rats given the control soy oil diet (Table 3.4). PCK rats also had higher urine protein levels and water intake, and lower feed intake and body weights than the normal rats, while serum creatinine, creatinine clearance, urine volume and urine pH were not different (Table 3.4).

PCK rats given fish and flax oil compared to soy oil had larger kidneys with higher water content, kidney cyst and lumen area and creatinine clearance. Providing fish oil compared to both flax and soy oil resulted in larger livers. Rats given flax oil had higher urine pH than those given soy, but not fish oil. There were no dietary oil effects on body weight, feed intake, water intake, serum creatinine, urine protein, urine volume, blood pressure, liver cyst area or liver fibrosis (Table 3.5 and, Figs 3.5 and 3.6).

In contrast to $Pkd2^{WS25/-}$ mouse models of ADPKD, providing soy protein compared to the casein protein found in the standard AIN93G diet resulted in higher relative kidney weights and lower liver weights in PCK rats. Dietary soy protein also resulted in higher water intake, creatinine clearance, urine pH and urine volume, and in lower urinary protein. Soy protein did not alter body weight, renal water content, renal cyst and lumen area, liver water content, liver cyst area, liver fibrosis, feed intake, serum creatinine or blood pressure (Table 3.5).

Table 3.4 Disease effects in male PCK rats

	Normal	**Diseased**
Body weight (g)	778±15	594±07*
Kidney		
weight (g)	3.8±0.1	4.4±0.1*
weight / body weight (g/100g)	0.49±0.01	0.75±0.01*
water content (%)	76.0±0.3	79.1±0.1*
lumen or cyst area area / section (pixels x 10^3)	16±5	40±5*
lumen or cyst area / kidney area (%)	6.6±2.3	17.6±2.4*
Serum creatinine (mg/dL)	0.46±0.03	0.45±0.03
Creatinine clearance (mL/min)	26.8±4.4	20.8±2.7
Liver		
weight (g)	26.7±0.9	28.3±0.9
weight / body weight (g/100g)	3.44±0.11	4.78±0.16*
water content (%)	61.8±1.0	69.2±0.7*
fibrosis area / liver area (%)	0.2±0.0	3.5±0.3*
lumen or cyst area / liver area (%)	1.4±0.4	4.7±0.2*
Feed intake (g/24h)	30.0±1.9	24.1±0.6*
Water intake (mL/24h)	17.2±0.9	21.0±0.6*
Urine	10.3±0.9	10.1±0.9
pH	5.9±0.0	6.2±0.3
protein/creatinine (mg/mg)	0.9±0.2	14.7±3.8*
volume (mL/24h)	9.0±0.7	9.2±0.7
Mean arterial pressure (mmHg)	117.5±6.9	116.9±5.2
N	8	8

Data from mice provided soy oil diets only. Values are mean±SE. *Significantly different from normal, $P<0.05$.

Table 3.5 Dietary oil and protein effects in diseased PCK rats

	Soy Oil		Flax Oil		Fish Oil			
	Casein	Soy protein	Casein	Soy protein	Casein	Soy protein	P<0.05	
Body weight (g)	594±7	592±11	604±7	569±9	576±7	579±13		
Kidney								
weight (g)	4.4±0.1^{B}	4.9±0.2	5.7±0.4^{A}	5.2±0.2	5.4±0.3^{A}	5.9±0.3	O	
weight / body weight (g/100g)	0.75±0.01^{C}	0.83±0.03	0.87±0.02^{B}	0.92±0.02	0.94±0.04^{A}	0.99±0.04	O	P
water content (%)	79.1±0.1^{B}	79.8±0.4	81.5±0.8^{A}	81.0±0.4	80.4±0.5^{A}	81.0±0.4	O	
lumen or cyst area/section (pixels x10^{3})	40±5^{B}	48±5	67±8^{A}	55±10	60±9^{A}	62±6	O	
lumen or cyst area/kidney area (%)	17.6±2.4	18.4±1.9	22.3±2.5	21.2±3.6	21.7±3.0	20.7±1.6		
Liver								
weight (g)	28.3±0.9^{B}	27.0±1.1	32.1±1.4^{B}	24.7±0.7	35.5±2.9^{A}	27.8±1.4	O	P
weight / body weight (g/100g)	4.78±0.16^{B}	4.56±0.14	5.32±0.26^{B}	4.34±0.09	6.16±0.49^{A}	4.81±0.26	O	P
water content (%)	69.2±0.7^{ab}	71.4±0.6^{a}	70.1±0.4^{ab}	68.8±0.4^{b}	70.5±0.8^{ab}	69.7±0.5^{ab}		I
fibrosis area / liver area (%)	3.5±0.3	4.0±0.5	4.6±0.7	3.3±0.3	4.7±0.9	3.7±0.4		
lumen or cyst area / liver area (%)	4.7±0.2	3.9±0.5	6.1±0.8	5.4±1.1	6.0±0.9	4.9±0.8		
Feed intake (g/24h)	24.1±0.6	23.3±1.4	25.3±0.5	24.0±1.0	25.6±1.0	25.3±1.1		
Water intake (mL/24h)	21.0±0.6	27.4±1.8	22.8±1.1	26.5±1.09	23.0±1.8	29.3±1.6		P
Serum creatinine (mg/dL)	0.45±0.03^{A}	0.44±0.02	0.49±0.04^{A}	0.41±0.01	0.40±0.03^{B}	0.39±0.04	O§	
Creatinine clearance (mL/min)	20.8±2.7^{B}	23.4±1.7	25.5±2.8^{A}	31.5±4.1	25.1±4.0^{A}	37.1±4.1	O	P
Urine								
pH	6.2±0.3^{B}	7.7±0.4	7.5±0.4^{A}	8.3±0.3	7.3±0.6^{AB}	7.9±0.4	O	P
protein / creatinine (mg/mg)	14.7±3.8	10.1±1.5	10.9±1.6	9.0±1.3	14.2±1.8	9.6±2.1		P
volume (mL/24h)	10.1±0.9	14.9±1.8	12.8±0.8	14.7±0.6	10.0±1.8	14.8±1.4		P
Mean arterial pressure (mmHg)	116.9±5.2	121.4±4.2	129.0±5.0	120.9±4.1	114.6±3.1	117.6±2.8		
N	8	8	8	8	6	8		

Values are mean±SE. With significant dietary oil effects, differing upper case superscript letters in casein columns indicate significant overall (casein and soy protein) differences between groups given different dietary oils. With significant interaction effects, differing lower case superscript letters indicate significant simple effect differences between values. I, interaction; O, oil; P, protein.

§P=0.057.

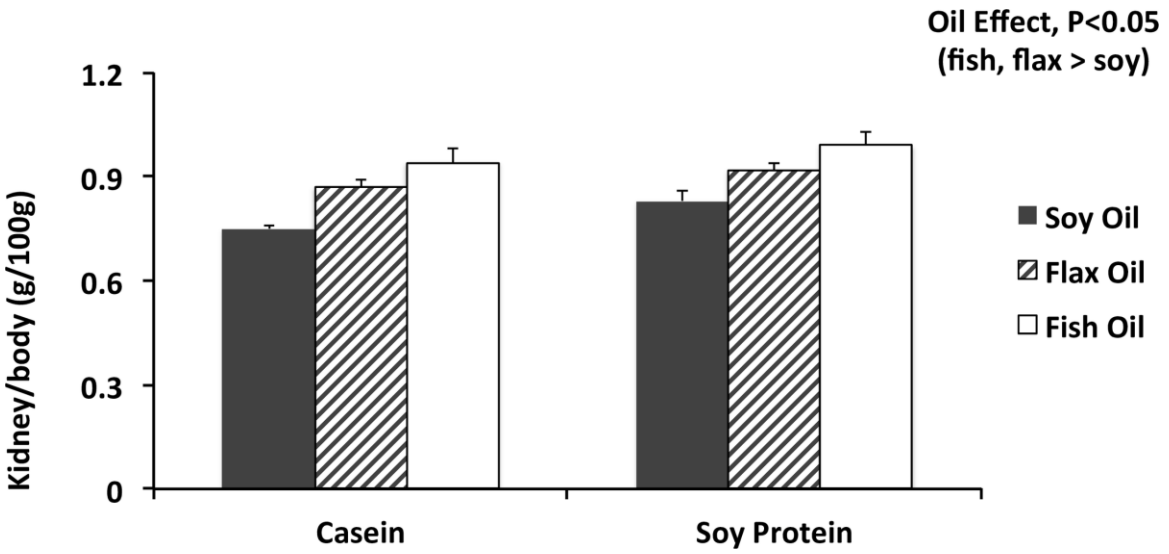

Fig 3.5 Dietary oil and protein effects on kidney size in diseased PCK rats. Significant diet effects are shown on figure. Data from Table 3.5

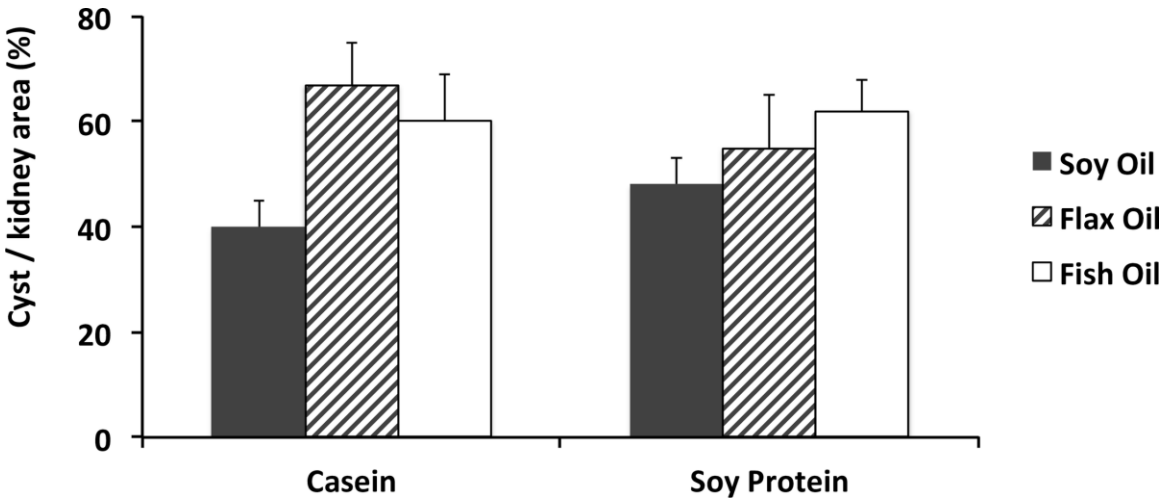

Fig 3.6 Dietary oil and protein effects on renal cyst area in diseased PCK rats. There were no significant diet or sex effects. Data from Table 3.5

3.5 Discussion

Overall, dietary interventions with oils enriched in n-3 fatty acids provided early in the development of PKD displayed no benefits and possible negative effects on disease in both orthologous models of PKD studied. This lack of benefit in male PCK rats and in Pkd2$^{WS25/-}$ mice is similar to the findings in female PCK rats (28). In non-orthologous models, fish oil has conflicting effects, with generally protective effects observed in the Han:SPRD-*Cy* rat (23,24), while in the *pcy* mouse beneficial, detrimental and no effects have been observed (21,26,27). With respect to liver effects, fish oil was recently shown to have no effects on liver cysts in female PCK rats, but complications due to cyst obstruction of the bile duct and hepatic vein were evident when these rats were given the fish oil diet (32). Dietary flax oil also displayed no beneficial effects on disease in either orthologous PKD model, which contrasts with the beneficial effects observed in both the Han:SPRD-*Cy* rat and in *pcy* mice (19,21,22). Although there were some effects of the oils containing n-3 fatty acids that were detrimental, these were small and not consistent in both models; as well, there were some potentially small positive effects, thus providing insufficient evidence to conclude that these dietary oils were harmful. Overall, these findings do not support dietary advice to increase dietary oils containing n-3 fatty acids for early treatment of PKD, and are consistent with a short-term study in human PKD that failed to demonstrate a beneficial effect of fish oil supplementation (33).

With respect to dietary protein source, there were no differences observed in the Pkd2$^{WS25/-}$ mouse. In the PCK rats, although kidney and liver histology were not affected, water intake, creatinine clearance and urine pH were higher, and proteinuria was lower in soy protein fed rats. Similarly, dietary soy protein exhibited no benefits in female PCK rats provided soy protein diets at similar ages (28). Increased water intake in the PCK rat is associated with

reduction in kidney disease progression (34), and increased urine pH via citrate administration is associated with protection from disease in the Han:SPRD-*Cy* rat (35), possibly indicating that there may be benefits of this dietary intervention that had not yet been manifested, but require further study.

While these studies provide no supporting evidence for dietary advice in the early stages of PKD to increase soy protein or oils enriched in n-3 fatty acids, it is important to determine whether interventions in later stages of disease would benefit from these treatments. Significant benefits of these identical dietary interventions were observed in non-orthologous models, in which disease progression is not only more rapid, but these animals also were terminated at a later stage of disease (10,11,21-23). Studies in the Han:SPRD-*Cy* rat when these dietary soy protein, flax or fish oil interventions were initiated after the disease had progressed to the equivalent of approximately stage 3 CKD, dietary interventions also were not as effective, indicating that the intervention may have been too late in the disease process (14). This suggests that there may be a window of opportunity during progressive cyst expansion in which dietary interventions may be more effective.

The amount of soy protein or n-3 enriched oil also may influence the dietary effect, as the amounts used in the current diets are higher than what would be achievable in analogous human diets. However, the diets used herein were identical in this regard to the studies showing considerable slowing of disease progression in the non-orthologous Han:SRPD-*Cy* rat and *pcy* mouse models (11,21).

These results also may suggest that dietary interventions may be more effective in models of NPHP compared to models of PKD. The *pcy* mouse is a model of NPHP3 (36), while the Anks6 protein mutated in the Han:SPRD-*Cy* rat appears to form complexes with NPHP proteins

as well (37). Nevertheless, these studies emphasize the need for studies in true models of PKD, and for replicating the results in multiple models, as no one model completely mimics the human form of the disease (38,39). Investigation of possible sex effects also warrants further investigation.

3.6 Conclusions

These studies show that dietary interventions with soy protein or n-3 fatty acid enriched oils are not effective when administered during early PKD development in human orthologous models. However, evidence from non-orthologous models indicate that these dietary interventions may possibly be more effective during the progressive growth phase of cyst development.

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3.8 Transition to next chapter

My objective in the present research was to determine whether the beneficial effects of dietary soy protein, flax or fish oil in NPHP models are replicated in the Pkd2 mouse model of ADPKD. In chapter 3 we showed that there was no beneficial effect of these dietary interventions in Pkd2 mice. This lack of benefit with soy protein, flax and fish oil was consistent with findings from a study in a rat model of ARPKD (28), as well as with a human study which provided fish oil for a short period of time to ADPKD patients (33). Dietary interventions were initiated in the early stage of disease, so, it can be concluded that there are no beneficial effects of early dietary interventions of soy protein, flax or fish oil in Pkd2 mice. Whether beneficial effects can be found in later stages of disease need to be explored in future studies.

Oxylipins are bioactive lipid metabolites that play important roles in renal homeostasis. In Han:SPRD-Cy rat and *pcy* mouse models of NPHP renal oxylipins are altered in disease (22, 23). Whether renal oxylipins are also altered in ADPKD is not clear. In the previous chapter we confirmed that by 16 weeks of age Pkd2 mice develop renal cysts and are a good model of human ADPKD. To determine whether disease specific alterations of renal oxylipins are also observed in the Pkd2 mouse model of ADPKD, the renal oxylipin profile of normal and diseased Pkd2 mice of both sexes were analyzed in the next chapter (hypothesis 2).

This study was done along with similar oxylipin profiling in models of ARPKD, NPHP and another model of ADPKD by my collaborators. My role in this collaborative study was as follows:

I conducted the animal feeding, tissue collection, oxylipin extraction and quantification, and statistical analysis of the Pkd2 mouse study. I was also primarily responsible for the writing

of the manuscript and responded to reviewer comments. For contributions of other authors please refer to Author Contributions section (p.vii-p.x).

Chapter 4

Distinct Oxylipin Alterations in Diverse Models of Cystic Kidney Diseases

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4.1 Abstract

Cystic kidney diseases are characterized by multiple renal cysts and are the leading cause of inherited renal disease. Oxylipins are bioactive lipids derived from fatty acids formed via cyclooxygenase, lipoxygenase and cytochrome P450 activity, and are important regulators of renal health and disease. Oxylipins are altered in nephronophthisis, a type of cystic kidney disease. To further investigate and to determine whether other cystic renal diseases share these abnormalities, a targeted lipidomic analysis of renal oxylipins was performed in orthologous models of autosomal dominant polycystic kidney disease 1 (*Mx1Cre*$^{+}$*Pkd1*$^{flox/flox}$ mouse) and 2 (Pkd2$^{WS25/-}$ mouse), autosomal recessive polycystic kidney disease (PCK rat) and nephronophthisis (*jck/jck* mouse).

Kidney cyclooxygenase oxylipins were consistently higher in all diseased kidneys, even in very early stage disease. On the other hand, cytochrome P450 epoxygenase derived oxylipins were lower only in the autosomal recessive polycystic kidney disease and nephronophthisis models, while lipoxygenase and cytochrome P450 hydroxylase derived oxylipins were lower only in nephronophthisis. Sex effects on renal oxylipin alterations were observed but they did not always coincide with sex effects on disease. For oxylipins with sex effects, arachidonic acid derived oxylipins formed via cyclooxygenases and lipoxygenases were higher in females, while oxylipins from other fatty acids and via cytochrome P450 enzymes were higher in males. The consistent and unique patterns of oxylipin alterations in the different models indicates the importance of these bioactive lipids in cystic renal diseases, suggesting that pharmacological agents (e.g. cyclooxygenase inhibitors) may be useful in treating these disorders, for which effective treatment remains elusive.

4.2 Introduction

Cystic kidney diseases are a group of inherited renal disorders characterized by proliferation of fluid filled renal cysts in the kidneys and other organs. In humans, the most common forms are autosomal dominant polycystic kidney disease (ADPKD) and autosomal recessive PKD (ARPKD) (1, 2). The prevalence of ADPKD is 1:400 to 1:1000, affecting approximately 12.5 million people worldwide (3, 4). Approximately 85% of ADPKD is caused by mutations in the *PKD1* gene, which codes for polycystin 1 (PC1), and the remaining 15% of cases are caused by mutations in the *PKD2* gene, which codes for polycystin 2 (5-7). ARPKD is much less common but is a more severe form of the disease occurring 1 in 20,000-40,000 live births (8, 9). ARPKD presents primarily in infancy and childhood, with 30-50% of patients dying shortly after birth (9-11). ARPKD results from a defect in the polycystic kidney and hepatic disease-1 (*PKHD1*) gene, which codes for polyductin/fibrocystin (12).Another pediatric form of a cystic kidney disease is known as nephronophthisis (NPHP), a type of cystic kidney disease caused by as many as 20 mutations in NPHP or related genes (13, 14). The prevalence of NPHP is 1 in 50-90,000, affects approximately 2.4% to 15% of children with end-stage renal disease and is the most common cause of renal failure in the first three decades of life (14-17).

Previous studies with NPHP models of cystic kidney disease have demonstrated alterations in oxylipins in diseased kidneys (18-20).Oxylipins are bioactive lipid metabolites of polyunsaturated fatty acids (PUFA), which are formed by the action of three classes of enzymes: cyclooxygenase (COX), lipoxygenase (LOX) and cytochrome P450 (CYP) (18, 21-23).Oxylipin formation is initiated when PUFA are released from tissue phospholipids by phospholipase A_2, and are subsequently converted into prostaglandins (PG) and thromboxanes (Tx) via the COX pathway, hydroxy fatty acids and their metabolites (i.e. leukotrienes and lipoxins) via the LOX

pathway, and epoxy and hydroxy fatty acids (e.g. epoxy- and hydroxy-eicosatetraenoic acids) via the CYP pathway(18, 24-26).

Oxylipins are critical for normal renal physiology and function, but also can have detrimental effects in disease. In the *pcy* mouse and Han:SPRD-*Cy* rat models of NPHP, COX enzyme activity and oxylipin levels are higher whereas LOX and CYP oxylipins are lower (18-20). The mechanism by which the elevated COX oxylipins lead to cyst formation and disease progression is not clear, but it is known that COX derived oxylipins such as PGE_2 mediate their effects by increasing the levels of cAMP (27-32). Elevated renal cAMP is common in cystic kidneys (33, 34), and several signaling pathways altered in cystic kidney diseases regulate and are regulated in part by cAMP (35-39). The COX oxylipin alterations in *pcy* mouse kidneys occur at a very early age, indicating that these bioactive lipids play a critical role in this disorder.

Whether oxylipins are altered in other types of renal cyst diseases is not clear, however. Human ADPKD displays alterations in some serum oxylipins (40), but renal changes are unknown. The current study therefore undertook a targeted lipidomic analysis of renal oxylipins in diverse models of cystic kidney disease. These analyses reveal that COX oxylipins are consistently elevated across disease models, while LOX and CYP oxylipin changes appear to be unique to specific types of cystic kidney diseases. Novel sex effects on renal oxylipins also were observed.

4.3 Materials and methods

4.3.1 Animal models

Orthologous models of ADPKD1, ADPKD2, ARPKD and NPHP were examined in five independent studies. In the first study, *Mx1Cre*$^{+}$*Pkd1*$^{flox/flox}$ (Pkd1) mice (ADPKD1 model) (41),

were obtained from breeders provided by Dr. Jing Zhou at Brigham and Women's Hospital and Harvard Medical School (Boston. MA, U.S.A). Male and female mice were injected i.p. with 250 μg of polyinosinic:polycytidylic acid (pI:pC) to induce somatic inactivation of *Pkd1*or were injected with saline to serve as controls, for five consecutive days beginning at 5 weeks of age. At 23 weeks of age kidneys were harvested. These mice are referred to as Pkd1 (5wk) mice.

The second study used the same mice as study 1 except that gene inactivation was at 1 week of age and mice were terminated at 9 weeks of age. These mice are referred to as Pkd1 (1wk) mice.

For the third study, $Pkd2^{WS25/WS25}$ and $Pkd2^{+/-}$ breeders (ADPKD2 model) (42), were obtained from Dr. Stefan Somlo at Yale University (New Haven, CT, U.S.A.).These genotypes were crossed to produce male and female mice with diseased ($Pkd2^{WS25/-}$) or normal ($Pkd2^{WS25/+}$) phenotypes that were terminated at 16 weeks of age. These mice are referred to as Pkd2 mice. These mice are the control diet mice used in a study published previously on dietary effects on disease (43).

For the fourth study, PCK rats (ARPKD model) (44), were purchased from a commercial breeder (Charles River, QC, Canada). Normal and PCK male (only) rats were terminated at 16 weeks of age. These rats are the control diet rats used in a study published previously on dietary effects on disease (43).

The fifth study used the *jck/jck* mouse (NPHP model) which harbors a mutation in the *Nphp9* gene (45). These mice were obtained from Dr. Shizuko Nagao at Fujita Health University (Toyoake, Japan). Kidneys from normal (+/+, *jck*/+) and diseased (*jck/jck*) male and female mice were harvested at 12 weeks of age and are referred to as *jck* mice.

Animals in all studies were housed in temperature and humidity controlled environments with a 12 hour day/night cycle, and were given free access to water and standard semi-purified or chow diets. At termination, body and kidney weights were measured and the right kidney was snap frozen in liquid nitrogen, and stored at -80°C until oxylipin analysis. The left kidney was fixed by placing it in 10% formalin for 24h, followed by transfer to PBS at 4°C until further processing. Formalin fixed kidneys were embedded in paraffin, sectioned at 5 µm and tissue sections were stained with Masson's trichrome as previously described (46). Kidney water content was determined by measuring the difference between wet and dry (lyophilized) kidneys weights. All animal procedures were approved by the Institutional Animal Care Committees and adhered to the guidelines of the Canadian Council on Animal Care.

4.3.2 Oxylipin analysis

Lyophilized whole kidney tissues were homogenized in ice cold Tyrode's salt solution (pH 7.6) in a 1:28 weight/volume ratio. After homogenization Triton X-100 was added to achieve a final concentration of 0.01%. Deuterated internal standards (10 µL) (Cayman Chemical, MI, USA) and 6.5µL of antioxidant cocktail [0.2 g/L BHT, 0.2 g/L EDTA, 2 g/L triphenylphosphine, and 2 g/L indomethacin in MeOH:EtOH:H_2O (2:1:1,by vol)] were added to 200 µL tissue homogenates used for analysis. Samples were adjusted to pH<3 and solid phase extraction was with Strata-X SPE columns (Phenomenex, CA, USA) preconditioned with methanol and pH 3 water. Samples were loaded onto the columns, rinsed with 10% methanol, and eluted with methanol. Evaporated samples were then re-suspended in solvent for analysis by HPLC-MS/MS (API 4000, AB Sciex, Canada) as described (20, 47, 48),based on methods developed by Deems et al. (49). Recoveries and intra-batch precision of four representative

oxylipins from kidney homogenates spiked with oxylipin standards were similar to recoveries reported with this method (49) (Table 4.1). Lower limit of detection (LLOD) and quantitation (LLOQ) were set at 3 and 5 levels above the background, respectively. Quantification of oxylipins was determined using the stable isotope dilution method (50), and amounts expressed as pg/mg of dry tissue. A list of all oxylipins screened for, detector response factors, internal standards, LLOQ and LLOD are listed in Appendix G.

Table 4.1 Recoveries of four representative standard oxylipins.

Oxylipin	**Recovery**	**Intra-batch precision**
$PGF_{2\alpha}$	Moderate (25-75%)	9.3%
5-HETE	Moderate (25-75%)	12.3%
LTB_4	Excellent (>75%)	9.4%
5,6-DiHETrE	Poor (<25%)	17.4%

4.3.3 Statistical analysis

Data were analyzed using the GLM procedure of SAS (SAS, version 9.4, Cary, NC, USA). When the data were normally distributed, disease and sex effects in mouse studies were tested by 2-way (disease x sex) ANOVA. Male rats only were used in the PCK rat study, so disease effects were tested using t-tests. Normality of data was tested using Shapiro-Wilk's Statistic ($W > 0.05$ for normally distributed data). If the data did not follow a normal distribution even if transformed, a nonparametric test (Kruskal-Wallis) was used in place of the parametric test. Post hoc analysis was done by Duncan's multiple range tests for simple effect comparisons when interactions were present or when the Kruskal-Wallis test indicated the presence of

differences. All data were presented as mean ±SEM. Significance was set at $p< 0.05$ for main, interaction, and simple effects.

4.4 Results

4.4.1 Disease progression

Cystic kidney disease was present in all models studied, although the extent of disease varied widely between models (Table 4.2). Small cysts were present in both models of ADPKD1 (*Mx1Cre*$^{+}$*Pkd1*$^{flox/flox}$ mice with gene inactivation at 5 weeks [Pkd1(5wk)] or 1 week [Pkd1(1wk)] of age, but kidney size was not affected in this early stage of PKD, and kidney water was only slightly (<1%) higher in Pkd1(5wk) mice with disease. Larger cysts were observed in ADPKD2 mice (Pkd2$^{WS25/-}$) and ARPKD rats (PCK), and this was reflected in 20-30% larger kidneys and 3-4% higher water content in diseased kidneys. Disease progression in the NPHP model (*jck* mouse) was much greater, with 250-500% higher kidney weights and 25% higher kidney water content in disease.

4.4.2 Disease effects on oxylipins

Over 100 oxylipins were analyzed for each mouse model and between 44 and 55 oxylipins were detected in normal and diseased kidneys (Tables 4.3-4.7). When oxylipins were examined as totals from the different biosynthetic pathways, COX oxylipins were consistently higher in diseased kidneys (Figure 4.1), being 30%, 21%, 40%, 32% and 303% higher in Pkd1(5wk), Pkd1(1wk), Pkd2, PCK and *jck* models, respectively. In contrast, total CYP epoxygenase derived oxylipins were only lower in PCK and *jck* diseased kidneys (by 58% and 42% respectively), whereas total LOX and CYP hydroxylase oxylipins were only different in *jck* mice, being lower

by 37% and 80%, respectively, in disease. The disease effect observed in total renal COX oxylipins reflected the differences in individual oxylipins. All oxylipin data are shown in Tables 5.3-5.7, and relative differences for oxylipins with differences due to disease are shown grouped by enzymatic pathway in Figure 4.1. Individual COX oxylipin differences ranged from 20% to >1100% higher in diseased kidneys, with PGE_2 (except for Pkd2) and 6-keto-$PGF_{1\alpha}$ being consistently higher in all models. The pattern of total LOX oxylipins only being lower in *jck* diseased kidneys also was reflected in individual LOX oxylipins, where 19/26 individual LOX oxylipins were significantly lower in the diseased kidneys. The only LOX oxylipin in *jck* mice that was higher was 11-hydroxy-eicosatetraenoic acid (HETE), which also is known to be produced by COX activity (57), possibly explaining this difference. There were few and inconsistent changes in individual LOX oxylipins in other models. The lower total CYP epoxygenase oxylipins in disease in PCK rat and *jck* mouse kidneys also were reflected in the significantly different individual oxylipins, in which 5/7 and 6/7 CYP epoxygenase oxylipins, respectively, were lower in disease. For CYP hydroxylase oxylipins, the only consistent individual oxylipin differences were observed in *jck* kidneys, in which 3/3 CYP hydroxylase oxylipins were significantly lower in disease, reflecting total CYP hydroxylase oxylipins in this model. In addition to these enzymatically derived oxylipins, 5-iso-$PGF_{2\alpha}$VI was 75% higher in Pkd2 diseased kidneys, and 8-iso-$PGF_{2\alpha}$III was 29% and 42% higher in disease in the PCK and *jck* models, respectively (Tables 4.4-4.7).

Table 4.2 Disease and sex effects in models of renal cystic diseases.

	Normal		Diseased		Effect	
	Male	Female	Male	Female	*P<0.05*	
Pkd1(5wk)						
Body weight (g)	30.2±2.2	25.1±1.2	27.8±0.9	22.1±0.6	D	S
Kidney						
weight (g)	0.28±0.02	0.24±0.01	0.28±0.01	0.23±0.01		S
weight / body weight (g/100g)	0.94±0.04	0.97±0.06	1.02±0.04	1.02±0.04		
water content (%)	74.6±0.1	75.1±0.3	74.9±0.2	75.9±0.2	D	S
Pkd1(1wk)						
Body weight (g)	21.7±0.9	18.8±0.4	21.85±0.5	19.8±1.0		S
Kidney						
weight (g)	0.24±0.01	0.22±0.01	0.24±0.01	0.24±0.01		
weight / body weight (g/100g)	1.10±0.05	1.19±0.04	1.12±0.05	1.22±0.07		
water content (%)	73.8±0.48	75.0±0.46	74.3±0.8	75.5±0.5		
Pkd2						
Body weight (g)	26.2±2.2	19.5±0.6	27.1±1.7	19.3±1.0		S
Kidney						
weight (g)	0.28±0.02	0.21±0.01	0.37±0.04	0.27±0.03	D	S
weight / body weight (g/100g)	1.05±0.04	1.11±0.04	1.37±0.01	1.40±0.12	D	
water content (%)	72.2±0.6	71.5±1.0	75.4±1.8	74.8±1.1	D	
PCK*						
Body weight (g)	778±15		594±07		D	
Kidney						
weight (g)	3.8±0.10		4.4±0.10		D	
weight / body weight (g/100g)	0.49±0.01		0.75±0.01		D	
water content (%)	76.0±0.3		79.1±0.10		D	
Jck						
Body weight (g)	24.5±0.87	20.2±0.59	23.1±0.98	19.8±0.38		S
Kidney						
weight (g)	0.27±.01^{c}	0.20±.01^{d}	1.51±.14^{a}	0.72±0.05^{b}		I
weight / body weight (g/100g)	1.10±0.04^{c}	1.01±0.03^{c}	6.81±0.89^{a}	3.63±0.25^{b}		I
water content (%)	71.3±0.44	69.3±0.30	89.4±0.47	87.5±0.47	D	S

*Male rats only were used in the PCK study. Values are mean±SE. Values within rows with differing lower case superscript letters indicate differences between values. D, disease; S, sex; I, interactions. Kidney sections from normal and diseased male kidneys of corresponding models are shown to the right. Bars represent 5.0 mm.

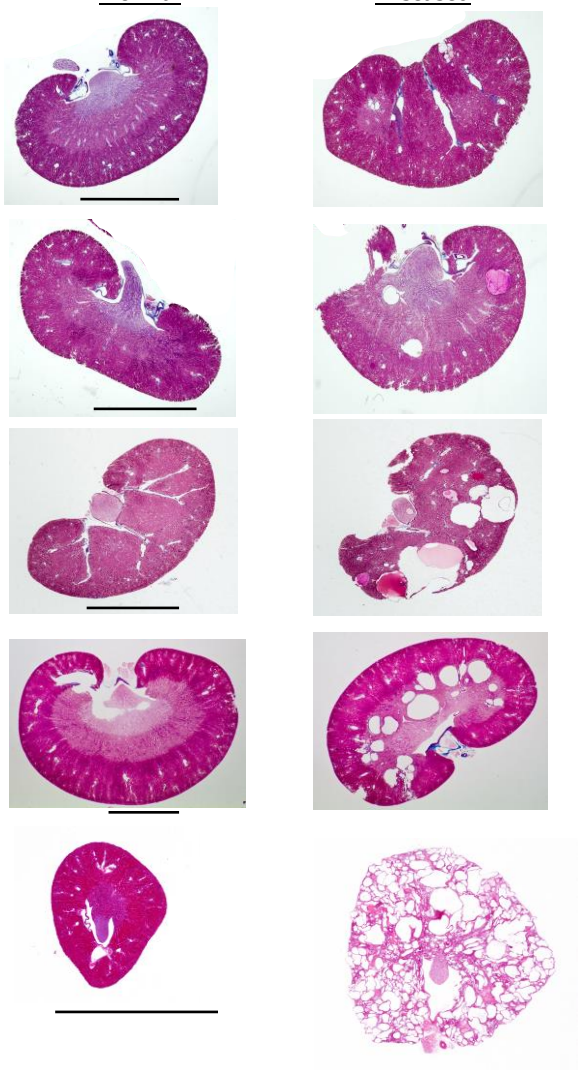

Table 4.3 Renal oxylipins in normal and diseased Pkd1 (5wk) (Mx1Cre^{+}*Pkd1*$^{flox/flox}$) mice.

Oxylipin (pg/mg dry tissue)	PUFA	Normal		Diseased		Effects (p<0.05)	
		Male (n=7)	Female (n=5)	Male (n=6)	Female (n=6)		
COX Pathway							
PGD_2	AA	175±31.4	192±18.6	166±33.3	203±31.5		
PGE_2	AA	202±20.1	346±34.7	279±24.8	419±17.6	D	S
$PGF_{2\alpha}$	AA	206±37.1	287±38.7	217±25.9	354±29.3		S
6-k-$PGF_{1\alpha}$	AA	310±71.5^{b}	332±66.1^{b}	302±50.4^{b}	760±122^{a}	I	
Total COX		894±103	1053±134	939±103	1576±148	D	S
LOX Pathway*							
9-HODE	LA	5941±379	3754±122	4451±270	4137±559		S
9-oxo-ODE	LA	2815±350	2175±426	1840±195	2531±304		
13-HODE	LA	5857±445	4080±247	5097±692	5256±586		
13-oxo-ODE	LA	2708±349	2690±657	2856±336	2212±438		
9,10,13-TriHOME	LA	3806±511	3069±706	3243±518	2869±330		
9,12,13-TriHOME	LA	1439±156	1110±286	1142±160	946±124		
8-HETrE	DγLA	286±26.6	213±63.7	265±29.4	152±15.5		S
15-HETrE	DγLA	117±21.0	96.4±16.3	88.7±20.6	113±15.5		
5-HETE	AA	1763±146	1623±94	1828±173	1740±197		
5-oxo-ETE	AA	371±34.0	306±16.3	411±50.5	390±53.4		
8-HETE	AA	353±56.8	510±106	445±78.4	424±37.8		
9-HETE	AA	785±164	1068±218	818±162	1177±110		
11-HETE	AA	813±151	1023±85.5	798±172	1299±100		S
12-HETE	AA	905±235	1152±163	1267±284	1657±269		
15-HETE	AA	1348±189	1717±205	1428±206	2008±137		S
15-oxo-ETE	AA	1874±322	1785±415	1556±282	1519±328		
9-HOTrE	ALA	678±119	368±76.1	528±76.9	303±59.0		S
9-oxo-OTrE	ALA	234±61.7	262±143	189±21.3	266±90.6		
13-HOTrE	ALA	43291±7088	40556±11761	39852±8629	28892±4349		
5-HEPE	EPA	405±88.7	413±138	382±59.8	300±106		
8-HEPE	EPA	109±35.7	175±106	79.8±9.40	72.5±26.1		
9-HEPE	EPA	653±188	639±233	667±130	519±178		
11-HEPE	EPA	224±61.5	363±143	237±34.4	214±59.0		
12-HEPE	EPA	524±77.5	485±74.3	761±88.3	509±126		
15-HEPE	EPA	264±39.7	214±48.1	285±52.4	300±50.3		
4-HDoHE	DHA	2147±158	1088±103	2330±184	1342±219		S
7-HDoHE	DHA	222±33.9	157±24.1	179±38.4	145±18.8		
10-HDoHE	DHA	231±60.1	197±34.8	158±23.2	139±27.3		
10S,17S-DiHDoHE	DHA	56.0±11.1	28.2±8.20	68.3±6.50	30.9±5.70		S
11-HDoHE	DHA	461±80.7	425±75.5	419±86.6	391±56.1		
13-HDoHE	DHA	177±28.1	127±15.2	185±26.6	141±13.2		
14-HDoHE	DHA	767±123	574±67.5	792±94.6	646±87.5		

16-HDoHE	DHA	776±86.7	526±53.8	846±43.9	565±59.6		S
17-HDoHE	DHA	2528±318	1492±79.0	2431±107	1666±181		S
17-k-DHA	DHA	2219±603	1167±218	3025±857	1013±141		S
Total LOX		82482±8547	71667±11580	76987±9008	64460±7685		
CYP-E Pathway							
9,10-DiHOME	LA	99.6±6.40	67.2±7.20	94.6±10.9	75.2±8.10		S
12,13-DiHOME	LA	63.4±4.70	42.9±5.50	61.3±7.60	45.8±3.10		S
5,6-DiHETrE	AA	125±15.0	78.7±10.9	126±12.7	96.6±15.6		S
8,9-DiHETrE	AA	19.6±1.80	15.5±1.50	17.3±1.80	17.3±2.10		
11,12-DiHETrE	AA	44.4±6.20	39.1±1.80	41.7±6.30	46.8±5.40		
14,15-DiHETrE	AA	41.7±4.20	51.1±5.70	50.4±6.20	59.0±4.70		
19,20-DiHDPE	DHA	330±48.7	100±13.3	454±59.7	150±9.70	D	S
Total CYP-E		663±48.3	356±23.4	619±117	444±27.5		S
CYP-H Pathway							
16-HETE	AA	198±24.3	168±19.5	184±17.8	183±13.8		
18-HETE	AA	45.9±5.40	39.1±6.50	42.2±5.50	30.6±2.20		
18-HEPE	EPA	549±20.6	358±50.7	586±40.2	499±39.2	D	S
20-HDoHE	DHA	14331±1406	7691±669	14926±1548	8832±1018		S
Total CYP-H		15067±1405	8222±712	15738±1556	9544±1055		S

Values are expressed as mean ± SEM. Values with differing lower case superscript letters indicate simple effect differences between values. D, disease; S, sex; I, interactions.
*Many LOX oxylipins, including HODE, 9- and 11-HETE, 9- and 11-HEPE, HDoHE can be formed both enzymatically and non-enzymatically (51-56).

Table 4.4 Renal oxylipins in normal and diseased Pkd1 (1wk) (Mx1Cre^{+}*Pkd1*$^{flox/flox}$) mice.

Oxylipin (pg/mg dry tissue)	PUFA	Normal		Diseased		Effects (p<0.05)	
		Male (n=6)	Female (n=8)	Male (n=6)	Female (n=4)		
COX Pathway							
PGD_2	AA	477±51.9	798±43.9	518±62.1	1064±78.2	D	S
PGE_2	AA	192±16.7	400±49.3	261±22.7	482±30.9	D	S
$PGF_{2\alpha}$	AA	241±23.4	333±29.9	240±19.5	419±42.9		S
6-k-$PGF_{1\alpha}$	AA	425±45.9^{c}	680±57.3^{b}	418±26.7^{c}	976±54.2^{a}	I	
TxB_2	AA	31.5±2.95	44.1±7.99	28.7±4.87	58.7±8.02		S
PGE_3	EPA	8.80±1.70	8.68±2.18	8.47±2.88	11.1±1.58		
Δ^{17}-6-k-$PGF_{1\alpha}$	EPA	1.24±0.13	1.26±0.24	0.94±0.13	1.48±0.18		
Total COX		1377±121	2154±143	1474±103	2648±289	D	
LOX Pathway*							
9-HODE	LA	3803±254	4090±395	4037±385	4201±256		
9-oxo-ODE	LA	1483±210	2051±158	1769±301	1973±243		
13-HODE	LA	4737±266	5068±684	4520±459	5265±610		
13-oxo-ODE	LA	4622±619	6174±553	4036±531	5939±783		S
9,10,13-TriHOME	LA	2047±235	1729±193	1523±102	1892±222		
9,12,13-TriHOME	LA	671±53.3	589±67.8	526±48.1	611±88.7		
13-HOTrE-γ	γLA	56.2±7.22	42.6±5.75	50.9±6.04	50.0±7.34		
8-HETrE	DγLA	156±18.5	153±13.2	163±8.02	164±13.9		
15-HETrE	DγLA	90.5±6.90	61.1±4.59	89.8±7.16	82.9±10.2		S
5-HETE	AA	2017±304	2225±159	1830±176	2669±244		S
5-oxo-ETE	AA	346±79.4	546±34.5	295±38.6	558±80.0		S
8-HETE	AA	737±101	955±83.6	847±69.0	913±175		
9-HETE	AA	1164±119	1195±124	1213±127	1603±71.8		
11-HETE	AA	542±116	879±65.9	625±45.1	945±196		S
12-HETE	AA	1077±154	877±104	1150±83.9	1332±127	D	
15-HETE	AA	746±80.6	1077±93.6	831±60.0	1427±131	D	S
9-HOTrE	ALA	325±45.2	228±38.6	277±75.1	236±38.8		
9-oxo-OTrE	ALA	210±51.2	201±38.6	138±42.8	256±145		
13-HOTrE	ALA	20187±1827	19023±2562	13979±2715	13385±1153	D	
5-HEPE	EPA	392±62.8	392±41.4	376±36.2	401±32.9		
8-HEPE	EPA	115±28.4	99.4±16.4	76.5±14.2	64.1±10.8		
9-HEPE	EPA	279±55.6	248±28.2	252±20.6	294±35.4		
11-HEPE	EPA	147±21.5	131±16.1	121±14.0	149±25.6		
12-HEPE	EPA	426±48.7	268±26.9	442±49.6	398±25.4	D	S
15-HEPE	EPA	96.5±15.5	78.7±11.3	72.1±13.8	81.6±11.7		
4-HDoHE	DHA	989±98.1	837±69.9	1098±115	1076±129		
7-HDoHE	DHA	230±51.6	149±28.6	203±25.6	190±13.5		
8-HDoHE	DHA	959±110	659±77.6	988±123	896±116		
10-HDoHE	DHA	134±16.7	114±16.2	136±10.3	145±21.1		

10S,17S-DiHDoHE	DHA	26.0±2.40	22.1.0±4.07	29.6±4.29	29.4±6.0		
11-HDoHE	DHA	218±33.4	175±22.7	238±29.2	219±15.9		
13-HDoHE	DHA	81.0±8.35[ab]	66.1±6.56[b]	82.4±5.44[ab]	97.5±4.37[a]	I	
14-HDoHE	DHA	531±7.9	340±38.0	511±45.1	505±40.3		
16-HDoHE	DHA	481±61.1	347±35.9	454±35.3	479±38.6		
17-HDoHE	DHA	1010±120	742±103	969±97.1	1001±70.2		
17-k-DHA	DHA	490±104	524±67.8	481±43.1	622±68.8		
RvD_2	DHA	97.2±16.2	108±15.5	102±11.8	93.2±17.2		
Total LOX		50173±4006	48210±3986	43972±5305	47615±2091		
CYP-E Pathway							
9,10-DiHOME	LA	102±8.11	79.9±9.74	83.9±10.0	82.0±5.31		
12,13-DiHOME	LA	1708±64.5	1457±167	1655±219	1421±70.0		
5,6-DiHETrE	AA	48.5±5.70	37.1±5.31	61.3±5.28	66.9±10.8	D	
8,9-DiHETrE	AA	14.1±2.22	14.4±1.71	13.4±1.11	18.8±2.56		
11,12-DiHETrE	AA	40.6±4.98	39.9±6.08	34.4±3.33	49.3±4.63		
14,15-DiHETrE	AA	36.4±3.82	36.2±3.97	38.1±2.02	44.7±4.06		
19,20-DiHDPE	DHA	207±22.8	85.4±8.06	222±22.6	94.1±10.7		S
Total CYP-E		2158±88	1749±194	2102±258	1777±52.9		
CYP-H Pathway							
16-HETE	AA	57.0±3.69	59.5±2.47	49.5±5.97	59.8±3.95		
18-HETE	AA	2.87±1.04	2.57±0.58	1.87±0.53	2.24±0.86		
18-HEPE	EPA	1233±137	1218±181	1382±158	1446±122		
20-HDoHE	DHA	17862±1478	14853±2044	21038±1899	20938±1975	D	
Total CYP-H		24585±1378	22574±2330	27931±2595	28250±2234		
Non-enzymatic products							
8-iso-$PGF_{2\alpha}$III	AA	588±47.8	810±81.2	620±50.4	1030±67.9		S

Values are expressed as mean ± SEM. Values with differing lower case superscript letters indicate simple effect differences between values. D, disease; S, sex; I, interactions.

*Many LOX oxylipins, including HODE, 9- and 11-HETE, 9- and 11-HEPE, HDoHE can be formed both enzymatically and non-enzymatically (51-56).

Table 4.5 Renal oxylipins in normal (Pkd2$^{WS25/+}$) and diseased (Pkd2$^{WS25/-}$) Pkd2 mice.

Oxylipin (pg/mg dry tissue)	PUFA	Normal		Diseased		Effects (p<0.05)	
		Male (n=6)	Female (n=4)	Male (n=3)	Female (n=3)		
COX Pathway							
PGD_2	AA	31.8±18.0	75.6±63.6	133±24.1	256±72.6	D	
PGE_2	AA	180±28.1	355±72.3	229±11.0	395±63.4		S
11β-PGE_2	AA	482±68	741±124	632±55	1054±353		S
$PGF_{2\alpha}$	AA	123±8.70	227±42.0	177±12.9	346±38.1	D	S
6-k-$PGF_{1\alpha}$	AA	283±23.7	654±130	672±188	895±120	D	S
TxB_2	AA	47.9±7.4	88.9±8.1	58.7±15.3	102±12.7		S
Total COX		1148±83.4	2342±147	1902±72.8	3124±447	D	S
LOX Pathway*							
9-HODE	LA	4170±533	4129±800	4755±1118	5480±1532		
9-oxo-ODE	LA	1672±189	949±264	918±116	602±31.9		
13-HODE	LA	4995±662	4910±853	5963±1598	5780±1409		
13-oxo-ODE	LA	2243±365	1447±662	3463±870	2432±702		
9,10,13-TriHOME	LA	2209±176	1692±329	2639±595	2936±312	D	
9,12,13-TriHOME	LA	1068±95.8	887±128	1032±79.5	1221±219		
8-HETrE	DγLA	193±31.0	109±24.2	123±53.3	111±6.30		
15-HETrE	DγLA	226±37.7	98±19.0	159±85.4	102±17.2		
5-HETE	AA	1119±179	1448±425	1121±445	2028±158		
5-oxo-ETE	AA	281±68.4	265±96.7	118±8.70	293±21.2		
8-HETE	AA	271±34.7	277±102	355±124	530±125		
9-HETE	AA	637±119	619±213	520±200	772±147		
11-HETE	AA	407±62.6	518±147	296±38.0	516±31.2		
12-HETE	AA	1885±471	556±159	1213±544	1624±619		
15-HETE	AA	987±130	1436±345	674±162	1117±57.7		
15-oxo-ETE	AA	574±44	660±370	624±157	629±128		
9-HOTrE	ALA	177±47.7	173±27.1	106±0.5	288±154		
9-oxo-OTrE	ALA	87.2±34.8	46.6±10.3	69.4±4.7	95.1±23.3		
13-HOTrE	ALA	10638±1944	8283±865	12127±5602	12497±7051		
5-HEPE	EPA	182±35.5	152±5.7	247±92.5	279±67.6		
9-HEPE	EPA	166±39.0	60.2±7.4	145±54.6	81.4±7.0		S
11-HEPE	EPA	603±116	191±28	275±134	308±100		
12-HEPE	EPA	603±116	191±28	275±134	308±100		
4-HDoHE	DHA	1357±214	751±152	750±288	901±116		
7-HDoHE	DHA	156±19.7	81.6±8.80	117±45.1	106±14.3		
8-HDoHE	DHA	649±97.3	374±130	628±221	551±34.2		
10-HDoHE	DHA	108±18.4	70.1±10.8	57.8±4.9	49.2±2.6	D	
11-HDoHE	DHA	344±58.3	186±21.6	137±26.8	128±7.60	D	
13-HDoHE	DHA	150±19.6	89.4±17.2	88.3±22.8	79.6±9.80		
14-HDoHE	DHA	926±160	376±69.8	395±99.2	383±64.0		

16-HDoHE	DHA	726±88.0	423±60.3	647±196	521±37.9		
17-HDoHE	DHA	1591±235	999±75.7	1125±187	1027±91.9		
17-k-DHA	DHA	2061±454	927±92.6	1077±351	848±143		
Total LOX		54465±4776	41724±5750	54533±11453	57247±9400		
CYP-E Pathway							
12,13-DiHOME	LA	67.3±5.9	37.9±5.5	111±24.9	93.0±34.7	D	
9,10-DiHOME	LA	73.1±6.70	46.8±8.70	77.2±10.7	99.5±34.7		
14,15-DiHETrE	AA	39.2±3.40	30.8±4.60	33.7±5.20	32.6±1.90		
5,6-DiHETrE	AA	91.7±10.4	77.6±12.9	61.5±31.4	84.6±4.70		
11,12-DiHETrE	AA	28.1±2.40	25.7±7.0	28.0±9.70	35.1±0.90		
19,20-DiHDPE	DHA	649±105	113±14.2	401±96.7	96.7±6.30		S
Total CYP-E		948±106	333±45.2	713±140	443±60.7		S
CYP-H Pathway							
16-HETE	AA	124±16.9	96.0±22.6	79.4±42.2	69.4±16.1		
18-HETE	AA	99.9±36.1	73.6±48.6	38.7±20.1	18.9±3.70		
19-HETE	AA	412±66.6	391±82.3	521±53.6	551±276		
18-HEPE	EPA	598±104	396±145	715±111	800±301		
20-HDoHE	DHA	18142±2121	11959±3212	17406±3124	20371±1616		
Total CYP-H		19376±2172	12916±3332	18760±3231	21810±1794		
Non-enzymatic products							
5-iso $PGF_{2\alpha}$VI	AA	14.2±3.41	26.1±5.93	24.6±4.89	45.3±5.23	D	S

Values are expressed as mean ± SEM. Values with differing lower case superscript letters indicate simple effect differences between values. D, disease; S, sex.
*Many LOX oxylipins, including HODE, 9- and 11-HETE, 9- and 11-HEPE, HDoHE can be formed both enzymatically and non-enzymatically (51-56).

Table 4.6 Renal oxylipins in normal and diseased PCK rats.

Oxylipin (pg/mg dry tissue)	PUFA	Normal (n=8)	Diseased (n=8)	Effect (p<0.05)
COX Pathway				
PGD_2	AA	248±45	283±34	
PGE_2	AA	180±13	250±12	D
11β-PGE_2	AA	552±131	572±30	
$PGF_{2\alpha}$	AA	355±45	382±29	
6-k-$PGF_{1\alpha}$	AA	239±19	366±24	D
TxB_2	AA	218±3	289±34	
Total COX		1792±103	2142±208	D
LOX Pathway*				
9-HODE	LA	8825±934	13512±1377	D
9-oxo-ODE	LA	8955±1252	7344±524	
13-HODE	LA	9818±539	15001±1249	D
13-oxo-ODE	LA	5332±763	3827±314	
9,10,13-TriHOME	LA	5198±401	4739±763	
9,12,13-TriHOME	LA	1825±168	1833±318	
13-HOTrE-γ	γLA	280±21	367±63	
8-HETrE	DγLA	389±63	483±23	
15-HETrE	DγLA	218±19	325±23	D
5-HETE	AA	4375±494	4719±352	
5-oxo-ETE	AA	1087±149	613±61	D
8-HETE	AA	1158±194	999±45	
9-HETE	AA	2876±440	3121±188	
11-HETE	AA	3784±508	3884±270	
12-HETE	AA	1595±219	1881±128	
15-HETE	AA	4850±653	6269±395	
15-oxo-ETE	AA	3992±484	2591±217	D
9-HOTrE	ALA	598±57	870±113	
9-oxo-OTrE	ALA	382±59	325±76	
13-HOTrE	ALA	61214±3753	90212±11000	
5-HEPE	EPA	2424±530	1573±190	
9-HEPE	EPA	522±86	416±57	
11-HEPE	EPA	191±32	166±21	
12-HEPE	EPA	154±21	205±15	
15-HEPE	EPA	424±51	823±111	D
4-HDoHE	DHA	948±138	880±73	
7-HDoHE	DHA	230±34	124±16	D
10-HDoHE	DHA	168±26	114±9	
11-HDoHE	DHA	423±57	376±31	
13-HDoHE	DHA	101±17	94±9	
14-HDoHE	DHA	472±81	499±43	
16-HDoHE	DHA	360±65	342±30	
17-HDoHE	DHA	1481±250	1520±122	

Total LOX		134649±453	170047±480	
CYP-E Pathway				
9,10-DiHOME	LA	190±22	141±21	
12,13-DiHOME	LA	94.0±11	32.0±4.0	D
5,6-DiHETrE	AA	181±30	51.0±8.0	D
8,9-DiHETrE	AA	103±31	41.0±3.0	D
11,12-DiHETrE	AA	130±15	74.0±10	
14,15-DiHETrE	AA	186±32	56.0±4.0	D
19,20-DiHDPE	DHA	157±55.0	48.0±5.0	D
Total CYP-E		1041±121	443±46.1	D
CYP-H Pathway				
16-HETE	AA	362±20	285±23	D
18-HETE	AA	126±15.0	121±20.0	
18-HEPE	EPA	801±124	1431±148	D
20-HDoHE	DHA	6083±995	6740±371	
Total CYP-H		7372±931	8577±462	
Non-enzymatic products				
5-iso-$PGF_{2\alpha}$VI	AA	313±30.2	259±20.2	
8-iso-$PGF_{2\alpha}$III	AA	844±126	1088±61.3	D

Values are expressed as mean ± SEM. D, disease.
*Many LOX oxylipins, including HODE, 9- and 11-HETE, 9- and 11-HEPE, HDoHE can be formed both enzymatically and non-enzymatically (51-56).

Table 4.7 Renal oxylipins in normal *(+/+, jck/+)* and diseased *jck* (*jck/jck*) mice.

Oxylipins (pg/mg dry tissue)	PUFA	Normal		Diseased		Effects (p<0.05)	
		Male (n=8)	Female (n=12)	Male (n=10)	Female (n=10)		
COX Pathway							
PGD_2	AA	457±52.6	972±52.4	2309±383	3555±379	D	S
PGE_2	AA	130±24.8	223±19.1	715±103	757±104	D	S
11β-PGE_2	AA	560±62.2	980±106	2745±524	3953±378	D	S
$PGF_{2\alpha}$	AA	247±22.9	459±30.9	555±80.1	1013±120	D	S
15-k-$PGF_{2\alpha}$	AA	78.7±14.5	129±21.4	415±50.1	457±55.4	D	
6-k-$PGF_{1\alpha}$	AA	580±75.2^{c}	998±69.8^{b}	11142±1049^{a}	8498±1173^{a}	I	
12-HHTrE	AA	3394±424	5352±473	9047±1098	13546±1601	D	S
Δ^{17}-6-k-$PGF_{1\alpha}$	EPA	3.20±1.20^{c}	4.50±0.9^{c}	34.1±4.2^{a}	21.6±2.4^{b}	I	
Total COX		5451±539	9117±564	26963±2679	31802±2986	D	S
LOX Pathway*							
9-HODE	LA	4956±593	5241±381	5450±619	5679±428		
9-oxo-ODE	LA	3351±709	3110±396	920±144	1194±153	D	
13-HODE	LA	4923±541	5097±364	4512±458	5012±358		
13-oxo-ODE	LA	2803±600	2641±313	680±107	1068±235	D	
9,10,13-TriHOME	LA	1997±357	2190±213	431±88.8	778±162	D	
9,12,13-TriHOME	LA	897±182	806±83.2	197±39.7	307±62.9	D	
15-HETrE	DγLA	188±20.9	209±17.2	119±13.9	183±29.0	D	S
5-HETE	AA	1656±136^{a}	1606±66.9^{a}	180±38.3^{c}	628±134^{b}	I	
8-HETE	AA	645±89.3	805±77.0	300±55.1	434±86.2	D	
9-HETE	AA	989±76.4	1217±84.7	803±146	1111±157		S
11-HETE	AA	1055±67.3	1373±77.0	2499±343	3240±420	D	
12-HETE	AA	2499±315	3633±434	2068±306	2734±453		S
15-HETE	AA	1353±118	1519±82.5	1102±126	1613±192		S
9-HOTrE	ALA	186±29.9	178±20.1	47±10.8	84±16.7	D	
5-HEPE	EPA	589±38.3^{a}	507±58.6^{a}	57.2±19.1^{b}	157±36.3^{b}	I	
9-HEPE	EPA	783±264	983±216	435±124	401±121	D	
11-HEPE	EPA	239±41.9	283±26.8	201±25.5	212±27.5		
12-HEPE	EPA	2217±336	3099±293	1103±121	1245±200	D	
15-HEPE	EPA	356±53.1	474±65.7	234±25.8	337±59.1	D	
4-HDoHE	DHA	3027±345^{a}	2059±196^{b}	111±28.0^{c}	564±141^{c}	I	
7-HDoHE	DHA	359±25.8^{a}	261±27.3^{a}	18.6±6.10^{c}	92.7±22.7^{b}	I	
8-HDoHE	DHA	1050±88.8^{a}	755±74.2^{b}	110±38.1^{c}	240±36.3^{c}	I	
10-HDoHE	DHA	318±37.0^{a}	240±25.2^{a}	39.1±12.2^{c}	101±15.8^{b}	I	
11-HDoHE	DHA	809±38.4^{a}	689±63.2^{a}	65.8±18.0^{c}	234±42.4^{b}	I	
14-HDoHE	DHA	1419±152	1155±91.8	468±97.2	548±68.5	D	
16-HDoHE	DHA	955±67.3^{a}	697±52.7^{b}	109±12.4^{d}	283±49.6^{c}	I	
Total LOX		39621±2227	40829±1897	22260±1847	28477±1989	D	
CYP-E Pathway							

9,10-DiHOME	LA	139±33.1	107±9.60	77.1±10.3	84.6±13.7	D	
12,13-DiHOME	LA	373±102	265±25.9	270±29.2	280±32.4		
5,6-DiHETrE	AA	70.2±5.30^{a}	49.1±3.70^{b}	15.2±2.80^{d}	32.4±5.2^{c}		I
8,9-DiHETrE	AA	15.7±2.60	14.5±1.0	7.50±1.70	10.6±2.20	D	
11,12-DiHETrE	AA	37.6±3.50^{a}	37.4±2.30^{a}	10.8±2.30^{c}	22.2±3.10^{b}		I
14,15-DiHETrE	AA	52.0±6.40^{a}	43.9±3.30^{a}	18.8±3.20^{c}	30.9±3.90^{b}		I
19,20-DiHDPA	DHA	393±24.0^{a}	198±24.6^{b}	97±12.4^{c}	100±10.3^{c}		I
Total CYP-E		1080±52.4^{a}	714±66.1^{b}	497±141^{c}	560±52.5^{bc}		I
CYP-H Pathway							
16-HETE	AA	173±20.5^{a}	193±15.1^{a}	48±9.90^{c}	111±22.6^{b}		I
18-HEPE	EPA	1702±188^{a}	1454±121^{a}	307±619^{b}	529±78.2^{b}		I
20-HDoHE	DHA	16917±1104^{a}	12050±1095^{b}	1421±281^{d}	4288±828^{c}		I
Total CYP-H		18793±2511^{a}	13696±1478^{a}	1776±343^{b}	4928±901^{b}		I
Non-enzymatic products							
8-iso-$PGF_{2\alpha}$III	AA	56±6	85.2±4.97	67.3±11.1	133±17.1	D	S

Values are expressed as mean ± SEM. Values with differing lower case superscript letters indicate simple effect differences between values. D, disease; S, sex; I, interactions.
*Many LOX oxylipins, including HODE, 9- and 11-HETE, 9- and 11-HEPE, HDoHE can be formed both enzymatically and non-enzymatically (51-56).

4.4.3 Sex effects on oxylipins

Analysis of oxylipin effects in the five mouse models revealed a sex effect. When main sex effects (effects both in normal and diseased kidneys) or sex effects only in normal kidneys were observed (in the case of interactions between sex and disease), the fatty acid precursor influenced the oxylipin differences in males vs females (Figure 4.2, Tables 4.4-4.7). Oxylipins that had a sex effect were higher in males, except for almost all oxylipins derived from arachidonic acid (AA). In Pkd1(5wk) mouse kidneys, for example, 6 AA derived oxylipins were affected by sex, and 5 of these were higher in females, while in comparison, all 13 oxylipins derived from other fatty acids that were affected by sex were higher in males. In Pkd1(1wk) and Pkd2 mice, the analogous numbers were 9/9 and 5/5 AA oxylipins with a sex effect, respectively, being higher in females, and 3/4 and 2/2 non-AA derived oxylipins, respectively, being higher in males. These ADPKD models displayed minor or no effects of sex on disease, and there were few interactions of sex with disease for oxylipins. In comparison, *jck* mice display a strong sexual dimorphism on disease and there were many interactions of sex effects with disease on oxylipin levels. Where there were main sex effects or sex effects only in normal kidneys, the *jck* mouse kidney oxylipins followed the same sex effect patterns as the other models (Figure 4.2). For the 9 oxylipins with main sex effects with no interactions, 8 were AA oxylipins and all were higher in females. For oxylipins that had sex by there were main sex effects or sex effects only in normal kidneys, the *jck* mouse kidney oxylipins followed the same sex effect patterns as the other models (Figure 4.2). For the 9 oxylipins with main sex effects with no interactions, 8 were AA oxylipins and all were higher in females. For oxylipins that had sex by disease interactions, 7 were different in normal kidneys; of these, 1/2 derived from AA was higher in females and 5/5 derived from docosahexanoic acid (DHA) were higher in males.

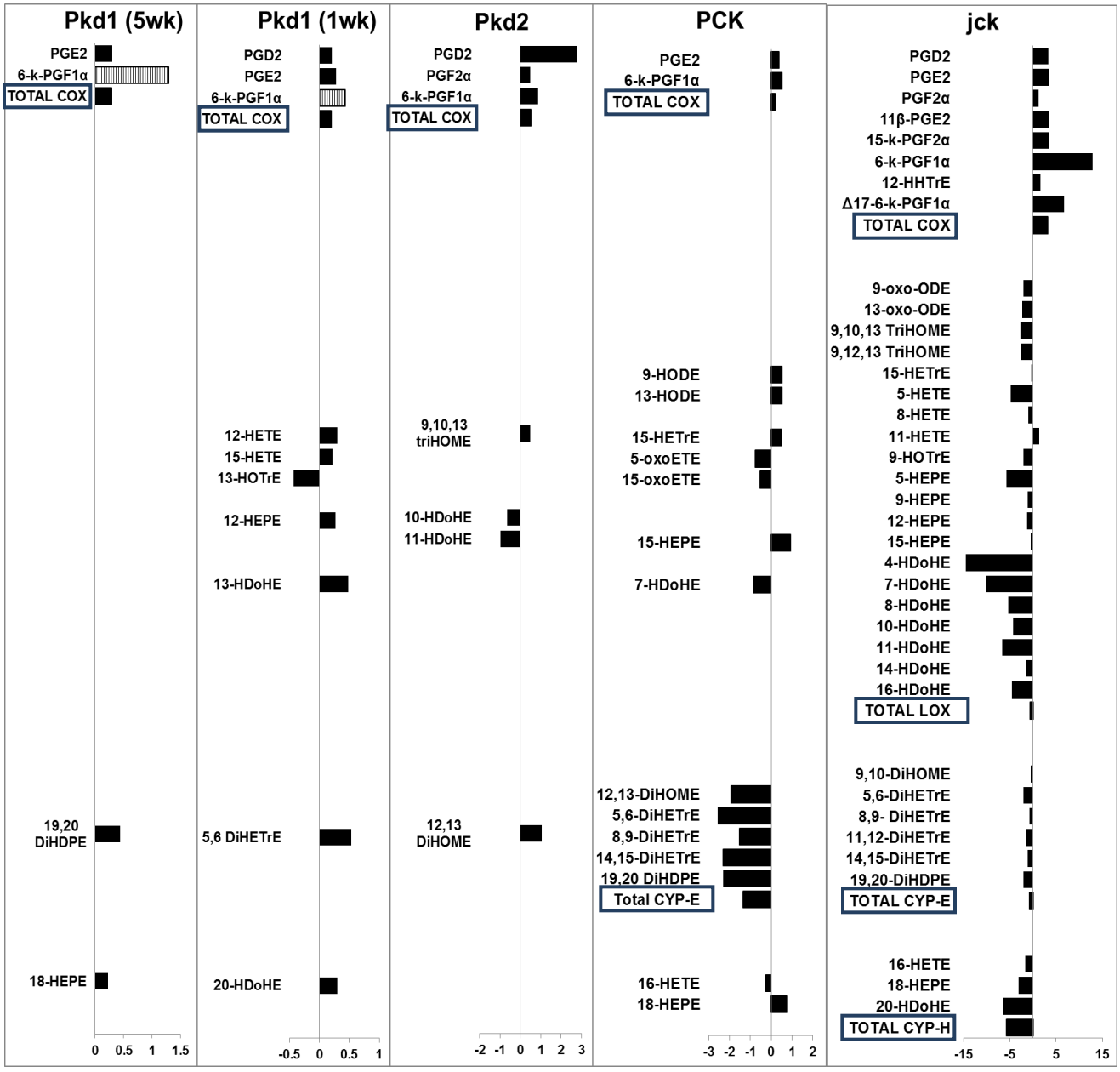

Figure 4.1 Renal oxylipins from different enzymatic pathways are altered by disease differentially in different models of cystic kidney disease.

Relative differences in renal oxylipins that displayed disease effects are presented, grouped by enzymatic pathway. Values were calculated by comparing the mean oxylipin concentration in diseased kidneys to the mean concentration in normal kidneys where the diseased kidney concentration was higher (bars in the positive direction), and vice versa when the disease concentration was lower (negative bars). Solid black bars indicate oxylipins with disease effects in both sexes and patterned bars indicate disease effects were present only in females. For full details of data and abbreviations used see tables 4.3-4.7.

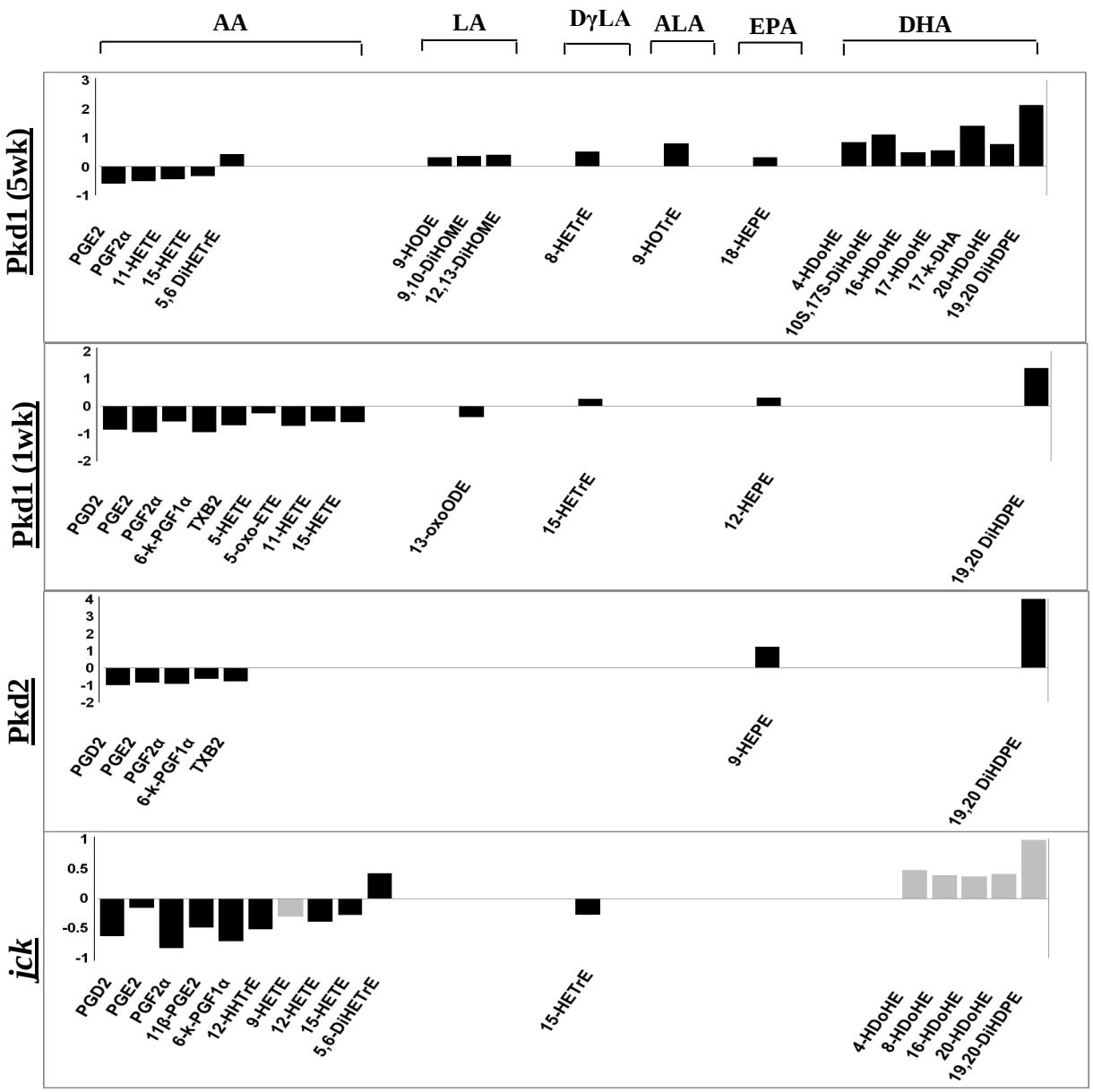

Figure 4.2 The relative levels of renal oxylipins in models of cystic kidney disease are dependent on sex and their fatty acid substrate.

Relative differences in renal oxylipins that displayed main sex effects (solid bars) or effects in normal mice (grey bars) are presented, grouped by their fatty acid precursor. Values were calculated by comparing the mean oxylipin concentration in males to the mean concentration in females where the male concentration was higher (bars in positive direction), and vice versa when the male concentration was lower (negative bars). Precursor fatty acids are indicated at the top of the figure. For full details of data and abbreviations used see tables 4.3-4.7.

When sex effects were observed in diseased kidneys with sex by disease interactions, however, the disease appeared to alter the pattern. Although all 5 AA oxylipins were higher in females and 1 EPA oxylipin was higher in males, all 5 DHA oxylipins were higher in females. These DHA oxylipins all were LOX or CYP oxylipins that are lower in disease, so the higher levels in females may reflect the lesser extent of disease in females compared to males in this model.

Interestingly, the AA oxylipins that were higher in females all were derived via the COX or LOX pathways or produced non-enzymatically. No AA oxylipins derived via the CYP pathways were higher in females, except in diseased females where there was a sex by disease interaction (*jck* mice only). For the non-AA oxylipins with a sex effect, they were derived from all enzymatic pathways, although few derived from the COX pathway were detected.

4.5 Discussion

The present findings demonstrate that COX oxylipins are consistently higher in diseased kidneys in orthologous models of ADPKD1, ADPKD2, ARPKD and NPHP, in agreement with previous findings in two other models of NPHP, the Han:SPRD-*Cy* rat (20, 58-60), and the *pcy* mouse (18,19]). This suggests that increased levels of these bioactive lipids are a common feature of all cystic renal diseases. Further, the disease phenotype varied widely between models, providing insight into the importance of these oxylipin changes. COX oxylipins were elevated even in the Pkd1 models which exhibited very minor disease expression. This is consistent with previous findings in early stage disease in *pcy* mice (19), suggesting that these alterations are critical during early disease development.

Oxylipins in the kidney play key regulatory roles in normal physiological function, maintaining glomerular filtration rate and salt/water homeostasis, as well as being involved in inflammatory and proliferative processes in response to renal injury (61-63). The precise roles of

COX oxylipins in cystic renal diseases are not known, but they are known to be involved in several key pathogenic pathways in the development of these disorders. One such pathway is related to cAMP and subsequent signaling pathways, as renal cAMP is elevated in several models of PKD (32-34), and cAMP stimulates epithelial cell proliferation and fluid secretion in primary cultured human kidney cyst cells (30). Lipid extracts containing COX oxylipins from *pcy* mouse renal cyst fluid stimulate secretion, cAMP production and cell proliferation in Mardin-Darby Canine Kidney cells (64). A recent study showed that down regulation of cAMP by inhibiting histone deacetylase 6 reduces cyst growth and proliferation of cyst-lining epithelial cells in a Pkd-conditional mouse (38). COX oxylipins may influence these cAMP mediated events via stimulation of their cognate G protein-coupled receptors and the resulting production of cAMP (27,28,35,37).This stimulation of cAMP production by COX oxylipins such as PGD_2, E_2 and I_2 also may influence several other altered signaling molecules in cystic kidney diseases, such as arginine vasopressin (AVP)(65-69), and microRNA-21 (35,38), that interact with the cAMP pathway and are known to influence cystic disease progression.

In contrast to increased production of renal COX oxylipins in all models, reduced levels of LOX oxylipins in diseased kidneys was observed only in *jck* mice. This is consistent with findings in other NPHP models (18-20), but the significance of these alterations remains to be determined. In the *pcy* mouse, LOX oxylipin changes were observed later in disease than the changes in COX oxylipins (19), indicating that these changes may be a consequence of disease. The reduced LOX oxylipin levels also could be due to reduction of fatty acid available for oxylipin formation due to the large increases in COX oxylipins in disease.

Reduced levels of CYP epoxygenase and hydroxylase oxylipins also were observed in the *jck* mouse, and reduced CYP epoxygenase oxylipins were observed in the PCK rat, suggesting

that there are unique differences in oxylipins in ADPKD, ARPKD and NPHP. The fact that the patterns in all 3 ADPKD models were similar (higher COX oxylipins only) and that oxylipin alterations in the *jck* mouse are consistent with findings in the *pcy* mouse and the Han:SPRD-*Cy* rat (18-20), (higher COX, lower LOX and CYP oxylipins) indicates that these two types of cystic renal disease have distinct patterns of oxylipins alterations. Studies with other models of ARPKD are needed to confirm whether the unique oxlipin pattern in the PCK rat (higher COX, lower CYP epoxygenase oxylipins) also occurs in other models of this cystic kidney disease.

It is possible that the differences in the extent of disease could have contributed to the different oxylipin patterns observed in the models used in this study. However, the fact that the level of disease in Pkd2 mice and PCK rats was similar (Table 4.1), but the oxylipin patterns were distinct, suggests that this was not likely a major contributor the different oxylipin patterns. Further evidence against this concept comes from a previous study in 60 day old *pcy* mice (19), in which LOX alterations were similar (5 out of 8 LOX oxylipins measured were lower in disease) to those observed in the *jck* mouse and different than in the Pkd2 mice and PCK rats in the current study, even though the disease severity in the 60 day old *pcy* mice was much less than in the *jck* mice and similar to that observed in the Pkd2 mice and PCK rats. Studies in the same models over time are needed to determine the precise role, if any, that disease severity has on the pattern of oxylipin alterations in each type of cystic kidney disease.

Non-enzymatic PUFA oxidation products such as isoprostanes have been shown to be elevated in kidney disease (70, 71), and several of these were higher in the diseased Pkd2, PCK and *jck* kidneys in the current study. As well, many of the LOX products can be produced both enzymatically and non-enzymatically: for example, 9- and 11-HETE may be produced by LOX activity (51, 52) or non-enzymatically (72). Since the current method does not separate oxylipins

such as these that are enzymatically derived from those that are non-enzymatically derived it is possible that some of the LOX oxylipins reported herein may be non-enzymatic PUFA oxidation products. Their contributions to disease as causative agents or as results of disease remain to be elucidated.

In all models which included both sexes, total oxylipins derived via the COX pathway were higher in females, consistent with the higher levels of renal COX oxylipins in females that has been reported previously in murine species (73-76). In contrast, in mouse zymosan-induced peritonitis and rat carrageenan-induced pleurisy, neutrophil PG levels were higher in males compared to females (77). Similarly in humans, COX oxylipins were higher in males in blood cells (77-79), as well as in emotional tears (80). Sex effects on COX oxylipins may therefore be tissue specific, but could also differ by species. Interestingly, AA derived oxylipins with a sex effect that were produced via the LOX pathways also were higher in females, while oxylipins with a sex effect derived from non-AA fatty acids were higher in males, suggesting that the sex effect may be influenced by the fatty acid substrate for these pathways. In contrast, all CYP oxylipins with a sex effect were higher in males. These sex differences in oxylipins may be due to sex differences in the level of available fatty acid substrate (81-83), enzyme preference for specific fatty acids for oxylipin synthesis or degradation (74, 75, 84, 85) , or differences in transporters and excretion of oxylipins from the kidney (86). Some evidence for all of these mechanisms has been reported; however, the evidence is scant and primarily restricted to AA oxylipin data. Further studies are clearly needed to confirm the current findings and to further elucidate potential mechanisms regulating the effect of sex on renal oxylipin formation.

Nevertheless, these sex effects on oxylipins do not appear to explain sex differences early in disease progression, as the very small sex effects on disease in the ADPKD models were not

consistent with sex effects on oxylipins. The only model that displayed strong sex effects on disease progression was the *jck* mouse, in which disease progression was slower in females. This also was the only model with sex by disease interactions in oxylipins derived from all pathways, exhibiting mitigation of disease associated oxylipin alterations in females with less disease. Whether this is a cause or consequence of disease differences in males and females in later stages of disease remains to be elucidated.

In conclusion, COX oxylipins are consistently elevated across different types and severity of cystic kidney diseases, suggesting that inhibition of these bioactive lipids may help slow disease progression. Effective treatment to prevent or slow cystic kidney diseases remains elusive (87-89), but the current findings and the fact that COX inhibitors reduce disease progression in the Han:SPRD-*Cy* rat model of NPHP (58, 90, 91), as well as other model of renal disease (92), suggests that use of these inhibitors in very early disease may offer a potential therapeutic approach in these disorders. The other unique patterns of oxylipin alterations involving the LOX and CYP pathways in the pediatric forms of disease also may provide opportunities for therapeutic interventions in these disorders.

4.6 References

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4.7 Transition to next chapter

In chapter 4, a comprehensive renal oxylipin profile of normal and diseased, male and female Pkd2 mice was presented. In diseased kidneys, total as well as select COX oxylipins were higher. The higher COX oxylipins in diseased kidneys was consistent with results from other orthologous models of ADPKD and ARPKD, as well as results from NPHP models. Interestingly, AA oxylipins were higher in female kidneys compare to males, while oxylipins derived from other fatty acids that displayed a sex effect were higher in males.

There is some literature showing that flax and fish oil alter renal oxylipins (18-20), but a comprehensive analysis of the renal oxylipin profile in response to fish and flax oil feeding side-by-side has not been done; neither has the effect of soy protein on renal oxylipins been examined. Since in chapter 3 we showed that dietary interventions containing soy protein, flax or fish oil, and sex, had minor effects on disease, in the next chapter kidney tissues were examined to determine the effects of these dietary interventions and sex on the comprehensive renal oxylipin profile (hypothesis 3).

The report in the next chapter includes results from similar studies in models of ADPKD, ARPKD and another model of ADPKD. My role in this collaborative project was as follows:

I conducted the animal feeding, tissue collection, oxylipin extraction and quantification, and statistical analysis of the Pkd2 mouse study. I prepared the results table for the Pkd2 mice, and I edited a draft of the manuscript. For contributions of other authors please refer to Author Contributions section (p.vii-p.x).

Chapter 5

Distinct effects of dietary flax compared to fish oil, soy protein compared to casein, and sex on the renal oxylipin profile in models of polycystic kidney disease

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5.1 Abstract

Oxylipins are bioactive lipids derived from polyunsaturated fatty acids (PUFA) that are important regulators of kidney function and health. Targeted lipidomic analyses of renal oxylipins from four studies of rodent models of renal disease were performed to investigate the differential effects of dietary flax compared to fish oil, soy protein compared to casein, and sex. Across all studies, dietary fish oil was more effective than flax oil in reducing n-6 PUFA derived oxylipins and elevating eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) derived oxylipins, whereas dietary flax oil resulted in higher α-linolenic acid (ALA) oxylipins. Dietary soy protein compared to casein resulted in higher linoleic acid (LA) derived oxylipins. Kidneys from females had higher levels of arachidonic acid (AA) oxylipins, but similar or lower levels of oxylipins from other PUFA. Modulation of the oxylipin profile by diet and sex may help elucidate their effects on renal physiology and health.

5.2. Introduction

Oxylipins are oxygenated metabolites of polyunsaturated fatty acids (PUFA) formed by mono- or dioxygen-dependent reactions of cyclooxygenase (COX), lipoxygenase (LOX) and cytochrome P450 (CYP) enzymes (1). Although eicosanoids formed from arachidonic acid (AA) are the most widely studied class of oxylipins, recent advances in lipidomics have revealed a large number of novel oxylipins formed from other PUFA with chain length varying from 18 (octadecanoids) to 22 carbons (docosanoids) (1-4). These bioactive lipids play significant roles in many key physiological processes in kidney health and disease, including maintaining blood flow, hemodynamics, renin secretion, and glomerular filtration rate (5-7). Oxylipins also are involved in inflammatory, fibrotic and proliferatory events in diseased kidneys (8,9).

Dietary oils influence the production of oxylipins in tissues, including the kidney. Increased dietary intake of n-3 PUFA has long been associated with an increase in beneficial n-3 prostaglandins (PG) and a reduction in n-6 PG (10), but the distinct effects of different n-3 PUFA (i.e. α-linolenic acid (ALA) vs. eicosapentaenoic acid (EPA)/docosahexaenoic acid (DHA)) on health (11-13) may be due to unique effects of their oxylipin metabolites. In kidney, few oxylipins have been studied, particularly in relation to dietary fish oil intake. In rats, a limited number of n-3 PUFA derived renal oxylipins were reported to be elevated and n-6 PUFA oxylipins were reduced with fish oil consumption in two recent studies (14,15). With flax oil feeding, elevated levels of several oxylipins from ALA and EPA and lower levels of some n-6 oxylipins have been reported in kidneys from obese rats (16). In a mouse model of renal disease, flax oil feeding lowered AA and linoleic acid (LA) oxylipin levels and elevated ALA, EPA and DHA oxylipins in renal tissues (17). These findings are generally similar to human plasma oxylipin studies with fish (11,18,19) and flax oil (20,21), which have examined a much wider

range of oxylipins. In the above studies, oxylipin alterations by dietary flax oil were associated with reduced renal disease (16,17), but fish oil induced oxylipin alterations did not consistently correlate with changes in disease (14,15). However, neither the human plasma studies nor the rodent renal studies have compared the effects of fish to flax oil on the oxylipin profile directly in the same model. Additionally, a detailed profile of oxylipins in the kidney is lacking.

Much less is known about effects of dietary protein on oxylipins. We recently reported that soy protein compared to casein feeding reversed some of the disease associated alterations in n-6 PUFA derived oxylipins in rat renal tissues (14). Peng et al also demonstrated a reduction in AA derived levels of thromboxane B_2 (TxB_2), and 6-keto $PGF_{1\alpha}$ in rat renal tissues with soy protein feeding that also was associated with disease mitigation (22). These results suggest that soy protein could alter renal oxylipins, but it is not clear whether this is an indirect effect due to its effects on disease. On the other hand, soy protein can reduce the activity of Δ6 desaturase, an enzyme that converts LA to longer chain PUFA, and raise the LA levels (23,24), and thus could potentially alter the formation of oxylipins from these fatty acids.

The effect of sex on oxylipins also is largely unknown, but there are some indications of differences between male and female oxylipin levels in the kidney. Female rats have higher renal levels of AA derived PGE_2, which may be due to lower levels of a PG-specific transporter responsible for PG clearance in rat renal tissues (25). Higher levels of PGE_2 and TxB_2 in the urine of diseased female rats have also been reported (26). Gender differences in enzymes that metabolize AA and AA oxylipins also may be responsible for these differences (27-31). These limited results indicate that there are sex specific differences in renal oxylipins, but a comprehensive analysis of differences in the male and female oxylipin profile has not been performed in the kidney, or any other tissue.

We recently reported that there were few effects of dietary fish and flax compared to soy oil, soy protein compared to casein, and sex on disease progression in a rat and a mouse model of polycystic kidney disease (32). We also examined these dietary and sex effects in 2 other studies with another model of this disease, but in these studies the disease progression was insufficient to examine dietary effects on disease. Since the dietary interventions and sex had only minor or no effects on disease in all four of these studies, the kidneys were examined herein to determine the effects of these diet interventions and sex on the comprehensive renal oxylipin profile. Findings across the different studies consistently revealed that fish and flax oil have distinct effects on the renal oxylipin profile, that soy protein increases LA derived oxylipins, and that AA derived oxylipins are uniquely higher in females.

5.3 Materials and methods

5.3.1 Animal models

Kidneys from four studies of rodent polycystic kidney diseases were used for the analyses. In the first study, weanling male PCK rats (33) purchased from a commercial breeder (Charles River, QC, Canada) were used. The second and third studies used Mx1Cre+Pkd1$^{flox/flox}$ (Pkd1) conditional knockout mice from our in-house colony, originally provided by Jing Zhou (Brigham and Women's Hospital and Harvard Medical School, Boston. MA, U.S.A) (34). To induce disease, male and female Pkd1 mice in the second study were administered i.p. with 250 µg polyinosinic polycytidylic acid (pI:pC) for five consecutive days beginning at 5 weeks of age (hereafter called Pkd1 (5wk) mice), and in the third study were injected at 1 week of age (hereafter called Pkd1 (1wk) mice). For the fourth study, Pkd2$^{WS25/WS25}$ and Pkd2$^{+/-}$breeders were obtained from Dr. Stefan Somlo (Yale University, New Haven, CT, USA) (35) and crossed to produce (Pkd2$^{WS25/-}$) mice with disease (hereafter called Pkd2 mice).

5.3.2 Diets

Diets were based on the American Institute of Nutrition (AIN) 93G standard diet for laboratory rodents (36) and reported in detail in the previously published study on disease effects in PCK rats and Pkd2 mice (32). All four studies had diets containing either soy oil, flax oil or fish oil with the only difference between these diets being that flax or fish oil replaced 80% of the soy oil in the standard soy oil diet (details in Table 3.1 and 5.1). Thus, dietary oil effects were examined in all four studies. The studies with PCK rats, Pkd1 (5wk) mice and Pkd2 mice also had diets that replaced the standard protein source (casein) with soy protein, resulting in 6 different diets (Table 3.1). In Pkd2 mice, however, diseased mice could only be identified upon termination and the number of mice in the protein groups was found to be too low in some subgroups to test protein effects on oxylipins, so protein effects were examined only in PCK rats and Pkd1 (5wk) mice. The three mouse studies also included both males and females, allowing the examination of sex effects in these studies. All female animals used were pre-menopausal for the duration of the studies. All diet ingredients were purchased from Dyets Inc. (Bethlehem, PA, USA). Oils contained 0.02% tert-butylhydroquinone (added by Dyets Inc) to prevent oxidation and diet ingredients were stored at 4°C. Diet was freshly prepared twice per month and stored in sealed containers at -20°C until feeding.

Animals were housed singly in a temperature and humidity-controlled environment with a 12-hour day/night cycle and were given free access to water and diet. The feeding period for each study was 12 weeks, (4 to 16 weeks of age), 16 weeks (6 to 22 weeks of age), 6 weeks (3 to 9 weeks of age) and 13 weeks (3 to 16 weeks of age), for the PCK rats, Pkd1 (5wk), Pkd1 (1wk) and Pkd2 mice, respectively.

Table 5.1 Fatty acid composition of the diets

	Casein			Soy protein		
	Soy oil	Flax oil	Fish oil	Soy oil	Flax oil	Fish oil
C14:0	0.23	0.24	5.69	0.34	0.43	7.38
C16:0	11.47	7.21	20.13	13.74	10.66	19.17
C16:1t	0	0.02	0.62	0.03	0.02	0.35
C16:1	0.13	0.12	10.42	0.27	0.41	9.4
C17:1	0.05	0.03	1.19	0.06	0.03	1.01
C18:0	3.97	3.48	4.1	4.26	3.71	4
C18:1	20.95	17.77	10.43	19.68	17.7	9.58
C18:1n7c	1.16	0.65	2.64	1.24	0.81	2.47
C18:2	53.28	23.24	14.29	51.39	24.96	17.58
C18:3n6	0	0.02	0.55	0	0.01	0.49
C18:3n3	7.39	46.01	3.14	6.85	39.98	3.47
C20:1	0.19	0.2	0.74	0.18	0.22	0.62
C20:4	0	0.01	0.93	0.01	0.02	0.88
C20:5n3	0	0	11.46	0.03	0	10.12
C22:5n3	0	0	1.92	0.05	0.01	1.82
C22:6n3	0	0.01	8.74	0.44	0.09	8.55

Values are g/100g fatty acid (n=2-3 per diet)

At the end of each study, animals were anesthetised with isoflurane. PCK rats were terminated by cardiac puncture and mice were terminated by decapitation. The right kidney was snap frozen in liquid nitrogen and stored at -80°C until analysis. All animal procedures were approved by the University of Manitoba Animal Care Committee and adhered to the guidelines of the Canadian Council on Animal Care.

5.3.3 Oxylipin analysis

Lyophilized whole kidney tissues were homogenized in ice cold Tyrode's salt solution (pH 7.6) in a 1:28 weight:volume ratio. After homogenization, Triton X-100 was added to achieve a final concentration of 0.01%. Deuterated internal standards (10 ng each, Cayman Chemical, MI, USA) and 6.5μL antioxidant cocktail (0.2 g/L BHT, 0.2 g/L EDTA, 2 g/L triphenylphosphine, and 2 g/L indomethacin in MeOH:EtOH:H_2O (2:1:1,by vol)) were added to 200 μL aliquots that were used for analysis. Samples were adjusted to pH < 3 and solid phase extraction was with Strata-X SPE columns (Phenomenex, CA, USA) preconditioned with methanol and pH 3 water. Samples were loaded onto the columns, rinsed with 10% methanol, and eluted with methanol. Evaporated samples were then resuspended in solvent for analysis by HPLC-MS/MS (API 4000, AB Sciex, Canada) as described (37) based on methods developed by Deems et al. (2). Details of all oxylipins screened, the deuterated internal standards used and the detector response factors are listed in Appendix G. Detection and quantification limits were set at 3 and 5 levels above the background, respectively. Quantities of oxylipins were determined using the stable isotope dilution method (38) and expressed as pg/mg dry tissue.

5.3.4 Statistical analysis

Data were analyzed using the GLM procedure of SAS (SAS, version 9.4, Cary, NC, USA). For the PCK rat study (n=5-8), protein and oil effects were tested by 2-way (protein x oil) ANOVA. For Pkd1 (5wk) mice (n= 3-7), a 3-way (sex x protein x oil) ANOVA was performed and for the Pkd1 (1wk) mice (n=3-7) and Pkd2 mice (n=4-7), a 2-way (sex x oil) ANOVA was used.

Normality of data was tested using Shapiro-Wilk's Statistic (W > 0.05 for normally

distributed data). Non-normal data was transformed to achieve normality. If the data did not follow a normal distribution even when transformed, a nonparametric test was used (Kruskal-Wallis). Post hoc analysis was done by Tukey-Kramer tests for simple oil effect comparisons when interactions were present or when the Kruskal-Wallis test indicated the presence of treatment differences. All data were presented as mean ± standard error (SE). Significance was set at $p < 0.05$ for main, interaction, and simple effects.

5.4. Results

A total of 158 oxylipins were scanned for in each study (Appendix G), of which 61 were detected at quantifiable levels (51 in PCK rats, 49 in Pkd1 (5wk) mice, 54 in Pkd1 (1wk) mice and 50 in Pkd2 mice).

5.4.1 Effect of dietary lipids on renal n-6 PUFA derived oxylipins

Kidney oxylipins derived from n-6 PUFA were generally lowest in the fish compared to the soy oil groups, with the levels in the flax oil groups being intermediate (Figure 5.1). Across all four of the studies, total LA oxylipins in all three dietary oil groups were different from each other. Compared to the soy oil groups, total LA oxylipins were 26-42% lower in the flax oil and 59-66% lower in the fish oil groups, while in fish compared to the flax oil groups, they were 40-54% lower (Tables 5.2-5.5). Of the 8 individual LA oxylipins quantified in each study, 25-75% were lower in the flax and 100% were lower in the fish compared to the soy oil groups, across all four studies. Fish oil had a greater lowering effect than flax oil in 25-100% of individual LA oxylipins.

Similar to the LA oxylipins, total renal AA oxylipins in flax compared to soy oil groups had 37-44% lower total AA oxylipins in the three mouse models, and 57% lower levels only in

the casein fed PCK rats. As well, individual AA oxylipins were 53-62% lower in the fish compared to soy oil groups across all studies. In contrast to the consistently lower LA oxylipins across all studies, total AA oxylipins were lower (by 20%) only in Pkd1 (5wk) mice in fish compared to flax oil fed animals (Tables 5.2-5.5). With respect to individual oxylipins, 17-19 individual AA oxylipins were quantitated in each of the four studies. 74-100% of these individual oxylipins were lower in flax, and 100% of were lower in fish compared to soy oil fed animals across all four studies, while 6-29% of individual AA oxylipins were lower in fish compared to flax oil fed animals.

Other n-6 fatty acid derived oxylipins (1 from gamma-linolenic acid (GLA), 2 from dihomo-gamma-linolenic acid (DGLA), 3 non-enzymatic AA products) had similar patterns to the LA and AA oxylipins. In general, they were lowest in the fish oil groups, with levels being intermediate in the flax oil groups (Tables 5.2-5.5).

5.4.2 Effect of dietary lipids on renal n-3 PUFA derived oxylipins

In contrast to the similar patterns among LA, GLA, DGLA and AA (n-6 fatty acids) derived oxylipins in response to dietary oil type, those derived from ALA, EPA and DHA (n-3 fatty acids) had differing patterns. Oxylipins derived from ALA were generally higher with flax oil feeding, while those derived from EPA or DHA were highest in the fish oil groups (figure 5.1). Total ALA oxylipins were 2-5.5 times higher in the flax compared to the soy oil groups in all four studies, although not always significantly in all subgroups. On the other hand, fish compared to soy oil consumption did not increase ALA oxylipins, but resulted in 18-79% lower ALA oxylipins, but this was only significant in PCK rats.

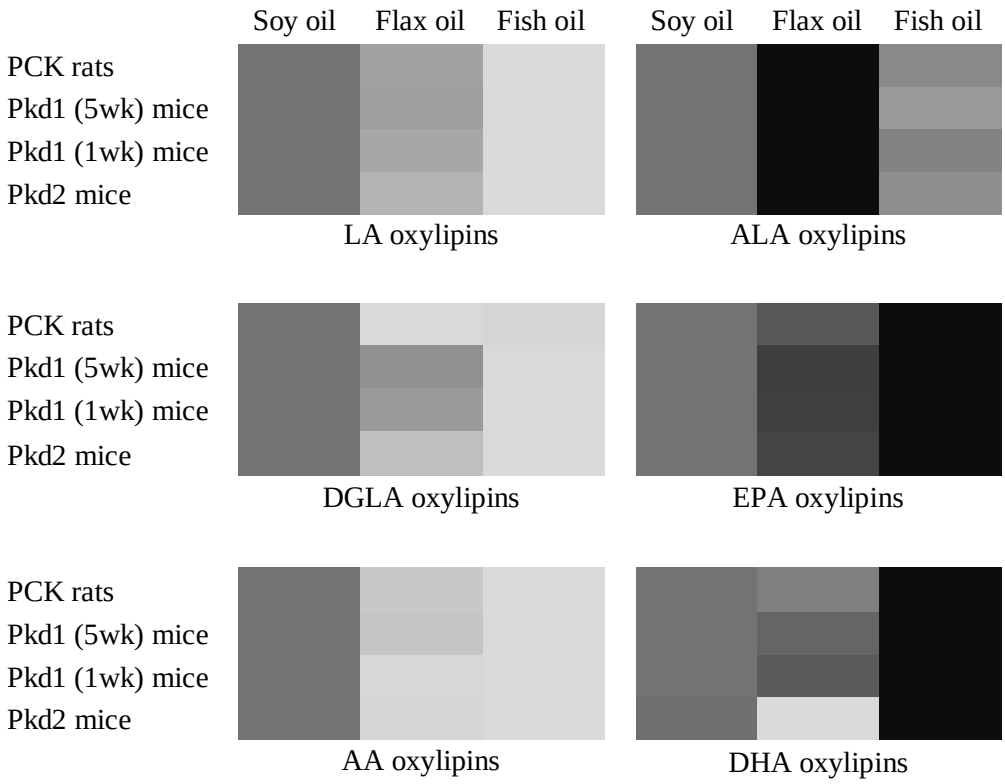

Figure 5.1 Flax oil and fish oil feeding results in lower n-6 oxylipins and higher n-3 oxylipins in the kidney. Heat map of dietary oil effects on total renal oxylipins from each PUFA are shown. Darker/lighter color indicates higher/lower levels, respectively, in flax and fish compared to soy oil groups. For full details see tables 5.2-5.5.

Compared to flax, the fish oil groups had 85-90% lower total ALA oxylipins across the studies, with most of the differences being significant (Tables 5.2-5.5). These patterns generally reflected the differences in the 2-3 individual ALA oxylipins present in each study.

Total EPA oxylipins were 4.5 to 9.1 times higher with flax compared to soy oil consumption in the mouse models, and 2.5-3.3 times higher in the PCK rats, although this latter difference was not significant. On the other hand, fish compared to soy oil consumption resulted in 8 to 16 times higher total EPA oxylipins across all four studies. Fish compared to flax oil differences were not as great, but total EPA oxylipins were 1.8 to 2.8 times higher in PCK rats,

Pkd1 (5wk) mice, Pkd2 mice and in the male subgroup of Pkd1 (1wk) mice (Tables 5.2-5.5). Of the 6-8 individual EPA oxylipins quantitated in each study, 100% were higher in both flax and fish compared to soy oil groups in all four studies, while fish compared to flax oil groups had higher levels in 38-100% of individual EPA oxylipins across studies.

Total DHA oxylipins were not affected by flax compared to soy oil consumption in any of these studies. On the other hand, fish compared to soy oil consumption resulted in 1.8 to 3 times higher total DHA oxylipins in PCK rats and in both Pkd1 models. Fish compared to flax oil consumption also resulted in 1.5 to 4 times higher DHA oxylipins in PCK rats, Pkd1 (1wk) mice, and in the soy protein subgroup of Pkd1 (5wk) mice. No oil effects on total DHA oxylipins were observed in Pkd2 mice (Tables 5.2-5.5). 10-14 individual DHA oxylipins were quantitated in each study. Flax compared to soy oil consumption only increased 14% of DHA oxylipins in Pkd1 (1wk) mice, but none in the other studies. However, fish compared to soy oil consumption resulted in 100% of these individual oxylipins being higher in PCK rats and Pkd1 (1wk) mice, while in Pkd1 (5wk) and Pkd2 mice, 50% and 25%, respectively, of these individual oxylipins were higher overall, with an additional 33 to 44% being higher in one of the subgroups. Fish compared to flax oil consumption resulted in higher levels in 42-100% of individual DHA oxylipins across all four studies.

5.4.3 Effect of dietary protein on renal oxylipins

Two sources of protein were provided to the PCK rats and Pkd1 (5wk) mice. Effects of soy protein compared to casein on renal oxylipins were observed, but only consistently in those derived from LA. Total LA oxylipins were 1.3 and 1.2 times higher in soy protein compared to casein fed PCK rats and Pkd1 (5wk) mice, respectively (Tables 5.2&5.3). Among 8 individual LA oxylipins quantified, 50% were higher in the soy protein groups in both studies (Figure 5.2).

Among these, 9,10-dihydroxy octadecenoic acid (DiHOME) and 12,13-DiHOME were always higher with soy protein consumption in both studies (Tables 5.2&5.3). There was no effect of dietary protein on total DGLA or AA oxylipins in either study.

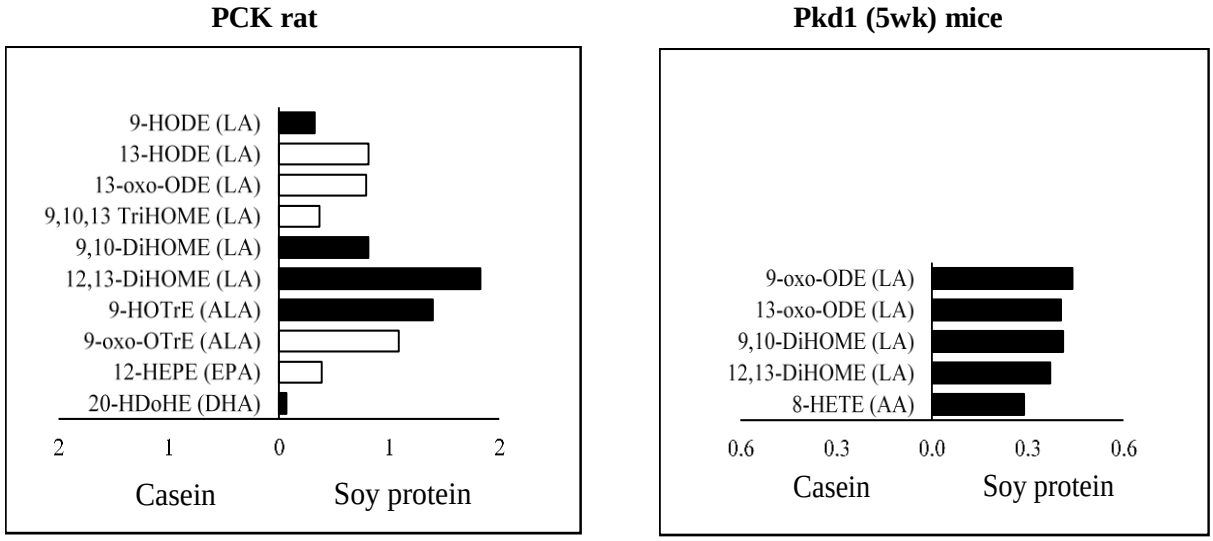

Figure 5.2 Dietary soy protein results in higher LA oxylipins in the kidney.

Relative differences in renal oxylipins that displayed protein effects are presented as fold difference values, calculated by comparing the mean oxylipin concentration in the soy protein group to the mean concentration in the casein group. Precursor fatty acids are shown in parentheses. Black bars indicate oxylipins with overall protein effects and open bars indicate protein effects only in the flax oil subgroup. For full details see tables 5.2-5.5.

The effect of dietary protein on n-3 PUFA derived oxylipins also was minimal. In PCK rats, total ALA, EPA, and DHA derived oxylipins were not affected by dietary protein, and only 1 of 19 n-3 fatty acid derived oxylipins were higher with soy protein feeding, with an additional 3 being higher in the flax oil subgroups (Tables 5.2). In Pkd1 (5wk) mice, total EPA oxylipin levels were not affected by soy protein feeding, while ALA oxylipins were 1.5 times higher only in the male flax oil subgroup and DHA oxylipins were 1.9 times higher in only in the male fish oil subgroup given soy protein. However, no individual n-3 fatty acid derived oxylipins were

affected by protein source in these mice (Tables 5.3).

5.4.4 Effect of sex on renal oxylipins

The 3 mouse studies included both males and females. In both Pkd1 mouse models, total AA derived oxylipins were 1.3 times higher in female compared to male kidneys (Tables 5.3&5.4). Of the 12 AA oxylipins quantitated in each of the Pkd1 studies, 59% and 29% were higher in female Pkd1 (5wk) and Pkd1 (1wk) mice, respectively (Table 5.3&5.4, Figure 5.3). An additional 35% of these AA oxylipins were higher in females in soy oil subgroups of Pkd1 (1wk) mice (Tables 5.4, Figure 5.3). In Pkd2 mice, although total AA oxylipins were not affected by sex, 6% of individual AA oxylipins were higher in females in all subgroups, and an additional 41% of AA oxylipins were higher in females in some subgroups (Table 5.5, figure 5.3).

In contrast to AA oxylipins, other n-6 PUFA derived oxylipins that were affected by sex were higher in male kidneys (Figure 5.3). Total oxylipins formed from LA, GLA and DGLA were not affected by sex in any of the 3 studies, but 12-50% of individual LA oxylipins were higher in males across all 3 studies (Tables 5.3-5.5).

The effect of sex on n-3 PUFA derived oxylipins also was minimal and opposite to its effect on AA oxylipins (figure 5.3). Among the totals of n-3 PUFA derived oxylipins, only total DHA oxylipins were (1.3 times) higher in males, but only in Pkd1 (1wk) mice (Table 5.4). Among the 3 individual ALA oxylipins quantitated in each study, only 1 was higher in males in each of the Pkd1 (5wk) mice, Pkd2 mice, and the flax oil subgroup of the Pkd1 (1wk) mice. With respect to EPA oxylipins, 2-4 out of 7 were higher in males across all three studies.

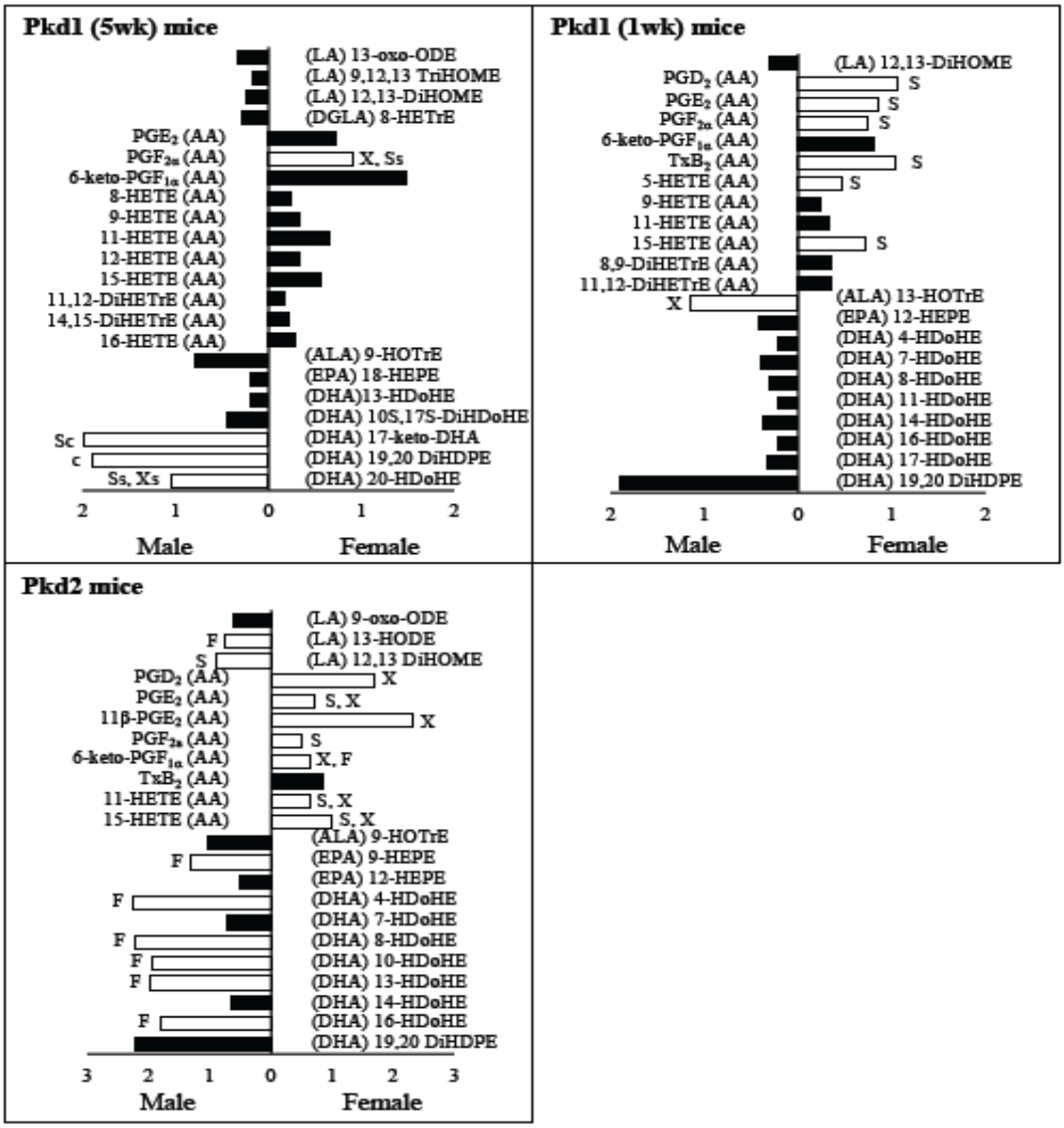

Figure 5.3 Females have higher AA oxylipins, while oxylipins from other PUFA are similar or higher in males

Relative differences in renal oxylipins that displayed sex effects are presented as fold difference values, calculated by comparing the mean oxylipin concentration in the sex that is higher to the mean concentration in the other. Precursor fatty acids are shown in parentheses. Oxylipins with overall sex effect are indicated by black bars and those with subgroup effects are shown with open bars with subgroups indicated as follows: S, soy oil; X, flax oil; F, fish oil; c, casein; s, soy protein. Open bars indicate the average of only the subgroups that showed effects. For full details see tables 5.2-5.5.

Among 12-14 individual DHA oxylipins, 17-57% of them were higher in males overall, with an additional 0-42% being higher in males in some subgroups (Tables 5.2-5.5).

5.5 Discussion

In all four studies, n-6 PUFA derived renal oxylipins were lower in animals provided flax or fish oil compared to soy oil, but the levels were generally much lower in the animals with fish oil diets. Reduction of n-6 PUFA derived oxylipins in response to fish oil consumption has been observed previously in rodent kidneys, but the number of oxylipins examined was limited (14,15). Similar effects of fish oil on a larger range of plasma n-6 oxylipins have been reported in humans (18,19). Flax oil also is effective in lowering n-6 PUFA derived oxylipins in rodent kidneys and human plasma (16,20). The current study extends these findings to a larger profile of renal oxylipins in a direct comparison of fish and flax oil in the same model. The greater effect of fish compared to flax oil occurred despite the fact that the EPA and DHA content of the fish oil diet was less than half that of the ALA content of the flax oil diet, indicating that EPA and DHA have a greater effect than ALA on the n-6 PUFA derived oxylipins.

In contrast to the reduction in the n-6 derived oxylipins, the n-3 derived oxylipins were increased with both flax and fish oil feeding, but the effect on ALA oxylipins was greater with flax oil, while the effect on EPA and DHA oxylipins was greater with fish oil. The elevation of ALA oxylipins with flax oil feeding is consistent with flax oil studies in rodent renal tissues and in plasma from human feeding studies (16,39,40). Flax oil feeding also increased EPA oxylipins, which can be attributed to the conversion of ALA to EPA in the tissue (41,42). This has been observed in rodent kidney and human plasma with flax oil feeding (16,20). However, fish oil

Table 5.2 Dietary oil and protein effects on renal oxylipins in PCK rats (pg/mg dry tissue)

Oxylipin	Casein			Soy protein			
	Soy oil	Flax oil	Fish oil	Soy oil	Flax oil	Fish oil	Effects
n-6 oxylipins							
LA							
9-HODE	13513±1377	6077±1152	4005±491	16128±1227 ^{A}	10241±1100 ^{B}	4893±650 ^{B}	P O
9-oxo-ODE	7344±524	4400±881	3675±745	7594±1169.2^{A}	7115±847 ^{B}	3897±517 ^{C}	O
13-HODE	15001 ±1249 ^{a}	7812±1347 ^{b}	5627±724 ^{b}	18878±1369 ^{a}	14167±1190 ^{a}	5899±760 ^{b}	Int
13-oxo-ODE	3827±314.2 ^{ab}	2782±556 ^{bc}	1997±397 ^{c}	3347±357 ^{abc}	4986±379 ^{a}	2001±279 ^{c}	Int
9,12,13 TriHOME	1833±318	1368±296	836±112	1723±250 ^{A}	1994±300 ^{A}	1063±76.3 ^{B}	O
9,10,13 TriHOME	4740±763	3800±520	2442±306	5414±691 ^{A}	6260±710 ^{A}	3356±331 ^{B}	P O
9,10-DiHOME	142±21.3	184±31.7	54.7±5.10	276±16.3 ^{A}	301±24.9 ^{A}	113±16.1 ^{B}	P O
12,13-DiHOME	31.8±4.10	29.2±7.50	15.3±1.60	79.9±8.60 ^{A}	80.7±9.10 ^{A}	55.3±7.50 ^{B}	P O
Total LA oxylipins	42566 ± 3997	25497 ± 4356	18651 ± 2466	51332 ± 3192^{A}	44091 ± 3201^{B}	20231 ± 2856^{C}	P O
GLA							
13-HOTrE-γ	367±62.5	140±31.9	101±8.90	335±57.1 ^{A}	204±32.6 ^{B}	104±21.0 ^{C}	O
DGLA							
8-HETrE	481±22.6	220±50.0	277±28.1	503±47.5 ^{A}	284±24.5 ^{B}	251±35.6 ^{B}	O
15-HETrE	325±22.7	145±27.9	164±18.3	322±48.3 ^{A}	230±23.0 ^{B}	187±29.4 ^{B}	O
Total DGLA oxylipins	807 ± 34.5^{a}	365 ± 75.5^{c}	441 ± 34.0^{c}	761 ± 117^{ab}	479 ± 50.4^{bc}	438 ± 60.0^{c}	Int
AA							
PGD_2	283±33.7	166±40.1	228±50.1	275±19.7^{A}	154±23.4^{B}	152±32.8^{B}	O
PGE_2	249±12.3 ^{a}	115±18.4 ^{c}	89.3±9.00 ^{c}	194±16.6 ^{ab}	141±17.9 ^{bc}	96.2±6.50 ^{c}	Int
11 β-PGE_2	572±30.2 ^{a}	231±36.2 ^{b}	200±19.7 ^{b}	468±28.4 ^{a}	289±36.8 ^{b}	214±20.1 ^{b}	Int
PGF2a	382±29.0	167±28.3	135±10.7	330±23.3 ^{A}	165±20.6 ^{B}	114±9.60 ^{B}	O
6-keto-$PGF_{1\alpha}$	366±23.6 ^{a}	166±18.0 ^{dc}	131±15.1 ^{d}	287±23.5 ^{ab}	271±33.4 ^{bc}	151±13.6 ^{d}	Int
TxB_2	289±33.8	149±21.5	113±23.4	200±20.4 ^{A}	162±19.7 ^{B}	87.5±8.30 ^{C}	O
5-HETE	4719±352	1916±424	1761±186	4145±341 ^{A}	2392±217 ^{B}	1858±103 ^{B}	O
5-oxo-ETE	613±61.1	297±69.2	285±60.2	525±109 ^{A}	440±85.1 ^{B}	265±34.8 ^{B}	O

8-HETE	999±44.8	477±95.4	382±70.6	822±139 ^{A}	497±53.6 ^{B}	374±52.7 ^{B}	O
9-HETE	3121±188	1098±271	1106±176	2866±192 ^{A}	1317±139 ^{B}	1100±115 ^{B}	O
11-HETE	3884±270	1754±320	1641±263	3266±268 ^{A}	2220±295 ^{B}	1384±180 ^{B}	O
12-HETE	1881±128	565±117	645±107	1524±195 ^{A}	794±111 ^{B}	624±72.8 ^{B}	O
15-HETE	6269±395	2278±511	2295±209	5524±460 ^{A}	3155±366 ^{B}	2055±260 ^{B}	O
15-oxo-ETE	2591±217	1886±288	1348±302	2166±502 ^{A}	1723±283 ^{AB}	1535±249 ^{B}	O
5,6-DiHETrE	50.5±8.30	28.5±6.30	23.3±5.60	59.9±15.8 ^{A}	51.9±10.4 ^{AB}	24.4±2.60 ^{B}	O
8,9-DiHETrE	40.8±3.20	26.6±5.30	15.7±1.80	43.1±4.70 ^{A}	31.6±3.30 ^{B}	15.4±2.40 ^{C}	O
11,12-DiHETrE	74.2±10.5	83.3±14.9	34.8±5.80	88.6±4.40 ^{A}	87.9±15.8 ^{A}	25.7±4.50 ^{B}	O
14,15-DiHETrE	55.8±3.60	38.9±8.30	26.2±3.80	64.3±10.4 ^{A}	42.7±5.80 ^{B}	27.8±3.70 ^{C}	O
16-HETE	285±23.4	143±31.9	129±11.2	261±37.5 ^{A}	195±33.5 ^{B}	102±16.1 ^{B}	O
Total AA oxylipins	26241 ± 1066^{a}	11208 ± 1972^{c}	10519 ± 1146^{c}	18860 ± 3054^{ab}	13350 ± 1350^{bc}	9891 ± 884^{c}	Int
n-3 oxylipins							
ALA							
9-HOTrE	870±113 ^{bc}	2314±613 ^{b}	307±56.6 ^{d}	861±117 ^{c}	5548±497 ^{a}	305±48.1 ^{d}	Int
9-oxo-OTrE	325±76.4 ^{cb}	806±237 ^{b}	179.6±30.7 ^{cd}	330±60.0 ^{cb}	1683±231 ^{a}	107±20.1 ^{d}	Int
13-HOTrE	90212±11000	289120±101067	43982±7719	117202±17767 ^{B}	534127±102839 ^{A}	35797±5057 ^{C}	O
Total ALA oxylipins	91407 ± 11133	291749 ± 101228	44468 ± 7778	118394 ± 17928^{B}	541357 ± 103304^{A}	36194 ± 5090^{C}	O
EPA							
5-HEPE	1573±190	2280±362	6282±284	1649±422 ^{C}	2837±360 ^{B}	5756±705 ^{A}	O
9-HEPE	416±56.5	2089±495	4713±378	342±60.8 ^{C}	1829±357 ^{B}	3998±547 ^{A}	O
11-HEPE	166±20.9	784±58.3	1717±52.6	154±22.4 ^{C}	832±73.3 ^{B}	1493±92.5 ^{A}	O
12-HEPE	205±14.9 ^{d}	686±103 ^{c}	2339±167 ^{a}	172±11.5 ^{d}	953±33.4 ^{b}	2049±134 ^{a}	Int
15-HEPE	823±111	1688±295	5428±402	531±91.9 ^{C}	2007±173 ^{B}	4186±634 ^{A}	O
18-HEPE	1431±148	5169±878	20424±3060	1545±298 ^{C}	6635±607 ^{B}	18858±1543^{A}	O
Total EPA oxylipins	4614 ± 364^{b}	11620 ± 1798^{b}	39856 ± 3120^{a}	4393 ± 602^{b}	14510 ± 601^{b}	31878 ± 4911^{a}	Int
DHA							
4-HDoHE	880±73.3	561±89.7	2686±346	788±117 ^{B}	841±95.4 ^{B}	2792±172 ^{A}	O
7-HDoHE	124±15.8	127±35.6	383±39.0	138±10.7 ^{B}	145±29.7 ^{B}	308±43.1 ^{A}	O

10-HDoHE	114±8.90	100±21.1	407±63.1	122±18.7 ^{B}	109±17.1 ^{B}	270±37.6 ^{A}	O
11-HDoHE	376±31.3	351±67.6	1130±150	305±25.3 ^{B}	352±52.8 ^{B}	1039±88.7 ^{A}	O
13-HDoHE	94.5±8.70	74.1±8.70	290±37.4	88.8±11.0 ^{B}	115±24.4 ^{B}	220±27.3 ^{A}	O
14-HDoHE	499±42.6 ^{b}	306±40.9 ^{c}	1610±207 ^{a}	480±39.0 ^{b}	428±28.8 ^{bc}	1279±111 ^{a}	Int
16-HDoHE	342±29.7	245±41.5	1141±132	349±51.7 ^{B}	328±24.6 ^{B}	945±66.6 ^{A}	O
17-HDoHE	1520±122	1241±237	4861±407	1423±185 ^{B}	1479±100 ^{B}	4597±349 ^{A}	O
19,20 DiHDPE	47.8±5.50	45.2±6.30	126±12.2	45.4±6.40 ^{B}	63.7±9.40 ^{B}	148±19.4 ^{A}	O
20-HDoHE	6740±371	4018±646	22501±2987	7381±611 ^{B}	5936±332 ^{C}	22221±1602 ^{A}	P O
Total DHA oxylipins	10736 ± 579^{b}	6466 ± 851^{b}	34136 ± 3245^{a}	10197 ± 1443^{b}	9650 ± 429^{b}	29708 ± 4535^{a}	Int
Non-enzymatic AA oxylipins							
5-isoPGF$_{2\alpha}$VI	259±20.3	105±26.3	84.0±13.5	204±32.4 ^{A}	120±13.9 ^{B}	85.2±10.1^{B}	O
8-iso-PGF$_{2\alpha}$	1088±61.2 ^{a}	392±56.4 ^{b}	286±22.6 ^{c}	906±69.3 ^{a}	554±69.8 ^{b}	294±28.6 ^{c}	Int

All values represent mean ± SEM (n=5-8). P, protein effect; O, oil effect, Int, interaction or Wilcoxin's test used for non-normal data. Oil subgroups were combined for determining protein effect. Differing uppercase superscripts (shown in soy protein groups) indicate differences in oil effects. Protein subgroups were combined for determining oil effects. Differing lowercase superscripts indicate differences among all six groups, when combining was not possible due to interactions or non-normal data.

Table 5.3 Dietary oil, protein and sex effects on renal oxylipins in Pkd1 (5wk) mice (pg/mg dry tissue)

Oxylipin	Female						Male								
	Casein			Soy protein			Casein			Soy protein					
	Soy oil	Flax oil	Fish oil	Soy oil	Flax oil	Fish oil	Soy oil	Flax oil	Fish oil	Soy oil	Flax oil	Fish oil	Effects		
n-6 oxylipins															
LA															
9-HODE	4137± 560	3068 ± 307	1164 ± 132	4840± 482	2854 ± 277	1542 ± 170	4451 ± 270	3119 ± 352	1306 ± 223	5654± 1111^{A}	3495 ± 460^{B}	2085 ± 534^{C}			O
9-oxo-ODE	2531± 304	1866 ± 255	755 ± 165	3212± 421	2129 ± 360	1316 ± 274	1840 ± 195	1491 ± 249	688 ± 184	3330 ± 702^{A}	2585 ± 690^{B}	637± 86.2^{C}		P	O
13-HODE	5256± 586	3608 ± 260	1239 ± 151	5456± 439	3215 ± 182	1916 ± 183	5097 ± 692	2943 ± 351	1334 ± 172	5773 ± 1120^{A}	4016 ± 444^{B}	2018 ± 573^{C}			O
13-oxo-ODE	2212± 438	2254 ± 391	1125 ± 208	3203± 484	2278 ± 673	1641 ± 410	2856 ± 336	3040 ± 555	905 ± 177	5174 ± 1032^{A}	3520 ± 660^{A}	1592 ± 575^{B}	S	P	O
9,10,13 TriHOME	2869± 330	2283 ± 208	1027 ± 184	3111 ± 340	2328 ± 255	1569 ± 214	3243 ± 518	2862 ± 252	1100 ± 149	3365 ± 508^{A}	3863 ± 645^{A}	1078 ± 127^{B}			O
9,12,13 TriHOME	946 ± 124	793 ± 83.2	369 ± 56.9	1126± 76.4	767 ± 66.4	563 ± 78.0	1142 ± 161	989 ± 100	411 ± 60.5	1132 ± 160^{A}	1295 ± 193^{A}	388 ± 44.3^{B}	S		O
9,10-DiHOME	75.2 ± 8.10	53.9 ± 4.70	30.2 ± 1.80	101 ± 4.50	76.7 ± 6.40	57.2 ± 5.50	94.6 ± 10.90	68.6 ± 4.60	30.6 ± 5.00	119 ± 17.2^{A}	84.3 ± 6.60^{B}	60.1 ± 12.6^{C}		P	O
12,13-DiHOME	45.8 ± 3.10	37.3 ± 4.50	20.5 ± 1.00	59.7 ± 5.90	52.5 ± 4.10	38.7 ± 5.60	61.3 ± 7.60	51.8 ± 7.30	23.4 ± 4.20	80.9 ± 10.5^{A}	55.2 ± 3.10^{B}	42.2 ± 6.20^{C}	S	P	O
Total LA oxylipins	18071 ± 1897	13963 ± 953	5723 ± 794	21109 ± 1523	13700 ± 1447	8643 ± 1247	18032± 1261	12296± 1543	5797 ± 884	23879 ± 4067 ^{A}	18913 ± 2617 ^{B}	7600 ± 1398 ^{C}		P	O
DGLA															
8-HETrE	152 ± 15.5	187 ± 16.8	79.4 ± 10.2	170 ± 17.4	141 ± 17.4	94.7 ± 4.20	265 ± 29.4	181 ± 19.9	94.4 ± 10.7	242 ± 35.9^{A}	177 ± 19.1^{B}	105 ± 14.3^{C}	S		O
15-HETrE	113 ± 15.5	98.4 ± 14.4	37.2 ± 3.60	89.3 ± 15.4	85.3 ± 12.8	55.8 ± 9.60	88.7 ± 20.6	61.0 ± 14.5	41.9 ± 5.50	96.7 ± 18.6^{A}	83.0 ± 11.5^{A}	43.9 ± 9.00^{B}			O
Total DGLA oxylipins	265 ± 21.3	286 ± 16.0	117 ± 13.0	259 ± 22.4	226 ± 22.0	150 ± 11.0	339 ± 35.9	242 ± 33.3	136 ± 15.4	338 ± 52.8 ^{A}	260 ± 28.3 ^{B}	149 ± 20.9 ^{C}			O
AA															
PGD_2	203 ± 31.5	87.8 ± 15.9	91.7 ± 14.0	204 ± 25.1	118 ± 23.0	108 ± 17.4	166 ± 33.3	117 ± 18.6	117 ± 34.7	178 ± 38.3^{A}	103 ± 29.1^{B}	120 ± 25.7^{B}			O
PGE_2	419 ± 17.6	191 ± 32.6	73.1 ± 5.70	434 ± 46.8	282 ± 53.2	96.3 ± 11.0	280 ± 24.8	112 ± 13.3	50.7 ± 3.70	229 ± 21.3^{A}	111 ± 18.5^{B}	87.2 ± 18.5^{C}	S		O
$PGF_{2\alpha}$	354 ± 29.3^{a}	165 ± 13.2^{bc}	76.3 ± 7.90^{d}	348 ± 34.2^{a}	184 ± 25.2^{b}	100 ± 6.50^{cd}	218 ± 25.9^{ab}	89.9 ± 6.60^{d}	61.9 ± 5.00^{d}	170 ± 23.2^{bc}	104 ± 15.2^{cd}	78.7 ± 7.80^{d}		Int	
6-keto-$PGF_{1\alpha}$	760 ± 122	271 ± 32.4	93.7 ± 9.30	727 ± 92.8	433 ± 142	138 ± 9.80	303 ± 50.4	131 ± 15.1	75.2 ± 8.20	244 ± 37.8^{A}	146 ± 24.0^{B}	80.5 ± 12.9^{C}	S		O
5-HETE	1740± 197	1128 ± 140	770 ± 53.4	1726± 100	1052 ± 298	1016 ± 86.0	1828 ± 173	765 ± 74.5	767 ± 91.9	1675 ± 190^{A}	779 ± 112^{B}	898 ± 115^{B}			O

5-oxo-ETE	390 ± 53.4	252 ± 35.9	243 ± 27.4	393 ± 56.0	153 ± 16.5	252 ± 16.5	411 ± 50.5	197 ± 29.1	200 ± 34.1	357 ± 37.5^{A}	204 ± 27.6^{B}	149 ± 21.7^{B}		O
8-HETE	424 ± 37.8	377 ± 60.5	179 ± 22.3	596 ± 136	404 ± 95.4	312 ± 31.8	445 ± 78.4	187 ± 67.8	190 ± 29.8	504 ± 122^{A}	266 ± 57.6^{B}	241 ± 60.8^{B}	S	P O
9-HETE	1177 ± 110	619 ± 75.0	411 ± 62.9	848 ± 112	549 ± 122	635 ± 163	818 ± 162	379 ± 127	398 ± 60.3	655 ± 148^{A}	357 ± 56.6^{B}	573 ± 173^{B}	S	O
11-HETE	1299± 100	815 ± 119	347 ± 37.5	1324± 97.9	907 ± 212	598 ± 101	798 ± 172	357 ± 70.4	318 ± 40.7	937 ± 190^{A}	419 ± 82.7^{B}	361 ± 83.2^{C}	S	O
12-HETE	1657± 269	791 ± 110	270 ± 29.5	1165± 178	1038 ± 280	592 ± 93.5	1270 ± 284.	367 ± 139	322 ± 39.0	1187 ± 368^{A}	674 ± 208^{B}	326 ± 68.0^{C}	S	O
15-HETE	2008 ± 137	1051 ± 103	625 ± 46.7	1958 ± 139	1376 ± 383	874 ± 119	1428 ± 206	564 ± 87.1	516 ± 57.9	1297 ± 182^{A}	613 ± 100^{B}	598 ± 110^{C}	S	O
15-oxo-ETE	1519± 329	1285 ± 224	1124 ± 186	1572± 244	1032 ± 184	1489 ± 414	1556 ± 282	1175 ± 186	923 ± 184	2497 ± 372^{A}	1242 ± 304^{B}	1007 ± 164^{B}		O
5,6-DiHETrE	96.6 ± 15.6	69.7 ± 7.80	52.1 ± 8.70	115 ± 12.3	61.2 ± 16.0	77.7 ± 5.30	126 ± 12.7	57.5 ± 6.50	65.1 ± 10.0	130 ± 14.6^{A}	58.0 ± 8.90^{B}	68.1 ± 12.4^{B}		O
8,9-DiHETrE	17.3 ± 2.10	8.50 ± 2.00	10.5 ± 2.00	17.2 ± 1.60	10.0 ± 0.50	12.2 ± 0.40	17.3 ± 1.80	10.9 ± 1.80	9.50 ± 2.20	14.3 ± 2.50^{A}	7.60 ± 1.60^{B}	9.60 ± 2.60^{B}		O
11,12-DiHETrE	46.8 ± 5.40	23.2 ± 1.90	20.2 ± 1.00	39.6 ± 3.40	21.4 ± 1.10	24.8 ± 1.80	41.7 ± 6.30	16.0 ± 2.30	18.7 ± 3.10	34.9 ± 5.00^{A}	24.1 ± 4.20^{B}	16.1 ± 1.40^{B}	S	O
14,15-DiHETrE	59.0 ± 4.70	28.4 ± 2.40	22.7 ± 2.40	47.3 ± 6.10	32.1 ± 7.70	30.0 ± 3.40	50.4 ± 6.20	21.4 ± 3.20	18.9 ± 2.80	43.9 ± 5.20^{A}	24.5 ± 2.80^{B}	20.5 ± 3.00^{B}	S	O
16-HETE	183 ± 13.8	102 ± 12.7	86.8 ± 12.4	215 ± 22.9	125 ± 13.7	121 ± 24.4	184 ± 17.8	75.2 ± 14.9	64.2 ± 13.4	176 ± 19.4^{A}	85.7 ± 12.6^{B}	65.5 ± 6.40^{B}	S	O
Total AA oxylipins	11924 ± 705	7161 ± 681	4495 ± 437	11729 ± 994	7606 ± 1358	6477 ± 980	9626 ± 846	4590 ± 762	4091 ± 493	10218 ± 1481 ^{A}	5216 ± 944 ^{B}	4665 ± 700 ^{C}	S	O
n-3 oxylipins														
ALA														
9-HOTrE	303 ± 59.0	1419 ± 285	103 ± 17.9	294 ± 32.2	1085 ± 263	96.8 ± 15.3	528 ± 76.9	2717 ± 488	101 ± 28.9	518 ± 88.0^{B}	1905 ± 307^{A}	160 ± 41.4^{C}	S	O
9-oxo-OTrE	266 ± 90.6	534 ± 131	119 ± 58.2	282 ± 66.3	511 ± 198	115 ± 45.1	189 ± 21.3	490 ± 122	40.2 ± 11.4	421 ± 93.7^{B}	707 ± 158^{A}	86.0 ± 46.2^{C}		O
13-HOTrE	2889± 4349	96328± 12726	9262 ± 1484	2773± 4112	9700± 23874	11189± 3372	39852± 8629	10240± 16508	8399 ± 1362	37707± 6567^{B}	14432±26919^{A}	1201± 3320^{C}		O
ALA oxylipins (3)	29461 ± 4459 ^{cd}	82137 ± 18945^{abc}	9433 ± 1535 ^{d}	28313 ± 4193^{dc}	98516 ± 24114^{ab}	11378± 3418 ^{d}	40569± 8697^{bcd}	71397± 23663^{bcd}	8540 ± 1397 ^{d}	38646 ± 6732^{bcd}	14694± 27343^{a}	12232 ± 3382 ^{d}	Int	
EPA														
5-HEPE	300 ± 106	2688 ± 535	4739 ± 485	265 ± 35.7	1411 ± 238	5077 ± 863	382 ± 59.8	3505 ± 412	4601 ± 1086	400 ± 54.7^{C}	1876 ± 316^{B}	4372 ± 559^{A}		O
8-HEPE	72.5 ± 26.1	459 ± 92.6	454 ± 37.7	92.4 ± 17.5	359 ± 109	744 ± 173	79.8 ± 9.40	461 ± 112	374 ± 99.5	85.3 ± 21.0^{B}	408 ± 72.4^{A}	385 ± 93.6^{A}		O
9-HEPE	519 ± 178	3902 ± 806	4377 ± 468	512 ± 94.7	2557 ± 527	6789 ± 1285	667 ± 130	3805 ± 702	4157 ± 1169	667 ± 123^{C}	2804 ± 860^{B}	3873 ± 689^{A}		O
11-HEPE	215 ± 59.0	1700 ± 395	1608 ± 131	217 ± 42.9	1319 ± 257	2850 ± 850	237 ± 34.4	1283 ± 348	1253 ± 471	267 ± 85.2^{B}	1113 ± 417^{A}	1244 ± 261^{A}		O

12-HEPE	509 ± 126^{b}	3777 ± 630^{a}	2647 ± 344^{a}	337 ± 45.0^{b}	2911 ± 474^{a}	5973 ± 1319^{a}	761 ± 88.3^{b}	2421 ± 504^{a}	2342 ± 360^{a}	574 ± 120^{b}	4085 ± 1632^{a}	2350 ± 350^{a}		Int	
15-HEPE	300 ± 50.3	881 ± 194	1405 ± 171	196 ± 20.6	1100 ± 74.9	1723 ± 609	285 ± 52.4	790 ± 262	1131 ± 234	252 ± 46.0^{C}	885 ± 306^{B}	1040 ± 149^{A}			O
18-HEPE	499 ± 39.2	4831 ± 609	1242± 1348	381 ± 59.2	3549 ± 573	12360 ± 2111	586 ± 40.2	7730 ± 1724	11976± 2449	501 ± 99.3^{C}	4981 ± 1258^{B}	1739± 4552^{A}	S		O
Total EPA oxylipins	2390 ± 508	18091 ± 2291	27654 ± 2130	1984 ± 178	13207 ± 1459	35515± 5969	2998 ± 336	19591± 949	25365 ± 4202	2732 ± 476 ^{C}	16070 ± 4685 ^{B}	30659 ± 4610 ^{A}			O
DHA															
4-HDoHE	1342± 219de	1935 ± 331^{cde}	2649 ± 150^{abc}	1078± 65.1^{de}	961 ± 132^{e}	3417 ± 424^{ab}	2330 ± 184^{bcd}	2352 ± 168^{bcd}	2563 ± 437^{abcd}	2317 ± 197^{bcd}	2143 ± 217^{bcde}	3917 ± 399^{a}		Int	
7-HDoHE	145 ± 18.8	197 ± 38.6	260 ± 22.8	171 ± 40.3	134 ± 23.6	426 ± 71.5	179 ± 38.4	203 ± 42.7	327 ± 77.9	252 ± 58.5^{B}	221 ± 49.7^{B}	297 ± 44.0^{A}			O
10-HDoHE	139 ± 27.3	241 ± 58.6	220 ± 12.8	217 ± 81.2	158 ± 14.7	381 ± 62.1	158 ± 23.2	281 ± 47.7	259 ± 62.8	247 ± 67.4^{B}	188 ± 42.6^{B}	335 ± 84.9^{A}			O
10S,17S-DiHDoHE	30.9 ± 5.70	57.0 ± 12.8	52.7 ± 7.00	36.6 ± 13.8	25.8 ± 3.00	80.7 ± 15.2	68.3 ± 6.50	57.6 ± 13.6	64.0 ± 16.4	51.8 ± 16.5^{B}	60.0 ± 10.1^{B}	107 ± 41.9^{A}	S		O
11-HDoHE	391 ± 56.1	605 ± 108	641 ± 42.1	387 ± 58.2	379 ± 29.0	1107 ± 200	419 ± 86.6	545 ± 102	665 ± 130	636 ± 165^{B}	558 ± 154 ^{B}	820 ± 207^{A}			O
13-HDoHE	141 ± 13.2	168 ± 21.5	227 ± 11.3	123 ± 12.0	120 ± 12.1	290 ± 36.9	185 ± 26.6	180 ± 19.7	247 ± 46.7	189 ± 23.1^{B}	166 ± 29.3^{B}	301 ± 59.4^{A}	S		O
14-HDoHE	646 ± 87.5	759 ± 109	795 ± 49.4	583 ± 117.2	605 ± 71.9	1215 ± 211	792 ± 94.6	716 ± 142	905 ± 194	871 ± 181^{B}	814 ± 200^{B}	1053 ± 226^{A}			O
16-HDoHE	565 ± 59.6^{cd}	711 ± 105^{bcd}	1106 ± 68.5^{ab}	526 ± 60.1^{cd}	493 ± 46.3^{d}	1273 ± 190^{a}	846 ± 43.9^{abcd}	836 ± 60.1^{abcd}	984 ± 201^{abc}	865 ± 82.3^{abc}	877 ± 117^{abcd}	1453 ± 194^{a}		Int	
17-HDoHE	1666± 181^{cd}	2252 ± 437^{bcd}	3232 ± 147^{ab}	1599± 233^{cd}	1440 ± 197^{d}	4186 ± 627^{a}	2431 ± 107^{abcd}	2587 ± 207^{abc}	3013 ± 453^{abc}	2660 ± 261^{abc}	2499 ± 302^{abcd}	4640 ± 757^{a}		Int	
17-keto-DHA	1013± 141^{d}	1137 ± 87.3^{cd}	2832 ± 318^{ab}	1673± 526^{bcd}	1230 ± 165^{cd}	4440 ± 847^{a}	3025 ± 857^{abc}	3028 ± 598^{abc}	3673 ± 828^{ab}	2976 ± 534^{abc}	1685 ± 238^{bcd}	3109 ± 272^{ab}		Int	
19,20 DiHDPE	150 ± 9.7^{de}	136 ± 15.0^{de}	210 ± 11.7^{cd}	94.0 ± 12.4^{e}	114 ± 24.1^{e}	234 ± 28.2^{bcd}	454 ± 59.7^{ab}	512 ± 49.6^{a}	471 ± 109^{ab}	360 ± 43.3^{abc}	359 ± 45.2^{abc}	375 ± 39.6^{abc}		Int	
20-HDoHE	8832± 1018^{ef}	13104± 2125^{def}	3427± 2459^{ab}	7450± 856^{f}	8974 ± 1532^{ef}	27179± 1093^{abc}	14926± 1548^{cde}	22950± 2683^{abcd}	29219± 3625^{abc}	14821± 1213^{cde}	18737± 2279^{bcd}	4817± 12146^{a}		Int	
Total DHA oxylipins	15061 ± 1703 ^{c}	21056 ± 2985 ^{bc}	41600 ± 5659 ^{ab}	13936 ± 1609 ^{c}	14634 ± 2120 ^{c}	44228± 3098 ^{ab}	24106± 1599 ^{bc}	30422± 4771 ^{bc}	33787 ± 5686 ^{bc}	26245 ± 2114 ^{bc}	28306 ± 3016 ^{bc}	64044 ± 12612^{a}		Int	

All values represent mean ± SEM (n=3-7). S, sex effect; P, protein effect; O, oil effect, Int, interaction or Wilcoxin's test used for non-normal data. P, protein effect; O, oil effect, Int, interaction or Wilcoxin's test used for non-normal data. Oil and protein subgroups were combined for determining sex effect and oil subgroups were combined for determining protein effect. Differing uppercase superscripts (shown in male soy protein groups) indicate differences in oil effects. Protein subgroups were combined for determining oil effects. Differing lowercase superscripts indicate differences among all twelve groups, when combining was not possible due to interactions or non-normal data.

Table 5.4 Dietary oil and sex effects on renal oxylipins in Pkd1 (1wk) mice (pg/mg dry tissue)

Oxylipin	Female			Male			
	Soy oil	Flax oil	Fish oil	Soy oil	Flax oil	Fish oil	Effects
n-6 oxylipins							
LA							
9-HODE	4201 ± 256	2753 ± 355	1473 ± 155	4037 ± 385^{A}	3199 ± 264^{B}	1432 ± 133^{C}	O
9-oxo-ODE	1973 ± 243	1050 ± 286	931 ± 8.50	1769 ± 302^{A}	1571 ± 175^{AB}	1123 ±195^{B}	O
13-HODE	5265 ± 610	3087 ± 364	1787 ± 162	4520 ± 459^{A}	3476 ± 678^{B}	1995 ±323^{C}	O
13-oxo-ODE	5939 ± 783	3128 ±1108	2101 ± 165	4036 ± 531^{A}	4126 ± 674^{A}	2251 ± 306^{B}	O
9,10,13 TriHOME	1892 ± 222^{a}	1096±184^{bc}	756 ± 40.4^{c}	1523 ± 102^{ab}	1660 ± 131^{ab}	904 ± 125^{c}	Int
9,12,13 TriHOME	611 ± 88.8	384 ± 59.2	222 ± 19.3	526 ± 48.1^{A}	507 ± 35.3^{A}	301 ± 34.4^{B}	O
9,10-DiHOME	82.0 ± 5.30	63.7 ± 11.2	35.6 ± 2.60	83.9 ± 10.0^{A}	79.3 ± 8.50^{A}	46.4 ± 7.70^{B}	O
12,13-DiHOME	1421 ± 70.0	966 ± 165	622 ± 30.6	1655 ± 219^{A}	1441 ± 128^{A}	834 ± 105^{B}	S O
Total LA oxylipins	20333 ±1377	12178 ±1903	7927 ± 515	18150 ± 1858^{A}	14823 ± 1204^{B}	8027± 887^{C}	O
GLA							
13-HOTrE-γ	50.0 ± 7.30	21.9 ± 2.70	10.1 ± 1.40	50.9 ± 6.00^{A}	21.5 ± 3.20^{B}	15.0 ± 2.70^{B}	O
DGLA							
8-HETrE	164 ± 14.0	125 ± 8.80	67.2 ± 2.80	163 ± 8.00^{A}	119.3 ± 8.4^{B}	78.9 ± 8.90^{C}	O
15-HETrE	82.9 ± 10.2	69.7 ± 8.20	32.8 ± 2.20	89.8 ± 7.20^{A}	68.2 ± 3.90^{B}	49.4 ± 5.10^{C}	O
Total DGLA oxylipins	247 ± 23.9	194 ± 11.3	100 ± 3.28	225 ± 25.7^{A}	187 ± 11.3^{B}	128 ± 13.0^{C}	O
AA							
PGD_2	1064 ±78.2^{a}	410 ±12.3^{bc}	257±32.7^{cd}	518 ± 62.1^{b}	245 ± 29.1^{cd}	166 ± 19.6^{d}	Int
PGE_2	482 ± 30.9^{a}	151 ± 22.2^{c}	74.9 ±14.4^{c}	261 ± 22.7^{b}	94.3 ± 11.0^{c}	70.6 ± 9.56^{c}	Int
$PGF_{2\alpha}$	419 ± 42.9^{a}	200 ±59.7^{bc}	117 ± 14.4^{c}	240 ± 19.5^{b}	147 ± 23.2^{bc}	89.9 ± 12.2^{c}	Int
6-k-$PGF_{1\alpha}$	976 ± 54.5	341 ± 8.0	246 ± 33.7	418 ± 26.7^{A}	279 ± 51.2^{B}	167.7 ± 21.6^{C}	S O
TxB_2	58.8 ± 8.00^{a}	28.6±1.30^{bc}	17.5±2.10^{bc}	28.7 ± 4.90^{b}	22.3 ± 2.70^{bc}	12.8 ± 1.26^{c}	Int
5-HETE	2669 ± 244^{a}	984 ± 53.2^{c}	1115±55.1^{c}	1830 ± 176^{b}	881 ± 45.5^{c}	982 ± 94.9^{c}	Int
5-oxo-ETE	558 ± 80.1^{a}	214 ±30.4^{bc}	187 ±26.9^{bc}	295 ± 38.6^{ab}	159 ± 19.9^{c}	286 ± 53.7^{bc}	Int

8-HETE	913 ± 175	351 ± 33.7	596 ± 187	847 ± 69.0^{A}	489 ± 79.1^{B}	420 ± 61.2^{B}		O
9-HETE	1603 ± 71.8	763 ± 88.2	758 ± 39.3	1213 ± 127^{A}	602 ± 59.2^{B}	707 ± 67.4^{B}	S	O
11-HETE	945 ± 197	454 ± 34.3	509 ± 30.2	626 ± 45.1^{A}	394 ± 45.1^{B}	418 ± 59.7^{B}	S	O
12-HETE	1332 ± 127	704 ± 180	480 ± 106	1150 ± 83.0^{A}	675 ± 53.7^{B}	532 ± 40.9^{B}		O
15-HETE	1427 ± 131^{a}	565 ±43.8^{bc}	552±11.2^{bc}	831 ± 60.0^{b}	438 ± 35.0^{c}	430 ± 54.0^{c}		Int
5,6-DiHETrE	66.9 ± 10.8	17.5 ± 0.70	25.5 ± 5.20	61.3 ± 5.30^{A}	27.0 ± 1.80^{B}	26.4 ± 3.80^{B}		O
8,9-DiHETrE	18.8 ± 2.60	11.1 ± 3.40	6.50 ± 1.30	13.4 ± 1.10^{A}	7.0 ± 0.80^{B}	6.70 ± 1.20^{B}	S	O
11,12-DiHETrE	49.3 ± 4.60	26.6 ± 5.10	25.1 ± 1.80	34.4 ± 3.30^{A}	19.7 ± 1.80^{B}	20.1 ± 1.90^{B}	S	O
14,15-DiHETrE	44.7 ± 4.10	17.9 ± 2.50	18.7 ± 1.00	38.1 ± 2.00^{A}	21.7 ± 1.60^{B}	22.7 ± 2.10^{B}		O
16-HETE	59.8 ± 3.00	32.7 ± 0.90	44.2 ± 6.10	49.5 ± 5.00^{A}	44.4 ± 4.90^{B}	42.5 ± 2.80^{B}		O
Total AA oxylipins	11455 ± 732	5270 ± 306	5028 ± 478	8445 ± 533^{A}	4437 ± 352^{B}	4400 ± 416^{B}	S	O
n-3 oxylipins								
ALA								
9-HOTrE	236 ± 38.8	648 ± 40.6	90.6 ± 11.0	277 ± 75.1^{B}	1474 ± 81.7^{A}	108 ± 19.8^{C}		O
9-oxo-OTrE	256 ± 145	394 ± 132	62.8 ± 27.7	138 ± 42.9^{B}	654 ± 116^{A}	124 ± 64.9^{C}		O
13-HOTrE	13385±1153^{b}	42021±6624^{b}	8804±1102^{b}	13979±2715^{b}	90309±15656^{a}	6875 ±822^{b}		Int
Total ALA oxylipins	13877 ± 1056^{b}	28840 ± 14863^{ab}	8957 ± 1074^{b}	14393 ± 2816^{b}	79021 ± 18446^{a}	7106 ± 882^{b}		Int
EPA								
PGE_3	11.1 ± 1.60	65.4 ± 5.90	96.0 ± 7.00	8.50 ± 2.90^{B}	62.9 ± 7.20^{A}	99.1 ± 17.2^{A}		O
5-HEPE	401 ± 32.9	3069 ± 282	5698±628.8	376 ± 36.2^{C}	3126 ± 466^{B}	5241 ± 605^{A}		O
8-HEPE	64.1 ± 10.8	376 ± 43.1	924 ± 277	76.5 ± 14.2^{C}	498 ± 98.9^{B}	885 ± 192^{A}		O
9-HEPE	294 ± 35.4	1951 ± 195	3541 ± 140	252 ± 20.6^{C}	2338 ± 320^{B}	4125 ± 672^{A}		O
11-HEPE	149 ± 25.7	957 ± 94.5	1511 ± 46.3	122 ± 14.0^{C}	1115 ± 147^{B}	1723 ± 272^{A}		O
12-HEPE	399 ± 25.4	3437 ± 484	3601 ± 541	442 ± 49.6^{C}	4191 ± 419^{B}	5928 ± 485^{A}	S	O
15-HEPE	81.6 ± 11.7	559 ± 46.2	878 ± 45.8	72.1 ± 13.8^{C}	551 ± 82.8^{B}	977 ± 102^{A}		O
18-HEPE	1446 ± 122	11917 ±150	20327±1440	1382 ± 158^{C}	12924±1606^{B}	25060±2460^{A}		O
Total EPA oxylipins	2830 ± 107^{c}	22330 ± 592^{b}	36575 ±1105^{ab}	2500 ± 336 ^{c}	21200 ± 4247^{b}	43289 ± 3809^{a}		Int
DHA								

4-HDoHE	1076 ± 129	987 ± 117	1490 ± 185	1098 ± 115^{B}	1316 ± 138^{B}	1903 ± 149^{A}	S	O
7-HDoHE	190 ± 13.6	151 ± 8.10	288 ± 45.8	203 ± 25.6^{B}	283 ± 40.2^{B}	398 ± 67.9^{A}	S	O
8-HDoHE	896 ± 116	903 ± 75.3	1473 ± 185	988 ± 123^{B}	1320 ± 117^{B}	1946 ± 197^{A}	S	O
10-HDoHE	145 ± 21.1	138 ± 12.5	220 ± 16.0	136 ± 10.3^{B}	179 ± 11.2^{B}	251 ± 35.5^{A}		O
10S,17S-DiHDoH	29.4 ± 6.00	21.7 ± 4.80	39.7 ± 7.70	29.6 ± 4.30	32.2 ± 4.50	47.6 ± 9.70		
11-HDoHE	219 ± 15.9	271 ± 31.3	422 ± 43.7	238 ± 29.2^{C}	330 ± 16.6^{B}	533 ± 44.4^{A}	S	O
13-HDoHE	97.5 ± 4.40	74.0 ± 5.30	120 ± 9.30	82.4 ± 5.50^{B}	111 ± 11.5^{B}	152 ± 15.3^{A}		O
14-HDoHE	505 ± 40.3	486 ± 59.4	624 ± 55.6	511 ± 45.1^{B}	723 ± 69.9^{B}	974 ± 93.1^{A}	S	O
16-HDoHE	479 ± 38.6	365 ± 30.0	622 ± 39.4	454 ± 35.3^{B}	583 ± 52.9^{B}	746 ± 72.4^{A}	S	O
17-HDoHE	1001 ± 70.3	751 ± 36.5	1301 ± 126	969 ± 97.2^{B}	1238 ± 126^{B}	1870 ± 243^{A}	S	O
17-keto-DHA	622 ± 68.8	501 ± 54.5	877 ± 178	481 ± 43.4^{B}	535 ± 65.8^{B}	988 ± 123^{A}		O
Resolvin D2	93.2 ± 17.2	82.1 ± 14.9	189 ± 38.7	102 ± 11.8^{B}	143 ± 20.3^{B}	202 ± 13.1^{A}		O
19,20 DiHDPE	94.1 ± 10.7	81.5 ± 6.10	131 ± 10.0	222.3 ± 22.6^{B}	313 ± 20.6^{A}	352 ± 39.2^{A}	S	O
20-HDoHE	20938±1975	21761±2117	32162±1991	21038 ± 1899^{B}	29300 ± 2918^{B}	44561 ± 5475^{A}		O
Total DHA oxylipins	24813 ± 2157	25147 ± 2388	37566 ± 2469	25053 ± 2225^{B}	34444 ± 3299^{B}	50994 ± 5584^{A}	S	O
Non-enzymatic AA oxylipins								
8-iso $PGF_{2\alpha}$III	1030 ± 68.3^{a}	527 ± 134^{bc}	243 ± 64.8^{c}	621 ± 50.7^{b}	342 ± 64.6^{c}	243 ± 30.2^{c}		Int

All values represent mean ± SEM (n=3-7). S, sex effect; O, oil effect, Int, interaction or Wilcoxin's test used for non-normal data. Oil subgroups were combined for determining sex effect. Differing uppercase superscripts (shown in male) indicate differences in oil effects. Sex subgroups were combined for determining oil effects. Differing lowercase superscripts indicate differences among all six groups, when combining was not possible due to interactions or non-normal data.

Table 5.5 Dietary oil and sex effect son renal oxylipins in Pkd2 mice (pg/mg dry tissue)

Oxylipin	Female			Male			
	Soy oil	Flax oil	Fish oil	Soy oil	Flax oil	Fish oil	Effects
n-6 oxylipins							
LA							
9-HODE	4454±1048	3087±563	1047±79.5	4529±666^{A}	2079±482^{B}	1554±182^{C}	O
9-oxo-ODE	788±127	485±70.1	412±167	1220±170^{A}	785±190^{B}	712±158^{B}	S O
13-HODE	5003±910^{ab}	3448±562^{bc}	1172±95.5^{e}	6168±888^{a}	2803±572^{cd}	2059±194^{d}	Int
13-oxo-ODE	2247±526	1475±467	839±189	3019±579^{A}	1467±362^{B}	1135±141^{B}	O
9,10,13 TriHOME	24430±350	1622±347	690±174	2127±402^{A}	1265±293^{B}	1020±147^{B}	O
9,12,13 TriHOME	1028±189	438±61.0	357±51.8	1167±199^{A}	638±149^{B}	522±72.8^{B}	O
9,10 DiHOME	82.5±21.7	84.4±17.8	39.2±3.80	122±21.7^{A}	52.7±10.0^{B}	57.3±7.60^{B}	O
12,13 DiHOME	76.6±21.6^{b}	82.3±14.6^{b}	42.1±2.10^{b}	146±16.4^{a}	53.9±10.7^{b}	56.8±5.80^{b}	Int
Total LA oxylipins	16121 ± 2484	10653 ± 1627	4599 ± 512	17834 ± 2292^{A}	9142 ± 1993^{B}	7116 ± 594^{C}	O
DGLA							
8-HETrE	96.7±14.6	54.3±16.0	18.3±6.8	119±32.3^{A}	48.9±14.4^{B}	50.9±6.40^{B}	O
15-HETrE	80.6±16.3	53.7±8.50	39.8±12.2	76.5±5.30	63.2±17.0	64.9±10.7	
Total DGLA oxylipins	177 ± 26.7	108 ± 24.2	58.1 ± 18.6	173 ± 21.4^{A}	112 ± 31.2^{B}	116 ± 15.8^{B}	O
AA							
PGD_2	236±56.6^{a}	124±5.90^{b}	61.3±6.30^{c}	163±15.6^{ab}	45.9±6.30^{c}	61.1±6.50^{c}	Int
PGE_2	360±42.5^{a}	176±10.5^{c}	70.2±12.3^{d}	251±19.2^{b}	59.2±4.60^{d}	73.5±7.50^{d}	Int
11β-PGE_2	1009±145^{a}	498±26.3^{b}	192±32.6^{c}	704±51.2^{ab}	150±15.8^{c}	176±26.9^{c}	Int
PGF_{2a}	298±36.2^{a}	160±10.1^{b}	84.3±8.10^{c}	196±11.1^{b}	74.9±6.30^{b}	85.5±11.6^{c}	Int
6-keto-$PGF_{1\alpha}$	820±66.2^{a}	725±64.5^{a}	384±47.0^{b}	724±90.9^{a}	257±45.6^{bc}	185±12.9^{c}	Int
TxB_2	106±7.30	58.9±4.50	23.0±4.20	53.1±7.70^{A}	28.4±6.90^{B}	19.6±4.30^{C}	S O
5-HETE	1720±237	561±196	281±135	1124±331^{A}	332±108^{B}	695±126^{B}	O
5-oxo-ETE	302±47.4^{a}	98.7±33.3^{c}	152±55.2^{bc}	314±145^{a}	130±39.7^{c}	252±46.6^{ab}	Int
8-HETE	430±95.8	188±44.3	118±29.0	362±53.0^{A}	153±39.7^{B}	177±31.2^{B}	O

9-HETE	626±128	205±39.9	206±89.5	519±116^{A}	190±51.5^{B}	318±38.5^{B}		O
11-HETE	485±53.8^{a}	278±18.3^{b}	142±19.9^{c}	332±29.1^{b}	137±15.8^{c}	175±21.2^{c}		Int
12-HETE	1219±420	456±113	378±115	1198±310	380±91.7	703±208		
15-HETE	1096±47.8^{a}	469±105^{bc}	216±98.5^{cd}	620±115^{b}	170±22.1^{d}	419±57.9^{bc}		Int
14,15 DiHETrE	31.3±1.40	12.1±2.00	10.3±1.40	30.0±4.30^{A}	9.00±1.30^{B}	19.5±2.10^{B}		O
5,6 DiHETrE	81.5±4.20	28.4±8.40	23.2±5.80	66.9±21.0^{A}	24.3±8.40^{B}	40.6±7.10^{B}		O
11,12 DiHETrE	32.1±3.10	13.3±2.30	8.70±1.20	26.5±5.60^{A}	6.80±1.00^{B}	16.6±2.20^{B}		O
16-HETE	80.4±14.1^{a}	37.5±8.70^{c}	42.8±14.9^{bc}	70.7±10.0^{ab}	37.8±9.70^{c}	44.2±9.50^{bc}		Int
18-HETE	56.9±8.10	27.2±7.20	35.6±18.0	86.5±19.3^{A}	53.4±15.7^{B}	22.3±6.50^{B}		O
Total AA oxylipins	9359 ±1084	4344 ± 613	2546 ± 611	6861 ±1042^{A}	2266±355^{B}	3644 ±575^{B}		O
n-3 oxylipins								
ALA								
9-HOTrE	222±93.5	503±149	28.1±5.30	299±94.3^{B}	1123±358^{A}	122±22.2^{C}	S	O
13-HOTrE	10585±4079	29927±7141	6072±2828	13392±2827^{B}	55429±18729^{A}	6040±856^{B}		O
Total ALA oxylipins	10894 ± 4185^{bc}	30586 ± 7269^{b}	3073 ± 2097^{c}	13833 ± 2947^{bc}	56719 ± 19057^{a}	5449 ± 1067^{c}		Int
EPA								
Δ17-6-keto-PGF$_{1\alpha}$	4.40±2.70	22.9±4.90	25.8±4.70	4.10±0.80^{B}	12.2±4.10^{A}	18.6±3.60^{A}		O
5-HEPE	222±53.0	816±286	813±279	215±66.0^{B}	669±205^{A}	1625±235^{A}		O
8-HEPE	43.6±25.1	133±24.2	93.9±14.6	48.7±12.7^{B}	145±28.7^{A}	194±20.9^{A}		O
9-HEPE	76.1±6.70^{d}	424±82.4^{bc}	538±132^{b}	157±33.9^{cd}	546±83.4^{b}	1238±137^{a}		Int
11-HEPE	66.7±11.6	248±16.7	365±50.5	71.7±9.40^{C}	272±43.1^{B}	547±80.0^{A}		O
12-HEPE	244±70.3	959±215	1630±366	503±168^{B}	1887±403^{A}	1932±209^{A}	S	O
18-HEPE	640±202	4056±866	6493±2022	798±277^{C}	3797±999^{B}	11789±1533^{A}		O
Total EPA oxylipins	1222 ± 256	5924 ± 1046	9837 ± 2758	1702 ± 489^{C}	7171 ± 1430^{B}	14730 ± 2210^{A}		O
DHA								
4-HDoHE	819±96.4^{b}	478±159^{b}	466±260^{b}	797±283^{b}	484±150^{b}	1520±259^{a}		Int
7-HDoHE	83.8±15.7	70.7±15.9	107±36.1	120±36.2^{B}	86.4±20.0^{B}	241±32.2^{A}	S	O
8-HDoHE	456±83.1^{b}	386±125^{b}	404±201^{b}	600±200^{b}	415±114^{b}	1306±221^{a}		Int
10-HDoHE	50.7±3.40^{b}	50.3±14.0^{b}	46.7±18.0^{b}	60.8±13.3^{b}	44.6±8.50^{b}	138±23.7^{a}		Int

11-HDoHE	122±6.10	106±35.0	101±48.5	144±46.3	96.6±27.3	372±75.7		
13-HDoHE	76.3±5.80^{b}	68.1±10.3^{b}	65.3±15.7^{b}	81.9±18.8^{b}	66.0±12.7^{b}	194±20.7^{a}		Int
14-HDoHE	329±48.4	282±55.2	370±74.6	397±89.3^{B}	378±75.0^{B}	849±117^{A}	S	O
16-HDoHE	463±44.9^{b}	373±102^{b}	450±164^{b}	582±142^{b}	429±97.3^{b}	1265±183^{a}		Int
17-HDoHE	979±70.7	796±217	1027±302	840±204^{AB}	499±110^{B}	1606±276^{A}		O
17-keto-DHA	845±81.2	776±267	792±310	1038±341	570±119	1674±348		
19,20 DiHDPE	94.7±3.90	71.2±9.10	160±21.2	376±62.0^{AB}	302±58.7^{B}	368±65.3^{A}	S	O
20-HDoHE	18172±2983	12412±4239	16078±9162	19588±7326	12535±3284	38313±6256		
Total DHA oxylipins	22491 ± 3129	14081 ±4252	20066±10547	24504 ± 8442	15904 ± 3939	42472 ± 7947		
Non-Enzymatic AA oxylipins								
5-iso PGF2aVI	38.9±4.50	5.70±2.70	23.1±3.80	26.6±2.80^{A}	9.20±2.60^{C}	19.5±3.40^{B}		O

All values represent mean ± SEM (n=4-7). S, sex effect; O, oil effect, Int, interaction or Wilcoxin's test used for non-normal data. Oil subgroups were combined for determining sex effect. Differing uppercase superscripts (shown in male) indicate differences in oil effects. Sex subgroups were combined for determining oil effects. Differing lowercase superscripts indicate differences among all six groups, when combining was not possible due to interactions or non-normal data.

feeding in the current study resulted in much higher levels of EPA derived oxylipins, even though the EPA level in the fish oil diet was present at a level one-fourth that of the ALA in the flax oil diet. Thus, directly supplying EPA in the diet is more effective in elevating EPA oxylipins than providing much higher levels of ALA. Another difference in the effect of fish vs flax oil on renal oxylipins was that while fish oil resulted in higher DHA oxylipins, flax oil did not. Dietary flax oil also did not elevate renal DHA oxylipins in a previous study in obese rats (16). In contrast, flax oil feeding increased the levels of 10 DHA derived oxylipins in a mouse model of cystic kidney disease in which DHA oxylipin concentrations were reduced with disease (17). Interestingly, 4-week consumption of flaxseed in humans decreased the levels of DHA oxylipins (20). On the other hand, consumption of fish oil raises the plasma levels of DHA derived oxylipins in healthy human subjects (19,43). In the four studies presented herein where fish and flax oil were directly compared, fish oil containing DHA elevated renal DHA oxylipins while flax oil did not. Conversely, the fish oil diet did not increase renal ALA oxylipins, suggesting that retro-conversion of EPA and DHA to ALA does not occur to a significant extent in the kidney with these diets. This is consistent with the effect of fish oil on tissue ALA levels (44-47).

The differential effects of flax and fish oil on n-3 PUFA derived oxylipins may help explain their differences in effects on kidney health. In some kidney diseases flax oil has beneficial effects (40,48-52), while fish oil has been shown to have beneficial effects (53-56), no effect (57-60) or in some cases detrimental effects (61,62). In one study that investigated the effects of flax oil and an algal oil rich in DHA on renal disease in the same model, flax oil slowed disease progression, while the DHA oil enhanced disease progression (61). While many studies have shown that oxylipins derived from EPA and DHA have anti-inflammatory and pro-

resolving properties (63,64), much less is known about ALA oxylipins. One study demonstrated that ALA derived HOTrEs have anti-inflammatory properties in-vitro (65) and another showed that 9,16-DiHOTrE inhibits human platelet aggregation (66). Thus, it is possible that distinct effects of flax vs fish oil may be due to the different oxylipin profiles that result from fish vs flax oil feeding. Further research is warranted to elucidate the effect of these two dietary oils on renal oxylipins and health.

There is very limited literature on the effect of dietary protein on the synthesis of oxylipins. In the two studies with different dietary protein sources, LA derived oxylipins were elevated with soy protein feeding, while oxylipins from other PUFA generally were not affected. Oxylipin changes in response to dietary soy protein previously have been observed, but these changes coincided with effects on disease, so whether these effects were secondary to disease effects could not be ascertained (14,23). In the current studies, dietary soy protein had minor (PCK rat) or no (Pkd1 (5wk) mouse) effect on disease, suggesting that the effect of dietary soy protein on oxylipins was independent of disease effects. Tissue LA levels have been shown to increase with soy protein consumption in renal and hepatic tissues of rats with renal disease (67) and in liver and lymphocytes of normal rats (68). This increase in LA levels has been attributed to the ability of soy protein to inhibit the activity of the Δ6-desaturase mediated conversion of LA to longer chain n-6 PUFA (23,24). This may help explain the increased formation of LA oxylipins in our studies. However, there is no evidence that this putative Δ6-desaturase inhibition reduced the levels of the oxylipins derived from the downstream PUFA, possibly because the fatty acid pool was not depleted sufficiently to affect oxylipin levels. Further studies examining tissue Δ6-desaturase and FA levels are warranted to further understand this novel effect of soy protein on LA derived oxylipins.

Implications of elevated levels of LA oxylipins, and the DiHOMEs in particular, on renal health are not well understood. However, it has been shown that epoxy-octadecenoic acids (EpOME) and DiHOMEs derived from LA have cytotoxic effects in renal proximal tubular cells (69,70) and DiHOMEs have been reported to induce chemotaxis in human neutrophils (71). Effects of other LA oxylipins have been demonstrated in other tissues as well, including anti-inflammatory and anti-proliferative properties in the skin (72), inhibition of tumor cell adhesion (73) and reduction in leukotriene B_4 secretion from leukocytes (74). In humans, LA oxylipins, HODE and oxoODEs have been implicated in oxidative stress and inflammation (75). The role of LA oxylipins in renal physiology largely remains to be elucidated.

In the current studies in which sex effects were examined, only AA derived oxylipins were higher in females compared to males. Studies investigating the effect of sex on the oxylipin profile are rare and have mostly examined a limited number of AA oxylipins. For example, plasma PGE_2 and prostacyclin levels are higher in female than in male rats (76) and female rats and mice have enhanced urinary excretion of PGE_2, and TxB_2 (26,27). These sex differences may be due to differences in formation and/or degradation of oxylipins, as female rats and mice have greater expression of COX-2 (27,28) and PGE_2 synthase (27), and estradiol suppresses the activity of 15-hydroxyPG dehydrogenase, a PG-degrading enzyme, in rat renal tissue (29,30) and in human fetal tissue (77). On the other hand, females also have a higher activity of prostaglandin 9-ketoreductase in rat renal tissues (31), suggesting that other factors also are involved. For example, tissue FA levels may be different in males and females, as suggested in human studies that indicate that females may have higher levels of plasma AA compared to males (78-80). Another potential factor affecting sex differences in renal oxylipin levels could be the role of transporters associated with clearance of oxylipins. Hatano et al recently reported that

the expression of a transporter responsible for clearance of PGE_2 was lower, and PGE_2 concentrations were higher in renal cortex of female compared to male rats (25). Thus, differential activity of enzymes involved in AA metabolism, higher levels of tissue AA and lower expression of transporters may have contributed to the higher levels of AA derived oxylipins in females in our studies.

It is notable that the effect of sex on AA derived oxylipins is unique and different from the effects on oxylipins from other n-6 and n-3 PUFA, which were either higher or not different in males compared to females. This is of particular importance because conclusions on oxylipins have almost exclusively been based on the literature available on the oxylipins produced from AA. The current study indicates that findings on sex differences in AA derived oxylipins may not be generalizable to other PUFA. Implications of these findings to health and disease need to be determined.

The diet induced changes on renal oxylipins in these studies are unlikely to be due to their effects on disease. As published elsewhere, diet had minimal effects on disease in the PCK rats and Pkd2 mice used in this study (32), and in both of the Pkd1 mouse studies disease progression was very minor, with no effect of diet or sex on disease progression. Thus, consistent sex and diet effects on oxylipins across varying degrees of disease in multiple studies herein suggest that the results are likely to be independent of disease. Nevertheless, whether these effects are present in normal models remains to be demonstrated.

In conclusion, these studies provide novel data directly comparing flax and fish oil on the renal oxylipin profile. Fish compared to flax oil resulted in greater reduction in n-6 PUFA derived oxylipins and greater increase in EPA and DHA derived oxylipins, whereas flax oil was more effective in elevating ALA derived oxylipins. Soy protein resulted in higher levels of

oxylipins from LA, while females compared to males displayed higher levels of AA oxylipins. Further studies are required to elucidate how these novel effects of diet and sex on the renal oxylipin profile affect renal health and disease.

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5.7 Transition to next chapter

In chapter 5, hypothesis 3 was addressed. Flax and fish oil feeding resulted in lower n-6 PUFA derived oxylipins; however, this level was much lower in animals with fish oil compared to those provided flax oil. Flax and fish oil feeding also resulted in increased n-3 derived oxylipins, with the effect on ALA oxylipins being greater with flax oil, while the effect on EPA and DHA oxylipins being greater with fish oil. Regarding sex effects, AA derived oxylipins were higher in female compared to male animals.

This study and previous chapters have shown that renal oxylipins are altered in diseased kidneys and that the renal oxylipin profile can be modulated by dietary interventions. Dietary HP also have been shown to increase renal disease progression, and *ex vivo* studies indicate that dietary HP compared to LP increases renal COX oxylipin (prostanoid) production (81-83). However, a recent short-term *in vivo* study did not find an effect of dietary HP on renal prostanoid production in normal rat kidneys (84). Therefore, in the next chapter, we examined the effect of a HP diet on renal oxylipin alterations and disease progression in normal and diseased mice (hypothesis 4). Due to the lack of availability of the Pkd2 mouse model of ADPKD, this study used the *pcy* mouse model of NPHP. This model displays elevated COX oxylipins in disease and responds to dietary protein intervention; therefore, this model was ideal to test our hypothesis 4 that the increased disease with HP feeding would be associated with further elevation of renal oxylipins.

I was responsible for all aspects of the study and was primarily responsible for the writing of the manuscript as outlined in the Author Contributions section (p.vii-p.x).

Chapter 6

Dietary high protein does not alter renal prostanoids and other oxylipins in normal mice or in mice with inherited kidney disease

This chapter is a portion of manuscript prepared for submission to the Journal of Nutritional Biochemistry

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6.1 Abstract

Ex vivo studies suggest that increased renal prostanoids may mediate effects of HP compared to LP diets on normal and diseased kidneys. However, a short-term HP feeding study in normal male rats failed to demonstrate higher renal prostanoids *in vivo*. Therefore, renal prostanoids in male and female mice, with and without kidney disease, were examined in a longer-term HP feeding study. Weanling normal mice and mice with kidney disease (CD1-*pcy/pcy* mice) were provided standard diets with NP (20%Efrom protein) or HP (35%Efrom protein) for 13 weeks. Renal disease was assessed by histomorphometric analysis of cysts and fibrosis, and measurement of serum urea nitrogen (SUN) and creatinine levels. Targeted analysis of renal oxylipins was performed by HPLC-MS/MS. The HP diet increased kidney size and water content of normal kidneys, and worsened disease (higher kidney weight and fluid content, greater cyst and fibrosis volume) and reduced renal function (elevated SUN). Diseased compared to normal kidneys had higher levels of prostanoids and lower levels of other oxylipins, consistent with previous findings. However, even though the HP diet had physiological effects, the HP diet did not alter renal prostanoids and other renal oxylipins in either normal or diseased kidneys. This study also showed that females have higher levels of renal prostanoids, but lower level of other oxylipins. In conclusion, the effects of HP diets on normal and diseased kidneys are independent of renal oxylipin alterations.

6.2 Introduction

HP diets are popular for the management of overweight and its complications. Several beneficial effects of HP diets include reduction of body weight, increased satiety, reduced hepatic steatosis and improved body composition, blood lipids and fasting insulin levels (1-5). However, HP consumption also results in renal and glomerular hypertrophy and increased glomerular filtration rates, fibrosis and glomerulosclerosis in normal kidneys (6-11), and further accelerates disease in diseased kidneys (12-15). The factor(s) which mediate these diet-induced alterations are poorly understood, but a group of bioactive lipid mediators known as oxylipins have been implicated.

Oxylipins are oxygenated metabolites of polyunsaturated fatty acids which are released from membrane phospholipid by phospholipase A_2 (PLA_2), followed by oxygenation via cyclooxygenase (COX), lipoxygenase (LOX) and cytochrome P450 (CYP) enzymes (16). In the kidney, COX derived oxylipins (prostanoids, such as prostaglandin (PG) E_2 and PGI_2) are important in regulating renal blood flow and glomerular filtration (17, 18). Higher levels of prostanoids also are associated with worsening renal function in experimental kidney disease (19-22), suggesting a potentially negative effect of these elevated COX derived oxylipins in disease. Further evidence for their role in disease is that reduction of prostanoid levels with the use of COX inhibitors or diet is associated with a slowing of renal disease progression (23-27).

It has been suggested that the effects of a HP diet in normal and diseased kidneys are mediated via increased production of renal prostanoids, based on studies from rats provided HP compared to LP diets that show that PLA_2 and COX activities and several prostanoids are elevated *ex vivo* in rat glomeruli isolated from rats provided the HP diets (19-22). However, a recent *in vivo* study showed that a short term (2 wk) HP compared to NP diet that was of

sufficient duration to cause renal hypertrophy, did not alter prostanoid levels in normal male rats (28). Therefore, the objective of the current study was to determine whether a HP compared to a NP diet increases *in vivo* prostanoid levels in normal mouse kidneys and in kidneys of mice with a chronic kidney disease. For this, we used the CD1-*pcy/pcy* (*pcy*) mouse model of nephronophthisis and its normal CD1 counterpart. Nephronophthisis is an inherited form of renal cystic disease that causes chronic kidney disease in children and adolescents (29). In previous studies we have shown that reducing dietary protein slows disease progression in the *pcy* mouse (30,31). This model displays elevated levels of renal prostanoids in disease (32,33), so this model was ideal to test our hypothesis that the increased disease with HP feeding would be associated with further elevation of renal prostanoids.

Another objective of this study was to re-examine sex effects on the renal oxylipin profile in mice. In recent studies we showed that most oxylipins that displayed a sex effect were higher in males compared to females in normal mouse kidneys (34,35), but that oxylipins derived from arachidonic acid (AA) were an exception, as these were higher in females. However, almost all of the AA oxylipins with a sex effect were derived via the COX pathway, so we were unable to determine whether the predominance of AA oxylipins in females was due to this specific fatty acid precursor, or whether it was due to the biosynthetic pathway. Due to the ability to detect and quantify more oxylipins, we re-assessed this issue in the current study.

6.3 Materials and methods

6.3.1 Animal procedures and diets

Weanling normal CD1 and diseased *pcy* mice were randomly assigned to a NP or a HP diet. The diets were based on the standard AIN-93G diet for growing laboratory rodents (36), with casein as the protein source. The NP diet was the AIN-93G diet and contained 200 g

casein/1000 g diet (i.e. 20% of calories from protein), and the HP diet contained 350 g casein/1000 g diet (i.e. 35% of calories from protein), which is the upper limit for protein according to the Dietary Reference Intakes (37). Cornstarch and casein were substituted for each other to make the diets isocaloric (diet details are provided in Table 6.1). Each diet group had 6 male and 6 female normal and diseased mice for a total of 48 mice. Diets were freshly prepared twice per month and stored in sealed containers at -20°C until feeding. All diet ingredients were purchased from Dyets Inc. (Bethlehem, PA).

Table 6.1 Diet formulations and macronutrient composition

Ingredients (g/1000g diet)	NP	HP
Casein (87%)	200	350
Cornstarch	397.5	247.5
Dextrinized cornstarch	132	132
Sucrose	100	100
Cellulose fibre	50	50
Soybean oil with TBHQ	70	70
AIN-93G mineral mix	35	35
AIN-93VX vitamin mix	10	10
L-Cystine	3	3
Choline bitartarate	2.5	2.5
Energy content		
Protein (% energy)	20	35
Carbohydrate (% energy)	59	44
Fats (% energy	21	21

NC, normal-protein; HC, high-protein; TBHQ, tert-butylhydroquinone.

The dietary interventions were carried out for 13 weeks and all mice were individually housed in temperature and humidity-controlled environments with a 12-hour day/night cycle. Mice were monitored daily and body weights were measured bi-weekly. Mice were anesthetized

to surgical plane using isofluorane and euthanized via exsanguination. Body and kidney weights were recorded, and the right kidney was snap frozen in liquid nitrogen and stored at -80°C until oxylipin analysis. The left kidney was fixed by placing it in 10% formalin for 24h, followed by transfer to PBS at 4°C until further processing. All animal procedures were approved by the Institutional Animal Care Committee and adhered to the guidelines of the Canadian Council on Animal Care.

6.3.2 Histology and renal function analysis

Formalin fixed kidneys were embedded in paraffin, sectioned at 5 µm and the kidney sections were stained with Masson's trichrome to measure cyst area and fibrosis as previously described (38, 39). A Nikon D600 FX DSLR camera equipped with macro rings between the body and a 60mm F2.8 Macro lens (Nikon Corporation, Mississauga, Canada) was used to capture 25X images of the whole kidney sections. Quantitative analysis of cyst and fibrosis areas of the whole kidney sections were carried out by ImageJ software (40). The ratios of the cyst and fibrosis areas related to the whole sections were multiplied by the left kidney weight to yield the cyst and fibrosis volumes, respectively, as described (39). Serum creatinine and serum urea nitrogen (SUN) were measured with a Cobas C111 auto analyzer (Roche Diagnostics, Indianapolis, IN, USA) using commercial kits (Roche Diagnostics).

6.3.3 Oxylipin analysis

Frozen kidneys were lyophilized and pulverized into a fine powder, and a representative portion was homogenized in ice-cold Tyrode’s salt solution (pH 7.6) in a 1:28 weight/volume (mg/mL) ratio. After homogenization, Triton X-100 was added to achieve a final concentration of 0.01%. Deuterated internal standards (Cayman Chemical, MI, USA) and 6.5µL of antioxidant

cocktail (0.2 g/L BHT, 0.2 g/L EDTA, 2 g/L triphenylphosphine, and 2 g/L indomethacin in MeOH:EtOH:H_2O (2:1:1,v/v/v)) were added to 200 µL tissue homogenates used for analysis. Samples were adjusted to pH<3 and solid phase extraction was with Strata-X SPE columns (Phenomenex, CA, USA) preconditioned with methanol and pH 3 water. Samples were loaded onto the Strata-X solid phase extraction columns (Phenomenex, CA, USA), washed with pH 3 water and hexane, and eluted with methanol. Nitrogen evaporated samples were then re-suspended in the mobile phase (water/acetonitrile/acetic acid, 70/30/0.02, v/v/v) for analysis by HPLC-MS/MS (QTRAP 6500; Sciex, Ontario, Canada), based on methods developed by Deems et al. (41). A complete description of methods, oxylipins that were scanned for, deuterated internal standards that were used and mass transition and retention times, is detailed in (35, 42). Detection and quantification limits were set at 3 and 5 levels above background, respectively. Quantification of oxylipins was determined using the stable isotope dilution method (43), and amounts expressed as pg/mg of dry tissue. Over 150 renal oxylipins were scanned for and 67 oxylipins were detected and quantified in normal and diseased kidneys.

6.3.4 Statistical analysis

Data were analyzed using the GLM procedure of SAS (SAS, version 9.4, Cary, NC, USA). Diet, disease and sex effects of all parameters except cyst volume was tested by 3-way ANOVA. However, as cyst development only occurs in diseased animals, diet and sex effect on cyst parameters were tested by 2-way ANOVA. Normality of data was tested using Shapiro-Wilk's Statistic ($W > 0.05$ for normally distributed data). If the data did not follow a normal distribution even if log transformed, a nonparametric test was used (Kruskal-Wallis). Post hoc analysis was done by Tukey's test for simple effect comparisons when interactions were present

or when the Kruskal-Wallis test indicated the presence of differences. All data were presented as mean ±SEM. Significance was set at $p< 0.05$ for main, interaction and simple effects.

6.4 Results

6.4.1 Dietary HP effects on normal and diseased kidneys

In normal mice the HP compared to the NP diet resulted in higher kidney weights (23% higher in male, 12% higher in female), water content (4% higher in male, 1.4% higher in female) and SUN (26% higher in male, 45% higher in female), and no effect on body weight, kidney weight relative to body weight and serum creatinine (Fig. 6.1A-F). Kidneys from diseased mice had significant cyst formation and fibrosis (Fig. 6.2), resulting in larger kidneys that contained more water than normal kidneys (Fig. 6.1B,D). Dietary HP compared to NP in diseased mice resulted in worsening of disease, as shown by the higher kidney weights (8% higher in male, 31% higher in female), water content (8% higher in male,10% higher in female) (Fig. 6.1B,D), cyst volume (36% higher in male, 60% higher in female) and fibrous volume (53% higher in male, 44% higher in female) (Fig. 6.2). Diseased female, but not male mice also had higher SUN levels, and dietary HP further increased SUN levels in both male and female diseased mice (Fig. 6.1E).

6.4.2 Dietary HP effects on prostanoids and other oxylipins

To examine the role of prostanoids in disease and with HP feeding, renal prostanoids and other oxylipins were quantified. In diseased compared to normal kidneys, a total of 42 oxylipins were altered in either or both sexes. COX oxylipins (prostanoids) were higher in diseased kidneys, i.e., 6 out of the 11 individual COX oxylipins were 20% to 159% higher in diseased compared to normal kidneys (either main or simple effects). In addition, the non-enzymatically

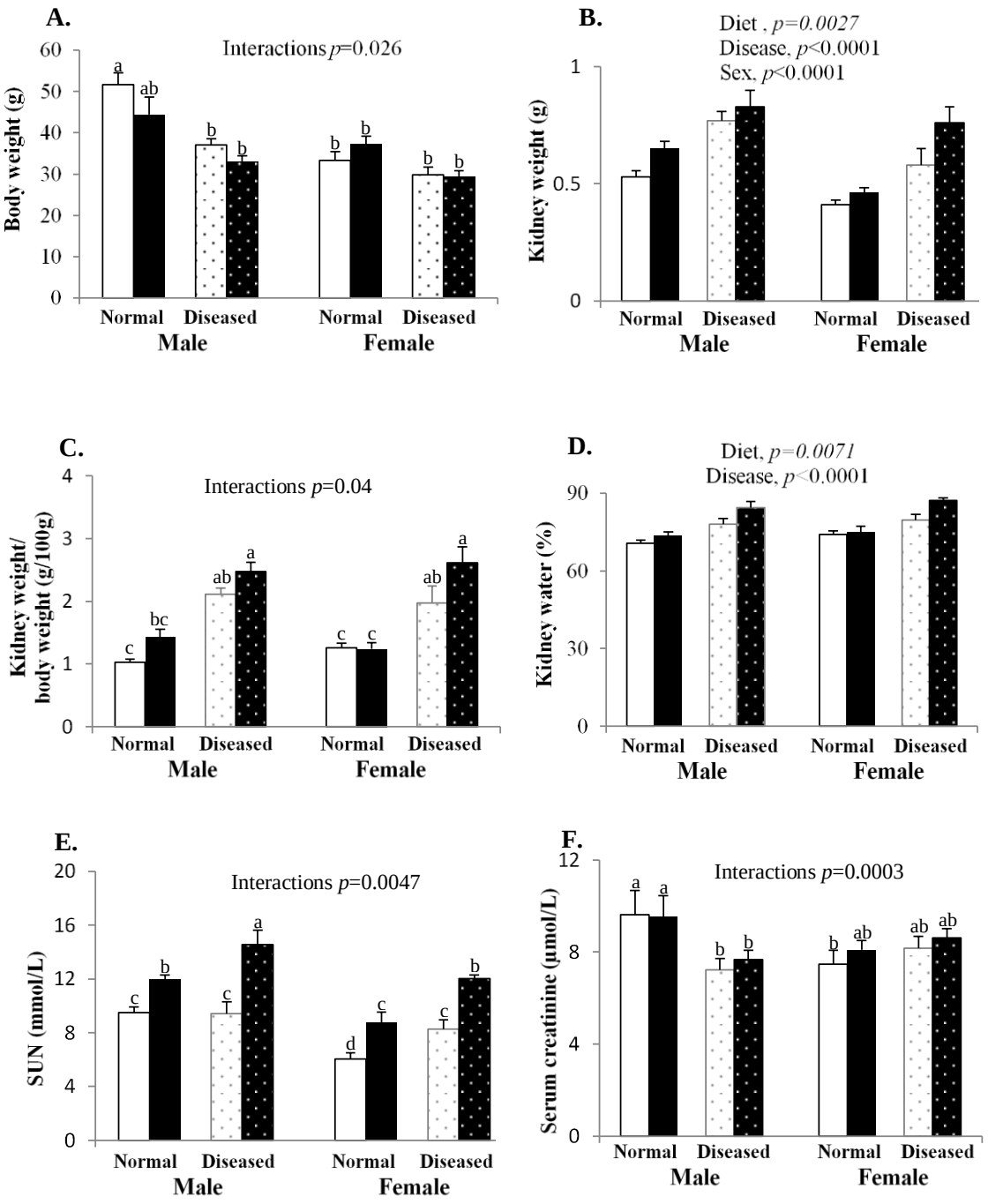

Fig. 6.1 Body weight (A), kidney parameters (B-D) and blood biochemistry (E-F) of normal (CD1) and diseased (*pcy*) mice provided NP (white bars) and HP (black bars) diets. Values are mean±SEM.

For interactions, values with differing lower-case letters are different.

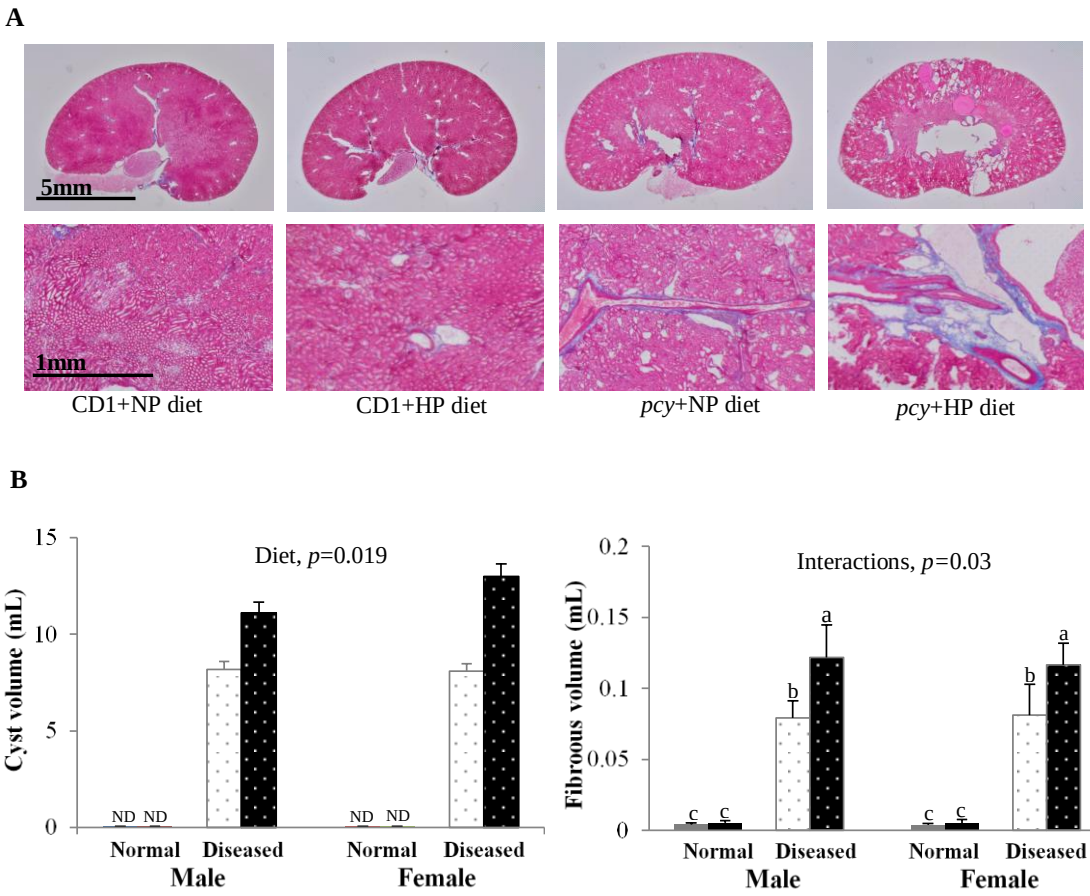

Fig. 6.2 A. Representative images showing cysts in whole kidney sections (upper panel), and fibrosis in enlarged section areas (lower panel) in normal (CD1) and diseased (*pcy*) mice provided NP (white bars) and HP (black bars) diet.

B. Cyst and fibrosis volume in normal (CD1) and diseased (*pcy*) mice provided NP (white bars) and HP (black bars) diets. Values are mean±SEM. ND, not detected.

For interactions, values with differing lower-case letters are different.

derived isoprostane, 8-iso-$PGF_{2\alpha}$III, was higher in the diseased kidneys. However, despite the fact that dietary HP increased the size of normal kidneys and worsened diseased kidney progression in diseased kidneys, HP feeding did not alter the levels of prostanoids or isoprostanes in normal or diseased kidneys. The only exception to this was the higher levels of 6-keto $PGF_{1\alpha}$ with HP feeding, but this only occurred in diseased female kidneys (Table 6.2).

With respect to LOX derived oxylipins, 27 out of 38 individual oxylipins were altered in diseased kidneys either in both sexes (24), males (1) or females (2); of these, 26 were lower and 1 was higher in diseased compared to normal kidneys. For CYP derived oxylipins, 6 out of 16 (1 in NP mice only) were lower and 3 out of the 16 (1 in males only) were higher in diseased kidneys. Similar to the lack of effect of HP feeding on prostanoids in normal and diseased kidneys, the HP diet had little effect on LOX and CYP derived oxylipins. Only the LOX oxylipin 12-HEPE and the CYP derived 18-HEPE in female kidneys were altered by HP feeding (Tables 6.3&4).

6.4.3 Sex differences in kidneys

Sex differences observed in these mice included higher body weights and serum creatinine in normal male mice provided the NP, but not the HP diet. Males also had higher kidney weights overall, but when kidney weights were normalized to body size there were no sex differences. SUN was higher in males compared to females, except in diseased mice provided the NP diet. No other parameters in normal or diseased mice were different in males compared to females.

6.4.4 Sex differences in kidney oxylipins

In contrast to the few sex differences on kidney parameters and disease progression, there were many kidney oxylipins that displayed sex differences in these mice. These differences appeared to be determined by the enzyme pathway responsible for their formation (Tables 6.2-4). Oxylipins derived via the COX pathway that displayed a sex effect were higher in females, while oxylipins with a sex effect formed via the LOX and CYP pathway were higher in males. For example, 9 out of 11 individual COX oxylipins were higher in females in both normal and

Table 6.2 Renal COX oxylipins and isoprostanes in normal CD1 and diseased *pcy* (CD1-*pcy/pcy*) mice.

	Sex	Male				Female							
	Genotype	Normal		Disease		Normal		Disease					
	Diet	NP (n=6)	HP (n=6)	NP (n=6)	HP (n=6)	NP (n=6)	HP (n=6)	NP (n=6)	HP (n=6)	Diet	Disease	Sex	Interactions
Oxylipins	PUFA												
$PGF_{1\alpha}$	DGLA	4.82±0.28^{c}	3.72±0.33^{c}	6.02±0.66^{b}	5.32±0.49^{b}	7.04±0.58^{b}	6.63±0.2^{b}	12.96±1.85^{a}	12.78±1.83^{a}				s*d
PGE_1	DGLA	21.8±2.21	14.0±0.73	19.7±3.02	17.7±1.42	31.6±6.09	24.7±1.90	29.9±3.51	30.9±5.24				
PGD_2	AA	297±118	150±17.2	367±97.0	228±30.6	412±118	264±11.9	483±85.1	762±139		↑	F	
PGE_2	AA	123±15.4	103±9.2	145±26.4	158±20.1	276±37.4	242±31.2	267±29.6	306±57.8			F	
15k-PGE_2	AA	1.80±0.29	2.08±0.42	2.42±0.22	2.22±0.41	14.8±3.91	18.4±3.73	37.1±10.2	25.1±2.31		↑	F	
$PGF_{2\alpha}$	AA	102±6.77	82.0±5.45	138±16.36	130±6.82	202±14.84	191±6.81	248±28.08	287±21.34		↑	F	
6k-$PGF_{1\alpha}$	AA	193±21.9^{d}	152±18.9^{d}	480±72.6^{c}	443±49.3^{c}	397±24.6^{c}	365±18.0^{c}	919±99.9^{b}	1417±167^{a}			F	p*d
TXB_2	AA	22.8±2.10^{c}	20.6±1.70^{c}	32.7±4.05^{c}	33.1±2.09^{c}	45.1±4.99^{bc}	41.2±2.53^{c}	76.5±13.7^{ab}	98.6±14.9^{a}				p*d*s
Δ^{17}6-k-$PGF_{1\alpha}$	EPA	6.21±0.81^{c}	4.74±0.71^{c}	11.7±2.18^{b}	9.97±1.45^{b}	9.17±0.89^{b}	11.6±1.94^{b}	28.1±4.72^{a}	41.0±3.45^{a}				d*s
$PGF_{3\alpha}$	EPA	16.5±1.45	14.2±2.08	16.3±1.67	12.7±0.81	13.9±1.08	15.3±1.07	16.6±0.98	16.2±1.64				
PGE_3	EPA	15.4±1.94	10.6±1.17	10.1±1.17	10.8±1.28	26.1±2.57	29.8±3.46	22.5±2.03	28.0±2.85				
Isoprostanes													
5-iso PGF2αVI	AA	12.9±1.49	10.1±1.41	10.7±2.3	6.8±0.32	19.6±1.37	19.4±2.18	16.2±4.0	17.1±0.98				
8-iso PGF2αIII	AA	19.4±1.48	16±0.51	19.6±1.28	19.7±0.59	36.4±3.1	32.6±2.33	40.9±4.43	44.1±3.51		↑	F	

Values are in pg/mg dry tissue and expressed in mean ± SEM. Values with differing lower-case superscript letters indicate simple effect differences. ↑, higher in disease; ↓, lower in disease; F, higher in female; d*s, interaction between disease and sex; p*d, interaction between diet and disease; p*d*s, interaction between diet, disease and sex, $p<0.05$.

Abbreviations: AA, arachidonic acid; COX, cyclooxygenase, DGLA, dihomo-γ-linolenic acid; EPA, eicosapentanoic acid, HP, high protein, k, keto; NP, normal protein; PG, prostaglandin; PUFA, polyunsaturated fatty acid, TX, thromboxane.

Table 6.3 Renal LOX oxylipins in normal CD1 and diseased *pcy* (CD1-*pcy/pcy*) mice.

	Sex	Male				Female							
	Genotype	Normal		Disease		Normal		Disease					
	Diet	NP (n=6)	HP (n=6)	NP (n=6)	HP (n=6)	NP (n=6)	HP (n=6)	NP (n=6)	HP (n=6)	Diet	Disease	Sex	Interactions
Oxylipins	PUFA												
9-HODE	LA	5561±455^{ab}	4822±386^{ab}	3995±457^{b}	4234±539^{b}	5086±643^{ab}	5494±407^{ab}	5751±613^{a}	6240±551^{a}				d*s
9-oxoODE	LA	1868±149	2128±182	1823±250	1599±197	1808±159	1914±171	1419±101	1502±205		↓		
13-HODE	LA	3812±257	3356±271	2712±288	2742±301	3903±677	3997±205	3517±381	3919±220		↓	F	
13-oxoODE	LA	1481±175	1614±234	851±93.9	1068±271	1223±104	1285±105	745±133	680±86.0		↓	M	
9,10,13-triHOME	LA	984±76.4	879±74.2	429±30.1	447±42.8	862±58.7	822±61.1	346±39.8	361±14.4		↓	M	
9,12,13-triHOME	LA	319±22.9	294±27.4	153±10.8	156±13.7	283±20.9	271±15.0	121±13.0	131±5.30		↓	M	
13-HOTrEγ	GLA	141±8.2	112±10.5	109±15.6	108±6.40	161±21.8	198±35.5	110±14.4	125±17.8		↓	F	
8-HETrE	DGLA	87.7±3.95	87.0±5.90	54.4±4.66	51.9±3.32	72.0±4.94	73.5±4.44	46.5±9.07	49.4±5.88		↓	M	
15-HETrE	DGLA	90.3±5.44^{a}	82.4±4.30^{a}	47.8±2.84^{b}	45.2±3.27^{b}	58.5±5.06^{b}	56.1±2.75^{b}	43.4±5.99^{b}	51.7±2.86^{b}				d*s
LTB_4	AA	3.22±0.39	3.50±0.22	2.28±0.31	2.32±0.28	2.92±0.25	3.12±0.38	1.99±0.15	2.14±0.13		↓		
5-HETE	AA	877±37.7^{b}	830±51.9^{b}	668±60.6^{b}	690±49.5^{b}	1142±33.9^{a}	1122±95^{a}	639±136.8^{b}	801±78.5^{b}				d*s
5-oxoETE	AA	81.4±6.44	109±18.81	109±21.8	94.1±16.53	84.2±7.71	84.5±9.66	75.5±6.4	74.2±7.7				
5,15 diHETE	AA	7.44±0.52	11.19±1.40	3.87±0.36	4.62±0.55	10.14±1.1	9.63±1.13	3.20±0.84	4.44±0.47		↓	M	
8,15 diHETE	AA	201±20.2	223±9.81	116±11.7	119±7.07	199±20.1	242±15.2	111±24.6	146±16.5		↓		
8-HETE	AA	199±17.0	214±13.1	158±25.5	172±22.2	233±6.90	275±20.0	166±24.8	179±12.5		↓	F	
9-HETE	AA	254±14.8	267±13.5	184±25.5	199±21.0	297±11.5	326±26.8	169±35.1	207±23.0		↓		
11-HETE	AA	354±18.4	340±16.5	363±23.5	376±22.4	522±21.9	543±22.9	603±15.2	614±19.2		↑	F	
12-HETE	AA	1279±114^{a}	1074±127^{a}	1583±109^{a}	1642±95.1^{a}	992±69.4^{b}	1067±67.1^{ab}	990±150^{b}	1390±73.3^{ab}				p*d
12-oxoETE	AA	614±106	950±170	891±261	674±155	603±89.1	486±81.9	518±65.1	451±36.1			M	
tetranor 12-HETE	AA	8.99±0.81	8.76±0.77	5.38±0.50	5.95±0.73	11.39±2.02	9.45±1.34	7.27±0.88	7.8±1.68		↓		
15-HETE	AA	1357±49.2	1297±58.2	844±20.3	872±49.1	1899±57.9	1855±165	1101±163.7	1436±71.1		↓	M	
15-oxoETE	AA	639±34.1	741±33.5	326±43.7	328±32.9	670±27.8	683±46.1	290±47.4	291±18.6		↓		
9-HOTrE	ALA	622±71.0	514±50.4	361±55.9	384±48.4	426±93.0	517±84.7	370±88.1	537±86.7		↓	M	
9-oxoOTrE	ALA	52.1±7.45	50.7±10.2	40.2±6.46	36.2±3.70	33.5±5.97	43.6±7.17	27.5±2.64	30.7±7.32		↓	M	
13-HOTrE	ALA	243±29.1	206±27.8	200±35.1	199±33.1	214±55.7	281±50.4	219±36.7	259±33.0				
5-HEPE	EPA	43.1±3.38	46.4±9.48	15.9±1.58	21.8±2.86	44.4±5.92	56.4±8.37	21.7±6.73	28.0±4.81		↓		
11-HEPE	EPA	31.3±1.82^{b}	29.7±3.81^{b}	26.4±1.32^{b}	27.8±1.16^{b}	37.8±2.78^{ab}	53.9±6.21^{a}	54.2±3.32^{a}	55±5.70^{a}				p*d*s
12-HEPE	EPA	322±27.9^{a}	240±35.7^{a}	317±19.5^{a}	337±15.8^{a}	187±12.4^{b}	272±46.7^{a}	210±40.5^{b}	296±27.8^{a}				p*s
15-HEPE	EPA	32.2±2.07	29.9±2.40	22.6±1.33	26.0±1.96	37.2±2.42	40.0±4.51	35.1±4.53	43.6±4.41			F	
4-HDoHE	DHA	769±41.8	582±56.9	817±90.8	742±54.8	539±97.3	621±90.3	488±57.9	485±46.7			M	
7-HDoHE	DHA	166±8.16	165±5.22	162±23.2	166±25.9	122±9.87	158±20.3	109±11.8	99.0±9.63		↓	M	
8-HDoHE	DHA	692±13.6	587±45.2	582±59.5	579±52.8	457±37.6	540±61.5	330±42.0	362±38.6		↓	M	
10-HDoHE	DHA	297±10.3^{a}	262±22.0^{a}	242±19.3^{a}	255±20.2^{a}	216±18.3^{a}	265±25.7^{a}	145±18.5^{b}	160±14.3^{b}				d*s
11-HDoHE	DHA	213±7.41	196±15.7	193±14.6	194±15.0	152±8.19	175±22.1	103±15.4	129±12.1		↓	M	
13-HDoHE	DHA	1041±30.5^{a}	909±60.1^{ab}	845±38.5^{a}	831±63.5^{ab}	799±66.6^{b}	948±68.0^{ab}	664±54.1^{b}	792±42.4^{ab}				p*s
14-HDoHE	DHA	610±41.6^{a}	475±38.8^{a}	693±37.6^{a}	693±44.1^{a}	427±45.1^{b}	527±71.2^{ab}	436±62.8^{b}	577±23.4^{ab}				p*s
16-HDoHE	DHA	395±18.6	332±22.2	224±18.2	241±25.8	275±28.0	295±21.5	156±17.9	189±12.1		↓	M	
17-HDoHE	DHA	1001±42.5	852±65.7	798±41.0	865±73.8	695±60.3	785±69.3	573±72.6	669±30.8		↓	M	

Values are in pg/mg dry tissue and expressed in mean ± SEM. Values with differing lower-case superscript letters indicate simple effect differences. ↑, higher in disease; ↓, lower in disease; F, higher in female; M, higher in male; d*s, interaction between disease and sex; p*d, interaction between diet and disease; p*s, interaction between diet and sex, p*d*s, interaction between diet, disease and sex $p<0.05$.

Abbreviations: AA, Arachidonic acid; ALA, α-linolenic acid; DGLA, Dihomo-γ-linolenic acid; DHA, Docosahexaenoic acid; DiHETE, Dihydroxy-eicosatetraenoic acid; DiHETrE, Dihydroxy-eicosatrienoic acid; DiHODE, Dihydroxy-octadecadienoic acid; EPA, Eicosapentaenoic acid; GLA, γ-linolenic acid; HDoHE, Hydroxy-docosahexaenoic acid; HEPE, Hydroxy-eicosapentaenoic acid; HETE, Hydroxy-eicosatetraenoic acid; HETrE, Hydroxy-eicosatrienoic acid; HHTrE, Hydroxy-heptadecatrienoic acid; HODE, Hydroxy-octadecadienoic acid; HOTrE, Hydroxy-octadecatrienoic acid; LA, Linoleic acid; oxo-ETE, oxo-Eicosatetraenoic acid; oxo-ODE, oxo-octadecadienoic acid; oxo-OTrE, oxo-octadecatrienoic acid; TriHOME, Trihydroxy-octadecenoic acid.

Table 6.4 Renal CYP oxylipins in normal CD1 and diseased *pcy* (CD1-*pcy/pcy*) mice.

	Sex	Male				Female							
	Genotype	Normal		Disease		Normal		Disease					
	Diet	NP (n=6)	HP (n=6)	NP (n=6)	HP (n=6)	NP (n=6)	HP (n=6)	NP (n=6)	HP (n=6)	Diet	Disease	Sex	Interactions
Oxylipins	PUFA												
9,10-EpOME	LA	6.55±1.05	5.03±0.42	10.1±1.66	9.84±1.16	5.83±1.04	5.71±0.97	9.13±1.74	8.14±1.96		↑		
9,10-DiHOME	LA	26.1±2.00	23.9±2.78	25.3±3.24	26.3±4.29	18.6±1.65	24.4±2.13	21.3±2.62	24.8±2.13				
12,13-EpOME	LA	10.7±1.50	8.80±0.84	14.4±1.96	14.4±1.86	10.5±1.49	8.76±0.93	13.7±2.60	13.6±2.75		↑		
12,13-DiHOME	LA	41.1±1.56	39.3±4.22	45.4±5.07	46.8±5.67	36.0±6.60	35.4±2.24	31.3±3.12	34.0±2.14			M	
8,9-DiHETrE	AA	9.54±0.89	9.61±0.61	8.38±0.59	9.96±0.94	10.7±0.58	11.3±1.06	8.15±1.49	10.4±1.22				
11,12-DiHETrE	AA	8.03±0.48	8.18±0.61	6.90±0.33	7.53±0.36	8.13±0.56	8.58±0.87	5.95±1.06	7.59±0.84		↓		
14,15-DiHETrE	AA	14.8±0.58	15.1±0.79	11.2±0.51	12.2±0.37	10.2±0.27	10.8±0.79	7.50±0.94	8.50±0.48		↓	M	
16-HETE	AA	155±10.1	161±6.99	66.7±2.82	80.1±3.24	165±14.5	164±14.4	58.3±5.15	68.6±4.41		↓		
17-HETE	AA	$4.56±0.32^{a}$	$4.32±0.2^{ab}$	$2.85±0.34^{c}$	$3.96±0.4^{bc}$	$4.83±0.74^{a}$	$3.94±0.16^{ab}$	$2.88±0.26^{c}$	$3.09±0.39^{bc}$				p*d
18-HETE	AA	5.79±0.48	6.48±0.53	5.36±0.29	5.97±0.43	5.85±0.26	5.81±0.56	4.29±0.46	5.17±0.39		↓		
14,15-DiHETE	EPA	41.9±3.47	44.6±1.81	41.2±2.25	41.0±2.52	12.0±0.75	16.5±2.59	17.2±2.86	15.1±1.38			M	
17,18-DiHETE	EPA	46.4±8.15	46.7±3.72	49.0±3.36	46.0±5.08	15.2±1.14	19.2±2.26	19.4±1.99	15.6±0.94			M	
18-HEPE	EPA	$104±3.42^{b}$	$92.6±5.47^{b}$	$41.9±3.11^{b}$	$47.9±3.03^{b}$	$99.6±7.2^{b}$	$127±18.8^{a}$	$55.6±12.7^{b}$	$84.4±10.4^{a}$				p*s
16,17-DiHDPE	DHA	24.24±2.29	19.28±1.98	21.24±0.68	21.27±0.67	3.40±0.19	4.43±0.55	3.47±0.47	3.70±0.24			M	
19,20-DiHDPE	DHA	$174±13.5^{b}$	$150±14.1^{b}$	$236±10.7^{a}$	$244±10.4^{a}$	$34.7±1.09^{c}$	$45.5±4.21^{c}$	$40.0±2.05^{c}$	$38.5±2.54^{c}$				p*d*s
20-HDoHE	DHA	637±34.6	531±81.1	473±36.8	498±15.8	416±34.2	465±50.8	289±51.5	341±30.0		↓	M	

Values are in pg/mg dry tissue and expressed in mean ± SEM. Values with differing lower-case superscript letters indicate simple effect differences. ↑, higher in disease; ↓, lower in disease; F, higher in female, M, higher in male; p*d, interaction between diet and disease; p*s, interaction between diet and sex, p*d*s, interaction between diet, disease and sex, $p<0.05$.

Abbreviations: AA, Arachidonic acid; DHA, Docosahexaenoic acid; DiHDPE, Dihydroxy-docosapentaenoic acid; DiHETE, Dihydroxy- eicosatetraenoic acid; DiHETrE, Dihydroxy-eicosatrienoic acid; DiHOME, Dihydroxy-octadecenoic acid; EpOME, Epoxy-octadecenoic acid; EPA, Eicosapentaenoic acid; HDoHE, Hydroxy-docosahexaenoic acid; HETE, Hydroxy-eicosatetraenoic acid; LA, Linoleic acid.

diseased kidneys (8), or only in diseased kidneys (1). In addition, 1 out of 2 non-enzymatically derived isoprostanes was higher in female kidneys (Table 6.2). Sex differences also were observed in 29 out of 38 individual oxylipins derived via the LOX pathway; of these, 21 were higher in males either overall (15), in those provided the NP diet only (4), in normals only (1) or in diseased mice only (1). Of the remaining 9 that were higher in females, 7 of these (9- and 13-HODE, 13-HOTrEγ, 11- and 15-HETE, and 11- and 15-HEPE) are also produced via the COX pathway (44-47) (Table 6.3). With respect to CYP oxylipins, 8 out of 16 oxylipins displayed a sex effect; of these, 7 were higher in males in both normal and diseased kidneys (6), or only in diseased kidneys (1) (Table 6.4).

6.5 Discussion

Kidney prostanoids have been implicated in dietary HP effects on kidney hypertrophy and disease progression based on comparisons to LP diets and studies of isolated glomeruli. These studies documented increased membrane-associated PLA_2 and COX enzyme activity, and higher production of COX derived prostanoids (e.g. PGE_2, 6-keto $PGF_{1\alpha}$ and TxB_2) in glomeruli isolated from normal or bilateral ureteral obstructed rats provided HP (40% of calories from protein) compared to LP (6% of calories) diets (19,22,48). Similar results were found in isolated glomeruli from rats with diabetes mellitus, Heymann nephritis or partial renal ablation that were provided 40% vs 8.5% protein diets (20), in partially nephrectomized rats provided 56% vs 9.5% protein diets (21), and in normal rats provided 50% vs 6% protein diets (49). In several of these studies, urinary production of prostanoids also was increased in the rats on the HP vs. LP diets (48,49), further suggesting a renal prostanoid inducing effect of HP diets.

The current study, however, demonstrated that a HP diet does not alter renal prostanoids, even though it increases kidney size in normal kidneys and worsens disease progression in

diseased kidneys. There may be several reasons for this apparent discrepancy. One such reason may be that the comparisons of HP diets in the prior studies were with LP diets that were deficient in protein. Consistent with the current findings, in a recent short-term study we found that kidney levels of prostanoids were not elevated in rats provided 53% protein compared to an adequate protein level (15%) in normal adult rats, even though the higher level of dietary protein also resulted in renal hypertrophy (28). Since HP diets are promoted in comparison to adequate protein diets, the current study examined the role of renal prostanoids and other oxylipins in HP compared to NP rats provided with adequate protein in both normal mice and mice with kidney disease.

The *pcy* model of nephronophthisis was used for this study because HP diets have greater and different effects on diseased compared to normal kidneys. Even with long-term dietary HP treatment the increased level of fibrosis is still minimal in normal animals (7,8), while HP feeding in diseased kidneys has greater effects on disease progression, including fibrosis (14,50). In the current study, disease in the *pcy* mice was significantly increased by the HP diet, as evidenced by the increased cyst and fibrosis volumes. Renal function also was affected, and worsened by HP feeding, as evidenced by the increased SUN levels. At the end of the study, however, renal function was not yet severely compromised, as indicated by normal serum creatinine levels. The higher serum creatinine level in normal males is likely a reflection of muscle mass (51,52), as this pattern closely reflected body weight.

Cystic kidney diseases display altered renal COX and PLA_2 protein and mRNA levels, as well as elevated renal prostanoids (53-56). Renal COX derived oxylipins are elevated even in early stages of disease, whereas LOX and CYP oxylipins are generally lower, especially in later stages of disease (33,35). Consistent with these prior findings, COX oxylipins were higher and

LOX and CYP oxylipins were generally lower in diseased compared to normal kidneys in the current study. If HP diets have a role in altering renal oxylipin levels, this may be more easily manifested in *pcy* mouse kidneys that already have perturbed oxylipin metabolism. However, this was not the case, as HP feeding also had few effects on oxylipins derived via the LOX and CYP pathways, consistent with our findings in rats provided HP vs NP diets in the short-term (28). This indicates that the effects of HP compared to NP diets on normal or diseased kidneys are not related to alterations in renal oxylipin metabolism.

In addition to comparing the HP diet to a NP rather than a LP diet, another reason for the lack of effect of dietary HP on prostanoids in the current study may be because the prostanoids and other oxylipins were measured in intact kidneys removed at termination, reflecting *in vivo* levels, whereas in prior studies the production of oxylipins were measured *ex vivo* from isolated glomeruli (19-22). The current results are consistent with our prior short-term study in which normal rats provided with 53%E compared to 8.5%E protein diets did not display increased renal prostanoids in intact kidneys (28), indicating differences between *in vivo* and *ex vivo* measurements, even when comparing HP to LP diets. Further, while the increased urinary prostanoids observed in HP vs LP fed rats in other studies (48,49), also may suggest increased renal prostanoid production with HP feeding, the kidney may not have been the source of these oxylipins. Other tissues also release prostanoids into the blood that is filtered by the kidney to produce urine, and altered urine prostanoid levels do not always correspond to alterations in renal prostanoids (55).

Therefore, the current study, along with the short-term HP study in normal rats (28), indicates that increased renal prostanoid or other oxylipin production does not occur with HP compared to NP feeding. The physiological effects of HP compared to NP diets in normal and

diseased kidneys therefore likely occur via other mechanisms. Although the exact mechanism not well known, it has been found that HP diets increase renal blood flow and glomerular hyperfiltration and enhance expression of proinflammatory genes (13-15). Excessive filtration of amino acids due to HP diets induces glomerular scarring and inflammation, and ultimately leads to fibrosis and renal hypertrophy (57-59). A strong correlation between proteinuria and renal lesions was observed when rats were provided 35%E from protein for a long time (60).

With respect to the cystic renal disease model used herein, branched chain amino acids enhance proliferation of cyst-lining cells and upregulate mTOR and MAPK/ERK pathways in another form of cystic kidney disease (61). HP diets increase renal fibrosis (7,8,62), whereas dietary protein restriction reduces renal fibrosis (63). How HP diet mediates these effects is not clear, but Kruppel-like factor-15 (KLF15) is associated with increased renal fibrosis (64), and its downregulation and renal fibrosis are reversed with dietary protein restriction (65).

There are few studies that have examined sex differences in the renal oxylipin profile. In previous studies (34,35) we observed that AA derived oxylipins that displayed a sex effect were generally higher in females, while oxylipins made from other fatty acids that displayed a sex effect were generally higher in males. Most of these AA oxylipins were derived via the COX pathway, but because of the limited number of oxylipins that displayed a sex effect, it was not possible to conclude whether the sex difference patterns were due to fatty acid precursor or due to biosynthetic pathway. With the availability of more sensitive instrumentation in the current study, 20-52% more renal oxylipins were detected compared to our previous studies (34,35), allowing us to re-examine this question. The current findings reveal that COX derived oxylipins and the non-enzymatically derived isoprostanes were higher in females, and LOX and CYP oxylipins were higher in males. Even though some oxylipins in the LOX pathway were higher in

females, most of these are known to also be produced by the COX pathway (44-47), providing further evidence of sex differences in oxylipins due to differences in pathway metabolism. Female rats and mice have greater expression of COX-2 and PGE_2 synthase (66,67), and estradiol suppresses the activity of 15-hydroxyPG dehydrogenase, a PG-degrading enzyme, in rat renal tissue (68,69) and in human fetal kidney tissue (70), consistent with the higher level of COX oxylipins in female compared to male kidneys. However, further studies are clearly needed to confirm the current findings and to further elucidate potential mechanisms regulating the effect of sex on renal oxylipin formation.

In conclusion, a HP compared to NP diet does not alter renal prostanoids and other oxylipins in normal and diseased kidneys, even though it increases normal kidney size and worsens disease in diseased kidneys. Further, renal COX oxylipins are higher in females, while oxylipins produced via LOX and CYP pathways are higher in males.

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6.7 Transition to next chapter

In chapter 6, we showed that dietary HP does not alter renal prostanoids and other renal oxylipins in normal as well as in diseased kidneys, despite having physiological effects on both normal and diseased kidneys. Our results are consistent with a previous *in vivo* study where a HP diet led to kidney hypertrophy without altering renal prostanoids in normal kidneys (28). We also showed that the sex difference in renal oxylipins was associated with pathway more than precursor fatty acid.

In chapters 4 and 6, we showed that COX oxylipins are consistently higher in diseased kidneys. A previous study with the Han:SPRD-Cy rat model of NPHP showed that selective inhibition of COX2 oxylipins ameliorated the COX oxylipin alterations and reduced disease progression (71-73). Whether similar effects occur in ADPKD was tested in the next chapter (objective 5).

I was responsible for all aspects of the study and was primarily responsible for the writing of the manuscript as outlined in the Author Contributions section (p.vii-p.x).

Chapter 7

Cyclooxygenase 2 inhibition slows disease progression and improves the altered renal lipid mediator profile in the Pkd2$^{WS25/-}$ mouse model of autosomal dominant polycystic kidney disease

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7.1 Abstract

Increased levels of cyclooxygenase (COX) derived oxylipins is the earliest and most consistent alteration in the renal oxylipin profile in diverse models of cystic kidney diseases. Therefore, we examined whether a COX2 inhibitor would reduce disease progression in the Pkd2$^{WS25/-}$ mouse model of autosomal dominant polycystic kidney disease (ADPKD).

Weanling normal and diseased male Pkd2 mice were provided diets that contained 0 or 50 mg celecoxib/kg body weight/day, for 13 weeks. Renal disease and function were assessed by histomorphometric analysis of renal cysts and measurement of serum creatinine and urea nitrogen (SUN) levels. Targeted lipidomic analysis of renal oxylipins was performed by HPLC-MS/MS.

Diseased mice had significant cyst involvement and reduced renal function as indicated by elevated serum creatinine and SUN. Celecoxib reduced cyst area by 48%, cyst volume by 70%, and serum creatinine and SUN by 20% and 16%, respectively. Consistent with our previous studies, 8 of the 11 COX derived oxylipins were higher in diseased kidneys. In addition, 24 of 33 lipoxygenase (LOX) derived oxylipins and 7 of 16 cytochrome P450 (CYP) derived oxylipins were lower in diseased kidneys. Celecoxib reduced total and 5 of the 8 individual elevated COX oxylipins and increased 5 of 24 LOX and 5 of 7 CYP oxylipins that were reduced by disease.

COX2 inhibition ameliorates disease progression, improves renal function and the altered oxylipins in Pkd2 mice. This represents a potential new approach for treatment of ADPKD, a disorder for which no effective treatment currently exists.

7.2 Introduction

Cystic renal diseases are a group of disorders characterized by proliferation of fluid-filled renal cysts. The most common form is autosomal dominant polycystic kidney disease (ADPKD), which manifests in adulthood and has a prevalence of 1:400 to 1:1000 (1). Other less common and largely pediatric cystic renal diseases include autosomal recessive PKD (ARPKD), which occurs in 1 in 20,000-40,000 live births (1, 2), and multiple forms of nephronophthisis (NPHP), which have a prevalence of 1 in 50-100,000 (3). ADPKD is the fourth leading cause of renal failure and is responsible for 10–15% of ESRD cases (2, 4). It can be caused by mutations in either the *PKD1* or *PKD2* gene, resulting in similar clinical phenotypes. Hypertension, flank pain, recurrent urinary tract infections are common in PKD patients and males develop disease slightly earlier than females (1, 5).

Previous studies in various models of cystic kidney diseases have demonstrated alterations in renal oxylipins with disease (6-9). Oxylipins are lipid mediators formed by monooxygenation of polyunsaturated fatty acids via cyclooxygenase (COX), lipoxygenase (LOX) and cytochrome P450 (CYP) activities (10). Oxylipins in the kidney play key regulatory roles in normal physiological function, maintaining glomerular filtration rate and salt/water homeostasis, as well as being involved in inflammatory and proliferative processes associated with renal injury (10, 11). Earlier studies showed that renal COX protein and mRNA levels are altered in models of NPHP (12, 13), and we have recently shown that increased COX derived oxylipins is the earliest and most consistent alteration in the renal oxylipin profile in diverse models of cystic kidney diseases, including models of ADPKD (7, 9). These oxylipins mediate their effects by binding to G protein receptors coupled to adenylyl cyclase, resulting in cAMP production and activation of protein kinase A mediated effects (14). Renal cAMP levels are

elevated in cystic kidney diseases and elevated cAMP increases epithelial cell proliferation and fluid secretion in human kidney cyst cells, as well as activating COX2 in several cell types (15-18). *In vitro*, PGE_2 has been shown to stimulate cAMP production, cell proliferation and cyst formation in primary cultured ADPKD and inner medullary collecting duct cells (19-21). Increased cAMP in cystic kidneys also is thought to contribute to the increased fluid secretion via activation of cystic fibrosis transmembrane conductance regulator (CFTR) and Ca^{2+}-activated potassium (KCa3.1) transporters (22, 23).

The elevated renal COX oxylipins in cystic kidneys may therefore contribute to disease by increasing cAMP levels, and reducing the levels of these oxylipins may reduce cystic kidney disease progression. This has been shown in the Han:SPRD-*Cy* rat model of NPHP. In this model the increased COX oxylipins in disease are formed mostly by the COX2 isoform (12), and COX2 inhibition appears to be more effective than a non-selective COX inhibitor (24-26). Furthermore, COX2 inhibitors have relatively fewer side effects with similar to more effectiveness than non-selective inhibitors (27). Therefore, the effectiveness of a COX2 inhibitor in an orthologous mouse model of ADPKD was investigated.

7.3 Materials and methods

7.3.1 Animal procedures

To generate mice with and without PKD, $Pkd2^{WS25/WS25}$ and $Pkd2^{WS25/+}$ mice were crossed with $Pkd2^{+/-}$ breeders originally obtained from Dr. Stefan Somlo (Yale University, New Haven, CT, USA). This resulted in a normal mice ($Pkd2^{WS25/+}$, $Pkd2^{+/+}$) that do not develop cysts and diseased mice ($Pkd2^{WS25/-}$) in which one of the Pkd2 alleles is mutated and the other contains the unstable *WS25* insert in the first intron. By 16 weeks of age these mice consistently develop renal

cyst disease (28), so drug treatment in the present study was carried out for 13 weeks, starting at weaning.

Weanling male Pkd2 mice were randomly assigned into either the control group that was provided the standard rodent diet (AIN-93G) or the intervention group in which celecoxib was incorporated into the diet to achieve a daily dose of 50 mg per kg body weight. The dose was selected based on previous studies which inhibited COX2 activity in mouse models of disease (29, 30). The amount of celecoxib in the diet was calculated based on feed intake and body weight data of Pkd2 mice from a previous study (31), and was confirmed by measuring feed disappearance and bi-weekly body weight. Diet was freshly prepared twice per month and stored in sealed containers at -20°C until feeding. All diet ingredients, except celecoxib (Ark Pharm, Inc., IL, USA), were purchased from Dyets Inc. (Bethlehem, PA, USA).

The mice were individually housed in temperature and humidity-controlled environments with a 12-hour day/night cycle. At the end of the study, mice were anesthetized using isofluorane and euthanized via exsanguination. Normal and diseased mice were identified by the absence or presence of renal cysts. Collected blood was centrifuged to obtain serum and stored at -80°C for serum creatinine and SUN analyses. Body, kidney and liver weights were recorded, and the right kidney was snap frozen in liquid nitrogen and stored at -80°C until oxylipin analysis. The left kidney was fixed by placing it in 10% formalin for 24h, followed by transfer to PBS at 4°C until further processing. All animal procedures were approved by the Institutional Animal Care Committee and adhered to the guidelines of the Canadian Council on Animal Care.

7.3.2 Histology and renal function analysis

Formalin fixed kidneys were embedded in paraffin and 5 µm tissue sections were stained with Masson's trichrome to measure cyst area and fibrosis as previously described (31, 32). The

entire section was captured using a Nikon D600 FX DSLR camera equipped with a 60mm F2.8 macro lens mounted using macro rings to achieve 2.5X magnification (Nikon Corporation, Mississauga, Canada). Quantitative analyses of cyst and fibrosis areas were carried out using NIH ImageJ software (33). The cyst and fibrosis area ratios were multiplied by the left kidney weight to yield the cyst and fibrosis volumes, respectively, as described (32). Renal function was assessed by determining serum levels of creatinine and SUN, using commercial kits for the Cobas C111 auto analyzer (Roche Diagnostics, Indianapolis, IN, USA).

7.3.3 Oxylipin analysis

Lyophilized whole kidney tissues were homogenized in ice cold Tyrode's salt solution (pH 7.6) in a 1:28 weight/volume ratio. After homogenization Triton X-100 was added to achieve a final concentration of 0.01%. Deuterated internal standards (Cayman Chemical, MI, USA) and 6.5µL of an antioxidant cocktail (0.2 g/L BHT, 0.2 g/L EDTA, 2 g/L triphenylphosphine, and 2 g/L indomethacin in MeOH/EtOH/H2O (2/1/1, v/v/v)) were added to 200 µL tissue homogenates used for analysis. Samples were adjusted to pH<3 and solid phase extraction was with Strata-X SPE columns (Phenomenex, CA, USA) preconditioned with methanol and pH 3 water.

Samples were loaded onto the Strata-X solid phase extraction columns (Phenomenex, CA, USA), washed with pH 3 water and hexane, and eluted with methanol. Nitrogen evaporated samples were then re-suspended in the mobile phase (water/acetonitrile/acetic acid, 70/30/0.02, v/v/v) for analysis by HPLC-MS/MS (QTRAP 6500; Sciex, ON, Canada). Detection and quantification limits were set at 3 and 5 times above background, respectively. Quantification of oxylipins was determined using the stable isotope dilution method (34), and amounts were expressed as pg/mg of dry tissue. Further details of methods and oxylipins that were scanned for

as well as the deuterated internal standards that were used, including their mass transitions and retention times, is detailed in (9).

7.3.4 Statistical analysis

Disease and drug effects were tested by 2-way (disease x drug) ANOVA using the GLM procedure of SAS (SAS, version 9.4, Cary, NC, USA), except for cyst development which was tested by Student's t-test since cysts are only present in disease. Normality of data was tested using Shapiro-Wilk's Statistic. If the data did not follow a normal distribution even if log transformed, a nonparametric test was used (Kruskal-Wallis). Post hoc analysis was done by Tukey's tests for simple effect comparisons when interactions were present or when the Kruskal-Wallis test indicated the presence of differences. All data are presented as mean ±SEM.

7.4 Results

7.4.1 Celecoxib slows disease progression and preserves renal function

Diseased mice provided the standard diet had significant cyst involvement, as shown in Figure 7.1. In diseased mice provided celecoxib, cyst area, cyst volume and the cyst area ratio were reduced by 48%, 70% and 53%, respectively, when compared to diseased mice not given the drug. Diseased mice provided the standard diet without drug also had higher kidney weight, kidney weight/body weight and percent kidney water content compared to normal mice. When diseased mice were provided celecoxib, these increases were 70% less for kidney weight, 57% less for kidney weight/body weight and 53% less for kidney water. This resulted in values for these parameters in diseased mice provided celecoxib that were not different than in normal mice with or without celecoxib.

Table 7.1 Physiological parameters in normal and diseased Pkd2 mice treated without or with celecoxib

Genotype	**Normal**		**Diseased**	
Diet	Control	Celecoxib	Control	Celecoxib
Body Weight (g)	30.9±0.84	30.0±1.02	30.2±1.90	26.8±1.21
Liver Weight (g)	1.21±0.04	1.26±0.05	1.25±0.08	1.12±0.06
LW/BW (g/100g body weight)	3.90±0.07	4.20±0.07	4.17±0.16	4.17±0.15
Feed intake (g/24h)	3.20±0.35	3.15±0.30	3.30±0.50	3.20±0.40
N	20	18	6	8

Values are means±SEM. There were no effects of disease or celecoxib on any of the parameters. Abbreviations: BW, body weight; LW, liver weight.

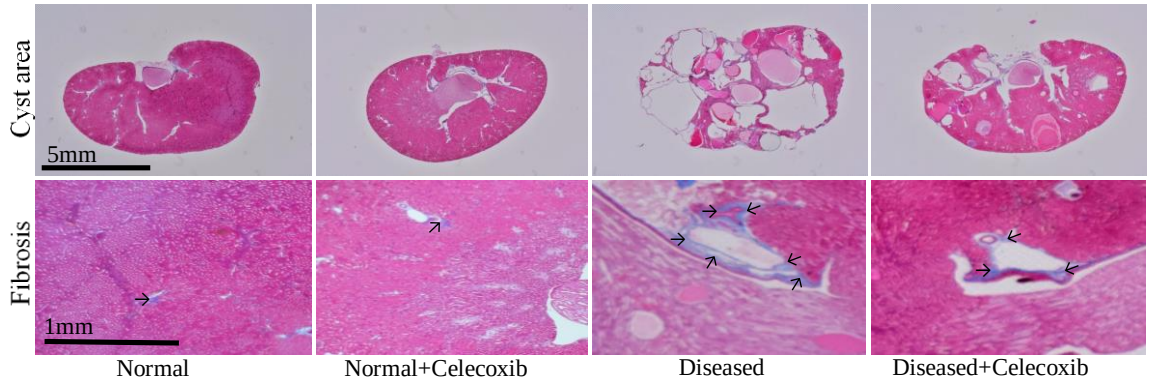
Similarly, fibrosis volume levels were 32% lower in treated vs. untreated diseased mice, although this did not reach statistical significance (Figure 7.1). It should be noted; however, that fibrosis in these kidneys was still minimal in this relatively early stage of disease.

The small increase in elevated SUN and serum creatinine indicated that renal function was starting to become impaired in diseased compared to normal mice, consistent with observations in the Han:SPRD-*Cy* rat and *pcy* mouse models of NPHP (6, 26), as well as in early stage PKD (35). For SUN, celecoxib provided to diseased mice reduced this increase by 79%, resulting in SUN levels not different than in normal mice provided the drug. For serum creatinine, disease tended (p=0.066) to increase, and celecoxib decreased these levels (Figure 7.1B). All mice displayed normal growth patterns throughout the study and there were no differences in body or liver weights between groups (Table 7.1).

7.4.2 Celecoxib ameliorates the alterations in oxylipin levels in diseased kidneys

Consistent with previous studies, total COX oxylipins were higher in diseased kidneys. This reflected the higher levels of 8 of the 11 COX oxylipins detected and lower levels of 2 minor COX oxylipins (PGE_3 and $PGF_{2\alpha}$). Efficacy of celecoxib treatment was evidenced by the

A. Kidney sections



B. Kidney disease and renal function

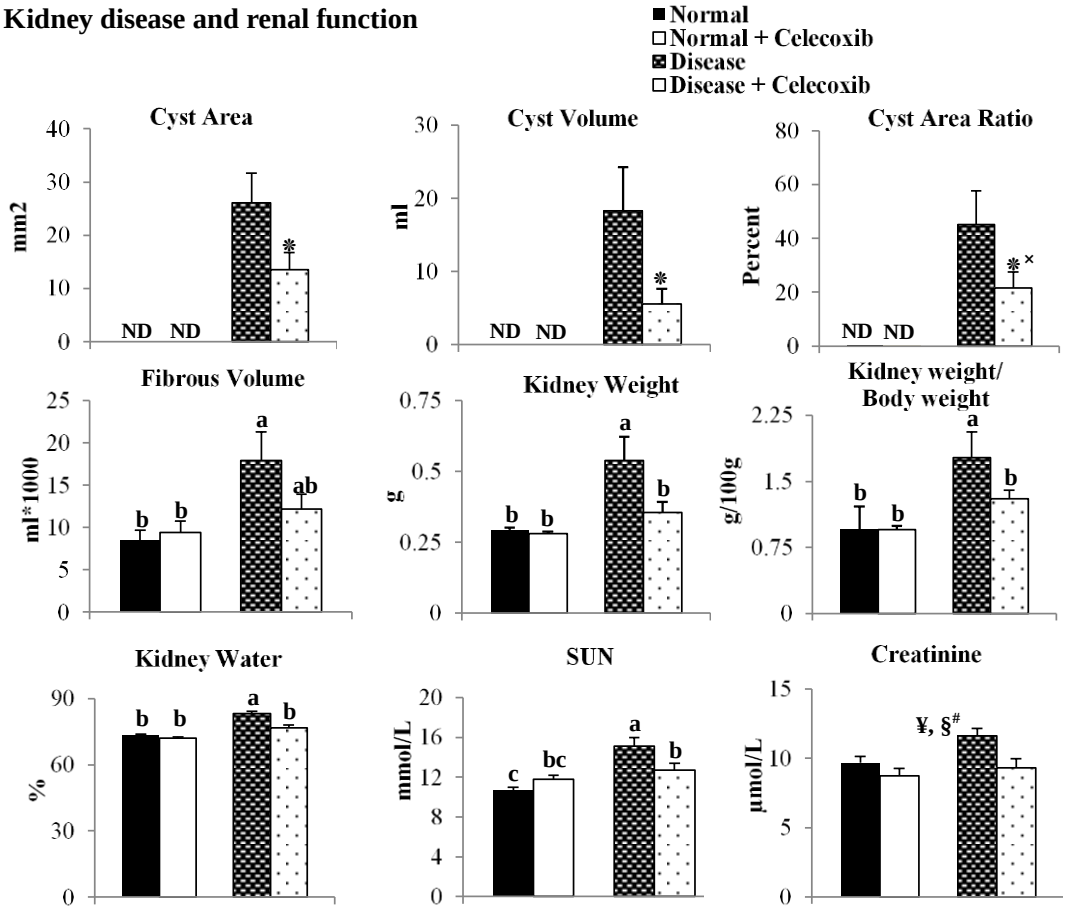


Fig 7.1 Celecoxib attenuates disease in Pkd2 mice.

Effect of disease and celecoxib on **A)** kidney cyst area and fibrosis, and **B)** kidney disease and function, in normal and diseased Pkd2 mice provided diet with or without celecoxib. Values are mean±SEM. ND, not detected; *, lower than diseased without celecoxib; ×, p= 0.057; #, p=0.066. For interactions, values with differing lower case letters are different.

For main effects, **¥** and **§** represent significant main effects of celecoxib and disease, respectively.

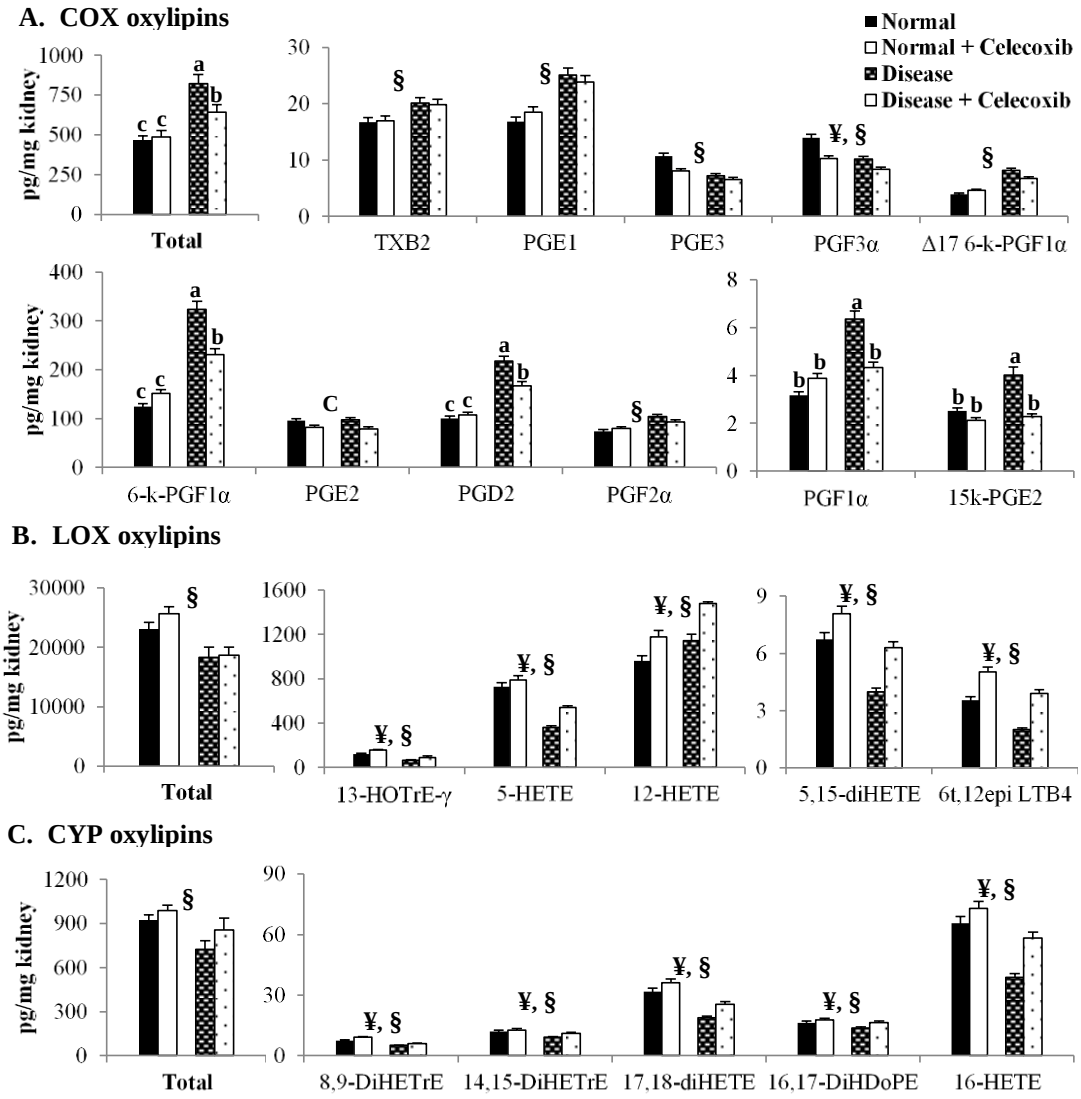


Fig 7.2 Celecoxib attenuates alterations in renal oxylipins in diseased Pkd2 mice.

Effects of disease and celecoxib on A) COX, B) LOX, and C) CYP oxylipins in normal and diseased Pkd2 mice provided diet with or without celecoxib. Values are mean±SEM, based on dry kidney weights.

For interactions, values with differing lower case letters are different.

For main effects, ¥ and § represent significant main effects of celecoxib and disease, respectively.

DiHETE, Dihydroxy-eicosatetraenoic acid; DiHETrE, Dihydroxy-eicosatrienoic acid; DiHDoPE, Dihydroxy-docosapentaenoic acid; PG, prostaglandin; HETE, Hydroxy-eicosatetraenoic acid; HETrE; HOTrE, Hydroxy-octadecatrienoic acid; LT, Leukotriene; TX, Thromboxane.

Table 7.2 Renal oxylipins in normal and diseased Pkd2 mice provided diets with or without celecoxib

Oxylipin (pg/mg kidney)	PUFA Precursor	Normal		Diseased		Effects $p<0.05$
		Control	Celecoxib	Control	Celecoxib	
COX Oxylipins						
$PGF_{1\alpha}$	DGLA	3.16±0.2[b]	3.88±0.26[b]	6.35±0.36[a]	4.33±0.46[b]	I
PGE_1	DGLA	16.4±1.01	18.5±1.10	25.1±1.39	23.8±2.35	D
PGD_2	AA	100±4.37[c]	108±5.23[c]	217±28.9[a]	167±25.0[b]	I
PGE_2	AA	95.5±4.52	81.9±3.92	97.1±8.86	79.0±8.04	C
15k-PGE_2	AA	2.51±0.18[b]	2.13±0.19[b]	4.02±0.40[a]	2.28±0.44[b]	I
$PGF_{2\alpha}$	AA	74.2±3.2	79.6±3.20	104±6.94	93.2±5.50	D
6k-$PGF_{1\alpha}$	AA	124±6.96[c]	152±6.61[c]	324±24.6[a]	231±15.1[b]	I
TXB_2	AA	16.7±0.68	17.0±0.80	20.1±1.87	19.8±0.65	D
Δ^{17}6-k-$PGF_{1\alpha}$	EPA	3.95±0.27	4.58±0.38	8.18±0.87	6.70±0.95	D
$PGF_{3\alpha}$	EPA	13.9±0.57	10.3±0.52	10.1±0.98	8.31±1.37	C, D
PGE_3	EPA	10.7±0.72	8.09±0.90	7.19±1.01	6.58±0.68	D
Total COX		465±27.1[c]	486±40.1[c]	821±56.2[a]	643±48.3[b]	I
LOX Oxylipins						
9-HODE	LA	5388±380	6543±505	4525±992	4360±375	D
9-oxoODE	LA	1923±172	2181±175	1492±311	1186±109	D
13-HODE	LA	3741±297	3903±333	3120±654	2928±194	
13-oxoODE	LA	1101±118[ab]	1265±114[a]	842±220[ab]	661±92.9[b]	I
9,10,13-triHOME	LA	814±58.2	863±68.7	704±98.84	611±53.6	D
9,12,13-triHOME	LA	269±21.0	292±24.4	229±31.5	205±16.6	D
13-HOTrEγ	GLA	121±7.38	155±5.43	63.5±5.01	89.3±13.7	C, D
8-HETrE	DGLA	86.2±6.00	93.2±4.08	49.0±3.70	61.1±10.0	D
15-HETrE	DGLA	66.4±3.16	69.3±3.27	46.7±2.64	50.7±5.52	D
5-HETE	AA	729±37.6	788±29.4	359±30.1	539±85.9	C, D
5-oxoETE	AA	68.6±5.29	62.7±4.45	55.7±11.2	48.2±6.36	
5,15 diHETE	AA	6.75±0.66	8.07±0.61	3.98±0.35	6.29±0.43	C, D
8-HETE	AA	175±10.8	191±7.86	136±26.5	153±13.8	D
9-HETE	AA	214±12.7	239±11.9	148±17.9	174±18.7	D
11-HETE	AA	260±11.7	267±9.53	262±14.6	286±24.3	
12-HETE	AA	961±61.5	1175±56.2	1144±62.3	1478±170	C, D
12-oxoETE	AA	345±31.4	332±20.9	280±44.1	363.1±26.7	
tetranor 12-HETE	AA	7.26±0.70	8.87±0.54	5.11±0.75	5.58±1.02	D
15-HETE	AA	962±45.8	999±39.3	742±36.9	852±93.9	D
15-oxoETE	AA	385±31.0	414±19.6	208±28.5	227±36.8	D
8,15-diHETE	AA	184±11.9	222±11.5	101±7.77	124±18.4	D
6t,12epi LTB_4	AA	3.56±0.39	5.04±0.43	2.00±0.22	3.90±0.58	C, D
9-HOTrE	ALA	735±60.5	1032±84.1	409±106	397±77.9	D
9-oxoOTrE	ALA	58.9±5.13	71.1±6.99	33.6±8.45	31.2±5.01	D

13-HOTrE	ALA	285.6±23.2	362.2±31.4	139±16.2	158±16.2	D
5-HEPE	EPA	37.4±3.64	37.9±2.34	13.8±2.46	21.3±5.38	D
11-HEPE	EPA	27.5±2.01	26.6±2.26	16.9±1.75	20.6±3.34	D
12-HEPE	EPA	262±18.0	312±17.9	240±17.2	290±41.7	
15-HEPE	EPA	28.0±1.44	28.8±1.39	19.1±1.11	19.7±3.27	D
RvE_1	EPA	1.12±0.21	1.57±0.25	1.58±0.55	0.86±0.42	
4-HDoHE	DHA	647±42.1	621.9±31.2	374±44.6	462±70.6	D
7-HDoHE	DHA	146±8.96	154±9.09	101±22.0	102±12.6	D
8-HDoHE	DHA	521±23.4	547±25.5	318±28.0	428±68.6	D
10-HDoHE	DHA	236±13.13	245±13.2	183±19.6	198±24.2	D
11-HDoHE	DHA	176±8.47	187±9.82	116±10.3	133±17.7	D
13-HDoHE	DHA	710±29.6	746±28.6	601±48.5	669±64.3	D
14-HDoHE	DHA	431±19.0	501±30.4	456±31.0	501±60.4	
16-HDoHE	DHA	225±10.2	238±12.4	186.5±20.5	223±25.6	
17-HDoHE	DHA	664±28.5	705.4±35.6	610±69.1	602±78.5	
Total LOX		23021±1188	25644±1044	18329±1980	18688±1233	D
CYP Oxylipins						
9,10-EpOME	LA	6.5±1.36	5.75±0.74	13.2±5.02	8.41±1.82	
9,10-DiHOME	LA	31.4±2.46	41.1±3.82	24.0±3.47	25.8±1.44	D
12,13-EpOME	LA	8.5±1.98	7.3±1.04	16.4±6.47	11.4±3.28	
12,13-DiHOME	LA	42.9±2.67	53.0±3.64	40.8±9.25	40.7±3.17	
5,6-EpETrE	AA	2.00±0.29	1.68±0.23	3.02±0.84	2.57±0.71	
8,9-DiHETrE	AA	7.41±0.43	9.07±0.51	5.01±0.33	5.99±0.91	C, D
11,12-EpETrE	AA	1.89±0.34	1.43±0.20	2.37±0.71	2.47±0.74	
11,12-DiHETrE	AA	6.14 ± 0.42^{ab}	6.93 ± 0.40^{a}	4.39 ± 0.69^{ab}	5.00 ± 1.08^{b}	I
14,15-DiHETrE	AA	11.9±0.41	12.7±0.33	9.05±0.49	11.1±0.92	C, D
12,13-EpODE	ALA	0.34±0.09	0.24±0.07	0.82±0.34	0.36±0.14	
17,18-DiHETE	EPA	31.8±2.04	36.2±1.78	18.7±0.91	25.5±2.71	C, D
16,17-DiHDPE	DHA	16.3±0.7	17.6±0.49	13.7±1.34	16.4±1.09	C, D
19,20-DiHDPE	DHA	151±9.27	165±6.66	128±17.6	153±12.2	
16-HETE	AA	65.7±4.20	72.9±3.98	38.8±3.07	58.3±9.19	C, D
18-HEPE	EPA	88.7±5.79	93.9±5.79	49.4±3.94	54.0±8.92	D
20-HDoHE	DHA	489±25.4	509±31.2	382±48.4	462±63.3	
Total CYP		920±37.7	987±38.0	726±56.2	857±80.8	D
Non-enzymatic Oxylipins						
5-iso $PGF_{2\alpha}$VI	AA	11.2 ± 1.00^{c}	13.7 ± 1.01^{bc}	20.4 ± 2.25^{a}	16.7 ± 1.53^{ab}	I
8-iso $PGF_{2\alpha}$III	AA	14.33±0.56	15.34±0.62	14.9±0.61	15.8±1.14	

Values are expressed as mean ± SEM, based on dry kidney weights. C and D represent significant main effects of celecoxib and disease, respectively. For interactions, values with differing lower case superscript letters indicate simple effect differences between values.

reduction in total COX oxylipins, as well as 6 individual oxylipins (6-keto-$PGF_{1\alpha}$, PGD_2, $PGF_{2\alpha}$, $PGF_{3\alpha}$, $PGF_{1\alpha}$, 15-keto-PGE_2) in diseased kidneys (Figure 7.2A and Table 7.2).

In contrast to the increased renal COX oxylipins in disease, total LOX and CYP derived oxylipins were lower in diseased mice (Figure 7.2B&C). This reflected the lower levels of 28 out of 39 individual LOX oxylipins and of 7 out of 16 CYP oxylipins. Celecoxib reversed a small number of these -5 of the 28 LOX oxylipins and 5 of the 7 CYP derived oxylipins that were reduced by disease were higher in diseased mice provided the drug. Total LOX oxylipins, total CYP oxylipins and the 10 oxylipins affected by celecoxib in these pathways are shown in Figure 7.2B&C; data for all oxylipins are shown in Table 7.2.

7.5 Discussion

This study demonstrates that COX2 inhibition significantly reduces disease progression and improves renal function in the Pkd2 mouse model of ADPKD. In addition to reducing cyst area and volume by ~50-70%, providing celecoxib also reduced the elevated kidney size, water content, serum creatinine and SUN by more than 50%, to values that were not different than normal. This is significant, given that there is currently no effective treatment to slow PKD progression, and therefore provides a potential therapeutic strategy for the treatment of PKD.

We recently demonstrated that increased levels of COX oxylipins is the earliest and most consistent alteration in diverse cystic kidney disease models, including the *Mx1Cre*$^{+}$*Pkd1*$^{flox/flox}$ mouse model of ADPKD1, the Pkd2$^{WS25/-}$ mouse model of ADPKD2, the PCK rat model of ARPKD and the *jck/jck* mouse model of NPHP (9). This was consistent with other rat and mouse models of NPHP that also display higher levels of COX oxylipins (6-8, 12, 25, 26). Over 80% of COX activity is due to the COX2 isoform in normal and diseased rat kidneys (12), and COX2 inhibitors attenuate the altered oxylipin production and reduce disease progression in the

Han:SPRD-*Cy* rat (25, 26). This model of NPHP displays much higher levels of fibrosis and inflammation, and selective COX2 inhibition by NS-398 reduced total fibrous area, macrophage infiltration and 2 of 3 COX oxylipins measured, but had a lesser effect on cystic expansion and no effect on renal function (26). Inhibition by celecoxib in this model reduced kidney weight, cystic and fibrosis index, serum urea nitrogen and creatinine, as well as 2 COX oxylipins measured in blood (25). COX2 inhibition also suppresses cell cycle progression, inhibits proliferation, and induces apoptosis in human ADPKD cyst-lining epithelial cells (25). Hence, the efficacy of COX2 inhibition in the Pkd2 model of ADPKD is consistent with findings in the Han:SPRD-*Cy* rat model of NPHP, and in ADPKD cells *in vitro*.

Cyst development is an important indicator of disease progression and plays a major role in the genesis of PKD by disrupting the renal architecture. It involves the combination of cell proliferation to form cyst cavities, fluid secretion into cysts and extracellular matrix remodeling surrounding expanding cysts (36). The mechanism by which the elevated COX oxylipins are involved in these processes is currently not known, but several possibilities exist. COX oxylipins mediate their effects via G protein-coupled receptors resulting in elevated intracellular cAMP and stimulation of PKA signaling (14). Renal cAMP is elevated in many models of PKD (17, 18), and COX oxylipin mediated production of cAMP stimulates cell proliferation and cyst formation in primary cultured ADPKD cells (19, 20). cAMP activates protein kinase A, which among other things stimulates B-Raf and the MAPK cascade in cyst epithelial cells, resulting in cell proliferation (37). Inhibiting COX oxylipin production may therefore reduce cyst growth by reducing cAMP, as is also the case with adenylyl cyclase 5 knockdown in Pkd2 mice, which results in reduced renal cAMP and lower kidney size, cyst index, kidney injury, and improved kidney function in these mice (38).

Inhibition of COX oxylipins also may act similarly to arginine vasopressin (AVP) receptor antagonists which inhibit disease development in animal models (39), as well as in ADPKD patients (40). These antagonists reduce adenylyl cyclase activity and inhibit ERK-dependent cell proliferation and *in vitro* cyst growth of human ADPKD cells (41), while activation of AVP receptors stimulates renal medullary COX-2 expression secondary to increasing medullary tonicity (42). Somatostatin (SST) analogs also reduce adenylyl cyclase activity and slow kidney expansion in human ADPKD patients and in rodent models of PKD (43, 44). In addition, cAMP transactivates renal cell miR-21 (45), which is upregulated in several orthologous mouse models of ADPKD as well as in human ADPKD kidneys (45, 46). In relation to the current findings, *in vitro* treatment with PGE_2 up-regulates miR-21 expression in colonic adenocarcinoma cells, while COX2 inhibition reverses this (47). Finally, cAMP may regulate fluid secretion into cysts by stimulating the CFTR and KCa3.1 transporters (22, 23) . Thus, COX oxylipins may play a role in the overall regulation of cAMP, a key regulator of the increased epithelial cell proliferation and fluid secretion in cyst growth.

A main limitation of the drugs described above for the treatment of PKD is the relative lack of benefit in the human population, combined with the significant side effects associated with their use. In this regard, NSAIDs are widely used drugs for pain and inflammatory conditions including arthritis, headache, dysmenorrhea, dental pain, gout, etc. (48). Approximately 15-30% of patients who regularly take NSAIDS, however, develop gastric or duodenal ulcers (49, 50), thus limiting their use. These ulcerogenic effects are thought to relate to their COX1 inhibitory activity, so COX2 inhibitors have become a viable alternative with similar to greater efficacy and fewer side effects (27). Nevertheless, renal impairment has been observed in older patients with pre-existing conditions such as heart failure or liver dysfunction, or who

are taking diuretics and/or ACE inhibitors (51, 52). Our dietary studies with the Han:SPRD-*Cy* rat model of NPHP have demonstrated that early intervention is more effective than treatment in the later stages of disease (53, 54), suggesting that early intervention may be the most effective and carry the least risk (27).

The dose used in this study is comparable to the dose of 200-400mg per day that is commonly used for human conditions (48). The average feed intake of the mice was ~3.2gm per day, an amount which provided 1 mg celecoxib to a 20 g mouse, or 1.5 mg to a 30 g mouse; this is equivalent to 243 mg drug per day for a 60 kg person (55), indicating that the dose used is readily achievable in humans. It also raises the possibility of combining a COX2 inhibitor with reduced levels of other drugs that are partially effective but have greater side effects.

In contrast to the elevation of COX oxylipins, LOX and CYP products were reduced in diseased compared to normal Pkd2 mouse kidneys. This is consistent with findings in models of NPHP and ARPKD (6-9). In the kidney, LOX derived oxylipins regulate renal blood flow, renal blood pressure and GFR (56), and CYP derived oxylipins have anti-inflammatory, vasodilatory, anti-apoptotic and anti-fibrotic effects, providing both cardiovascular and renal benefits (57). Interestingly, celecoxib supplementation increased several individual LOX and CYP oxylipins that were reduced in disease, but it did not alter the total levels of these groups of oxylipins. This finding was unexpected, as celecoxib is not known to stimulate either LOX or CYP activity. However, it is possible that increased COX oxylipin formation in diseased kidneys decreases the availability of fatty acids for the LOX and CYP enzymes, and that inhibition of COX oxylipin production mitigates this, but this remains to be elucidated.

In conclusion, this study demonstrates that renal COX oxylipins are elevated whereas LOX and CYP oxylipins are reduced in diseased compared to normal Pkd2 mouse kidneys.

Celecoxib at a dose commonly used in humans mitigated the elevated COX oxylipins in diseased kidneys, reduced renal cyst growth and disease, and improved renal function in this model of ADPKD. Further studies on potential risks and long-term effects of COX2 inhibition are needed to determine whether such drugs alone or in combination with other drugs are viable therapeutic options in ADPKD.

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Chapter 8

Overall summary, discussion and conclusions

8.1 Summary

In this thesis we hypothesized that dietary soy protein, flax oil, fish oil and a selective COX2 inhibitor will reduce and dietary HP will enhance PKD progression. These effects will be accompanied by parallel effects on the altered renal oxylipins in PKD.

The present research investigated the effects of dietary soy protein, flax oil, fish oil and celecoxib in the Pkd2 mouse model of ADPKD, and a HP diet in the *pcy* mouse model of NPHP on disease progression and the renal oxylipin profile. The current research also examined if there were any sex specific differences in disease or oxylipin alterations. A comprehensive profile of 50-67 oxylipins were quantified in normal and diseased Pkd2 and *pcy* mice kidneys. Consistent disease specific alterations of renal COX derived oxylipins were observed.

In chapter 3, it was hypothesized that dietary soy protein, flax and fish oil would reduce disease progression in male and female Pkd2 mice with ADPKD. We showed that dietary soy protein, flax and fish oil provides no beneficial effect on disease progression in the Pkd2 mouse model of ADPKD. In the Han:SPRD-Cy rat dietary fish oil provided general protective effects (1, 2), whereas in the *pcy* mouse dietary fish oil provided beneficial, detrimental and no effects (3-5). In a human trial, short-term fish oil supplementation did not provide any beneficial effect on renal function and kidney volume (6). On the other hand dietary flax oil consistently showed beneficial effects in the Han:SPRD-Cy rat and *pcy* mouse models of NPHP (3, 7, 8). Dietary soy protein also showed beneficial effects in the Han:SPRD-Cy rat and *pcy* mouse models (9-15). The lack of benefit in Pkd2 mice is similar to findings in which dietary fish oil or soy protein in female PCK rats provided no beneficial effects (16). Our results indicate the importance of using

orthologous animals in intervention studies, since beneficial effects observed in non-orthologous or other disease models may not be directly applicable to ADPKD. Our data suggest that there is no evidence to support dietary recommendations for n-3 PUFA or soy protein to human ADPKD patients. Prior to this study, the US PKD foundation advised ADPKD patients to increase dietary n-3 PUFA or soy protein, but after this manuscript was published, this recommendation was removed from the website (17).

In chapter 4, we hypothesized that the renal oxylipin profile will be altered by disease and sex in the Pkd2 mice model of ADPKD. In previous studies in Han:SPRD-Cy rats and *pcy* mice, it was shown that renal oxylipins are altered in disease (1, 8, 18-21). Consistent with these findings, we have shown that oxylipins are also altered with disease in Pkd2 mouse kidneys. This, along with the findings in several cystic kidney disease models (including ADPKD, ARPKD and NPHP), provides strong evidence for the consistency of these findings of oxylipin alterations across various types of cystic kidney diseases.

In chapter 5, we hypothesized that dietary interventions with soy protein, flax or fish oil and sex would alter renal oxylipins in the Pkd2 mouse model of ADPKD. In the Pkd2 mice, dietary flax and fish oil reduced renal n-6 PUFA derived oxylipins, and this effect was greater for fish oil compared to flax oil. Reduction of n-6 PUFA derived oxylipins in response to fish and flax oil has been observed in rodent kidneys as well as in human plasma; however, the number of oxylipins examined was limited (1, 22-24), and fish and flax oil were not directly compared to each other. In contrast to reduced n-6 PUFA derived oxylipins, the n-3 derived oxylipins were increased with both flax and fish oil feeding. Flax oil feeding increased ALA derived oxylipins while fish oil feeding increased EPA and DHA oxylipins. Due to the low number of animals in the soy protein group we could not test the effect of soy protein on renal

oxylipins in Pkd2 mice. However, this study was done in conjunction with other models and in those models dietary soy protein increased LA derived oxylipins. This is consistent with previous studies that have shown that soy protein feeding increases linoleic acid levels in rodent kidneys (25, 26).

In chapter 6, it was hypothesized that in the *pcy* model of NPHP, a HP diet would enhance disease progression and exacerbates disease specific alterations of renal oxylipins. Studies of prostanoid production *ex vivo* indicated that dietary HP increases selective prostanoid production, enhance disease progression and worsen renal function (27-29). However, a short-term *in vivo* study with normal rats showed that although HP increases renal hypertrophy, it did not alter renal prostanoids. Therefore, we carried out our *in vivo* study using normal and diseased animals. Consistent with the previous *in vivo* study we also found that the HP diet had very little effect on renal prostanoids as well as other oxylipins. The exact mechanism by which HP enhances disease progression and cyst expansion are not clear; however, a recent study showed that branched chain amino acids enhance proliferation of cyst-lining cells and upregulate the mechanistic target of rapamycin (mTOR) and MAPK/ERK pathways (30). In the Wistar rat, a HP diet increased interstitial connective tissue deposition (31), whereas dietary protein restriction reduced renal fibrosis (32). KLF15 is associated with increased renal fibrosis (33), and dietary protein restriction down-regulates KLF15 and renal fibrosis (34). These studies indicate that a HP diet furthers disease progression by other mechanisms, apart from oxylipin alterations. These HP effects on hyperfilteration, alteration of kidney hemodynamics, increasing renal protein load, electrolyte and acid-base imbalance, metabolic and blood pressure imbalance and enhanced expression of proinflammatory genes (35-37).

In chapter 7, we hypothesized that a selective COX2 inhibitor (celecoxib) would attenuate disease progression and disease specific alterations in renal COX oxylipins in the Pkd2 mouse model of ADPKD. We had shown that COX oxylipins are consistently higher in diseased cystic kidneys and that in previous studies with NPHP models as well as with other models of renal disease, selective or non-selective COX inhibition reduces disease progression (21, 38-40). In this chapter, we demonstrated that selective COX2 inhibition by celecoxib reduces disease progression as well as improves renal function in the Pkd2 model of ADPKD. This study provides a potential therapeutic strategy for the treatment of ADPKD. In this regard, combining COX inhibitors with other treatments may increase their effectiveness in reducing PKD progression. For example, combining celecoxib with tolvaptan, a highly selective vasopressin V2 receptor antagonist approved for the treatment of ADPKD (41), may be advantageous , since both inhibitors reduce cAMP levels via different mechanisms, and their effects may be additive.

8.2. Discussion

Overall, in this thesis a comprehensive renal oxylipin profile from normal and diseased kidneys of Pkd2 and *pcy* mice has been established. We have shown that renal oxylipins are altered in disease; specifically, COX derived oxylipins are higher in diseased kidneys of various PKD models. The exact mechanism by which the elevated COX oxylipins enhance disease progression is not clear. However, COX oxylipins increase the production of cAMP (42-45), and in cystic kidney diseases elevated renal cAMP is a common feature (46, 47). This elevated cAMP may influence and regulate several signaling molecules that are being altered in cystic kidney diseases (48-52), such as arginine vasopressin (AVP) and microRNA-21 (miR-21). AVP is a major regulator of adenylyl cyclase activity and a source of cAMP production in the distal nephron (52). AVP receptor antagonists inhibit *in vitro* cyst growth and ERK-dependent cell

proliferation in cultured human ADPKD cells (53), whereas AVP receptor agonists restore the full cystic phenotype in the AVP null PCK rat (54). AVP receptor activity also is dependent on functional COX2 activity (55). AVP receptor agonists increase renal cAMP levels and aggravate disease development in animal models (56), whereas, antagonists inhibit disease development in animal models (57-62) and in ADPKD patients (63).

Another effect of potential COX oxylipin-induced increases in cAMP may be on renal cell miR-21(48). In several orthologous mouse models of ADPKD as well as in kidneys from human ADPKD patients, miR-21 was upregulated (48, 51). MiR-21 targets the programmed cell death 4 (*PDCD4*) tumor suppressor gene and suppresses its expression (64), while in cyst epithelial cells, expression of *PDCD4* is increased by miR-21 inactivation (48). *PDCD4* is a pro-apoptotic gene and a subset of *PDCD4* null (*Pdcd4*$^{-/-}$) mice spontaneously develop kidney cysts (65). In relation to the current findings, *in vitro* treatment with PGE_2 up-regulates miR-21 expression and down-regulates PDCD4 proteins in colonic adenocarcinoma cells, while selective COX2 inhibition reverses this (64).

In PKD, interstitial inflammation is common in which macrophages infiltrate cystic kidneys, and lead to cyst growth and functional impairment of kidneys (66). The macrophage migration inhibitory factor (MIF), a proinflammatory cytokine, plays an important role in regulating innate immunity and inflammation (67). Higher levels of MIF have been found in human and experimental kidney disease, and lead to progressive renal injury by contributing macrophage and T cell accumulation in kidneys (68). Conversely, anti-MIF antibody treatment prevents renal injury in rats with crescentic glomerulonephritis (69). MIF regulates several proteins and signaling pathways including ERK/MAPK, mTOR, tumor necrosis factor-α and monocyte chemotactic protein 1 in different cell types, and these proteins and pathways are

hyperactive in PKD (66). CREB is a positive regulator of MIF gene expression in human monocytic, epithelial and keratinocytic cells (70); further, pituitary expression of MIF was induced by corticotropin-releasing factor in a cAMP-dependent manner (71). Thus, increased cAMP levels as a result of elevated COX oxylipins in cystic kidneys could contribute to disease as well.

The cystic fibrosis transmembrane conductance regulator protein (CFTR), a cAMP-regulated chloride channel, increases epithelial chloride secretion and fluid accumulation in human primary ADPKD cells (72, 73), while CFTR inhibitors slow cyst expansion in *in vitro* and *in vivo* models of PKD (74). CFTR expression was increased by forskolin, a cAMP agonist that stimulates adenylate cyclase, in cultures of ADPKD, normal human kidney cortex, and T84 cells (72). Addition of cAMP to cultured PKD cyst epithelial cells also increases fluid secretion and ATP release into the cyst fluid, which may be due to the activation of CFTR (75). Further, a recent study with mouse inner medullary collecting duct (mIMCD)-K2 cells demonstrates that PGE_2 activates basolateral EP4 receptors to increase Cl^- secretion through CFTR (76). Therefore, elevated COX oxylipins may activate CFTR and contribute to PKD progression.

Another way that COX oxylipins could affect PKD via increased production of cAMP is by regulating the renin-angiotensin system (RAS) (77). In progressive kidney disease RAS is activated and may contribute to ADPKD progression and hypertension (78). Direct activation of adenyl cyclase or cAMP analogs triggers renin release in juxtaglomerular cells (79, 80). PGE_2 or PGI_2 act on their respective Gs-coupled receptors and can stimulate adenyl cyclase activity, cAMP production and renin release (77). $G_{s\alpha}$ deficient mice display lower levels of plasma renin and reduced renin stores in juxtaglomerular cells, and secretion of renin is diminished in

response to PGE_2 in those cells (81). In contrast, administration of selective cAMP phosphodiesterase inhibitors increases renin secretion (82, 83).

In contrast to the elevated level of COX oxylipins, LOX and CYP products were generally lower in diseased compared to normal Pkd2 and *pcy* mouse kidneys. This was consistent with findings in previous studies with the Han:SPRD-Cy rat and the *pcy* mouse (1, 8, 18). Compared to COX oxylipins, the physiological roles of LOX and CYP oxylipins are less understood. In the kidney, LOX derived oxylipins regulate GFR, renal blood flow and renal blood pressure (84), whereas CYP derived oxylipins provide cardiovascular and renal benefits by their vasodilatory, anti-inflammatory, anti-fibrotic and anti-apoptotic effects (85). In one study (chapter 4), we failed to show consistent LOX and CYP oxylipins alterations in diseased kidneys, while in chapter 6 and chapter 7, we found LOX and CYP oxylipins to be reduced in the *pcy* and Pkd2 kidneys, respectively. This discrepancy may be due to the fewer animals in the diseased groups (chapter 4), resulting in less power to show the difference between normal and diseased animals.

In the present thesis we have also investigated sex effects on renal oxylipins. Very few studies have examined sex effects on renal oxylipins and only a limited number of AA oxylipins were investigated in these studies (86-88). In the Pkd2 mouse we found that AA derived oxylipins were higher in females compared to males, while other oxylipins were higher in males. However, the number of AA oxylipins with a sex effect was limited, so we could not determine whether it was the pathway or the precursors that determined sex effects. In chapter 6 and 7, however, more oxylipins were analyzed and sex effects were revealed. These results suggest that sex effects are likely pathway determined as COX oxylipins are higher in females, whereas LOX and CYP oxylipins are higher in males. These sex differences in renal oxylipins may be due to

differences in formation, degradation and/or clearance of oxylipins. For example, female rats and mice have greater renal expression of COX2 (87, 89) and PGE_2 synthase (87), and estradiol suppresses the activity of 15-hydroxyPG dehydrogenase, a PG-degrading enzyme in rat renal tissue (90, 91), and in human fetal tissue (92). Females might also have higher renal AA compared to males (93-95). Moreover, renal expression of a transporter responsible for clearance of PGE_2 was lower in female compared to male rats (96). Our data with a larger number of analyzed oxylipins demonstrated that renal COX oxylipins were higher in females compared to males, and that LOX and CYP oxylipins tended to be higher in males. Therefore, our data demonstrate that there are sex differences across the renal oxylipin profile. Further research on the implications of these differences is thererfore needed.

Since COX oxylipins were consistently higher in disease kidneys, and in NPHP models reducing the elevated COX oxylipin production by selective COX2 inhibitor reduced disease progression (97-99), we wanted to replicate these findings in an ADPKD orthologous animal. We have showed that selective COX2 inhibition by celecoxib not only reduced disease progression but also increased renal function. The celecoxib dose used in our study is comparable to commonly used human doses of 200-400mg per day (79), indicating that the dose used is readily achievable in humans. Although celecoxib has been previously reported to associated with renal dysfunction and cardiovascular events (100-102), a large observational study showed that relative risks of myocardial infarction was similar for celecoxib and other NSAIDs (e.g. naproxen), while rofecoxib seemed to be a significant outlier (103). Another recent systemic review and meta-analysis showed that the risk of myocardial infarction with celecoxib was similar when compared to either placebo or other NSAIDs, including naproxen. Moreover, the risk of stroke was significantly lower with celecoxib when compared to nonselective

NSAIDs. Rofecoxib was again outlier in this study (104). Similar outcomes were also observed in another study where celecoxib was compared with naproxen and ibuprofen in patients with either established, or at high risk of circulatory disease (105). This study also showed that the risk of renal events was significantly lower with celecoxib when compared to ibuprofen; however, not with naproxen. In a meta-analysis Zhang et al. showed that there was significant increased risk of renal outcomes with rofecoxib use, while, celecoxib use was associated with a decreased risk of renal outcomes when compared to controls (106). Nonetheless, our study is important because there is a scarcity of effective treatments for PKD and the only drug that is approved for PKD is tolvaptan, which has considerable side effects (107). Therefore, our study provides a potential therapeutic strategy for the treatment of PKD.

8.3 Conclusions

In conclusion, our data do not provide evidence to support an increase in dietary soy protein or oil containing n-3 PUFA in PKD. Because of our findings, dietary recommendations for PKD patients in US have already been changed. On the other hand, our studies do provide a comprehensive renal oxylipin profile in normal and diseased Pkd2 mice. We found that renal COX oxylipins are elevated whereas LOX and CYP oxylipins are reduced in diseased compared to normal Pkd2 mouse kidneys, and this led to a potential alternative treatment of this disease. We found that selective COX2 inhibition by celecoxib, at a dose commonly used in humans, mitigates the elevated COX oxylipins in diseased kidneys, ameliorates disease progression, and improves renal function in the Pkd2 model of ADPKD, providing a potential novel therapeutic strategy for treating ADPKD.

Our study also provided novel renal oxylipin data in response to fish oil and flax oil supplementation, which largely did not alter disease progression. Fish oil resulted in increased

EPA and DHA derived oxylipins, and greater reduction of n-6 PUFA derived oxylipins when compared to flax oil, while flax oil resulted in greater elevation of ALA derived oxylipins. On the other hand, a diet containing HP (35%E from protein) enhances disease progression in the *pcy* mouse model of NPHP without altering oxylipins in disease as well as in normal kidneys. These studies indicate that these diets alter disease progression independent of oxylipin alterations.

Finally, these studies provide novel fundamental data on sex differences in renal oxylipins. There is very little data on renal oxylipins, and the data that are present include few oxylipins. Our studies show that there appear to be patterns of sex differences in renal oxylipins that warrant further investigation.

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Chapter 9

Strengths, limitations and future research directions

9.1 Strengths

We have investigated the effect of dietary SP, flax and fish oil and celecoxib in the Pkd2 mouse model of ADPKD, and a HP diet in *pcy* mouse model of NPHP. The following are strengths of these studies:

The studies were done in conjunction with other models to reveal patterns in renal oxylipin alterations in disease.

We used orthologous models of cystic kidney diseases.

A comprehensive renal oxylipin profile was examined both in normal and diseased animals.

We have tested the effect of flax and fish oil side-by-side on disease and renal oxylipins.

We have included males and females in our studies.

We have examined sex effects on the renal oxylipin profile and disease progression in an ADPKD and a NHPH model.

We have shown that the selective COX2 inhibitor celecoxib ameliorates disease progression, improves renal function and oxylipin alterations in the Pkd2 mouse, which provides a potential therapeutic approach in ADPKD. The dose used in our study is comparable to normal human doses.

9.2 Limitations

For Pkd2 mice, identification of diseased animals was not possible prior to termination and we did not get the expected number of diseased animals after breeding. Because of this, some groups had lower sample sizes than expected.

Since, Pkd2 mice were in the early stage of disease when treatments were initiated, we do not know whether diet and drug effects would be different in the later stages of disease.

We have used high levels of dietary soy protein, fish oil and flax oil. Also, these dietary interventions were carried out only in diseased animals, so we do not know the effects in normal animals.

Because of the unavailability of sufficient of Pkd2 mice, we carried out our HP study using *pcy* mice. Also, we could have included another group providing LP to compare with the HP diet (like in older studies) in addition to comparing the HP to a NP diet.

We have tested only one dose of celecoxib on disease progression and oxylipin alterations because of limited availability of diseased animals. It is not clear whether a higher or lower dose will have the same beneficial effects on disease. Also, we used only male Pkd2 mice since sufficient female mice were not available.

In the present thesis we analyzed many oxylipins in several studies. This increases the risk of type I error due to the many comparisons made within each study. To minimize this, in all studies post-hoc analysis was done by the more conservative Tukey's test. Moreover, we also place much more emphasis on oxylipin patterns rather than a single or particular oxylipin change.

In this thesis, we did not measure tissue PUFA composition. However, tissue PUFA composition may not necessarily provide an accurate reflection of oxylipins in renal tissues as

observed in several studies (1-3). The tissue PUFA levels are several times higher than the oxylipin level in tissues and only a very small portion of PUFA is converted to oxylipins. Moreover, tissue phospholipid is not the only possible source of tissue oxylipins (4). Since the effects of PUFA are considered to be mediated via their derived oxylipins, the oxylipin profile provides more direct evidence of their potential effects.

Whether the oxylipin alterations are due to higher/lower expression and activity of specific enzymes and/or degradation and clearance of oxylipins cannot be determined from the present thesis. Measuring the levels and expression of these enzymes, as well as oxylipin flux analyses would be informative in this regard and would provide more mechanistic insights.

9.3 Future research directions

There are several novel findings from our research which need to be further investigated. Dietary interventions in the Pkd2 mouse with soy protein, flax or fish oil were carried out in the early stage of disease and showed no benefits. However, several studies with other models of cystic kidney disease showed beneficial effects of these dietary interventions in more advanced stages of disease (5-7), which need to be investigated in the Pkd2 model.

This thesis also demonstrated that COX oxylipins are consistently elevated in the Pkd2 mouse model of ADPKD and *pcy* mouse of NPHP. COX oxylipins are thought to be involved in several key pathogenic pathways in the development of these disorders; however, the exact mechanism by which these elevated COX oxylipins enhance disease progression is not known and need to be investigated further. For example, cAMP activates protein kinase A, which stimulates B-Raf and the MAPK cascade in cyst epithelial cells, resulting in the phosphorylation of ERK. ERK and MAPK in turn activate CREB and promote transcriptional activity related to renal cell proliferation (8-13). Therefore, to determine the importance of the B-Raf/ERK

pathway, the levels of cAMP, protein and mRNA levels of COX enzymes and these signaling molecules, their expression pattern and activity should be measured in renal tissue of Pkd2 and other PKD models.

MIF is an important regulator of several proteins and signaling molecules in PKD progression. It has also been shown that MIF gene expression can be regulated by cAMP (14,15). Therefore, future studies can be done to determine whether elevated COX oxylipins also increase MIF via the cAMP pathway in PKD. In this regard, renal tissue from PKD compared to control mice can be analyzed to determine levels of cAMP, protein levels of COX, ERK/MAPK, CREB, and their expression patterns and activities in the Pkd2 or *pcy* mouse kidneys.

CFTR increases epithelial chloride secretion and fluid accumulation in human primary ADPKD cells. A cAMP agonist forskolin increased CFTR gene expression in several cell types. And PGE_2 increases Cl^- secretion by activating basolateral EP4 receptors through CFTR (16,17). Therefore, elevated COX oxylipins may activate CFTR and lead to PKD progression. This can be investigated in future studies by measuring renal levels of cAMP, protein levels of COX, CFTR, EB4, and their expression patterns and activities in normal compared to Pkd2 or *pcy* mouse kidneys.

Another possible pathway to be investigated is the RAS pathway. Activated RAS leads to hypertension and contributes to ADPKD progression and renal damage (18,19). cAMP also is an important regulator of RAS (20). Direct activation of adenyl cyclase or cAMP analogs triggers renin release in JG cells (21,22). In addition, administration of selective cAMP phosphodiesterase inhibitors also increases renin secretion (23,24). Therefore, to determine whether COX oxylipins also regulate the RAS pathway, the levels of cAMP, protein and mRNA

levels of COX enzymes, and expression and protein levels of renin could be measured in renal tissue of normal compared to Pkd2 and other PKD models.

Our results also suggest the involvement of the LOX and CYP pathways in cystic kidney diseases, however, how they affect disease need to be examined in the future. For example, the effects of LOX and/or CYP inhibitors/activators on disease progression can be determined in PKD models.

We also showed that flax oil and fish oil affect renal oxylipins differently. In our side-by-side comparison, we showed that flax oil increases ALA derived oxylipins whereas fish oil increases EPA/DHA oxylipins. ALA, EPA and DHA derived oxylipins might have differential effects on renal health. However, effects of these PUFA derived oxylipins on kidneys need to be examined further. For example, ALA derived HOTrEs show anti-inflammatory properties *in-vitro.* Their *in vivo* effect is still unknown. Therefore, study can be designed to assess the anti-inflammatory effects of HOTrEs *in vivo.* For example, a short-term study can be done with direct injection of HOTrEs in a PKD model and the measurement of inflammatory markers. An indirect study can be done by providing an ALA rich diet to a PKD (i.e. *pcy* mice, Han:SPRD-Cy rat) model and by measuring changes in inflammatory markers, since PKD displays increased inflammation.

In chapter 6 we showed that a HP diet containing 35%E from protein enhances cyst and fibrosis expansion in diseased kidneys. Interestingly, in both normal and diseased kidneys, the HP diet did not alter renal oxylipins, despite having physiological effects on the kidneys. The mechanisms involved are largely unknown and need to be investigated in the future. For example, Branched chain amino acids enhance proliferation of cyst-lining cells and upregulate mTOR and MAPK/ERK pathways (25,26). Also, KLF15 is associated with increased renal

fibrosis, and dietary protein restriction down regulates KLF15 and renal fibrosis (27,28). Whether HP diet increases expression and protein level of MAPK/ERK signaling molecules and KLF15 should be investigated in PKD animals. For example, Pkd2 or *pcy* mice can be provided HP diets, and then disease progression and expression pattern and protein levels of these signalling molecules can be determined from kidney tissue.

Celecoxib, a selective COX 2 inhibitor, reduces disease progression in Pkd2 mouse model of ADPKD, however, benficial effects of celecoxib need to be tested in other orthologous models of PKD before extrapolated in humans. Also, we carried out our study in the early stages of disease in this model, and whether a similar effect can be found in later stages of disease needs to be tested in the future. In addition, further studies on potential risks of long-term use need to be determined in this and in other models. Also, whether celecoxib in combination with other drugs, such as with tolvaptan, can provide more disease reducing effects needs to be investigated further.

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APPENDICES

Appendix A: Animal ethics approval

UNIVERSITY OF MANITOBA | Research Ethics and Compliance
Office of the Vice-President (Research and International)

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veterinaryservices@umanitoba.ca

15 April 2016

TO: Dr. H. Aukema
HNS/FAFS

FROM: Dr. M. Fry, Acting Chair, Fort Garry Campus Animal Care Committee

RE: Your protocol entitled **"Effect of diet on oxylipins"**

Please be advised that your Animal Use Protocol form was reviewed by the Fort Garry Campus Animal Care Committee (FG ACC) at its meeting of **March 24 2016**. The committee recommended **APPROVAL** of your protocol **SUBJECT TO A SATISFACTORY RESPONSE TO THE QUERIES NOTED BELOW.**

Protocol Reference Number: **F16-006 (AC11047)**
Animals approved for use:

Number	Common Name	Sex	Age or Weight	Formal Name
30 rats – older than 21 days	sprague dawley	male only	10 weeks	sprague dawley
36 mice – older than 21 days	CD1-pcy/pcy (name is subject to change)	male and female	weanling	CD1-pcy/pcy (name is subject to change)
36 mice – older than 21 days	CD1	male and female	weanling	CD1
90 mice – older than 21 days	$Pkd2^{ws25/-}$ and $Pkd2^{ws25/+}$ (name subject to change)	male and female	weanling	$Pkd2^{ws25/-}$ and $Pkd2^{ws25/+}$ (name subject to change)

Protocol approval is valid from: **April 15 2016 – April 14 2017**

Category of Invasiveness: **D**

As indicated above, your protocol has been approved, and as such, you are authorized to begin the work described. However, the Committee requires your written response on or before **April 29 2016** to the following queries under which this approval is subject:

1. Please consult with Mr. Terry Germscheid regarding the completion of Schedule 13s and Request to Establish forms for the Pkd2$^{ws25/-}$ /Pkd2$^{ws25/+}$ mice and the CD1-pcy/pcy mice.
2. Training for A. Islam:
   This individual needs to complete the Lab Animal Allergen and Zoonosis training. This should be completed no later than May 14 2016. Please note that the training module can be found at
   http://umanitoba.ca/admin/human_resources/ehso/media/Lab_Animal_Allergen_and_Zoonosis_Online_Training_2012_SCC.pdf

   In addition, A. Islam is required to complete the On-Line Ethics course prior to the initiation of any animal work. If they have not already done so, please ensure they self-register for this course. See the link below for the self-registration instructions.
   http://umanitoba.ca/research/orec/ethics/animalcare_education_training.html.
   This course must be completed no later than May 14 2016.

   As well, this individual must register for the rat and mouse intro wet labs. Again, please register by completing the registration form found at
   http://umanitoba.ca/research/orec/ethics/animalcare_education_training.html
3. Schedule 5 section 5: Please indicate why no preconditioning will be used.
4. The committee would like to commend you for submitting a very well written protocol.

If your written response is not received by the date noted above, the protocol will be suspended and no animal experimentation will be allowed to continue until further clarification by you is provided.

Please direct your response to Ms Tracy VanOsch, Co-ordinator, Animal Care, Office of Research Services, 208 Crop Technology Centre, 194 Dafoe Road.

The protocol reference number must be used when ordering animals. It is understood that these animals will be used only as described in your protocol. Failure to follow this protocol will result in the termination of your ability to use animals.

The protocol must be kept current. Minor modifications to the protocol must be submitted in the form of an amendment. Major changes would necessitate preparation and submission of a new protocol. Failure to renew this protocol prior to the expiry date will result in the termination of your ability to continue ordering animals.

On behalf of the Fort Garry Campus Animal Care Committee, I would like to extend our best wishes for the successful completion of your research.

MF/tvo

copy: Ms J. Nelson, Department of Biological Sciences
Mr T. Smith, Department of Biological Sciences

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26 September 2014

TO: Dr. Harold Aukema
Department of Human Nutritional Sciences

FROM: Dr. R. Hodges, Acting Chair, Fort Garry Campus Animal Care Committee

RE: **Your Renewal entitled "Dietary interventions in animal models of polycystic kidney disease"**

Please be advised that the above noted renewal was reviewed by a sub-committee of the Fort Garry Campus Animal Care Committee (FG ACC). The sub-committee has recommended **APPROVAL** of your renewal of 11-007/1/2.

New Protocol Reference Number: **11-007/1/2/3 (AC10393)**
Animals approved for use:

# Approved	Description
0	

Protocol approval is valid from: **May 1 2014 to April 30 2015**
Category of Invasiveness: **D**

The protocol reference number must be used when ordering animals. It is understood that these animals will be used only as described in your protocol. Failure to follow this protocol will result in the termination of your ability to use animals.

The protocol must be kept current. Minor modifications to the protocol must be submitted in the form of an amendment. Major changes would necessitate preparation and submission of a new protocol. Failure to renew this protocol prior to the expiry date will result in the termination of your ability to continue ordering animals.

Please be advised that this is the final renewal allowed. Subsequently, a full application must be submitted.

HA/ck

copy: Veterinary Services
Animal Health Technician, RCFFN

Appendix B: Methods of histological analysis

Step A: Acquiring pictures of whole kidney

1. Pictures of whole kidney sections are taken to ensure complete coverage of the cyst area.
2. Mount the Nikon DSLR camera on the tripod.
3. Connect 60mm macro lens, and use spacers (teleconverter) between the camera and lens to achieve maximum magnification.
4. Invert the camera with lens facing down.
5. Keep the slide on a back lit surface under the lens (about 10 cm away).
6. Focus the camera on the tissue by half pressing the button.
7. Once focus is achieved, turn the focus switch to manual focus position.
8. Take pictures of all the kidneys from the same postion. Be careful not to change the distance between the lens and the slide, as this will change the magnification.
9. Take a picture of a micrometer under the same conditions, to convert pixel dimensions to engths later on.

Step B: Measuring cyst area from whole kidney pictures

1. Open Image-Pro Plus Software 6.0. Select ‘Complete’. Select ‘Done’.
2. First you must create a colour standard. To do this open a kidney photograph that has a lot of cysts. Click the measure tab and then click “count/size” Click “select colours”
3. Under the colour cube based tab click the left button that looks like a pen. Now put the mouse over your kidney photograph and select the truest colours representing cyst area. 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.

4. Click Save file and save your colour standard under a recognizable name. You must load this standard for every picture you analyze.
5. Now open your kidney picture.
6. Under the measure tab, click the data collector.
7. 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.
8. Where it says “image” in the drop down box, select “count/size” then select “area” from the list below.
9. The dropdown box below select sum, then click the arrow to add it to the list to the right.
10. 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”.
11. Click “File”.
12. Click “Load File”.
13. Select the colour standard that you just created.
14. Click “yes” to replace the ranges.
15. Close the segmentation window.
16. One the count/size window, select “count”.
17. In the data collector window, select “collect now”.
18. The area sum of the cyst area from your photo should appear in the data list.
19. Repeat for all kidneys. Once finished, click the “export” tab in the data collector window.
20. Select export target.
21. Select export now.

22. Data can be exported to the active excel sheet. To change theexport options, click “export options”.

**If you wish to exclude certain elements of your photograph such as tubular lumen, you can simply crop it out using paint and open the modified picture in the Image pro plus software.

Appendix C: Lyophilisation Protocol

Materials:

Small vial of 70% (v/v) ethanol
Small vial of purified H2O
Scalpel with sterile blade
Kimwipes
Styrofoam container of ice
15 ml plastic centrifuge tubes
Balance
Small beaker, large enough to hold tubes
Disposable, small scintillation vials
Metal angled scoopula
Small, wax, weigh sheets
15ml disposable scintillation vials

1. Fill Styrofoam container with ice.
2. Gather the amount of 15 ml tubes required.
3. Poke 4 small holes in each of the lids
4. Label the tubes to correspond with the samples.
5. Remove 6 kidneys at a time from the -80 freezer and put on ice.
6. Place beaker on balance and tare.
7. Weigh tube, including lid, recording all decimal places, now and throughout. Recording all decimal places becomes important as the kidney nears dryness.
8. Using a sterile blade, slice kidney into 3 or 4 sections, depending on fit, and place in corresponding tube. Screw on lid and record mass.
9. Place tube on ice.

10. Clean blade in 70% ethanol and wipe dry. Rinse blade in H2O and wipe dry.
11. Repeat steps 6 to 9 with the remaining 5 samples.
12. Store samples in the -20 freezer near the scale.
13. Remove 6 new samples from the -80 and repeat same procedure for these samples.
14. Repeat steps 6 to 13 until all kidneys are weighed and stored in the -20 freezer.
15. If the samples are not being lyophilized right away, store in the -80 until they are ready to be freeze dried.
16. See Dennis regarding the use of the freeze dryer.
17. Samples will be put in the freeze dryer at the end of the day.
18. The next morning ask Dennis to remove the samples from the freeze dryer.
19. Keeping about 15 samples on ice, place the rest of the samples in the -20 freezer near the scale.
20. Using a Kim wipe, ensure all ice and water has been removed from the outside of the tubes. Weigh the 15 samples, recording mass to the last decimal place, and place in the -20 freezer.
21. Take out 15 new samples and repeat the procedure.
22. Repeat steps 20 and 21 until all samples have been weighed.
23. Leave samples in the -20 freezer for approximately 30 minutes before returning to the freeze dryer.
24. At the end of the day, repeat steps 19 to 23.
25. Repeat weighing the samples at time intervals until samples have the same mass as the previous reading. The mass may actually increase due to the dried kidney absorbing moisture from the atmosphere or leftover ice from the container.

26. The kidneys are now freeze dried. They should be transferred to labelled, small, disposable scintillation vials or 2.0ml microcentrifuge tubes.
27. To pulverize the sample, take an angled scoopula cleaned in 70% Ethanol and rinsed in purified H2O. Make sure the scoopula is wiped completely dry so as not to add water to the sample. Carefully use it to mash the kidney sample until no chunks remain in the tube.
28. When pulverizing, if static attracts sample to scoopula, wipe off sample into a folded, small wax weigh sheet where it can be transferred to the vial. Using a dryer sheet, wipe gloves on both hands and in between fingers. This should take care of the static.

Appendix D: Oxylipin analysis methods

Step A: Measuring Kidney Tissue and Tyrode's

200uL homogenate x 2 for duplicate = 400uL homogenate required for oxylipins.

Make extra to account for losses. Therefore, made approximately 700 uL in total.

Use Tyrode's (pH 7.6) salt solution to homogenize dried kidney tissue. For every 70 mg of dried (lyophilized) kidney tissue approximately 2000uL of Tyrode's (pH7.6) is required.

Therefore, 25 mg of dry (lyophilized) tissues = 700 uL of Tyrode's required

Step B: Homogenizing

1. Ensure there is enough prepared of:
    - Tyrode's salt solution (pH 7.6) (Check for deterioration. (See Solutions Preparation below)
    - 1.4X whole cell buffer – only if doing westerns (see western blotting buffer solutions)
    - 1% Triton Solution as per instructions below (see Solutions Preparation).
    - 12 mL test tubes with lids that have been soaked overnight in Contrad solution, rinsed and dried. *see p. 3 for details
    - 100:1 Methanol and Formic Acid
    - pH 3 water – (water that has had pH adjusted to 3.0 using 1M HCL)
    - Antioxidant Cocktail

2. Obtain a large container of ice
3. Remove the required lyophilized kidney samples from the -80C freezer and keep on ice.
4. Label 16 x 125mm disposable glass test tubes with sample ID's
5. If samples appear to be a fine powder proceed to weighing. If there are clumps in the sample, pulverize before weighing out sample.
6. Weigh and record **25 mg** (can be +/- 2? mg –make sure that the weight is recorded in lab book) lyophilized kidney sample into labeled tubes (prepared in step 4), cover with parafilm and immediately place on ice.
7. Return the remaining lyophilized kidney samples to the -80 freezer
8. Calculate, record, and add required amount of Tyrode's (pH7.6) to each massed kidney sample.

    25 mg of tissues = 700 uL of Tyrode's required

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. Clean homogenizer before use, after use, and in between each sample by:
    - 3 tubes ethanol x 30 seconds each at **speed 15**
    - 3 tubes ultrapure water x 30 seconds each at **speed 15**
    - dab with kimwipe to dry
11. Place test tube containing lyophilized kidney tissue in a small plastic container (yogurt container) containing an ice slurry (ice plus water).
12. Insert rotor into test tube and homogenize at **speed 20** 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.

13. Homogenize again for another 30 seconds (speed 20).
14. Repeat steps 10 – 13 for each sample. Remember to keep tubes covered with parafilm and on ice as much as possible
15. When finished all samples, take apart rotor to wash thoroughly inside. When putting rotor back together do not tighten too firmly.
16. Any tools contaminated with biological hazards can be wiped off with 10% bleach then washed normally with Contrad / other detergent.

Step C: Aliquoting homogenate

From each sample, remove an aliquot of 200 uL for the oxylipin analysis and place in a microcentrifuge tube. Go to Step D for further preparation.

Step D: Preparation of fraction for solid phase extraction of Oxylipins

1. Add 1 uL of a 1% Triton solution for every 100 uL aliquot for oxylipin analysis. (Final concentration of Triton in homogenate should be 0.01%: 0.01x 100=1ul)
2. Vortex for 10 seconds
3. Incubate, covered, on ice for 10 minutes
4. Vortex again for 10 seconds
5. Incubate, covered, on ice for 10 minutes
6. Vortex again for 10 seconds
7. Incubate, covered, on ice for 10 minutes
8. For the oxylipin fraction, vortex and aliquot 200 uL of tyrode's homogenate (this represents 30mg of wet tissue) into two labeled 2 mL microtubes (if doing duplicates).

9. Vortex each 2 mL microtube for 10 seconds.
10. Working quickly, add in the same order below to the samples;
    a. 500 uL of 100:1methanol formic acid
    b. 800 uL of pH3 water
    c. 90 uL of 100% ethanol
    d. 10 uL of antioxidant cocktail
    e. Vortex for 5 seconds.
11. Store samples in -80C freezer for future oxylipin extraction – go step E.

Step E: Solid phase extraction of oxylipins

Ensure that there is enough of the following:

- Internal Standard (the internal standard will be prepared specific to your tissues)
- Strata-X SPE columns (CAT# 8B-S100-UBL)
- pH3 water
- methanol (neat)
- 10% methanol in pH3 water
- Hexane
- Solvent A

*Samples should be kept on ice whenever possible.

***Do not** allow the column to run dry during steps 12,13, or 14

1. Remove homogenized samples from -80ºC freezer and defrost on ice.
2. Cool-down centrifuge to 4C.

3. Turn on nitrogen evaporator water bath to 37C. Make sure evaporator has enough water in it, if not add distilled water. The temperature knob is already set to this temperature.
4. Vortex to combine and continue on with the protocol.
5. One thawed vortex and transfer 1ml of the sample into the test tube.
6. Add 20 uL of internal standard to each glass tube. Vortex.
7. Before putting your internal standard back into the freezer run some nitrogen gas over it before closing the cap. Use the nitrogen gas from the Nitrogen Evaporator in the fume hood.
8. Acidify samples to pH 3 with 1N HCl if necessary (usually 4 uL of 1N HCl suffices for rat kidney and 14 uL for hamster kidney). ATTENTION: Vortex to mix before reading pH. Use pH-indicator strips to test pH.
9. Centrifuge for 5 min at 3000 rpm at 4C to remove debris.
10. In the fume hood, set-up and label a Strata-X SPE (Phenomenex) (33u, 60 mg/3mL) column for each sample using the wooden rack designed for columns. The SPE columns are found in the dessicator.
11. Place a waste vial under each column.
12. Pre-condition the column with 3.5ml Methanol. Allow the methanol to drip through for 1 minute, then gently apply pressure with a BD 10ml syringe to increase flow. Do not allow column to go dry.
13. Pre-condition with 3.5ml pH3 water. Push through after 1min in the same way as the previous step.

14. Apply sample to the column, avoiding the pellet at the bottom. The entire sample will be too much volume for the column. Let the sample drip through then add the remainder when there is enough room.
15. Add 1ml of 10% methanol in pH3 water to the sample vial. Vortex, then centrifuge at 4°C, 3000rpm for 5 minutes. Apply this to the column avoiding the pellet.
16. Rinse column with 2 ml pH3 water, and then with 1 ml Hexane. The Hexane will not flow through without pressure applied. Push through until dry.
17. Remove waste vials and place 1.5ml microtubes underneath columns. Elute with 1ml methanol. Apply some pressure the is covering the sorbent, and allow to soak for at least 1 min. Push the methanol through and give a few additional pushes to get the last drops.
18. If the samples are not being run that day on the LCMS (ask Tanja), displace air with nitrogen gas and store at -80C until the day they will be run. Then dry down. When you remove the samples from the -80C, vortex then spin down with minicentrifuge to get all solvent/analyte out of the cap.
19. Drying down in the nitrogen evaporator:
    a. Make sure that the water bath in the nitrogen evaporator is at 37C.
    b. Open one or two of the needles (so that when you turn on the pressure it has somewhere to go)
    c. Open the valve on top of the nitrogen tank, the pressure on the right gauge should increase
    d. Open the regulator on the far left (the one that says “Parker”), the pressure on the left gauge should increase

e. You can adjust the amount of nitrogen gas coming out of the needles with the knob that is attached to the side of the water bath (LPM AIR)

f. Open a needle for each tube that you will be drying.

g. Clean the needle with 100% chloroform, which should be in a 20 mL vial, labeled, next to the evaporator or in Flammables cabinet. Dip the needles into the chloroform. You can watch the bubbles to see how strong your flow of nitrogen gas is.

h. Put your tubes into the evaporator, and lower the needles so that they are *gently* blowing nitrogen gas on the surface of the solution in the tube. Do not let the needles touch the solution.

i. Leave your samples to dry for about an hour. Check on them every 15 minutes to ensure the temperature is kept at 37C. As the samples evaporate you can lower the needle.

20. Adding Solvent A (water-acetonitrile-formic acid [70:30:0.02 v/v/v] *LC-MS Grades):

a. Once your samples have dried (there should be no methanol left in the tubes, just dried residue from the sample), take them out of the water bath.

b. Take the solvent A out of the fridge (4C) and add 100 uL to each tube.

c. Vortex each tube so that all the dried sample is mixed in with the solvent A.

d. Centrifuge the samples at 14000g (rcf) for 10 minute at 4C.

e. Transfer into labeled GC/LC vials containing a 200ul **polypropylene** conical insert.

f. Run on LCMS that day.

21. Clean up:
    a. Clean each needle with 100% chloroform and close the needle, leaving at least one needle open.
    b. Turn off the nitrogen tank by closing the valve, leave the regulator on so that the pressure can decrease, once both of the pressure gauges reach zero, close the regulator.(the one that says “parker”)
    c. Turn off the switch on the water bath, unplug the water bath.

Make sure all solvents and tools are put away.

SOLUTIONS PREPARATION

<u>To Reconstitute Tyrode’s Salts Without Sodium Bicarbonate Product# T2145 (Sigma)</u>

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 plastic graduated cylinder. Water temperature should be 15-20C
2. Transfer about 800 mL of measured water into a 2000 mL flask. 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 200 mL measured water to remove all traces of powder. Add to solution in step 3. Rinse the package 3 times.
5. Transfer solution to a 1L volumetric flask and bring to volume using some of the remaining 200 mL measured water in graduated cylinder from step 1. Rinse the flask 3 times. Can use a pipette to bring the flask up to volume w/ water.
6. Insert volumetric stopper and invert 10x to mix. Before inverting, ensure the stopper and mouth of the volumetric flask are completely dry to prevent leaks.
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).

<u>To Make 100mL of Tyrode's (pH 7.6)</u>

1. Measure 100 mL of reconstituted Tyrode's salt solution into a graduated cylinder.
2. Weigh 100mg of powdered sodium bicarbonate (Sigma, S5761) into a 125 mL Erlenmeyer flask.
3. Cover flask with tin foil to protect from light. Add a stir bar.
4. Transfer about 80 mL 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 20 mL 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 20 mL scintillation vial
2. Add 2.0 mL of room temperature Tyrode's (pH 7.6) using a 1.0 mL 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 0.01% Triton final solution

i.e. Volume 1% Triton to add (uL) = (0.01 final concentration)(1000uL kidney homogenate)

Antioxidant Cocktail

0.2 mg/mL BHT, 0.2 mg/mL EDTA, 2mg/mL TPP, 2 mg/mL Indomethacin in a solution of 2:1:1 MeOH:EtOH:H_2O).

Make a minimum of 100 mL Antioxidant Cocktail. Measure out 50 mL of Methanol and 25ml Ethanol in separate graduated cylinders. Mix together in a 250 mL beaker. Cover the outside of the beaker with tinfoil and place on magnetic stirrer. Put stir bar in beaker and cover the top with tinfoil to minimize volatilization. Weigh out 20 mg BHT, 20 mg EDTA, 200 mg TPP and 200 mg Indomethacin onto separate weigh paper. Add the ingredients to the MeOH:EtOH solution and stir solution until all dissolved. This will take a while. Keep beaker completely covered with tinfoil to also minimize exposure to light. When all dry ingredients are dissolved, transfer the mixture to a 100ml volumetric flask. Using a small amount of ddH2O, wash down the sides of the beaker and pour into the 100ml flask. Do this 3 times to ensure you transfer all of the solvent and antioxidants. Fill up the 100ml volumetric flask to the mark with ddH_2O. Stopper flask and invert 10x to mix. Transfer into a clean, tinfoil covered, labeled 125 mL bottle. Aliquot the appropriate amount of antioxidant cocktail into covered scintillation vials for individual users.

Solvent A

Water – Acetonitrile – Acetic Acid [70:30:0.02; v/v/v]
*MS Grade
*Prevent evaporation of prepared solutions using paraffin around cap seals

To make 1000 mL:

700 mL water
300 mL acetonitrile
200 uL acetic acid

Vacuum filter through Whatman #4 filter paper

Solvent B

Acetonitrile – Isopropyl Alcohol [50:50; v/v]
*MS Grade

To make 1000mL:

500 mL Acetonitrile
500 mL Isopropyl alcohol

Vacuum filter through Whatman #4 filter paper

Appendix E: Assay principle for serum urea nitrogen measurement using commercial kit (Cat. No.# 04657616 190, Roche Diagnostics, Indianapolis, IN, USA) in Cobas C111 autoanalyzer.

Kit contents: TRIS buffer: 220 mmol/L, pH 8.6; 2-oxoglutarate: 73 mmol/L; nicotinamide adenine dinucleotide (NADH): 2.5 mmol/L; Adenosine-diphosphate (ADP): 6.5 mmol/L; urease (jack bean): ≥ 300 µkat/L; glutamate dehydrogenase (GLDH) (bovine liver): ≥ 80 µkat/L; preservative.

Assay Principle: "In the first reaction urea is hydrolyzed by urease to form ammonium and carbonate.

$$\text{Urea} + 2H_2O \xrightarrow{\text{Urease}} 2NH_4^+ + CO_3^{2-}$$

In the second reaction 2-oxoglutarate reacts with ammonium in the presence of glutamate dehydrogenase (GLDH) and the coenzyme NADH to produce L-glutamate. In this reaction two moles of NADH are oxidized to NAD for each mole of urea hydrolyzed.

NH_4^+ + 2-oxoglutarate + NADH $\xrightarrow{\text{GLDH}}$ L-glutamate + NAD^+ + H_2O

The rate of decrease in the NADH concentration is directly proportional to the urea concentration in the specimen and is measured photometrically at 340/409 nm wavelength (1)".

Sample used: Undiluted serum (50ul)

Reference:

1. https://www.gundersenhealth.org/app/files/public/6700/Lab-Policies-Urea-Nitrogen-BUN---Cobas-c501-Lab-4255.pdf

Appendix F: Assay principle for serum creatinine measurement using commercial kit (Cat. No.# 05401755 190, Roche Diagnostics, Indianapolis, IN, USA) in Cobas C111 autoanalyzer.

Kit contents: Potassium hydroxide: 900 mmol/L; phosphate: 135 mmol/L; pH ≥ 13.5; preservative; stabilizer, picric acid: 38.2 mmol/L; pH 6.5; non-reactive buffer.

Assay Principle: "This kinetic colorimetric assay is based on the Jaffé method (1). In alkaline solution, creatinine forms a yellow-red complex with picrate.

$$\text{Creatinine + picric acid} \xrightarrow{\text{Alkaline pH}} \text{yellow-red complex}$$

The rate of dye formation is proportional to the creatinine concentration in the serum (2)."

Sample used: Undiluted serum (50ul)

References:

1. Jaffé M. Ueber den Niederschlag, welchen Pikrinsäure in normalem Harn erzeugt und über eine neue Reaktion des Kreatinins. Z Physiol Chem 1886;10:391-400.

2. http://repository.sustech.edu/bitstream/handle/123456789/12435/Crea.pdf?sequence=3

Appendix G: Oxylipins screened for, source PUFA, deuterated internal standards (IS), their detector response factors (DRF), lower limit of detection (LLOD) and lower limit of quantitation (LLOQ).[1]

Oxylipin	PUFA	IS[2]	DRF[3]	LLOD (pg/mg dry tissue)	LLOQ (pg/mg dry tissue)
COX derived oxylipins					
PGD_1	DGLA	PGD_2-d4	0.203	0.162	0.271
PGE_1	DGLA	PGE_2-d4	0.306	0.252	0.420
$PGF_{1\alpha}$	DGLA	$PGF_{2\alpha}$-d4	3.454	0.085	0.142
PGK_1	DGLA	PGE_2-d4			
TXB_1	DGLA	TXB_2-d4	1.275	0.013	0.021
15-k-$PGF_{1\alpha}$	DGLA	$PGF_{2\alpha}$-d4			
dihomo-15deoxy-PGD_2	ADA	15deoxy-PGJ_2-d4			
dihomo-PGD_2	ADA	PGD_2-d4			
dihomo-PGE_2	ADA	PGE_2-d4			
dihomo-$PGF_{2\alpha}$	ADA	$PGF_{2\alpha}$-d4			
dihomo-PGJ_2	ADA	15-deoxy-PGJ_2-d4			
bicyclo-PGE_2	AA	PGE_2-d4	0.097	0.161	0.269
dihydro-$PGF_{2\alpha}$	AA	$PGF_{2\alpha}$-d4	0.934	0.428	0.713
dihydro-k-PGD_2	AA	PGD_2-d4	0.719	0.021	0.035
dihydro-k-PGE_2	AA	PGE_2-d4	0.117	0.855	1.424
dihydro-k-$PGF_{2\alpha}$	AA	dihydro-k-$PGF_{2\alpha}$-d4	1.372	0.261	0.435
PGA_2	AA	15-deoxy-PGJ_2-d4	0.47	0.172	0.287
PGB_2	AA	15-deoxy-PGJ_2-d4	0.029	2.595	4.324
PGD_2	AA	PGD_2-d4	0.882	0.129	0.216
PGE_2	AA	PGE_2-d4	1.606	0.068	0.113
$PGF_{2\alpha}$	AA	$PGF_{2\alpha}$-d4	2.223	0.118	0.197
PGJ_2	AA	15-deoxy-PGJ_2-d4	0.331	0.418	0.696
PGK_2	AA	PGE_2-d4	0.95	0.015	0.026
tetranor-PGDM	AA	PGD_2-d4			
tetranor-PGEM	AA	PGE_2-d4	0.146	0.063	0.105
tetranor-PGFM	AA	$PGF_{2\alpha}$-d4			
TXB_2	AA	TXB_2-d4	1.518	0.143	0.238
2,3-dinor-11β-$PGF_{2\alpha}$	AA	$PGF_{2\alpha}$-d4	5.01	0.01	0.018
2,3-dinor-TXB_2	AA	TXB_2-d4			
2,3-dinor-6-k-$PGF_{1\alpha}$	AA	6-k-$PGF_{1\alpha}$-d4			
6-k-PGE_1	AA	PGE_2-d4	1.095	0.007	0.012
6-k-$PGF_{1\alpha}$	AA	6-k-$PGF_{1\alpha}$-d4	1.85	0.110	0.183
6,15-diketo-dihydro-$PGF_{1\alpha}$	AA	$PGF_{2\alpha}$-d4	2.973	0.138	0.231

11β-$PGF_{2\alpha}$	AA	$PGF_{2\alpha}$-d4	0.944	0.068	0.113
11β-dihydro-keto$PGF_{2\alpha}$	AA	$PGF_{2\alpha}$-d4	2.612	0.272	0.454
11β-PGE_2	AA	PGE_2-d4	0.527	0.324	0.540
11-dehydro-TXB_2	AA	TXB_2-d4	0.052	0.623	1.038
12-HHTrE	AA	15-HETE-d8	0.425	0.274	0.457
15-deoxy-PGA_2	AA	15-deoxy-PGJ_2-d4	0.048	6.625	11.041
15-deoxy-PGD_2	AA	15-deoxy-PGJ_2-d4	1.328	0.108	0.181
15-deoxy-PGJ_2	AA	15-deoxy-PGJ_2-d4	1.597	0.145	0.242
15-k-PGD_2	AA	PGD_2-d4			
15-k-PGE_2	AA	PGE_2-d4	0.527	0.1	0.166
15-k-$PGF_{2\alpha}$	AA	$PGF_{2\alpha}$-d4	1.354	0.198	0.330
19-hydroxy-PGE_2	AA	PGE_2-d4	0.222	0.027	0.045
19-hydroxy-$PGF_{2\alpha}$	AA	$PGF_{2\alpha}$-d4			
20-cαrboxy-AA	AA	ARA-d8			
20-hydroxy-PGE_2	AA	PGE_2-d4			
20-hydroxy-$PGF_{2\alpha}$	AA	$PGF_{2\alpha}$-d4			
Δ17-6 k-$PGF_{1\alpha}$	EPA	$PGF_{2\alpha}$-d4	12.354	0.009	0.015
PGD_3	EPA	PGD_2-d4	0.147	0.103	0.171
PGE_3	EPA	PGE_2-d4	0.669	0.051	0.085
$PGF3_\alpha$	EPA	$PGF_{2\alpha}$-d4	1.128	0.079	0.131
TXB_3	EPA	TXB_2-d4	1.637	0.01	0.016
LOX derived oxylipins					
9-HODE	LA	9-HODE-d4	1.536	0.157	0.261
9-oxoODE	LA	5-oxoETE-d7	1.272	0.673	1.121
9,10,13-TriHOME	LA	9,10-DiHOME-d4	1.443	0.133	0.221
9,12,13-TriHOME	LA	12,13-DiHOME-d4	0.463	0.197	0.328
13-HODE	LA	13-HODE-d4	1.445	0.096	0.160
13-oxo-ODE	LA	5-oxoETE-d7	3.861	0.542	0.903
13-HOTrE-γ	γLA	13-HODE-d4	1.278	0.092	0.153
8-HETrE	DγLA	5-HETE-d8	1.175	0.026	0.044
15-HETrE	DγLA	15-HETE-d8	2.299	0.056	0.093
HXA3	AA	LTB_4-d4			
HXA3	AA	LTB_4-d4			
HXB3	AA	LTB_4-d4			
LTB4	AA	LTB_4-d4	0.194	0.150	0.250
LTC4	AA	LTB_4-d4			
LTD4	AA	LTB_4-d4			
LTE4	AA	LTB_4-d4			
LXB4	AA	LTB_4-d4	0.896	0.055	0.092
5-HETE	AA	5-HETE-d8	1.152	0.169	0.282
5-oxoETE	AA	5-oxoETE-d7	2.306	0.178	0.296
6R-LXA_4	AA	LTB_4-d4	0.955	0.101	0.169
6S-LXA_4	AA	LTB_4-d4			

6-trans-LTB_4	AA	LTB_4-d4	0.717	0.070	0.116
6-trans,12epi-LTB_4	AA	LTB_4-d4	1.046	0.058	0.097
8-HETE	AA	5-HETE-d8	0.756	0.206	0.344
9-HETE	AA	5-HETE-d8	0.357	0.974	1.623
11-HETE	AA	5-HETE-d8	4.123	0.160	0.266
12-HETE	AA	15-HETE-d8	1.727	0.202	0.337
tetranor-12-HETE	AA	15-HETE-d8	1.12	0.003	0.006
12-oxoETE	AA	5-oxoETE-d7	11.52	0.097	0.162
12epi-LTB_4	AA	LTB_4-d4	2.053	0.042	0.070
12-oxo-LTB_4	AA	LTB_4-d4	1.42	0.042	0.070
14,15-LTC_4 (EXC_4)	AA	LTB_4-d4			
14,15-LTD_4 (EXD_4)	AA	LTB_4-d4			
14,15-LTE_4 (EXE_4)	AA	LTB_4-d4			
15-HETE	AA	15-HETE-d8	1.572	0.22	0.367
15-oxoETE	AA	5-oxoETE-d7	5.877	5.214	8.691
15R-LXA_4	AA	LTB_4-d4			
20-hydroxy-LTB_4	AA	LTB_4-d4			
20-carboxy-LTB_4	AA	LTB_4-d4	0.29	0.079	0.132
5,15-DiHETE	AA	LTB_4-d4	1.497	0.021	0.035
5,6-DiHETE	AA	LTB_4-d4	0.002	26.176	43.626
8,15-DiHETE	AA	LTB_4-d4	0.646	0.082	0.137
9-HOTrE	ALA	9-HODE-d4	1.342	0.017	0.028
9-oxoOTrE	ALA	5-oxoETE-d7	2.396	0.049	0.081
13-HOTrE	ALA	13-HODE-d4	0.004	11.751	19.584
13-oxoOTrE	ALA	5-oxoETE-d7	0.295	10.218	17.029
LXA_5	EPA	LTB_4-d4	0.027	1.75	2.916
RvE_1	EPA	LTB_4-d4			
5-HEPE	EPA	5-HETE-d8	0.828	0.097	0.162
8-HEPE	EPA	5-HETE-d8	0.593	0.116	0.193
9-HEPE	EPA	5-HETE-d8	0.377	0.294	0.489
11-HEPE	EPA	5-HETE-d8	0.78	0.302	0.503
12-HEPE	EPA	15-HETE-d8	1.491	0.042	0.070
15-HEPE	EPA	15-HETE-d8	0.426	0.235	0.391
15-oxoEDE	EPA	5-oxoETE-d7	0.928	0.367	0.611
17 keto-DPA/17oxo-DPA	DPA	LTB_4-d4			
4-HDoHE	DHA	5-HETE-d8	1.396	0.061	0.101
7-HDoHE	DHA	5-HETE-d8	1.029	0.104	0.174
7R-Maresin-1	DHA	LTB_4-d4	0.05	0.617	1.029
8-HDoHE	DHA	5-HETE-d8	0.455	0.830	1.383
PD_1	DHA	LTB_4-d4			
RvD_1	DHA	LTB_4-d4	0.692	0.057	0.094
RvD_2	DHA	LTB_4-d4	0.517	0.304	0.506
10-HDoHE	DHA	5-HETE-d8	2.411	0.095	0.158

10S,17S-DiHDoHE (PDX)	DHA	LTB_4-d4	2.76	0.048	0.079
11-HDoHE	DHA	5-HETE-d8	1.783	0.456	0.760
13-HDoHE	DHA	15-HETE-d8	3.104	0.052	0.087
14-HDoHE	DHA	15-HETE-d8	1.059	0.172	0.287
15-trans-PD_1	DHA	LTB_4-d4			
16-HDoHE	DHA	15-HETE-d8	3.131	0.022	0.036
17-HDoHE	DHA	15-HETE-d8	0.318	0.599	0.999
17-k-DHA/ 17-oxo-DHA	DHA	LTB_4-d4	0.536	0.967	1.612
5-HETrE	MA	5-HETE-d8	1.213	0.008	0.013
CYP-E derived oxylipins					
9,10-EpOME	LA	9,10 EpOME-d4	0.944	16.597	27.661
9,10-DiHOME	LA	9,10 diHOME-d4	2.784	0.029	0.049
12,13-EpOME	LA	12,13 diHOME-d4	0.393	1.066	1.777
12,13-DiHOME	LA	12,13 diHOME-d4	2.358	0.047	0.078
5,6-DiHETrE	AA	11,12 DiHETrE-d11	0.690	0.005	0.009
5,6-EpETrE	AA	11,12 DiHETrE-d11			
8,9-DiHETrE	AA	8,9 DiHETrE-d11	1.519	0.032	0.053
8,9-EpETrE	AA	8,9 DiHETrE-d11			
11,12-DiHETrE	AA	11,12 DiHETrE-d11	1.371	0.023	0.038
11,12-EpETrE	AA	11,12 DiHETrE-d11			
14,15-DiHETrE	AA	14,15 DiHETrE-d11	1.177	0.014	0.023
14,15-EpETrE	AA	14,15 DiHETrE-d11			
9,10-diHODE	ALA	9,10 diHOME-d4			
9,10-EpODE	ALA	9,10 diHOME-d4			
12,13-DiHODE	ALA	12,13 diHOME-d4	0.637	0.469	0.781
12,13-EpODE	ALA	12,13 diHOME-d4	0.255	0.074	0.124
15,16-DiHODE	ALA	12,13 diHOME-d4			
15,16-EpODE	ALA	12,13 diHOME-d4			
14,15-EpETE	EPA	14,15 DiHETrE-d11			
17,18-EpETE	EPA	14,15 DiHETrE-d11			
16,17-EpDPE	DHA	14,15 DiHETrE-d11	0.017	1.674	2.790
19,20-DiHDPA	DHA	14,15 DiHETrE-d11	0.210	0.069	0.114
19,20-EpDPE	DHA	14,15 DiHETrE-d11	0.131	0.180	0.299
CYP-H derived oxylipins					
16-HETE	AA	15-HETE-d8	2.038	0.084	0.140
17-HETE	AA	15-HETE-d8	0.007	13.629	22.716
18-HETE	AA	15-HETE-d8	1.406	0.030	0.050
19-HETE	AA	20-HETE-d6	0.812	1.168	1.947
20-HETE	AA	20-HETE-d6	1.302	0.032	0.054
18-HEPE	EPA	20-HETE-d6	0.662	0.649	1.081
20-HDoHE	DHA	20-HETE-d6	1.118	1.001	1.668
Non-enzymatic oxylipins					
5-iso-$PGF_{2\alpha}$VI	AA	$PGF_{2\alpha}$-d4	2.814	0.083	0.139

8-iso-$PGF_{2\alpha}$III	AA	$PGF_{2\alpha}$-d4	0.841	0.208	0.347
8-iso-$PGF_{3\alpha}$	EPA	$PGF_{2\alpha}$-d4			
8-iso-15k-$PGF_{2\alpha}$	AA	$PGF_{2\alpha}$-d4			
2,3-dinor-8-iso-$PGF_{2\alpha}$	AA	$PGF_{2\alpha}$-d4			
10-Nitrooleate	OA	ARA-d8			
9-Nitrooleate	OA	ARA-d8			

[1]Abbreviations: ADA, Adrenic acid; DiHEPE, Dihydroxy-eicosapentaenoic acid; DiHETE, Dihydroxy-eicosatetraenoic acid; DiHODE, Dihydroxy-octadecadienoic acid; DiHOTrE,Dihydroxy-octadecatrienoic acid; DPA, Docosapentaenoic acid; EpDPE, Epoxy-docosapentaenoic acid; EpEDE, Epoxy-eicosadienoic acid; EpETE, Epoxy-eicosatetraenoic acid; EpETrE, Epoxy-eicosatrienoic acid; EpODE, Epoxy-octadecadienoic acid; EpOME, Epoxy-octadecenoic acid; Ex,Eoxin; HpODE, Hydroperoxy-octadecadienoic acid; Hx,Hepoxilin; Lt,Leukotriene; Lx,Lipoxin; MA, Mead acid; MaR, Maresin; OA, Oleic acid; oxo-EPE, Oxo-eicosapentaenoic acid; PD, Protectin; PGEM, Prostaglandin E metabolite; for remaining abbreviations see tables 2-6.

[2]Concentration of all IS was 1ng/ µL, except PGD_2 (2ng/ µL).

[3]Detector response factors, retention times, LLOD and LLOQ could not be experimentally determined for those oxylipins where primary standards were not commercially available. In such instances, retention times from the following 2 publications were used for screening these oxylipins.

[1]. Dumlao DS, Buczynski MW, Norris PC, Harkewicz R, Dennis EA.High-throughput lipidomic analysis of fatty acid derived eicosanoids and N-acylethanolamines.BiochimBiophysActa. 2011 Nov;1811(11):724-36. doi: 10.1016/j.bbalip.2011.06.005.

[2]. Wang Y, Armando AM, Quehenberger O, Yan C, Dennis EA. Comprehensive ultra-performance liquid chromatographic separation and mass spectrometric analysis of eicosanoid metabolites in human samples. J Chromatogr A. 2014 Sep 12;1359:60-9. doi: 10.1016/j.chroma.2014.07.006.