OXYLIPIN PROFILES IN AN ORTHOLOGOUS MOUSE MODEL OF AUTOSOMAL DOMINANT POLYCYSTIC KIDNEY DISEASE

by

Roxanna Yasmin Koohgoli

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Department of Food and Human Nutritional Sciences

University of Manitoba

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DEDICATION

I dedicate this thesis to

My parents, *Farzi Khazai* and *Mahshad Koohgoli*

My brother and his wife, *Sacha Koohgoli* and *Grace Rowen*

and

The best support I could've asked for (my cat), *Nimmy*

ABSTRACT

Autosomal dominant polycystic kidney disease (ADPKD) is characterized by the growth of renal cysts, which ultimately causes renal failure. Previous studies have suggested that whole flaxseed or flax oil can slow disease progression. The first analysis in this thesis examined the effect of a flax protein isolate (FPI) on disease progression in an orthologous ADPKD2 model (*Pkd2*$^{WS25/-}$ mice). Male and female mice were provided diets containing FPI or casein protein for 13 weeks. Heart, kidney, liver, and serum were then taken. There were no differences in kidney weight or water content between groups, indicating that there was no difference in the effect of FPI compared to casein on disease progression in this model.

Oxylipins are bioactive lipids that are formed from polyunsaturated fatty acids (PUFAs) and have proliferative and inflammatory properties, which are key elements of the pathogenesis of ADPKD. Altered levels of kidney oxylipins, liver cysts and heart abnormalities can occur in ADPKD. Serum oxylipins may provide a useful biomarker for disease, so in the second analysis of this thesis the oxylipin profiles in serum, heart, kidney, and livers of healthy and diseased mice that were provided the control diet in the first analysis were analyzed. Cytochrome P450 derived oxylipins in serum were reduced in the diseased animals, while lipoxygenase derived oxylipins were largely lower in the diseased heart and kidney, with no consistent pattern of differences in liver oxylipins observed.

Little is known about sex effects on tissue oxylipin profiles, so in the third analysis of this thesis, sex differences in oxylipins from healthy male and female mice given the standard diet were examined. Serum oxylipins were generally higher in females, while heart and liver oxylipins were generally higher in males. In kidneys, cyclooxygenase derived oxylipins were generally higher in females, while oxylipins derived from other pathways were higher in males.

These studies demonstrate that FPI and casein have similar effects on ADPKD disease progression. In addition, tissue oxylipins are uniquely altered by disease and sex, and the serum oxylipin profile has potential as a biomarker of disease progression in ADPKD.

ABBREVIATIONS

AA	Arachidonic acid
ADPKD	Autosomal dominant polycystic kidney disease
ALA	Alpha-linoleic acid
ARPKD	Autosomal recessive polycystic kidney disease
cAMP	Cyclic adenosine monophosphate
CO	Corn oil
COX	Cyclooxygenase
CVD	Cardiovascular disease
CYP	Cytochrome P450
DHA	Docosahexaenoic acid
DiHDoPE	Dihydroxy-docosapentaenoic acid
DiHETE	Dihydroxy-eicosatetraenoic acid
DiHETrE	Dihydroxy-eicosatrienoic acid
DiHODE	Dihydroxy-octadecadienoic acid
DiHOME	Dihydroxy-octadecenoic acid
DGLA	Dihomo-gamma-linolenic acid
EPA	Eicosapentaenoic acid
EpDPE	Epoxy-docosapentaenoic acid
EpETE	Epoxy-eicosatetraenoic acid
EpETrE	Epoxy-eicosatrienoic acid
EpODE	Epoxy-octadecadienoic acid
EpOME	Epoxy-octadecenoic acid

ESRD	End-stage renal disease
FA	Fatty acid
FDA	Food and Drug Administration
FLAP	Five-lipoxygenase-activating protein
FO	Flax oil
FPI	Flax protein isolate
GLA	Gamma-linolenic acid
GPCR	G-protein coupled receptor
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
LOX	Lipoxygenase
Lt	Leukotriene
Lx	Lipoxin
NPHP	Nephronophthisis
OxoETE	Oxo-Eicosatetraenoic acid
OxoODE	Oxo-Octadecadienoic acid
OxoOTrE	Oxo-Octadecatrienoc acid
PC	Polycystin
PCK rat	Rat model of ARPKD

PG	Prostaglandin
PKD	Polycystic kidney disease
PUFA	Polyunsaturated fatty acid
RAAS	Renin-angiotensin-aldosterone system
Rv	Resolvin
SDG	Secoisolariciresinol diglycoside
TriHOME	Trihydroxy-octadecenoic acid
Tx	Thromboxane

CHAPTER 1: LITERATURE REVIEW

1.1 INTRODUCTION

Autosomal dominant polycystic kidney disease (ADPKD) is a slow, progressive genetic disease resulting in the loss of kidney function, affecting approximately 1:1000 to 1:2500 individuals worldwide[1]. It is characterized by the presence of numerous, progressively enlarging fluid-filled cysts scattered throughout the kidney parenchyma[2–4]. Clinical manifestations of ADPKD often commence with abdominal, flank, or back pain, elevated calcium or phosphorous in the serum, and hypertension[5,6]. However, none of these symptoms are ADPKD specific. The exact mechanism of cyst formation is still unknown despite the identification of various abnormalities in the extracellular matrix, gene expression, cell polarity, fluid secretion, and apoptosis[7].

In addition to kidney growth due to the presence of cysts, ADPKD affected individuals may exhibit extrarenal defects such as hepatic cysts, cerebral aneurysms, and cardiac valvular lesions[8]. Tolvaptan, a vassopressin receptor (V_2) blocking agent, is the only FDA-approved treatment for ADPKD, other than renal transplantation, and has been approved in both the United States and Canada [9–11]. Medications used to treat PKD, such as ACE inhibitors and/or statins, are prescribed to relieve symptoms but not treat the cause of the disease[12]. There has been progress utilizing diet and nutraceuticals as new potential therapeutic targets to slow or halt disease progression[13]. Studies have exploited several different animal models to understand the effect of diet and disease progression, discovering that the oxidized lipid metabolites of polyunsaturated fatty acids (PUFA), oxylipins, may play a key role regarding disease indication, severity, and progression[13–17]. It is hypothesized that oxylipin profiles can reflect disease severity and prognosis. Previous studies compared alterations in oxylipin profiles in diseased rodent models of polycystic kidney disease (PKD) which were given an animal based protein (i.e. casein) versus a plant-based protein, commonly soy [16,18,19]. These studies concluded that soy protein can be considered beneficial in decreasing cyst progression in PKD animal models. A separate study found that whole flaxseed may impede the progression of PKD[20]. Due to the similarity in composition to soy protein, it is

reasonable to speculate that flax protein isolate (FPI) can also be used as a nutraceutical towards PKD.

Furthermore, sex is a significant factor in the rates of progression of many forms of ADPKD; however the role of sex in disease states and in response to nutritional intervention has not been deeply explored[21]. This review focuses on disease pathogenesis, progression, sex, and the possible role of oxylipins in human and animal models of ADPKD with a *PKD2* mutation.

1.2 GENETICS AND PATHOGENESIS

ADPKD is a genetic disease and is caused by mutations of either *PKD1* (in 78% of disease pedigrees) or *PKD2* (in 15% of disease pedigrees) genes, encoding polycystin-1 (PC1) and polycystin-2 (PC2), respectively[7,22]. Located on chromosome 16p13.5 and chromosome 4q21, respectively, these two genes are the principal genes known to cause ADPKD, with an overall mutation rate of ~90% [23]. Evidence indicates that PC1 and PC2 inhibit cystogenesis in a dose-dependent way, with cystogenesis occuring when the concentration of these proteins drop below a certain threshold[24]. Cyst formation in ADPKD is believed to occur through a cellular recessive somatic "2-hit" mechanism [10]. ADPKD is usually heterozygous, with one PKD allele having a germ-line mutation (1st hit) while the other is normal (2nd hit); the homozygous mutations of either PKD genes in mammals are considered embryonically lethal[9].

PKD2 is mutated in approximately 15% of ADPKD individuals and the disease phenotype is often less severe than a *PKD1* mutation [25]. *PKD2* encodes PC2, a ~110 kDa multispanning tansmembrane protein that is part of the transient receptor potential (TRP) family [26]. Transmembrane protein PCI is comprised of a large extra-cytoplasmic N-terminal domain, believed to function as a receptor, and a C-terminal cytoplasmic tail[27,28]. In the cilium of renal epithelial cells, PC2 forms a protein complex with PC1 via C-terminal coiled domains, creating the PC1-2 complex, which regulates various signalling pathways involved in cell proliferation, degradation

and apoptosis. Cysts arise when *PKD2* is inactivated, resulting in the loss of PC2 expression in all renal lining cells [29]. In 2015, a study found in mice that the functional restoration of PC2 significantly decreased the rate and progression of cyst growth[10,30]. PC1 and PC2 localize in the cilium of renal epithelial cells through their C-terminal domains, sensing fluid flow and pressure in the kidney and regulate intracellular Ca^{2+} and cyclic-AMP (cAMP) levels[30,31]. PC2 is a nonselective cation channel that is highly permeable to Ca^{2+}; the channel is primarily found in the endoplasmic and sarcoplasmic reticulum membrane and the primary cilia where it colocalizes with PC1 [32,33]. Both the intrinsic channel generated by activated PC2 or the emergent channel formed by the PC1-PC2 complex are key factors for creating a functional ion channel[34].

Cells cultured from ADPKD cysts have lower Ca^{2+} concentrations as compared to their unaffected counterparts, indicating that the deregulation of PC1 and PC2-mediated cellular Ca^{2+} signaling may be important in disease development [26]. Mutations in cilia-associated proteins, such as PC1 and PC2, lead to renal epithelial proliferation and cyst growth. Primary cilia are usually non-motile and are thought to serve as either a chemosensory or mechanosensory function, detecting alteration in neural tubular flow and reacting accordingly [35]. A study involving PC2 knockdown in zebrafish, showed that non-functional PC2 induces kidney cysts, hydrocephalus, left/right asymmetry defects, and strong dorsal axis curvature, suggesting that polycystins could function in the maintenance of exctracellular matrix integrity [36]. The study concluded that PC2 deficiency may cause increased collagen II synthesis through the upregulation of secretory pathway coat protein complex II components. The relationship between the polycystin complex and ciliary signaling remains complicated; while ablation of primary cilia by mutation of core ciliary components results in cyst growth, the same mutations of *PKD2* results in significant reduction of cystic disease [37].

Some patients with ADPKD may also develop cardiovascular abnormalities including hypertension[38]. Renal structural changes have an important role in the pathogenesis of hypertension in ADPKD. Patients with ADPKD and hypertension have greater renal volume than patients with

normal blood pressure[39,40]. It is suggested that activation of the renin-angiotensin-aldosterone system (RAAS) results in cyst expansion and local renal ischemia, causing disease associated hypertension. Nephrectomy specimens from patients with ADPKD suggests chronic stimulation of RAAS through hyperplasia of renin-secreting cells of the juxtaglomerular apparatus[41]. Other components of the RAAS, including angiotensinogen, angiotensin-converting enzyme, angiotensin II receptor and angiotensin II peptide are also present in the cysts and dilated tubules of ADPKD kidneys[42]. Asides from RAAS, muscle sympathetic nerve activity is increased in hypertensive patients with ADPKD, suggesting that sympathetic hyperactivity could contribute to the pathogenesis of hypertension in this disease[43].

Polycystic liver disease is the most common extrarenal manifestation of ADPKD. Somatostatin analogues have a liver volume-reducing effect by inhibiting adenosine 3',5'-cyclic monophosphate production, resulting in reduced cell proliferation and fluid secretion[44]. A study using somatostatin analogue octreotide or placebo found that liver volume increased with placebo and decreased with octreotide, with the reduction of liver volume maintained for 2 years after treatment ended[45].

1.3 ANIMAL MODELS

Animal models have been used to understand the complexities of cystic kidney disease. In models of nephronophthisis (NPHP), a pediatric form of cystic kidney disease, *pcy/pcy* and *jck/jck* mice, and Han:SPRD-*Cy* rats have been used. The *pcy/pcy* mouse phenotype is caused by mutations in *Nphp3*, which is orthologous to human NPHP type 3[46]. In *pcy/pcy* mice, the kidneys grow several times their normal size, and progressive decline in renal function begins to develop by 18 weeks, a disease course similar to human cystic kidney disease[47]. The *jck/jck* (juvenile cystic kidney) mouse model harbours a mutation in the *Nphp9* gene and its products affect normal expression of *PKD1* and *PKD2* gene products, PC1 and PC2[48]. Initial renal cysts begin to show at 4 weeks of age and the lifespan of *jck* mice is approximately 20-25 weeks[49]. The Han:SPRD-*Cy* rat strain causes renal cysts by a missense mutation (C to T, R823W) in exon 13 of the *Pkdr1* (also called *Cy, Samd6,* or

Anks6) gene on rat chromosome 5[49]. In this model, the expression of *Pkdr1* is increased in either the immature or mature stages of kidney development, suggesting that gain-of-function by the mutated gene product may cause the disease. From a Sprague Dawley outbreeding colony, a mutant rat was discovered that had a mutated *Pkhd1* gene, which orthologous to the human autosomal recessive PKD[50,51]. In this model (named the PCK rat), polycystic kidney and liver disease results from a frameshift mutation in the *Pkhd1* gene and the lifespan of the PCK rat is approximately 1.5 years[52].

ADPKD is a heterogenetic disease, in which the individual must contain one copy of a mutated PKD allele from an affected or carrier parent, and one normal PKD allele from an unaffected or carrier parent. The normal allele masks the effects of the mutated allele and it is only once this "protector" allele becomes inactivated by a somatic event, the disease begins to manifest [29]. Individuals who are homozygously mutated for the genes (i.e. receives a mutated allele from both parents), die embryonically. Current rodent models of *PKD1* use conditional knockouts for the analysis of ADPKD pathogenesis[24]. Targeted deletion of exon 34 in a mouse mutant of *PKD1* found that homozygotes were perinatally lethal due to renal cystic disease while heterozygotes were viable, developed multiple bilateral renal cysts by 16 months of age as well as liver cysts but no cardiac manifestations[53,54]. Limited functional and biological characterization suggested this *PKD1* model resembles the human disease phenotype with regard to renal cystic disease and hepatic cystic involvement[53,54]. Another *PKD1* model with targeted deletion of exon 17-21 of mouse *Pkd1* exhibited renal and hepatic cysts in heterozygotes, and cardiovascular abnormalities in embryonically lethal homozygotes[55]. The introduction of hypomorphic *Pkd1* allele reduced normal levels of *Pkd1* to 13-20% which was sufficient to induce a renal cystic phenotype as well as liver dilations similar to those seen in ADPKD[56]. Animal models that have been used to understand the complexities of ADPKD have been heterozygous for the mutation; however, none have been orthologous to human *PKD2* mutations until recently. An orthologous model refers to a model in which the mutated gene, such as *Pkd2* in mice, has a strong similarity and functionality to the

human *PKD2* after a speciation event [7,29]. Mouse *Pkd2* cDNA is predicted to encode an integral membrane protein with 91% sequence identity and 98% similarity to human PC2; it is this similarity which makes a mouse *Pkd2* model orthologous and therefore, superior to other types of animal models [10]. A unique strain of mice carries the unstable *Pkd2* allele, WS25, causing it to be prone to genomic rearrangement, resulting in enhanced cyst formation when the allele is present [7]. Double heterozygote *Pkd2*$^{WS25/-}$ mice with one unstable allele and one mutated allele, reliably develop renal cysts within 3 months, illustrating that a Pkd2$^{WS25/-}$ model is a good animal model for experimental therapies for ADPKD.

1.4 DIETARY INTERVENTION

There has been a steady increased interest towards plant-based diets as a prophylaxis for ADPKD. ADPKD patients with larger kidneys excrete higher amounts of urea, a surrogate for protein intake, as they progress to ESRD; however, the mechanisms that render animal-sourced protein intake harmful towards PKD are still unknown[57,58]. Plant-based proteins have been found to be effective in reducing urinary urea, protein excretions, and blood pressure in spontaneously hypertensive and normotensive rats[14,59]. A study shows that plant-based protein, soy, is more beneficial than casein when slowing down progression of cyst growth; however, the argument between plant vs. animal protein is not black and white[57]. In 2011, Aukema *et al.* used the Han:SPRD-*cy* rat model of NPHP and compared three different plant-based proteins (soy, hemp and pea) to animal protein, casein, in relation to its effects on PKD. They found greater kidney enlargement in rats given the casein and pea protein when compared to soy and hemp protein; the hemp and soy protein diet rats had kidney weights (relative to body weight) that were 17% and 21% lower in comparison to diseased kidneys in rats given the casein diet[16]. Those that consumed pea protein had kidney weights that were 27% higher than the casein diet, proving to be even more detrimental to kidney health in affected animals. In addition, the study found an increase in heart weight in diseased rats given the casein and pea protein when compared to the hemp and soy; an

overall 15% elevation in blood pressure of diseased rats on all diets compared to normal was also observed[16]. Therefore, there is an indication that disease severity can be influenced by dietary protein and its effects are primarily seen in the kidney but can also be witnessed in the heart.

A separate study tested the hypothesis that dietary flaxseed supplementation can modify the clinical course and renal pathology of NPHP[60]. Male Han:SPRD-*cy* rats were pair fed either a 10% flaxseed or the control diet for 8 weeks from weaning. They found the flaxseed-fed animals had lower serum creatinine, cystic change, renal fibrosis and macrophage infiltration compared to the control diet. However, renal tubular epithelial cell apoptosis and proliferation did not change between groups. These results suggest that whole flaxseed retards the progression of ADPKD due to the maintenance of renal tubular structure when compared to controls. Ogborn *et al.* (2006) fed both male and female Han:SPRD-*cy* rats either corn oil (CO) or flax oil (FO) diets, with or without flaxseed derivative secoisolariciresinol diglycoside (SDG) to measure flaxseed derivative effects on renal injury. FO reduced cystic change and renal inflammation in male PKD rats. In females, the same FO effects were observed in addition to reduced tissue lipid peroxides and epithelial proliferation[21]. SDG decreased renal inflammation in all animals and lipid peroxides in PKD females. The differences could arise from the relationship between hormone pathways and flax derivatives, stage of disease progression, or differences regarding dietary metabolism between genders. An in-depth review of the role of proteins, lipids and phytochemicals in the prevention of PKD progression highlights how the role of nutrition in PKD can contribute to diet based therapies towards cyst growth (discussed in [13]).

Recently, a pilot intervention study aimed to determine if a relatively complex dietary prescription targeting dietary salt, protein, acid precursors, and fluid intake would be adopted by adult patients with ADPKD[61]. At baseline, the eleven subjects were eating reasonably well-balanced amounts of protein, carbohydrates and fat. The diet in the trial was designed to mitigate risk factors shown to be associated with the development of large cystic kidneys in ADPKD which rapidly

progress to renal failure. Patients were advised to decrease their intake of animal-based protein and increase ingestion of fruits and vegetables, among other factors[61]. Urea excretion decreased by 28% in response to decreased dietary animal-protein intake, suggesting healthier ADPKD kidneys. Despite the variety of study limitations such as small sample size, short duration of treatment and lack of kidney function markers to monitor renal damage, the results address the extent to which dietary modifications may ameliorate hypertension, renal pain, hematuria and biomarkers of renal dysfunction in ADPKD clinical trials.

There has been an increase in the use of plant-based proteins to slow ADPKD progression but, as described above, the advantages are not clearly defined as hoped. Benefits of soy protein have been measured, but studies using other plant-based proteins are scarce. Research which utilizes a wide variety of plant-based proteins, such as flax protein, must be conducted to determine whether dietary interventions are beneficial in slowing disease progression.

1.5 OXYLIPINS

Saturated and monunsaturated fatty acids can be synthesized in the liver as they have either no double bonds or a single double bond whereas PUFA have multiple double bonds and must be consumed as part of the human diet[62]. Oxylipins, a subclass of lipid mediators, are oxidized metabolities of PUFA and are formed when fatty acids are released from membrane phospholipids via phospholipase A_2[63]. They are found in all tissues in the body and in the serum. Originating from various PUFA precurosors such as arachidonic acid (C20:4n-6, AA), eicosapentaenoic acid (C20:5 n-3, EPA), linoleic acid (C18:2 n-6, LA), docosahexaenoic acid (C22:6n-3, DHA), and alpha-linoleic acid (C18:2n-3, ALA), oxylipins are metabolized via cyclooxygenase (COX), lipoxygenase (LOX), and cytochrome P450 epoxygenase (CYPe), and hydroxylase (CYPh) enzymatic pathway activity[64]. Once formed, oxylipins are released into circulation, becoming involved in various

pathophysiological conditions such as proliferation, inflammation, vasoconstriction, renal disease, and cardiovascular disease[63,65–68]. The aforementioned pathways will be briefly discussed below.

1.5.1 CYCLOOXYGENASE PATHWAY (COX)

Within the COX pathway, COX enzymes convert PUFA such as dihomo-γ-linoleic acid (DGLA), arachidonic acid (AA), and eicosapentaenoic acid (EPA), into prostanoids[69]. Additionally, COX enzymes can also produce select hydroxy-FAs from AA [e.g. 11-hydroxy-eicosatetraenoic acid (11-HETE)], docosahexaenoic acid (DHA) [e.g. 13-hydroxy-docoahexaenoic acid (13-HDoHE)], and LA [e.g. 9-hydroxy-octadecadienoic acid (9-HODE)][64,70,71]. In the kidney, COX derived oxylipins maintain normal renal function; however, elevated levels are associated with worsening of renal function[72]. Studies which utilize COX inhibitors support the association of elevated COX derived oxylipins with worsening renal function, suggesting the potential negative effect of certain oxylipins[19,73–75].

In mammals, two isoforms of COX exist, namely COX-1, which is expressed in kidney vasculature, glomerular mesangial cells, and the collecting duct, and COX-2, which is constitutively expressed in the kidney and mediated by inflammation and glucocorticoids[76]. Severity of ADPKD is accompanied with noteable changes in COX-mediated eicosanoid synthesis. Within the kidney, COX2 inhibition significantly reduces disease progression and improves renal function in a *Pkd2* mouse model of ADPKD[77]. A study using 5 different models of cystic kidney disease recently demonstrated that increased levels of COX oxylipins is the earliest and most consistently observed alteration in diverse murine models of cystic kidney disease[77]. Renal cAMP is elevated in many models of PKD, and in primary cultured ADPKD cells, COX oxylipin mediated production of cAMP stimulates cell proliferation and cyst formation[78–81]. Inhibition of COX oxylipin production may reduce cyst growth by reducing cAMP, resulting in reduced renal cAMP and therefore, lower kidney size, kidney injury, and improved kidney function.

Serum from ADPKD patients show an increase in COX-mediated LA metabolites; these metabolites are pro-inflammatory lipids involved in the regulation of angiotensin II, angiogenesis, and various cell signaling and cell proliferation pathways[82]. In the Han:SPRD-*Cy* rat cystic kidneys, COX1 activity is elevated and responsible for increased production of AA-derived TXB_2 and 6-keto $PGF_{1\alpha}$; higher COX2 activity results in greater AA-derived TXB_2, 6-keto $PGF_{1\alpha}$, and PGE_2 production when compared to normal kidneys[19]. Maintenance of normal kidney function is dependent on prostaglandins (PGs), mainly PGE_2, in both animal and human models of renal insufficiency, heart failure, and liver cirrhosis[83]. COX-2 expression is increased in liver cirrhosis, possibly contributing to overproduction of PGs and a major component in inflammation associated with cirrhosis[84].

As previously mentioned, virtually all cell types employ oxylipins as signals. Oxylipins induce the blood clotting cascade (platelets), promote vasodilation (smooth muscle cells), and signal the recruitment, activation, and adherence of immune cells[69]. During acute and chronic inflammation, COX-2 is up-regulated and involved in stimulus induced prostanoid synthesis[76]. Cardiovascular disease (CVD), often characterized by thrombosis, is the leading cause of premature mortality in produce prostanoids (TxA_2, PGE_2, and PGD_2)[85]. Increased PGD_2 is responsible for cardiomyocyte apoptosis in a model of transient occlusion of the left anterior descending coronary artery; however, inhibition of the COX pathway is found to reduce PG release, attenuate apoptosis and improve cardiac function[86].

1.5.2 LIPOXYGENASE PATHWAY (LOX)

The lipoxygenase pathway containes LOX enzymes which catalyze the formation of hydroxy FAs and their metabolites, including leukotrienes, lipoxins, resolvins, protectins, maresins, hepoxilins, and eoxins[87]. Leukotrienes have a role in pro-inflammatory signalling and are activated via the metabolism of AA by 5-lipoxygenase activating protein (FLAP)[88]. Hydroxy FAs (e.g. 5-HETE)

produced via LOX are further metabolized to their keto or dihydroxy derivatives [e.g. 5,15-dihydroxy-eicosatetraenoic acid (5,15-DiHETE)][64]. These combinations of sequential LOX activities results in the formation of di- and tri- hydroxy FAs[89]. Animal LOXs are classified according to their positional selectivity when oxygenating AA, noteably 5-LOX, 8-LOX, 12-LOX, and 15-LOX[90]. Some LOX-dependent products include AA-derived HETEs, LA-derived HODEs, and EPA-derived hydroxyeicosapentaenoic acids (HEPEs)[69].

ADPKD patients have higher serum 12- and 15-LOX metabolites 12-HETE, and 15-HETE[82]. These metabolites cause further oxidative stress, vascular inflammation, cell proliferation, and endothelial dysfunction, resulting in acceleration of the disease. In the kidney, LOX derived oxylipins regulate renal blood flow, renal blood pressure, and glomarular filteration rate[77]. Similar to prostanoids, the LOX-derived oxylipins appear to mediate their effects by binding to G-protein coupled receptors (GPCRs) and intracellular effectors; however, receptors for all oxylipins have yet to be identified. Additionally, interstitial inflammation is a better prognostic marker than glomerular health for PKD advancement; inflammatory markers are present earlier than detectable cyst growth and linked to cyst progression[91]. Macrophages are inflammatory cells and have been reported in both human and animal models of PKD, showing a correlation between degree of inflitration and disease severity in humans[92]. The 15-LOX metabolite, 15-HETE, has been shown to activate intracellular effector PPARγ, a nuclear transcription factor involved in epithelial differentiation and arrest of cell growth[93]. Using PCK rats as an orthologous model of human autosomal recessive PKD, a study found that a PPARγ agonist, pioglitazone, decreases disease progression in a polycystic rat kidney suggesting that agonists inhibit disease progression by inhibiting cell proliferation and fibrosis[93]. PPARγ is expressed in both the kidney and liver, and is activated by 9-HODE and 13-HODE, oxylipins which are known to promote differentiation of human monocytes and macrophages[94]. It has been reported that the interaction between 15-HETE and PPARγ controls

the effects of 15-LOX products that inhibit renin release, have renal cytoprotective effects and, in glomerular nephritis, preserve renal function[95]. Furthermore, 5-LOX-mediated inflammatory properties in the distal nephron are antagonized by 15-LOX[96].

Cystogenesis in ADPKD is regulated by G-proteins to promote abnormal epithelial cell expansion[97]. Alterations in cAMP levels is a hallmark of G protein signalling; binding of LOX metabolite, 9-HODE, to GPCR, G2A, inhibits cAMP accumulation[98]. 9-HODE is an oxidized derivative of LA and, in its free fatty acid form, causes G2A activation, resulting in intracellular Ca^{2+} mobilization, inhibition of cAMP formation, and MAP kinase activation[98]. 12-LOX expression has been reported in rat distal tubules and its AA-derived metabolite, 12-HETE, acts as a vasoconstrictor in small renal arteries and modulates angiotensin II-induced intracullular calcium transients in rat vascular smooth muscle cells[99,100]. Renal tubular cells play a critical role in the fibrogenic processes accompanying chronic renal disease. Enhanced cAMP levels in mouse distal convoluted tubule regulates 12-LOX activity, enhancing the production of 12-HETE[101]. The pro-thrombotic and pro-inflammatory properties of 12-HETE in PKD casuse a) proliferation of glomerular endothelial cells and b) predisposition to hypertension and progressive atherosclerosis, contributing towards ADPKD-associated CVD [101]. It is reasonable to speculate that the serum concentrations of 12-LOX metabolites, primarily 12-HETE, in ADPKD2 patients will be elevated, acting as indicator of kidney damage and secondly, predicting susceptibility to cardiovascular disease.

In the kidney, LOX oxylipins regulate renal blood flow, renal blood pressure, and glomerular filteration rate[102]. In rodent models of NPHP and ARPKD and in *Pkd2*$^{WS25/-}$, LOX products were reduced in diseased compared to healthy kidneys[15,77,103–105]. Supplementation with the drug celecoxib in a *Pkd2*$^{WS25/-}$ model increased individual LOX oxylipins that were reduced in disease, but it did not alter the total levels of LOX oxylipins[77].

1.5.3 CYTOCHROME P450 PATHWAY (CYP)

The cytochrome P450 pathway involves a diverse array of membrane bound CYPh and CYPe enzymes, which obtained their name due to their unique absorbance at 450nm when reduced and bound by carbon monoxide[106]. The kidney has the highest abundance of CYP enzymes among all extrahepatic organs, with the highest concentration found in the proximal tubules[107]. CYP enzymes that form oxylipins can have CYPe or CYPh activity – enzymes via CYPe can convert AA, EPA, and DHA into epoxy-eicosatrienoic acids (EpETrEs), epoxy-eicosatetraenoic acid (EpETEs), and epoxy-docosapentaenoic acid (EpDPEs) and, through CYPh, to HETEs, hydroxy-eicosapentoenoic acid (HEPEs), and HDoHEs[108]. 11,12-EpETrE and 14,15-EpETrE are primarily associated with cardiovascular and renal function[109]. Furthermore, epoxygenase products are rapidly metabolized via soluble epoxide hydrolase (sEH) to form dihydroxy FAs from AA, EPA, and DHA, forming dihydroxy-eicosatrienoic acid (DiHETrEs), DiHETEs, and dihydroxy-docosapentaenoic acid, respectively[108]. Increased sEH activity and decreased epoxide levels correspond with increased hypertension associated inflammation and renal injury; studies have also shown that epoxides decrease vascular inflammation through inhibition of phospho-IκB kinase derived nuclear factor kappa-light-chain-enhancer of activated B cells activation[109]. LA products following either CYP pathways appear to be cytotoxic in renal cells and are elevated in inflammation[110]. Additionally, pro-inflammatory oxylipins may pre-dispose individuals to chronic disease conditions[111]. Human serum samples indicate higher 20-HETE production in ADPKD patients when compared to healthy subjects[82]. The vasoconstrictor effects of this metabolite promotes natriuresis by inhibiting Na^+/K^+ ATPase activity in proximal tubule and, in the thick ascending limb, inhibiting the Na^+-K^+-$2Cl^-$ co-transporter[112]. Also, due to its vasoconstriction properties, it is reasonable to speculate that inhibition of its formation may reduce cyst cell proliferation in ADPKD patients[113]. In the liver, 20-HETE is a potent activator of PPARα and an inflammatory mediator, playing an important role in

inflammatory disease[114]. During cirrhosis, the excretory rates of 20-HETE is significantly higher than PGs and TxB_2, compared to relatively similar excretory rates in healthy livers[115].

Overall, oxylipin levels in disease is dependent on their pathway of metabolism – COX, LOX, or CYP. In a mouse model of NPHP, a juvenile type of cystic kidney disease, total oxylipins derived from the COX pathway were elevated in the kidney while those formed by LOX- and CYP enzymes were lower in disease state[103]. These results correspond to previous findings of elevated COX- and LOX- derived oxylipins and decreased CYP associated oxylipins in renal disease states[19,76,82,84,101,109,113]. Therefore, it is reasonable to speculate COX- and LOX- oxylipins will be elevated and CYP oxylipins will be lower in kidney, liver, heart, and serum of ADPKD2 models.

1.6 OXYLIPINS IN KIDNEY, LIVER, HEART, AND SERUM

Oxylipins in the kidney play a regulatory role in normal physiological function and are involved in inflammatory and proliferative processes associated with renal injury. Increased COX derived oxylipins is the earliest and most consistent alteration in the renal oxylipin profile in diverse models of cystic kidney disease, including models of ADPKD[15,104]. In diseased kidneys, oxylipins derived from AA via the LOX and CYP pathways can be inflammatory in the kidney and LA-derived oxylipins such as EpOME and DiHOME appear to be cytotoxic in renal and other cells[116–119]. It is important to study oxylipin profiles in the kidney of ADPKD to provide a deeper understanding of cystic disease development, allowing development of effective therapies.

Oxylipin profiles in the liver and serum have been scarcely reported, with the majority of studies focused on dietary interventions and its effects on the kidney and its oxylipins[14,87,93,120]. The majority of oxylipins in the liver and serum are derived from AA and in the rat liver, oxylipins derived from LA were proportionately higher than LA itself while AA oxylipins were also proportionately higher than the level of their AA precursor in the serum[120]. However, the data obtained from the study was conducted in normal rat tissues and serum and there have been no comprehensive oxylipin profile reports done on mouse tissues and serum. Oxylipin data from

ADPKD liver may help explain why some ADPKD patients develop secondary cysts in the liver while others do not. Furthermore, oxylipin profiles in the serum could act as biomarkers for disease if a pattern of difference between disease and oxylipin profiles exist.

Many oxylipins, especially those derived from AA, influence cardiac physiology in health and disease; for example, 19-HETE reduces cardiomyocyte hypertrophy while 20-HETE and EpETrE induce cardiac hypertrophy[121,122]. EPA-derived oxylipins such as 18-HEPE, also have anti-inflammatory effects on the heart[123]. CYPe derived oxylipins are reported to be highly expressed in hearts[124,125]. Their products are cardioprotective, with roles such as vasodilation, mitochondrial protection of cardiomyocytes, and decreased cardiac fibrosis and inflammatory effects[126–128]. If ADPKD patients have low CYPe derived oxylipins in the heart, it could indicate the link between ADPKD and cardiovascular disease, potentially facilitating future therapeutic interventions.

1.7 SEX DIFFERENCES IN OXYLIPINS

The effect of sex on oxylipins is largely unknown; however, there are some indications that there is a difference between male and female oxylipin levels in the kidney. Understanding oxylipin profiles may be helpful in advancing our knowledge of sex differences in health and disease [129]. Oxylipin profile studies have demonstrated higher levels of renal, hepatic, and serum oxylipins in male rats[120,130]. In ~40% of total oxylipins, sex differences in Sprague-Dawley rats were observed in the kidney (30/69), liver (32/71), and serum (23/60), of which almost all were higher in males[120]. Furthermore, rat oxylipin/PUFA ratios in males were greater than or similar to females for all oxylipins for all PUFA substrates, with the exception of 12,13-DiHODE/ALA ratio in the serum[120]. Mouse models of cystic kidney disease found that total AA-derived oxylipins were higher in the kidneys of females than males[131]. However, other n-6 PUFA derived oxylipins in mice that were affected by sex were higher in male kidneys[131]. AA-derived oxylipins produced via the COX pathway are higher in females, while oxylipins derived from non-AA fatty acids are higher in males in mouse models of *Pkd1* and *Pkd2*[15]. This suggests that sex effects may be influenced by the fatty

acid substrate. Moreover, in various rodent models of kidney disease, total oxylipins from LA, GLA, and DGLA were not affected by sex; however, up to 50% of individual LA oxylipins were elevated in males[15].

In the heart, AA oxylipins have been associated with various functional effects; for example, lipoxygenase-generated HETE are increased in mitochondria from failing human hearts and vasoconstriction in small porcine coronary arteries via 20-HETE[132,133]. Until 2019, the sex effects on the heart oxylipin profiles had not been examined[134]. The study found that oxylipins in DHA fed rats were similar in females and males, but if given a diet enriched with ALA, the oxylipins derived from DHA were higher in females – a novel finding which may be due to hormones; however, further studies must be conducted[134]. Furthermore, CYPe enzymes are highly expressed in the heart, and their epoxygenated products appear to be cardioprotective with roles such as vasodilation via activation of Ca^{2+}- sensitive K^{+} channels, mitochondrial protection of cardiomyocytes, and decreased cardiac fibrosis and inflammation effects[124–128].

A study reported higher levels of 20-HETE or its metabolites (20-carboxy-AA) in female renal tissue[120]. However, previous studies have reported that while renal 20-HETE levels increase differentially during different phases of pregnancy in rats, a separate report demonstrated that androgen treatment in male CYP 4a14 knockout mice increased 20-HETE levels[135,136]. The contrast between these studies highlights that there is much more work to be done to understand and explain these differences. In various models of cystic kidney disease which included both sexes, total oxylipins derived via the COX pathway were higher in females[15,137]. In Sprague-Dawley rats, sex differences were observed in 21/70 oxylipins in the liver and 49/70 in the serum[130]. Of those observed, 16/21 were higher in males in the liver and 46/49 were higher in males in the serum. Recent findings in mouse kidneys display that oxylipins derived from AA are lower and oxylipins from EPA and DHA are higher in males, contrasting the results found in rat kidney where DHA and EPA oxylipins were higher in females[15,130]. This data suggests that there are species-specific effects

of sex on renal oxylipins and more research needs to be conducted to understand and elucidate these differences.

CHAPTER 2: EFFECT OF DIETARY FLAX PROTEIN ISOLATE ON DISEASE PROGRESSION IN THE *Pkd2*$^{WS25/-}$ MOUSE MODEL OF ADPKD2

2.1 INTRODUCTION

Studies have exploited various animal models of human disease to understand the relationship between diet and disease, with increased interest in plant-based diets as prophylactic treatments for ADPKD. Plant-based proteins have been found to be effective in reducing urinary urea, protein excretions, and blood pressure in spontaneously hypertensive and normotensive rats[14,59,138]. The recommended dietary protein consumption in humans is 0.8 – 1.5g protein/kg body weight[13]. However, protein intake exceeding 0.8 protein/kg body weight in ADPKD patients has been suggested to negatively affect cyst progression, resulting in higher renal perfusion and kidney hyperfilteration of the compromised nephron[13]. Dietary soy protein has been studied intensely and has been suggested as a safe alternative or partial substitute for animal protein-based diets in ADPKD[13,16]. Soy protein is the only widely available plant protein shown to be comparable to animal protein pertaining to nutritive effects based on numerous nitrogen balance studies using isolated soy protein or soy protein concentrate[139]. However, not all plant-based proteins are comparable when discussing ADPKD therapeutics. In 2011, Aukema *et al.* used a Han:SPRD-*cy* rat model of PKD and compared three different plant based proteins (soy, hemp and pea) to animal protein, casein, in relation to its effects on ADPKD[16]. Hemp and soy protein diet mice had lower kidney weights (relative to body weight) compared to those given the pea and casein diets ($p<0.05$). Additionally, the pea protein was found to be more detrimental than casein-protein regarding kidney health in ADPKD[16]. Increased heart weight in diseased casein or pea-protein diet mice were reported alongside with a 15% blood pressure elevation in diseased rats when compared to normal. Rats with cystic kidney disease have been shown to have larger hearts, augmented left ventricular mass, and elevated blood pressure[140]. Ingestion of soy protein vs. casein results in slight improvements in systolic and mean arterial pressure however, the mechanism responsible for these effects are unknown.

A separate study tested the hypothesis that dietary flaxseed supplementation can modify the clinical course and renal pathology of ADPKD[60]. They found the flaxseed-fed animals had lower serum creatinine, cystic change, renal fibrosis, and macrophage infiltration as compared to the control diet, suggesting that whole flaxseed hinders the progression of ADPKD. However, renal tubular epithelial cell apoptosis and proliferation was not different between groups. Flax (*Linum usitatissimum L.*) is commercially cultivated oilseed crop[60]. Compared to other plant proteins, such as soy, flaxseed proteins have a greater capacity to absorb water and oil[141]. This is likely due to the two major fractions of flaxseed proteins, one salt-soluble with high moelcular weight (11-12S) and another water-soluble with low molecular weight (1.6-2S)[142]. Due to the lack of effective extraction methods, the use of flaxseed protein isolate (FPI) in the food industry has been primarily limited to its oil and whole seeds. Defatted flax meal, which is the residue after extraction of flaxseed oil, is presently being used as livestock feed while limited attention has been given to the physicochemical and functional properties of the constituent proteins[142]. A study compared the amino acid profile of FPI, to whey protein isolate and soy protein isolate and found that the essential amino acid profile of FPI was similar to those observed in whey and soy[143]. Due to the similarity in composition to soy protein, and the demonstrated benefit of whole flaxseed in an NPHP model[60], it is reasonable to speculate that FPI can be used as a nutraceutical towards treatment of ADPKD.

2.2 OBJECTIVES

To determine whether dietary FPI can slow disease progression in an orthologous ADPKD2 mouse model (*Pkd2*$^{-/WS25}$).

2.3 HYPOTHESIS

We hypothesize that the *Pkd2*$^{WS25/-}$ mice given the FPI in replacement of casein protein will exhibit delayed cyst development.

2.4 MATERIALS AND METHODS

2.4.1 GENOTYPING PROTOCOL

Polymerase chain reaction (PCR) was used to determine the genotypes of the mice to be included in the study. Ear notches were taken after weaning to extract DNA and mixed with three primers before being placed in the BioRad thermal cycler with the pre-programmed PKD2 genotyping protocol. The components in the PCR tubes alongside extract mouse genomic DNA was the RED extract-N-amp PCR reaction mix (10μL), McF15b Primer (2μL) 5'GCG CCG GCC TAG CTG TCC 3', McR14 Primer (2μL) 5' GTT GTC GCG GCT CCA CG 3', NeoARL Primer (2μL) 5'GTG CTA CTT CCA TTT GTC ACG TCC TGC 3', and the tissue extract (4μL), creating a total volume of 20μL.

Upon completion in the thermal cycler reactions, the PCR products were electrophoresed in an agarose gel. An electric current was applied to the gel, causing the negatively charged DNA to move towards the positive end, resolving the DNA amplicons by size. The gel contained SYBR safe, allowing the visualization of the bands. Genotyping of these mice resulted in the production of either 1 or 2 bands. Two bands are observed if the genotype is either +/-, WS25/-, WS25/+ or WS25/WS25, whereas if only the lower band is observed the genotype is +/+ and these mice are not used for the study (Figure 1).

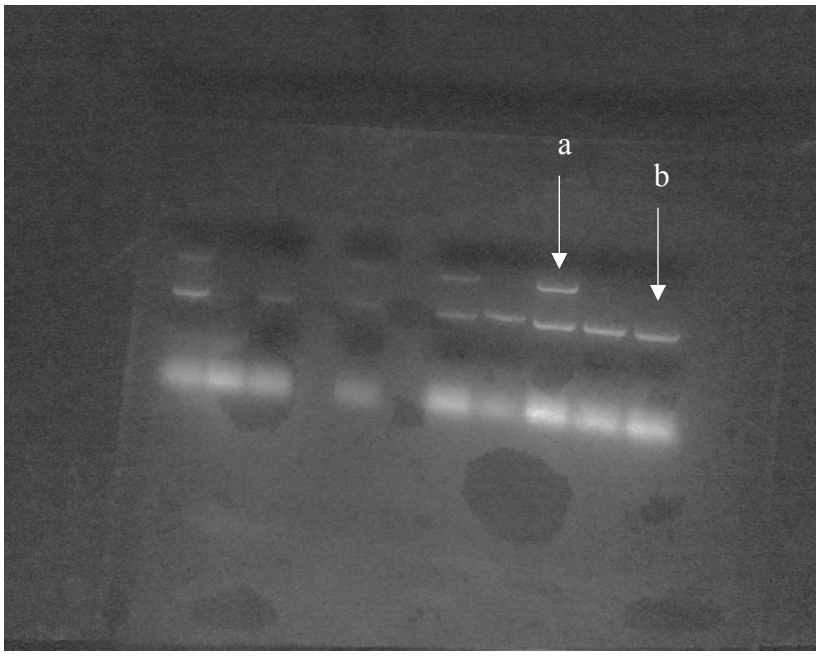

Figure 1: Genotyping for the WS25 or mutant allele. The lane indicated by ‘a’ with 2 bands signifies either the +/-, WS25/-, WS25/+, or WS25/WS25 genotype. The lane indicated by ‘b’ signifies the +/+ genotype.

2.4.2 ANIMAL MODEL

The study mimicked human ADPKD2 by utilizing an orthologous mouse model (*Pkd2*). *Pkd2*$^{WS25/WS25}$ and *Pkd2*$^{WS25/+}$ mice were crossed with *Pkd2*$^{+/-}$ breeders originally obtained from the Dr. Stefan Somlo lab (Yale University, New Haven, CT, USA) to form both normal mice (*Pkd2*$^{WS25/+}$, *Pkd2*$^{+/+}$) that do not develop cysts and diseased mice (*Pkd2*$^{-/WS25}$). Double heterozygote *Pkd2*$^{WS25/-}$ mice with one mutated *Pkd2* allele and one unstable allele (WS25), have been proven to reliably develop renal and hepatic cysts within 9 weeks and 11 weeks, respectively [33,144]. These features make this specific orthologous mouse a good model to test experimental therapies for ADPKD.

The study involved 76 male and female mice which were divided into 2 groups, those who received the FPI (n=37) and those given the standard AIN-93G diet (n=39). The genotypes of the mice could not be determined until necropsy; therefore, the mice were randomly allocated to the diets. The AIN-93G standard diet contained casein, an animal protein, whereas the diet for the intervention group had the casein replaced with FPI provided by the Aluko lab (Department of Food and Human Nutritional Sciences, University of Manitoba, Winnipeg, MB, Canada). Mice were placed on their respective diets 4 weeks after weaning and housed individually in a temperature and humidity-controlled environment with a 12-hr day/night cycle with free access to water and diet. The feeding period for the study lasted 13 weeks. At the end of the study, the mice were anesthetized in a gas chamber with isoflurane and terminated by decapitation to collect blood. Final body, kidney, liver, and heart weights were recorded, and blood was collected and centrifuged to obtain serum. The right kidney, liver, and heart were snap frozen in liquid nitrogen and stored, along with serum, at -80°C until analysis. All animal procedures have been approved by the University of Manitoba Animal Care Committee and adhere to the guidelines of the Canadian Council on Animal Care (Appendix A).

2.4.3 DIET COMPOSITION

The diets were formulated to ensure proper nutrient to energy ratio during feeding. Diet preparation protocol can be found in Appendix B. Compared to casein, FPI was significantly richer in carbohydrates, fibre, and sodium; therefore, these ingredients were adjusted to the standard diet, in order to ensure that both diets remained isocaloric and had similar amounts of these ingredients (Table 1; Table 2). Table 1 describes the amino acid concentration in the protein isolates. Sodium and seven essential amino acids where added to the standard and flax diet respectively, to remove the differences in the diet. The AIN-93G standard diet contains animal derived protein source, casein (87%)[145]. The flax diet had similar components to that of the AIN-93G; however, flaxseed protein (62.9%) constituted the main protein source. FPI was obtained from Dr. Aluko's lab at the University of Manitoba. Diet was given 3x/week between the hours of 7am -9am to maintain consistency and in quantities of 3.5g/day. Diet was a powdered mix and presented to the animals in a small glass container within a metal bowl for both ease of access and to prevent the mice from wasting food.

2.4.4 STATISTICAL ANALYSIS

Data was analyzed using R software (R Development Core Team, New Zealand). Diagnostic tests to evaluate assumptions of normality and uniformity of variance was performed and if necessary, the data was transformed prior to analysis. Shapiro-wilks test was used to measure normality, followed by Kruskal-Wallis if normality is not achieved. A 3-way ANOVA (sex x diet x genotype) was used to test the interactions between the groups and determine significance. Data was considered significant a p-value less than 0.05.

Table 1: Amino acid composition of casein and flax protein isolate (FPI).

Amino Acids	Casein (g/1 kg)	FPI (g/1 kg)
Arginine	4	7
Aspartic acid	7	7
Glutamic acid	21	15
Histidine	3	3
Leucine	9	**4**
Methionine	3	**1**
Phenylalanine	5	**3**
Threonine	4	**1**
Tryptophan	1	1
Alanine	3	2
Cysteine	0	1
Glycine	2	4
Isoleucine	5	**2**
Lysine	7	**2**
Proline	12	3
Serine	5	4
Tyrosine	5	2
Valine	6	**3**

Listed in red are the amino acids which were added to the FPI diet to ensure that the essential amino acids were balanced between the two diets.

Table 2: Composition of the experimental diets.

Nutrient Composition	*AIN-93G Diet (g/100g)*	*Kcal*	*Flax Diet (g/100g)*	*Kcal*
Cornstarch	39.75	1.59	32.08	1.28
Protein*	**20.00**	**0.80**	**27.67**	**1.11**
Dextrinized Cornstarch	13.20	0.53	13.20	0.53
Sucrose	10.00	0.40	10.00	0.40
Soybean Oil	7.00	0.63	7.00	0.63
Fibre	5.00	-	4.86	-
Mineral Mix (AIN 93G)	3.50	-	3.50	-
Vitamin Mix (AIN 93G)	1.00	-	1.00	-
Total	**100.52**	**3.96**	**100.19**	**3.96**

*Protein in the AIN-93G diet is casein (87% protein) and in the flax diet is flaxseed protein isolate (62.9% protein).

2.5 RESULTS

2.5.1 GENERAL RESULTS

After 13 weeks of feeding, body weights of the mice presenting with disease compared to healthy animals were not different, but female mice had lower overall body weights as compared to males (Table 3). Dry kidney weights were calculated via lyophilization (Table 3); sex effects were deemed significant while diet effects were not. Once normalized to body weight, male kidney weights were higher in disease presenting mice compared to their healthy counterparts; the heart and liver showed no difference.

2.5.2 STANDARD DIET VS. FLAX PROTEIN ISOLATE

There was no difference between kidney, liver, heart, and body weights between mice given the control diet or the FPI. When expressed relative to body weight, the only effect of FPI was on heart weight relative to body weight. Overall, this parameter was higher in FPI compared to control fed mice.

Table 3: Effects of diet and genotype (healthy v disease) on body weights and tissues of male and female mice.

	Male				Female						
	Control		Flax		Control		Flax		Effects (P values)		
	Normal (n=13)	Disease (n=12)	Normal (n=19)	Disease (n=8)	Normal (n=12)	Disease (n=2)	Normal (n=6)	Disease (n=4)	Diet	Sex	Genotype
Body Weight (BW)(g)	29.3 ± 0.78	27.2 ± 0.81	26.4 ± 0.65	26.1 ± 1.0	19.6 ± 0.81	19.8 ± 1.99	20.8 ± 1.15	20.1 ± 1.41	0.089	**<0.0001**	0.35
Kidney Weight (KW)(g)	0.28 ± 0.040	0.41 ± 0.042	0.29 ± 0.033	0.63 ± 0.052	0.29 ± 0.042	0.39 ± 0.10	0.25 ± 0.060	0.31 ± 0.073	0.56	**0.043**	0.56
KW/BW (g/kg)	8.24 ± 1.04	11.13 ± 1.08	7.64 ± 0.86	16.42 ± 1.32	5.76 ± 1.08	7.64 ± 2.6	5.21 ± 1.52	6.14 ± 1.87	0.74	**<0.0001**	**<0.0001**
Liver Weight (LW)(g)	1.19 ± 0.056	1.13 ± 0.058	1.19 ± 0.046	1.15 ± 0.071	0.83 ± 0.058	0.87 ± 0.14	0.96 ± 0.082	1.11 ± 0.10	0.15	**<0.0001**	0.84
LW/BW (g/kg)	35.8 ± 2.26	31.0 ± 2.35	31.8 ± 1.87	30.0 ± 2.88	16.3 ± 2.35	17.2 ± 5.75	20.2 ± 3.32	22.3 ± 4.07	0.95	**<0.0001**	0.52
Heart Weight (HW)(g)	0.12 ± 0.0048	0.12 ± 0.005	0.12 ± 0.004	0.12 ± 0.006	0.10 ± 0.005	0.10 ± 0.120	0.010 ± 0.007	0.09 ± 0.009	0.16	**<0.0001**	0.62
HW/BW (g/kg)	3.63 ± 0.17	3.28 ± 0.18	3.06 ± 0.14	3.04 ± 0.22	1.96 ± 0.18	1.99 ± 0.44	2.04 ± 0.26	1.75 ± 0.31	**0.047**	**<0.0001**	0.38
Dry Kidney Weight (g)	0.073 ± 0.05	0.048 ± 0.01	0.044 ± 0.006	0.051 ± 0.010	0.033 ± 0.006	0.041 ± 0.009	0.036 ± 0.006	0.037 ± 0.003	0.38	**0.041**	0.67
Kidney Water Weight (g)	0.07 ± 0.04	0.16 ± 0.005	0.101 ± 0.03	0.264 ± 0.016	0.12 ± 0.03	0.154 ± 0.05	0.089 ± 0.003	0.118 ± 0.028	0.81	0.052	0.81

The kidney weight is the combined weight of both the left and right kidney. Dry kidney and kidney water weight are from one kidney only.

1) Values are means + SD. The main and interaction effects were analyzed by 3-way ANOVA. Significant if $p \leq 0.05$. There were no interactions between diet, sex, and genotype for any of the parameters.

2.6 DISCUSSION

In ADPKD, pharmacological therapies typically target cyst pathogenesis; however, their efficacy is inconsistent[146]. Bioactive compounds found in plant-based food have anti-proliferative, antioxidant, and anti-inflammatory properties, suggesting that dietary intervention may provide as an additional therapeutic option[13]. Current literature displays a lack of human trials on ADPKD, with the majority of studies using genetic rodent models[13,146]. The current study aimed to create a better understanding of the role of nutrition, specifically FPI, on ADPKD and its potential contribution towards diet-based therapies to reduce ADPKD disease progression and severity.

On average, plant proteins are considered to be lower-quality proteins when compared to animal proteins[147]. However, various PKD animal models have shown a reduction in cyst proliferation and growth when given soy protein isolate (SPI), in place of regular casein, in the diet[16,19,105]. Flax (*Linum usitatissimum L.*) is commercially cultivated as a field crop and generally considered a non-edible oilseed[60]. Compared to other plant proteins, such as soy, flaxseed proteins have a greater capacity to absorb water and oil[141]. This is likely due to the two major fractions of flaxseed proteins, one salt-soluble with high molecular weight (11-12S) and another water[142]. Moreover, flaxseed protein is known to release bioactive peptides upon enzymatic hydrolysis, particularly antihypertensive peptides during simulated GI ingestion[148]. Due to the lack of effective extraction methods, the use of flaxseed protein isolate (FPI) in the food industry has been primarily limited to its oil and whole seeds. A study tested the hypothesis that dietary flaxseed supplementation can modify the clinical course and renal pathology of ADPKD[60]. They found the flaxseed-fed animals had lower serum creatinine, cystic change, renal fibrosis, and macrophage infiltration compared to the control diet; however, renal tubular epithelial cell apoptosis and proliferation did not change between groups. Overall, this data suggests that whole flaxseed slows the progression of ADPKD.

In the present study, across all main factors tested by three-way ANOVA (diet x sex x genotype) no interactions were observed. There was one diet effect that was observed – heart weight

normalized to body weight (HW/BW) illustrated a difference ($p \leq 0.05$) between the two diets in both sexes (Table 3). All tissue weights and body weights differed between sexes while only kidney weight normalized to body weight (KW/BW) was affected by genotype – kidney weights were higher in both diseased male and female mice compared to healthy. Similar to what was found in a 2016 dietary intervention study on the development of ADPKD, there were no benefits and possible negative effects on disease in *Pkd2*$^{-/WS25}$ mice given a soy protein diet[18].

Previous studies have used either whole flaxseed or flaxseed oil to measure its effects on disease progression whereas little is known about the effects of FPI. Studies have shown that dietary flax oil reduces kidney size, water content, cyst growth, and fibrosis in both the Han:SPRD-C*y* rat and *pcy* mouse[21,103]. In contrast, dietary flax oil shows no beneficial effects on disease in orthologous rodent models of ADPKD[18]. A study using the *Pkd2*$^{WS25/-}$ mouse found that, with respect to dietary protein source such as soy protein, no differences were observed regarding disease progression[18]. As earlier established, FPI is similar in amino acid composition to soy protein; therefore, the results of our study are in-line with previously obtained data[18]. Various studies have shown significant benefit of dietary protein intervention in non-orthologous models, where disease progression is more rapid and animals are terminated at a later stage of disease[105,149,150]. This suggests that the data from our study which show no effect of FPI may be due to duration of study or the fact that it was an orthologous mouse model, i.e. there may have been an effect if termination occurred at an older age, in a later disease stage, in a non-orthologous model.

2.7 CONCLUSIONS

The current data was the first of its kind to use an orthologous mouse model of ADPKD2 in a FPI study. The aim of the study was to determine whether a FPI would be an effective agent to slow the progression of ADPKD2 as the isolate has similar properties to that of soy protein, a protein with proven benefits in this disease model. Larger tissue weight is a normal ocurance in tissues from diseased compared to healthy mice. Based on preliminary analysis regarding tissue weight

normalized to body weight, our study found no differences between tissue size, diet, and presence of disease. This finding suggests that FPI does not act in a similar manner as soy protein in regard to decreasing disease severity in ADPKD2 and it also suggests that the FPI component of flaxseed may not be responsible for the nutraceutical properties previously reported. Despite the lack of benefit of FPI on decreasing the progression of ADPKD2, FPI may contain nutraceutical properties in other models of cystic kidney disease or other types of disease. Future research investigating the beneficial properties of FPI should be conducted to provide a better understanding on how it can be used for treatment of disease.

CHAPTER 3: OXYLIPIN PROFILE DIFFERENCES BETWEEN HEALTHY AND DISEASED TISSUES IN AN ORTHOLOGOUS MALE MOUSE MODEL OF *Pkd2*$^{WS25/-}$

3.1 INTRODUCTION

Autosomal dominant polycystic kidney disease (ADPKD) is a genetic disorder characterized by the presence of fluid-filled cysts, leading to a progressive decline in renal function[151]. Previous studies with ADPKD models have demonstrated alterations in oxylipins, such as an increase in cyclooxygenase (COX) products in diseased kidneys[4,15,22,82,131,131]. Oxylipins are PUFA derived oxidation products formed via one or more mono- or dioxygen- dependent reactions and are critical for normal renal physiology and function but can also have detrimental effects in disease. The metabolism of PUFAs into oxylipins occurs through three primary enzymatic pathways: COX, lipoxygenase (LOX), and CYP (CYPe/CYPh). Once formed, oxylipins act as signaling molecules and are involved in the regulation of many cell and tissue responses. They have been attributed to inflammation, vasoconstriction, and chronic disease while also having anti-inflammatory and vasodilatory properties – playing an important role in maintaining body homeostasis. For example, CYP products of fatty acids demonstrate anti-inflammatory effects by preventing the activation of nuclear factor κB[152]. 20-HETE is involved in the regulation of blood pressure, and cardioprotection while LOX metabolites such as 12-HETE, 15-HETE and 13-HODE exhibit additional effects on cell proliferation and regulation of apoptosis[153–155]. In 2017, a targeted lipidomic analysis of renal oxylipins in diverse models of cystic kidney diseases examined whether oxylipins were altered in the renal tissue in those with cystic kidney disease[15]. The data revealed that COX oxylipins were consistently elevated across all cystic kidney disease models – including ADPKD – while LOX and CYP oxylipin changes were dependent on specific cystic kidney diseases. Another study focused on NPHP, an inherited juvenile type of PKD, used a *pcy* orthologous mouse model to human NPHP to determine whether there were alterations in renal fatty acid and oxylipin levels in early stages of the disease[104]. The findings showed that renal COX-derived oxylipins were increased while those that derived from LOX were decreased in NPHP. The findings allow us to speculate that in cystic

kidneys, there will be an increased in COX associated oxylipins and a decrease will occur in the LOX metabolities.

However, the oxylipin profile alterations in the kidney may not be the same in the heart. Compared with other tissues, the heart is much more resistant to changes in oxylipins derived from omega-3 PUFA[134]. Platelet 12-LOX, an oxygenase predominatly expressed in human platelets, uses AA released from phospholipids as a substrate to form 12-HETE, an oxylipin shown to regulate biological processes such as integrin activation and vascular hypertension[156]. It is important to understand the oxylipin profiles in the heart in ADPKD patients as CVD is one of the main causes of morbidity and mortality in patients with ADPKD[157].

There are only a few studies which have measured oxylipin profiles in the liver and serum. Secondary cysts in the liver commonly occur in many cases of ADPKD but the causality of this is unknown[8]. Oxylipin data from ADPKD liver could eludicate as to why some ADPKD patients develop secondary cysts in the liver while others do not. Furthermore, oxylipin profiles in the serum could act as biomarkers for disease if a pattern of difference between disease and oxylipin profiles exist. Overall, lower levels of LA oxylipins have been reported in the serum of PKD patients and the liver has higher levels of CYP oxylipins relative to other tissues[87,158].

By analyzing oxylipins in the kidney, heart, liver and serum, we may be able to achieve a better understanding of oxylipins and their potential roles in ADPKD. Specifically, we would be able to determine whether there are differences in profiles in serum or tissues known to be affected in this disease. The current study identifies oxylipins in both healthy and disease heart, kidney, and liver of ADPKD2 mice and investigates whether the oxylipin profiles in the serum can be used as a biomarker for ADPKD.

3.2 OBJECTIVES

Investigate serum, heart, kidney, and liver oxylipin profiles in an orthologous ADPKD2 mouse model (*Pkd2*$^{-/WS25}$). Determine whether serum oxylipin profiles can be used as a potential biomarker for disease.

3.3 HYPOTHESIS

The oxylipin profiles in diseased kidney, liver, heart, and serum will differ when compared to healthy mice, with a higher presence of COX-derived oxylipins.

3.4 MATERIALS AND METHODS

3.4.1 GENOTYPING PROTOCOL

Refer to Chapter 2, section 2.4.1 Genotyping Protocol.

3.4.2 MICE

Male healthy and diseased mice given the standard diet from Chapter 2 (n=8) were used for this analysis. These mice were provided the AIN-93G standard diet from the age of 4 weeks for a period of 13 weeks. At the end of the study, the mice were anesthetized in a gas chamber with isoflurane and terminated by decapitation to collect blood. Final body, kidney, liver, and heart weights were recorded, and blood was collected and centrifuged to obtain serum. The right kidney, liver, heart, and serum were 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 adhere to the guidelines of the Canadian Council on Animal Care (Appendix A).

3.4.3 OXYLIPIN ANALYSIS

Oxylipins were quantified by HPLC-tandem mass spectrometry, an established method in the Aukema lab (Protocols in Appendix C-J)[77,87,130,134]. Liver, heart, and lyophilized kidney tissues were homogenized in ice-cold Tyrode's buffer (pH 7.6) and homogenates and serum oxylipins were

extracted using Strata®X solid phase extraction columns (Phenomenex, CA). Eluted samples were analyzed by liquid chromatography-tandem mass spectrometry using a Shimadzu HPLC System coupled to API 6500 triple quadrupole tandem mass spectrometer with turbospray (Sciex Instruments, ON). Oxylipins were quantified by reverse-phase LC on a Bead Homogenizer. With this methodology, it is possible to detect and quantify over 150 oxylipins in a single run, which are identified by retention time and transition ions, using stable isotope dilution internal standards (Cayman Chemical, MI). Internal standards and external standard curves were used to quantify the oxylipins, as outlined in Appendix H.

3.4.4 STATISTICAL ANALYSIS

Data was analyzed using R software (R Development Core Team, New Zealand). Diagnostic tests to evaluate assumptions of normality and uniformity of variance were performed and if necessary, the data was transformed prior to analysis. Shapiro-wilks test was used to measured normality, followed by Student t-Test or the Mann Whitney test to determine significance. Data was considered significant a p-value less than 0.05.

3.5 RESULTS

3.5.1 GENERAL RESULTS

At the age of 17 weeks, body weights of mice presenting with ADPKD2 compared to healthy were not different. Kidney weights were higher in disease presenting mice compared to their healthy male counterparts; the weights of their heart showed no difference (Table 4).

Table 4: Final body and tissue weights of diseased and healthy male *Pkd2*$^{WS25/-}$ mice at 17 weeks.

Tissue Weight			**Effects (P values)**
	Healthy	*Disease*	*Disease vs Health*
Body Weight (g)	30.3 ± 4.8	27.4 ± 1.85	0.06
Kidney (g)	0.135 ± 0.019	0.218 ± 0.09	**0.004**
Liver (g)	1.22 ± 0.28	1.15 ± 0.18	0.46
Heart (g)	0.121 ± 0.017	0.123 ± 0.022	0.73

Values are mean ± SD (n=8).

Student's t-tests determined significance at $p \leq 0.05$.

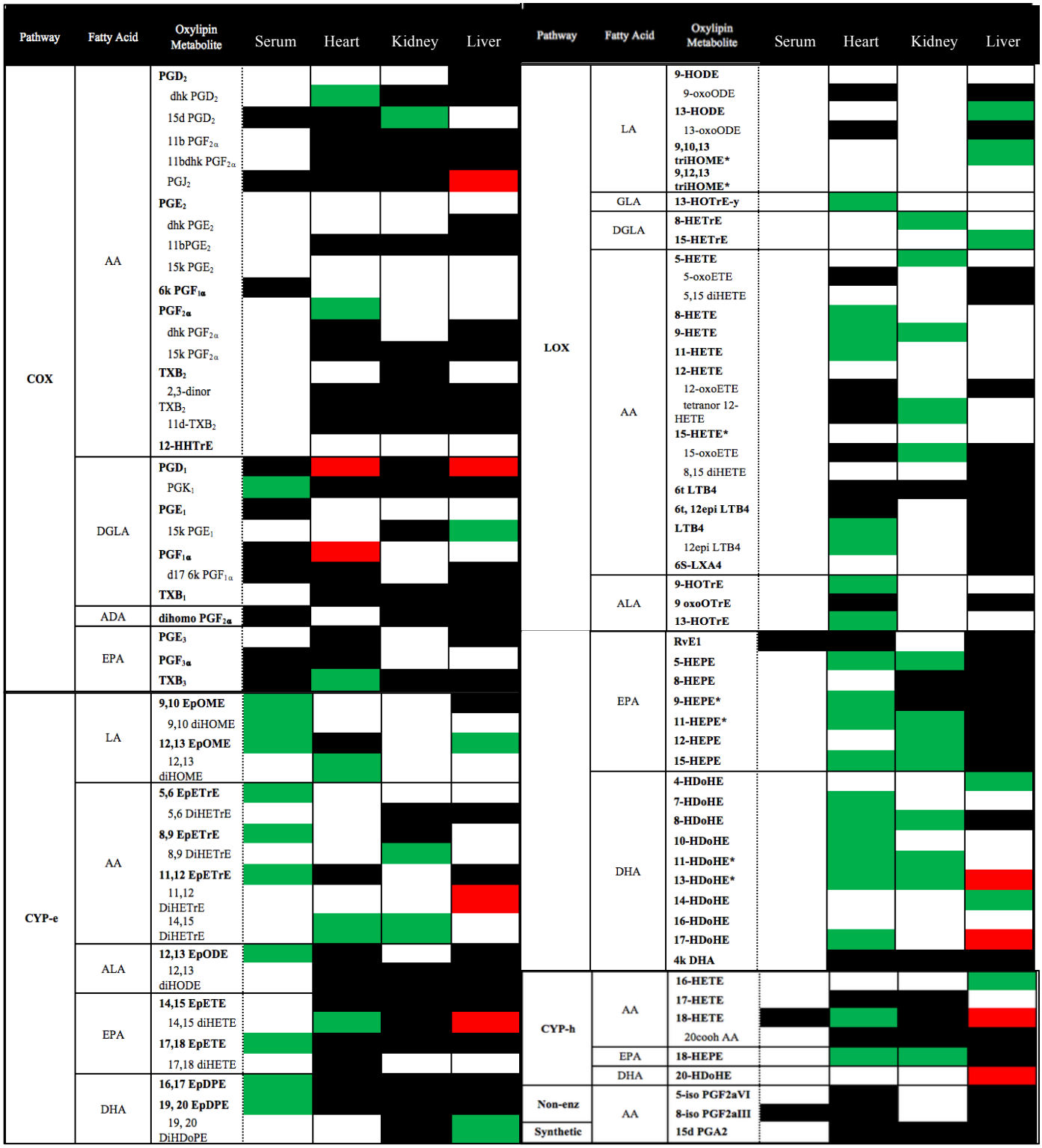

Figure 2: Oxylipins quantified in male Pkd2$^{-/WS25}$ mice. For healthy compared to disease, green refers to lower oxylipin levels, red refers to higher oxylipin levels, white refers to alterations deemed not significantly different, and black refers to oxylipin not detected. Significance at $p < 0.05$.

3.5.2 SERUM CYP DERIVED EPOXYGENATED PUFAS WERE LOWER IN DISEASE

In the serum, 89 oxylipins were detected and 11 were significantly different between healthy and diseased *Pkd2*$^{-/WS25}$ mice (Supplementary Material Table 6; Figure 2). Of the 11, 1 was derived from the COX-pathway (PGK1) and the remaining 10 were metabolized via the CYPe pathway (9,10 EpOME; 9,10 diHOME; 12,13 EpOME; 5,6 EpETrE; 8,9 EpETrE; 11,12 EpETrE; 12,13 EpODE; 17,18 EpETE; 16,17 EpDPE; 19,20 EpDPE). All the oxylipins that were different were lower in the diseased mice. Serum oxylipin directly formed via the CYPe pathway were lower in diseased compared to healthy mice; alternatively, the oxylipins formed from these CYPe derived oxylipins by sEH (apart from 9,10 DiHOME) did not display significant alterations in profile levels between healthy and disease. Overall, of the 20 oxylipins that were detected in both healthy and diseased serum derived from the CYPe pathway, 8 of which had both the oxylipin substrate (epoxide) and its corresponding product (diol) detected. Individual (Figure 3) and total (Figure 4) sEH product/substrate (diol/epoxide) ratios were calculated and 3 diol/epoxide ratios (Figure 3) were higher in disease (17,18 diHETE:EpETE, 11,12 DiHETrE:EpETrE, and 9,10 diHOME:EpOME); this is due in part to the lower epoxide levels in normal serum (Figure 4). No significant profile changes were observed from the LOX nor CYPh pathways. These results indicate that serum oxylipin profile changes between healthy and disease are pathway dependent, specifically those formed by the CYPe pathway.

3.5.3 HEART AND KIDNEY LOX OXYLIPINS WERE LOWER IN DISEASE

Both heart and kidney tissue from diseased mice had an overall trend of lower levels of primarily LOX-derived oxylipins as compared to healthy mice. Within the heart, 65 oxylipins were detected with 27 oxylipins illustrating a significant difference in concentration ($p \leq 0.05$) between healthy and disease (Supplementary Material Table 7; Figure 2).

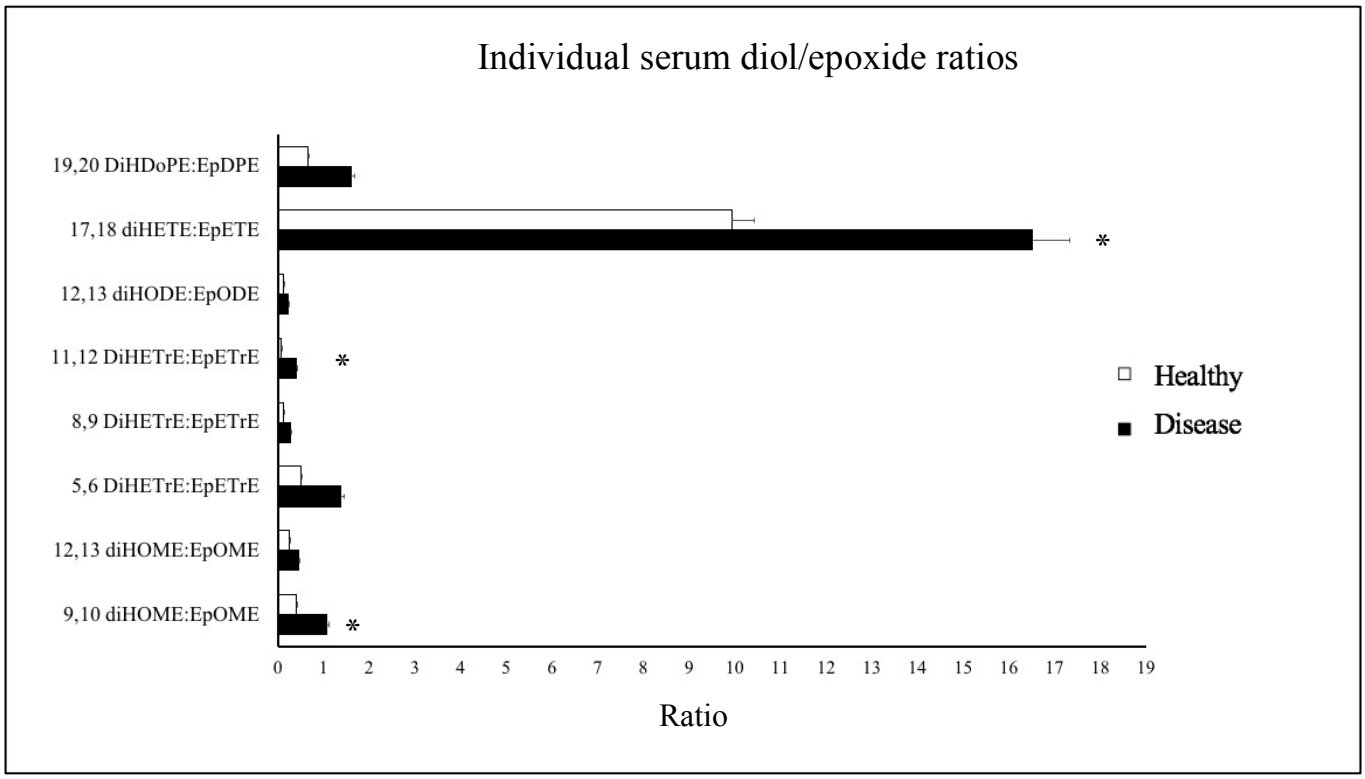

Figure 3: Individual serum sEH product/substrate (diol/epoxide) ratios in healthy and diseased *Pkd2*$^{WS25/-}$ mice. Significance (*) at $p < 0.05$.

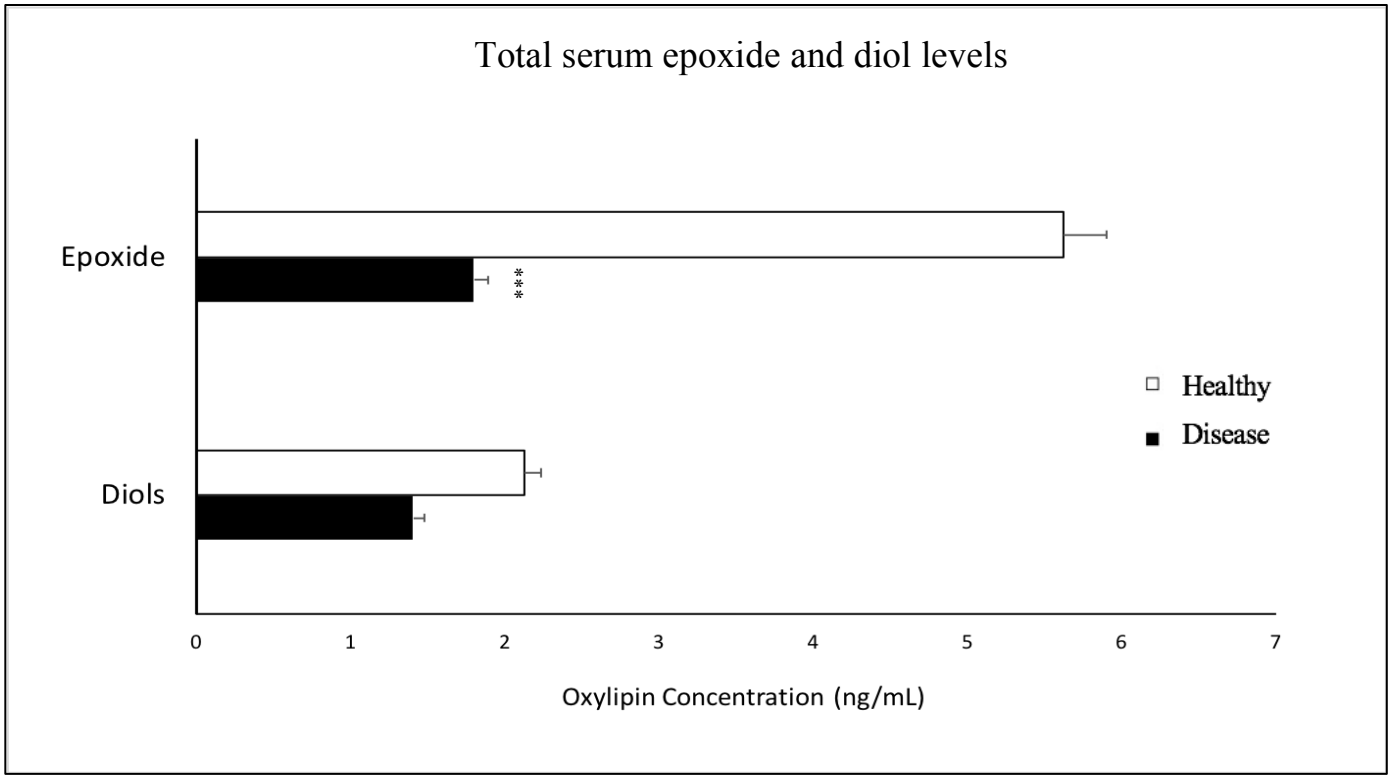

Figure 4: Total serum epoxide and diol levels in healthy and diseased *Pkd2*$^{WS25/-}$ mice. ***Epoxide, p = 0.00471; Diol, p=0.08.

Of the 27 oxylipins, 17 (63%) were LOX-derived, 5 (19%) were COX-derived, 3 (11%) were CYP-e derived, and 2 (7%) were CYP-h derived. Oxylipins were lower in disease as compared to healthy mice across all enzymatic pathways, with the exception of 2 COX-derived enzymes (PGD1 and PGF1α) which were higher in the hearts of diseased mice.

In the kidney, 72 oxylipins were detected, of which 16 were altered in disease – all of which were lower (Supplementary Material Table 8; Figure 2). Of the 16 altered oxylipins, 12 (75%) were LOX-derived, 2 (13%) were CYPe-derived, 1 (6%) was CYPh-derived, and 1 (6%) was COX-derived.

3.5.4 LIVER OXYLIPINS DISPLAYED NO SPECIFIC PATTERN OF ALTERATION BETWEEN HEALTHY AND DISEASED

Within the liver, 50 oxylipins were detected, of which 17 displayed differences in profile concentrations between healthy and diseased mice. Of these, 9 were significantly lower ($p<0.001$) in disease compared to healthy, while the remaining 8 were higher (Supplementary Material Table 9; Figure 2). Of the 9 which were lower, 5 (56%) were LOX-derived, 2 (22%) were CYPe-derived, 1 (11%) was CYPh-derived, and 1 (11%) was COX-derived. Of the 8 which were higher, 2 (25%) were LOX-derived, 2 (25%) were CYPe-derived, 2 (25%) were CYPh-derived, and 2 (25%) were COX-derived.

3.6 DISCUSSION

Oxylipins are key regulators of normal physiological function and are involved with inflammation and proliferation[63], processes that are associated with ADPKD2[15,91]. Our study found that alterations in oxylipin levels in the tissues and serum were enzyme-pathway specific, not reflections of phospholipid fatty acid levels. The alterations were enzyme-pathway specific as they did not exhibit any trends in relation to the fatty acid precursors (i.e. AA, EPA, etc.) but they did show trends which were linked to the enzymatic pathways from which they were derived (i.e. a

decrease in heart and kidney LOX-derived oxylipins in disease). Therefore, the regulation of oxylipin metabolite production is likely enzymatic rather than occurring at the substrate level.

Studies have shown that reduced COX activity is correlated to reduced renal fibrosis and cyst volume in PKD, highlighting the importance of COX metabolites in the progression of this disease[64,77,159]. In the kidney, COX derived oxylipins maintain normal renal function and studies have shown that COX inhibitors can reduce renal disease progression[72–74,160]. Unlike previous PKD studies, our study showed little change in overall COX derived oxylipins between healthy and disease but displayed a decrease in LOX derived oxylipins within the kidney[15]. Metabolites formed as a result of LOX catalytic activity are tissue specific and can have both pro- and anti-inflammatory effects[161]. Actions of LOX metabolites are mediated through G-protein coupled receptors and intracellular effectors; however, receptors for all oxylipins have yet to be identified[82]. The majority of animal models have associated increased LOX products with renal inflammation and injury[89,105,161]. A low dose of LOX inhibitor NDGA was administered in a PKD study to determine the role of LOX on disease progression[160]. They found that inhibition of the pathway did not alter disease development or severity, suggesting that lower levels of LOX products are a result of and not caused by PKD – i.e. lower levels of LOX products is produced by PKD, it does not cause PKD. A study using *Pkd2*$^{-/WS25}$ mice similar to our study also found lower concentrations of LOX derived oxylipins in diseased kidneys[77]. LOX produced hydroxy FAs, hydroxy-eicosatetraenoic acid (HETEs) and oxo-eicosatetraenoic acid (oxo-ETEs), are primarily proinflammatory but can also modulate response to injury through mediation of apoptosis, inflammation and decrease cell proliferation[89]. Inflammation occurs through cellular and molecular signaling, acting to protect host organisms against pathogens and damaged cells or tissues[162]. Within the kidney, LOX derived oxylipins are involved with the regulation of renal blood flow and pressure, affecting both cardiovascular and renal functions. A report found that changes in renal

cysts and fibrosis was associated with lower HETE concentration in a rat model of kidney disease, possibly reflecting the loss of anti-inflammatory roles of specific LOX metabolites[105].

Oxylipins derived via the CYP pathways have vascular, cardiac and renal functions which are both unique and can be opposing[64,108,163,164]. Our study found that serum CYP derived epoxygenated PUFAs were lower in disease. CYPe inserts an oxygen atom on a carbon atom attached to one of the double bonds of the PUFA, reducing it to its epoxide form such as the EpOMEs, EpETrEs, EpODEs, EpETEs, and EpDPEs[165]. Epoxides have high biological activity and have anti-inflammatory and vasodilatory properties[166]. Furthermore, epoxides can act as long-chain fatty acids, binding to fatty-acid-binding proteins and nuclear peroxisome-proliferator-activated receptors, suggesting an intracellular mechanism[165]. Epoxides are unstable therefore are rapidly hydrolyzed by sEH to its more stable, but less biologically active, diols (products)[68,108,109]. Unlike their epoxide substrates, these diols carry pro-inflammatory and vasoconstrictive activity. Interestingly, product:substrate ratios for sEH in the serum were higher in disease in our study, suggesting an increase in sEH activity, although this could also be due to lower formation of the epoxides by CYPe. Generally, inhibition of sEH has potential physiological benefits as it prolongs the functional effects of epoxides[167]. sEH has been shown to have high tissue expression in liver, kidneys, and vasculature[168,169]. Subcellular localization of sEH is variable, however; it is mostly localized to the cytosol with peroxisomal involvement in hepatocytes and renal proximal tubule epithelial cells [162]. It is important to note that the biological activity and significance of different cell types and subcellular localization of sEH remains unclear.

The use of plasma oxylipins as biomarkers for disease prediction is emerging. Our current study found that healthy *Pkd2*$^{-/WS25}$ mice had higher levels of EpETrEs in the serum compared to diseased, suggesting less sEH activity in disease or increased CYPe activity. The 11,12 DiHETrE:EpETrE ratio was significantly higher in the serum of disease mice in our study. EpETrEs are generally considered to exert anti-inflammatory properties, while the DiHETrEs are pro-inflammatory. sEH

converts 11,12-EpETrE to its more stable diol, 11,12-DiHETrE, resulting in a significant loss of vasodilation. EpETrEs formed by CYPe have hypotensive effects via their vasodilatory properties, allowing us to speculate that our diseased mice had hypertension due to lower levels of these metabolites[163]. 9,10-EpOME and 12,13-EpOME have anti-inflammatory properties while their diols have been indicated to have toxic effects[68]. Serum of our diseased *Pkd2*$^{-/WS25}$ mice had lower levels of these EpOMEs compared to their healthy counterparts, demonstrating a loss of anti-inflammatory characteristics. Additionally, there were lower concentrations of EpODE, EpETE, and EpDPEs serum oxylipins which carry vasodilatory, anti-inflammatory, and antiarrhythmic properties in healthy mice[109,170–172]. However, oxylipin differences in the serum did not reflect the alterations found in the heart, kidney, and liver of the diseased mice; this finding supports the current literature which found that serum profiles do not reflect tissue profiles in disease[120,130,173].

The kidney findings are not consistent with other models of ADPKD2 but comparable to mouse models of NPHP, a pediatric form of cystic kidney disease[15], and ARPKD[15]. In the *pcy* mouse, an orthologous model of human NPHP, lower concentrations of LOX oxylipins HETEs and hydroxy-octadecadienoic acids (HODEs) were detected in affected kidneys, suggesting that an increase of LOX oxylipin activity may have beneficial effects for NPHP[104]. Various studies have shown that both LOX and CYP products are reduced in diseased mouse models of PKD compared to healthy[15,77,103–105]. Furthermore, hydroxyeicosapentaenoic acid (HEPE) and hydroxydocosahexaenoic acid (HDoHE) oxylipins possess anti-inflammatory properties and were lower in diseased kidneys in *pcy* mice[174]. In our study, the majority of HEPEs and HDoHEs detected in the heart and kidney were lower, speculating that there may be a loss of anti-inflammation and renoprotection. 5-HEPE and 9-HEPE in the heart, and 5-HEPE in the kidney are known to suppress the expression of pro-inflammatory cytokines, which is helpful to attenuate disease[75].

Liver oxylipins in our study displayed no specific pattern of alterations between health and disease. LA derived oxylipins may be factors in the progression of liver injury. In the liver in rats given a high protein diet, higher levels of EpOMEs were observed while their sEH products, DiHOMEs, were largely unaffected[87]. In mice with chronic alcohol exposure induced hepatocyte apoptosis, 13-HODE was elevated in the liver[175]. Oxylipins such as HODE and oxo-octadecadienoic acids play a role in oxidative stress, vasodilation, proliferation and inflammation[173,176,177]. In the liver, PGs appear to be associated with protective effects in a variety of liver diseases[178,179]. The current study displayed very few liver cysts, higher COX-derived PGs (PGJ_2 and PGD_1) and lower 15k PGE_1; however, whether these changes have direct protective effects in ADPKD is unclear.

3.7 CONCLUSIONS

Our study focused on the oxylipin differences between healthy and diseased kidney, liver, heart and serum of male ADPKD2 mice to fill the knowledge gap within the current literature. After analysis, oxylipin profile differences were found to be enzyme-pathway specific with lower profiles of LOX oxylipins in the heart and kidney in disease, suggesting increased inflammation in these tissues. Unlike previous reports, the COX oxylipins in the tissues of diseased mice were not altered in our model; however, further investigation using the *Pkd2*$^{WS25/-}$ mouse model should be conducted.

Mechanistically, diols have been shown to disrupt mitochondrial disfunction, leading to cellular apoptosis; therefore, sEH may play a key role in the regulation of inflammatory responses, degrading the anti-inflammatory epoxides and producing pro-inflammatory diols. Future studies investigating the regulation of sEH and oxylipin profiles under multiple inflammatory states should prove informative in further understanding oxylipin profiles in disease as well as determine if profile changes can be disease specific. The lower serum epoxide levels that were found in the disease may be used as a preliminary indicator of ADPKD2 via a simple blood test, however further

studies must be conducted to ensure that the alterations seen in this study are ADPKD2 specific, and whether these alterations also occur in human ADPKD. Currently, the diagnosis of ADPKD2 generally occurs when the patient has stage 2 – 3 kidney disease with limited treatments. The mice in our study would likely be near the beginning stages of stage 3 kidney disease and we are already seeing alterations in their oxylipin profiles. By determining whether these changes we observe, especially in the serum, are ADPKD2 specific, it can prove to be an early diagnostic tool that allows for earlier medical intervention which hopefully improves mortality and morbidity rates.

CHAPTER 4: OXYLIPIN PROFILES IN SERUM, KIDNEY, HEART AND LIVER OF HEALTHY MALE AND FEMALE MICE

4.1 INTRODUCTION

Oxylipins are oxygenated PUFA metabolites formed by reactions of COX, LOX, and CYP enzymes and have bioactive lipid properties. These lipids play significant roles in maintaining overall physiological health as well as roles in kidney, cardiovascular, and liver disease. The effect of sex on oxylipin levels is an emerging area where the majority of information has yet to be uncovered; however, there are indications of differences between male and female oxylipin levels.

Previous studies of oxylipin profiling in rats have shown that levels of oxylipins are generally higher in males, with the exception of those derived from DHA[120,130]. A study reported oxylipin profiles in rat kidney, liver, and serum and examined the effect of sex on these profiles[120]. With the exceptions of 20-HETE, 5,6-DiHETrE, and all DHA-derived oxylipins (kidney); PGA_2, tetranor-12-HETE, 12-oxoETE, 20-carboxy-AA, $PGF_{1\alpha}$, and 13-HDoHE (liver); and 12,13-DiHODE and $PGF_{3\alpha}$ (serum), all oxylipins were higher in males. Furthermore, when levels of oxylipins are standardized relative to their precursor PUFA level (i.e. oxylipin/PUFA ratios), almost all oxylipins with expressions that were sex dependent were higher in males[120]. A separate study had similar findings where almost all oxylipins that displayed a sex effect were higher in males, with the exception of all DHA and most EPA oxylipins in the kidney[130]. On the other hand in the mouse kidney, studies have reported elevated AA-derived oxylipins in females, while oxylipins from other PUFAs are either similar or higher in males[131]. Furthermore, in various models of kidney disease, total oxylipins from LA, GLA, and DGLA were not affected by sex, but up to 50% of individual LA oxylipins were elevated in males[15,131]. Thus studies which report sex differences in oxylipin profile have divergent findings – oxylipin profiles in rat kidney differs from findings in mouse kidneys, suggesting species effects of sex oxylipins[15,120,130].

Oxylipin profiling in the heart may be relevant for further understanding of sex differences in normal and diseased hearts[66]. Levels of oxylipins have been reported to be higher in the heart in female rats while serum oxylipins were higher in males[120,130,134]. Sex differences were examined in

the heart of healthy Sprague-Dawley rats and analyses revealed that oxylipins with a main sex effect were higher in females[134]. This was the first study which looked at the sex effects on the heart oxylipin profile. The study reported in several adipose depots that oxylipin sex effects were observed in fewer oxylipins, with many being higher in females[134]. Sex effects on heart oxylipins in the mouse have not been reported.

The rationale for this study is to determine baseline sex differences in oxylipin profiles in a healthy rodent model. By establishing these differences, they can be used as a reference tool to understand the effect of oxylipin profile alterations in disease states as well as determine whether sex is a factor in modulating oxylipin function. As there is inconsistency in findings between species, this study aims to provide further understanding of oxylipin profiles in the kidney, liver, serum and heart of healthy male and female mice. This novel study will establish baseline oxylipin profiles which can be used to measure oxylipin profile differences in disease states.

4.2 OBJECTIVES

Investigate oxylipin profiles in healthy serum, heart, kidney, and livers of male and female mice.

4.3 HYPOTHESIS

We hypothesize that there will be different concentrations of oxylipins between female and male healthy mice.

4.4 MATERIALS AND METHODS

4.4.1 GENOTYPING PROTOCOL

Refer to Chapter 2, section 2.4.1 Genotyping Protocol.

4.4.2 ANIMAL MODEL

Normal male and female mice from the study in Chapter 2 were used for this study. Only mice provided the control diet were used. Due to the lack of female mice with disease, the study only

looked at the tissues and serum of healthy male and female mice. Healthy male and female mice (n=8) were given the AIN-93G standard diet from the age of 4 weeks and were fed for a period of 13 weeks. At the end of the study, the mice were anesthetized in a gas chamber with isoflurane and terminated by decapitation to collect blood. Final body, kidney, liver, and heart weights were recorded, and blood was collected and centrifuged to obtain serum. The right kidney, liver, and heart were snap frozen in liquid nitrogen and stored, along with serum, at -80°C until analysis. All animal procedures were approved by the University of Manitoba Animal Care Committee and adhere to the guidelines of the Canadian Council on Animal Care (Appendix A).

4.4.3 OXYLIPIN ANALYSIS

Refer to Chapter 3, section 3.4.3. Oxylipin Analysis.

4.4.5 STATISTICAL ANALYSIS

Data was analyzed using R software (R Development Core Team, New Zealand). Diagnostic tests to evaluate assumptions of normality and uniformity of variance were performed and if necessary, the data was transformed prior to analysis. Shapiro-Wilks was used to measure normality and Student's t-test was used to determine differences. Data was considered significant a p-value less than 0.05.

4.5 RESULTS

4.5.1 GENERAL RESULTS

A total of 105 oxylipins were detected overall in the serum, heart, kidney, and liver in healthy male and female mice (Figure 5); the complete data set of all oxylipins quantified can be found in the Supplementary Material. Throughout the study, all mice grew well, and males had consistently higher overall weight when compared to females. When normalized to body weight, healthy female mice had lower kidney, liver, and heart weights as compared to males (Table 5; Figure 5).

Table 5: Final body and tissue weights of healthy male and female mice at 17 weeks of age.

Tissue Weight	Male	Female	Effects (P values)
Body Weight (g)	29.29 ± 0.78	19.63 ± 0.81	**≤0.0001**
Kidney (mg)	8.24 ± 1.04	5.76 ± 1.08	**≤0.0010**
Liver (mg)	30.98 ± 2.35	16.30 ± 2.35	**0.0012**
Heart (mg)	3.28 ± 0.18	1.96 ± 0.18	**0.0025**

Values are mean ± SD (n=11). Significance was determined using Student's t-test.

4.5.2 OVERALL SERUM OXYLIPINS WERE HIGHER IN FEMALES

A total of 89 oxylipins were detected within the serum of male and female mice, with 27 being markedly different between healthy males and females (Supplementary Material Table 10; Figure 5). Three oxylipins (PGK_1, TXB_1, and 12,13 diHOME) were higher in males while the remaining 24 oxylipins were higher in females (Figure 5). Oxylipin differences were primarily evident in the LOX-derived pathway (15 detected), followed by the COX-derived pathway (7 detected), CYP-e/CYP-h pathways (4 detected overall), and 1 oxylipin detected from a synthetic pathway (15d PGA_2 which can also be formed via COX). Alterations did not appear to be precursor specific and overall LOX pathway products had the most differences in expression.

4.5.3 HEART AND LIVER OXYLIPINS WERE HIGHER IN MALES

Overall, both heart and liver oxylipin profiles were higher in males compared to females. In the liver, a total of 50 oxylipins were detected of which only 1 did not significantly differ between sexes ($PGF_{2\alpha}$, COX-derived) (Supplementary Material Table 13; Figure 5). 45 (90%) of the oxylipins were higher in males. Of those which were higher in females, 2 from the COX-pathway (PGE_2, dhk PGE_2), 1 from the LOX-pathway (12-HEPE), and 1 from the CYPe pathway (9,10 EpOME).

A total of 70 oxylipins were detected in the heart of male and female mice (Supplementary

Table *11*; Figure 5). All oxylipins that were LOX-derived in the heart were higher in males compared to females. Additionally, only 6 oxylipins were higher in females – 12-HHTrE, PGD_1, and $PGF_{1\alpha}$ (COX-derived), 12,13 EpOME and 12,13 EpODE (CYP-e derived), and 8-iso $PGF_{2\alpha III}$ (no enzymatic pathway defined). No pattern between fatty acid precursors and differences in oxylipins were detected in the heart.

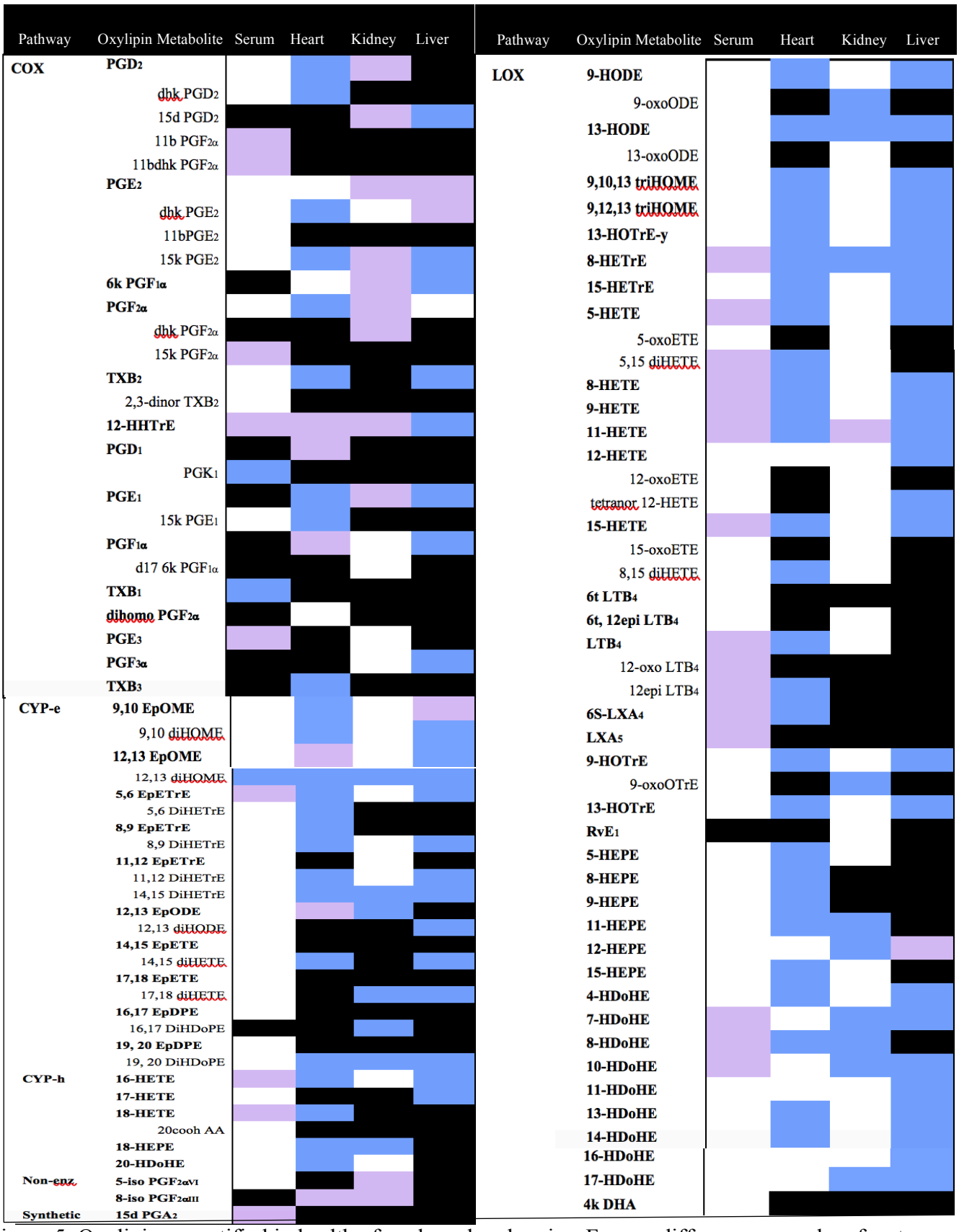

Figure 5: Oxylipins quantified in healthy female and male mice. For sex differences, purple refers to higher levels in females, blue refers to higher levels in males, white refers to alterations not significantly different, and black refers to oxylipin not detected. Significance determined using Student's t-test.

4.5.4 FEMALE KIDNEY OXYLIPINS PRODUCED BY THE COX PATHWAY WERE HIGHER

A total of 74 oxylipins were detected in the kidney of healthy male and female mice (Supplementary Material Table 12; Figure 5). 29 of those oxylipins displayed a significant difference between sexes. In males, 17 oxylipins, independent of enzymatic pathway or PUFA precursor, were higher compared to females and the remaining 12 oxylipins were higher in females. In addition, higher female oxylipin profiles were generally associated with the COX-pathway derived metabolites, while LOX and CYP oxylipins were generally higher in males.

4.6 DISCUSSION

This study provides fundamental data on the oxylipin profiles of healthy male and female mice, providing baseline values to be used as a reference tool for future studies on oxylipin sex differences in the kidney, liver, heart, and serum. Information on the effects of sex on oxylipin profiles in the kidney, liver, heart, and serum was limited to rat data and no general oxylipin profile pattern on tissues has been reported. Studies have shown conflicting results between species, where some have observed higher levels of oxylipins in males while others have not[15,120,130,180].

The majority of studies report the effects of PUFA patterns on oxylipins rather than the oxylipin pathways. Studies exploring sex effects on PUFAs have indicated an association between estrogen and increased DHA levels[181–183]. There is a higher concentration of DHA oxylipins in female rat kidney; however, oxylipin-to-parent PUFA ratios were similar or higher in males versus females, suggesting that there is higher formation and/or lower degradation of oxylipins in males[130]. Estrogen has been associated with increased DHA levels when looking at sex effects on PUFAs but little has been reported on sex effects on oxylipins[181–183]. In the heart, EPA oxylipins have beneficial effects. Studies have shown that 18-HEPE can prevent cardiac remodeling, and epoxygenated EPAs have antiarrhythmic effects in neonatal cardiomyocytes and vasodilatory effects in coronary smooth muscle cells[123,184,185]. Data from our study found both heart and liver

oxylipin profiles were higher in males compared to females. Until recently, sex effects on the heart oxylipin profile had not been examined[134]. Sex effects on oxylipins that are not attributed to dietary PUFAs have yet to be explored. Our study found no detectable pattern between fatty acid precursors and oxylipin levels in the heart or liver between sexes.

Overall, our data in mice showed that overall serum oxylipins are higher in females as compared to males. Our study found 27 oxylipins were markedly different between healthy male and female mice, with 24/27 of the oxylipins being higher in the serum of females. The higher serum oxylipin levels did not follow a pattern specific to fatty acid precursors but rather appeared to be pathway specific, with the LOX-pathway most consistently involved. A previous study found 46/49 oxylipins with a sex effect were higher in the serum of healthy male rats compared to females[130]. Another study using rats found 23/60 oxylipins displayed sex differences in the serum of which 21/23 were higher in males[120]. There are studies documenting fundamental differences in oxylipin levels between cystic models of kidney disease and species[15,64,120,130,134]. In male Sprague-Dawley rat kidney, liver, and serum, overall oxylipins were higher in males[130]. A separate rat study had similar results to previously reported data, showing that oxylipin/PUFA ratios in males were greater than or similar to females for all oxylipins for all PUFA substrates, with the exception of serum 12,13-DiHODE/ALA[120]. The differences between our results and what has been previously reported in rats may be due to species variation.

Consistent with other studies, our data found that total female COX derived oxylipins in the kidney were higher than males[15]. Similar to what was detected in the serum, all oxylipins increased in females – with the exception of PGE_1 (DGLA as fatty acid) – were formed using AA as the fatty acid precursor. This may suggest that oxylipin profile changes in the kidney between healthy male and female *Pkd2*$^{-/WS25}$ mice may not be only pathway specific, but perhaps fatty acid dependent as well. Weanling Sprague-Dawley rats displayed sex differences in ~40% (30/69 oxylipins in kidney, 32/71 in liver, and 23/60 in serum) of oxylipins[120]. With a few exceptions, almost all oxylipins were

higher in males in these rats. In the kidneys of *Pkd1* mice, almost all AA-derived oxylipins that had a sex effect were higher in females and almost all non-AA derived oxylipins were higher in males[15]. This ADPKD model displayed minor to no effects of sex on disease kidneys, and there were few interactions of sex with disease for oxylipins found in the kidney[15]. Other studies using rats have shown females having higher renal levels of AA derived oxylipins – sex differences in AA metabolizing enzymes may be responsible for these transformations [186,187]. Few studies of sex differences in the heart oxylipins exist, but some studies indicate some individual AA-derived oxylipins may be higher in vascular tissue of females[188,189].

4.7 CONCLUSION

Sex differences in healthy ADPKD2 mice may be relevant in our further understanding of oxylipin profiles and their presence in tissues and normal physiology. The oxylipin profiles of kidney, liver, heart, and serum of mice presented in this study provide insight on the nature of these bioactive lipids. Although serum oxylipins were higher in females compared to males, the profiles were different from other tissues, suggesting that serum may not reflect tissue levels of oxylipins. Additionally, heart and liver oxylipins were higher in males while kidney oxylipins derived from the COX pathway were higher in females. This data provides baseline oxylipin data in healthy male and female mice which can be used as a reference tool for interpreting oxylipin alterations in various disease states. The differences between sexes suggest that there may be other components which play a role in oxylipin profiles, such as hormone levels. Further studies should investigate whether hormones, or other components in sexes, play a role in oxylipin function. The data obtained complement the current literature which suggest that the oxylipin profile differences between sexes can be pathway specific. Research regarding sex effects on oxylipin levels in kidney, liver, heart, and serum is limited; thus, our current analysis provides novel insight into the of oxylipin status in these mouse tissues. Our study gave insight on baseline oxylipin levels in both healthy male and

female mice, providing a stepping stone towards other related studies. It will allow future researchers to understand and comprehend the complexities of sex differences in tissues.

CHAPTER 5: STRENGTHS, LIMITATIONS, FUTURE DIRECTIONS

Chapter 2: This study was the first of its kind that looked at the effect of FPI on the progression of PKD in an orthologous animal model. It identified that although flax oil and whole flaxseed have previously been found to be beneficial in a ADPKD model, FPI does not significantly affect disease progression. This finding suggests that the beneficial nutraceutical component of flax is not in the flax protein but in other flax components, such as its oil or PUFA content. Despite being a true othologous model, mice cannot provide an accurate representation on what occurs in human *PKD2* affected individuals. This study only focused on one plant based protein, flaxseed, and one animal based protein, casein, limiting the analysis to these protein sources. In addition, the effects may be due to an increased sodium content in the FPI and despite adjusting for this variation and the differences in essential amino acid compositions between the two diets, there are still likely to be other differences between these proteins. Another limitation is that there were only 2 females on the control diet which had the disease. The lack of female disease representation in the control diet limits the analysis of the differences between sexes and progression of disease in our results. Finally, the effects observed may only be applicable to *Pkd2* mice and not to other forms of PKD. Future studies which use the FPI on $Pkd2^{WS25/-}$ mice should be conducted for a longer duration, to determine if there would be an effect in the longer term. Furthermore, FPI should be compared with whole flaxseed and flax-oil in the same animal models to have a more accurate understanding of the effects of FPI.

Chapter 3: The data obtained from this study indicates that there are oxylipin differences between healthy and diseased mice in an ADPKD2 model. Specifically, our data displayed a decrease in LOX derived oxylipins in the diseased heart and kidney while oxylipins found in the liver displayed no disease specific pattern. Our findings differed from previous studies which showed that the COX oxylipins are altered in diseased kideys. The variation between results may be due to species effects and the use of different models of cystic kidney disease. Serum CYPe PUFAs were lower in disease, making its presence a possible biomarker for the disease. The current study

only used male mice. It is likely that we would see sex effects on oxylipins if both male and female mice were used. Measurement of glomerular filteration rate and fatty acid analysis of kidneys would provide additional insight regarding disease pathology and oxylipin differences in the tissues. Furthermore, a more accurate understanding of cardiovascular abnormalities could be obtained if blood pressure was measured in all mice. It is likely that, if the study extended beyond its 13 week duration, that more mice may have developed the disease and/or those which did develop the disease, would have done so more severely. However, this is speculation and further studies must take place to answer these questions while also creating new ones.

Chapter 4: The study measured the levels of baseline oxylipin profiles in both male and female healthy mice. Overall, oxylipins are higher in the serum of female mice as compared to males however, oxylipin levels in the serum are not reflective of those in the tissues. Oxylipins levels were found to be higher in males within the heart and liver while COX oxylipins were higher in the kidney in females. Similar to previously reported data in rats, overall oxylipin were higher in males compared to females in the liver. In the heart and serum, oxylipins were higher in male mice in our study compared to previous studies which showed they were higher in female rats. The results provide further insights in baseline sex differences regarding oxylipins. Further studies which measure the pathway of the oxylipins that were altered would provide more insight towards understanding oxylipin regulation and determining whether other components, such as hormones, play a definitive role in their physiology and function. Future studies which measure the oxylipin profiles between healthy and diseased male and female mice should be conducted to have a better understanding on disease pathology between sexes, contributing to more accurate therapeutics towards disease prevention among sexes.

Overall conclusions and implications: The studies described in this thesis expand on the current literature regarding plant-based protein as a method of treatment for ADPKD, the differences between healthy and diseased tissues and serum in mice with *Pkd2*, and attempts to outline the

baseline sex differences of oxylipins in a healthy mouse model. The benefits of whole flaxseed and flax oil have been reported in various models of cystic kidney diseases; however, use of its protein isolate has been scarcly reported most likely due to difficulties in obtaining FPI. Soy protein has been reported in multiple studies to be advantageous in decreasing the progression of renal cysts in models of ADPKD. FPI composition has been shown to be comparable to soy protein therefore, it was reasonable to determine whether FPI could be used as a possible therapautic treatment in a mouse model of ADPKD2. Tissue weight is a reliable preliminary method to determine the presence of disease – i.e. if the kidney weight and water content is higher this reflects greater cyst growth and disease progression. The results of kidney weights and water content from the flax study signify that the use of FPI in replacement of animal protein does not alter disease progression in mice with ADPKD2, suggesting the the nutracutical component of flaxseed is not due to its protein. However, this is not to say that FPI has no beneficial qualities. Future studies should analyze FPI in different models of polycystic kidney disease and other disease states as it may prove beneficial.

The findings in Chapter 3 outlined the differences between oxylipin profiles in healthy and diseased tissues and serum in a mouse model of ADPKD2. We hypothesized that there would be higher levels of tissue and serum COX derived oxylipins in disease and that overall oxylipins would differ between healthy and disease. We found that the COX oxylipins were not altered in the diseased state while LOX oxylipins in the heart and kidney were lower in disease as compared to healthy. Furthermore, in the serum, CYPe oxylipins substrates were lower in disease as compared to healthy; however, the sEH products exhibited few changes. The higher levels of epoxide oxylipins in the serum of normal mice suggests decreased sEH activity or increased CYPe activity in disease. Epoxides have anti-inflammatory properties and also have hypotensive effects, allowing us to speculate that our diseased mice had hypertension due to lower levels of these metabolites. These findings are important as they suggest the use of serum as a possible early biomarker of disease as the mice in our study were likely in the early stages of kidney failure. In humans, many ADPKD

cases are diagnosed at a later stage or reported at autopsy. Although further research is required, this study suggests that the measurement of serum oxylipins may be used as an early detection tool for disease, allowing for ealier medical intervention therefore reducing ADPKD2 mortility and morbidity.

The findings in Chapter 4 establish baseline oxylipin profile differences between sexes in a healthy mouse model. Throughout the study, mice grew well and males had consistently higher overall body and tissue weight compared to females. A total of 105 oxylipins were detected in the serum, heart, kidney and liver between healthy male and female mice. In the serum, overall oxylipins were higher in females while overall heart and liver oxylipins were higher in males. In the kidney, there was an equal divide between oxylipin detection between sexes however, female oxylipin profiles were generally associated with oxylipins derived from the COX pathway, while LOX and CYP oxylipins were generally higher in males. These findings have several implications. Firstly, the differences between oxylipin profiles in the serum compared to other tissues suggests that the serum may not be an accurate tool in determining tissue oxylipin status. Secondly, the sex differences in oxylipin profiles in the tissues and serum may be due to a variety of physiological differences such as the role of hormones. Finally, the establishment of baseline sex differences in oxylipin profiles in a healthy mouse model creates a tool of reference when measuring and interpreting oxylipin profiles in non-homeostatic states such as disease. It allows researchers to identify the degree of oxylipin alteration to then further investigate its causality.

Overall, this thesis further investigated the effects of a plant-based protein as a therapeutic tool for disease, provided a better understanding regarding the complexity of oxylipins in disease states, and established baseline oxlipin profiles in healthy male and female mice which can be used as a reference tool for the interpretation of future oxylipin studies.

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APPENDICES

A. Animal Ethics Approval Letter

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

Animal Care & Veterinary Services
208-194 Dafoe Road
Winnipeg, MB
Canada R3T 2N2
Phone [redacted]
Fax +[redacted]
veterinaryservices@umanitoba.ca

9 March 2017

TO: Dr. Harold Aukema
Department of Human Nutritional Science[redacted]

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

RE: **Your Renewal entitled "Effect of dietary flaxseed in 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 F16-016.

New Protocol Reference Number: **F16-016/1 (AC11175)**
Animals approved for use:

Number	Common Name	Sex	Age or Weight	Formal Name
90 mice – older than 21 days	N/A	male & female	weanling	$Pdk2^{ws25/-}$ and $Pkd2^{ws25/+}$

Protocol approval is valid from: **June 1 2017 to May 31 2018**
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 only three renewals are allowed. Subsequently, a full application must be submitted.

RH/ck

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

Protocol Approved By:

[redacted] March 8/17

Chair, Animal Care Committee — Date

[redacted] Feb 23/17

Clinical Veterinarian — Date

[redacted] Feb 20/17

Community Representative — Date

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September 2016

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

Animal Care & Veterinary Services
208-194 Dafoe Road
Winnipeg, MB
Canada R3T 2N2
Phone [redacted]
Fax [redacted]
veterinaryservices@umanitoba.ca

1 June 2016

TO: Dr. H. Aukema
[redacted]n Nutritional Sciences

FR [redacted] Ivanco Acting Chair, Fort Garry Campus Animal Care Committee

RE: Your protocol entitled **"Effect of dietary flaxseed in kidney disease"**

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 **26 May 2016**. The committee recommended **APPROVAL** of your protocol **SUBJECT TO A SATISFACTORY RESPONSE TO THE QUERIES NOTED BELOW.**

Protocol Reference Number: **F16-016 (AC11175)**
Animals approved for use:

Number	Common Name	Sex	Age or Weight	Formal Name
60 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: **June 1 2016 to May 31 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 **June 15 2016** to the following queries under which this approval is subject:

1) Block 6: Please remove the reference to carbon dioxide overexposure and revise this section so that it correctly reflects the use of isofluorane (as per Block 13A).

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.

TI/tvo

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

B. Diet Preparation Protocol

All ingredients are found in the walk-in refrigerator. Labeled containers for weighing ingredients can be found in the refrigerator as well and must be placed back in their correct spots after use.

1. Measure out all ingredients according to the recipe
2. In a large bowl, add all dry ingredients which have a weight less than 200g (e.g. Min mix, Vit Mix, L-cysteine, etc.). Using your hands, mix until all ingredients are thoroughly combined – this ensures that the lower quantity ingredients are well distributed in the diet.
3. Using the Hobart Mixer bowl, add half of all remaining ingredients and half of the smaller ingredient mixture. Again, mix thoroughly by hand to break up any large clumps. Once mixed, add the remaining half of all ingredients and mix by hand.
4. Attach the Hobart Mixer bowl to the machine, locking it into place. Attach the plastic shield and raise the bowl up using the lever.
5. Set the timer to 3 minutes at mixer speed level 1. Turn the power on once the timer has been set.
6. At the end of the timer, turn off the mixer power and lower the bowl, scraping the sides and the beater to ensure that you have all the product. Make sure the ingredients at the bottom of the bowl have been well mixed – use your hands if necessary. Once finished, return the bowl back to its position as outlined in Step 4.
7. Set the timer to 5 minutes and mixer speed level to 2. Do not start the mixer until you have added the oil.
8. Place the oil container (without the lid) on the scale and tare the scale. Measure oil volume according to recipe requirements.
9. Turn on the mixer and slowly add the oil, ensuring that it is properly mixed with the dry ingredients.

10. Turn power off when mixing stops, once again lowering the bowl and scraping the sides, the beater, and the bottom to ensure all the product is well mixed. Raise the bowl back into position as described in Step 4.
11. Repeat Step 7 without the addition of any oil.
12. Repeat Steps 10 and 11 one last time.
13. Once mixing is finished, turn off the power, lower the bowl and mix contents by hand, breaking up any remaining clumps. The diet should be grainy in consistency.
14. Place diets in large Ziploc bag, labeled as either Casein protein or Flax Protein, and store it in the walk-in freezer.
15. Clean all equipment, wash down counters, sweep floor, and wash weight containers.

C. Lyophilisation Protocol of Kidney

1. Collect the amount of 2mL tubes required for your study.
2. Using a Styrofoam container, fill it with ice.
3. Taking aluminum, create lids for each of the tubes, poking 4 small holes in each lid.
4. Label the tubes to correspond to each sample.
5. Remove 4 kidneys at a time from the -80°C freezer and put on ice.
6. Tare beaker on scale.
7. Weigh the tube, including lid. Record all the decimal places as this will be an important indicator of weight as the kidney becomes dry.
8. Place kidney in the tube, secure the lid, and record the mass.
9. Place tube on ice.
10. Repeat steps 6 to 9 with the remaining samples.
11. Store the samples in the -20°C freezer.
12. Remove 4 new samples from the -80°C freezer and repeat Steps 6 – 11.
13. Repeat Steps 5 – 11 until all kidneys are weighed and stored in the -20°C freezer.
14. If samples are not being immediately lyophilized, store in the -80°C until their turn.
15. Talk to one of the Dennis' regarding the use of the freeze dryer.
16. Samples are put in the freeze dryer at the end of the day and remain there overnight.
17. The next day, ask Dennis to remove the samples.
18. Keeping about 10 samples on ice, place the remaining samples in the -20°C freezer.
19. Ensure all ice and water has been removed from the outside of the tubes using a Kim wipe.
20. Weigh the 10 samples, recording all the decimal places of the mass. Place the samples in the -20°C freezer.
21. Repeat Steps 18 – 20 until all samples have been weighed.

22. Before returning samples to the freeze dryer, ensure they were in the -20°C freezer for approximately 30 minutes prior.
23. At the end of the day, repeat Steps 15 – 22.
24. Keep weighing the samples at time intervals until the sample mass no longer differs between weigh-ins.
25. Once the mass remains consistent, the kidneys have become freeze dried. Transfer the samples to labeled, corresponding scintillation vials or 2mL microcentrifuge tubes.
26. Pulverize the samples using an angled scoopula cleaned in 70% Ethanol and rinsed in purified H_2O. Scoopula should be completely dry to prevent the addition of water to the sample. Carefully use the scoopula to crush the kidney sample until no chunks remain.
27. Final dry kidney weights are listed in Table *3*.

D. Tyrode's (pH 7.6) Protocol

1. In a graduated cylinder, measure 100mL of reconstituted Tyrode's salt solution.
2. Weigh 100mg of powdered sodium bicarbonate into a 125mL Erlenmeyer flask.
3. Protect the flask from light by covering it with tin foil. Add a stir bar.
4. From the Tyrode's in Step 1, transfer about 80mL into the Erlenmeyer flask.
5. Place the flask on a stir plate and stir for approximately 15 minutes until the powder is completely dissolved into the solution.
6. While continuing to stir, adjust the pH using 1N HCl or 1N NaOH until the solution reaches pH 7.6.
7. Transfer the solution into a 100mL volumetric flask. Add the remaining 20mL from Step 1.
8. Insert volumetric stopper and invert 10x to mix.
9. Transfer solution into a 100mL glass bottle covered with aluminum foil to protect it from light. Clearly label the bottle as Tyrode's salts (pH 7.6) and include the date made.
10. Store in the -8°C refrigerator.

NOTE: Tyrode's that have both sodium bicarbonate and have been pHed can deteriorate. This can be recognized by:

- pH change
- precipitate or particulates
- cloudy appearance
- colour change

Check all these signs before using.

E. 1% Triton By Using Tyrode's (pH 7.6) Protocol

1. Take Tyrode's pH 7.6 out of refrigerator and measure out 2.0mL. Put rest of Tyrode's pH 7.6 back in fridge and let the 2.0mL reach room temperature.
2. Weigh out 0.02g of Triton solution in a 20mL scintillation vial.
3. Add 2.0mL of Tyrode's pH 7.6 at room temperature.
4. Place the cap on the scintillation vial and vortex well.
5. Cover the vial with aluminum foil to protect from light and chill in fridge.

The 1% Triton is required in the homogenate to disrupt lipids and release proteins. This will be added to the LC-MS/MS fraction.

Add 10μL of 1% Triton (pH 7.6) to 1000μL of tissue homogenate for the LC-MS/MS fraction to make a 0.01% Triton final solution.

Volume 1% Triton to add (μL) = (0.01 final concentration) (1000μL tissue homogenate)

F. Antioxidant Cocktail Protocol

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

Procedure makes 100mL Antioxidant Cocktail.

1. Measure out 50mL of Methanol and 25mL of Ethanol in separate graduate cylinders.
2. Combine the two liquids in a 250mL beaker.
3. Cover the beaker in aluminum foil to protect it from light and place on magnetic stirrer.
4. Put stir bar in beaker and cover the top with foil to minimize volatilization.
5. On separate weigh papers, weigh out 20mg BHT, 20mg EDTA, 200mg TPP and 200mg Indomethacin.
6. Add the dry ingredients to the MeOH:EtOH solution and stir until completely dissolved. Make sure that the beaker is completely covered with aluminum foil to minimize exposure to light.
7. When all the dry ingredients are dissolved, transfer the mixture to a 100mL volumetric flask.
8. Wash down the sides of the beaker using a small amount of ddH_2O and pour into the 100mL flask. Repeat 3x to ensure the transfer of all the solvent and antioxidants.
9. Use ddH_2O to fill the 100mL volumetric flask to the mark.
10. Add a stopper to the flask and invert 10x to thoroughly mix.
11. Transfer solution into a clean, aluminum foil covered, labeled 125mL bottle.

G. Solvent A and Solvent B Protocol

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 1000mL:

700mL water

300mL acetonitrile

200µL acetic acid

Vacuum filter through Whatman #4 filter paper

Solvent B:

Acetonitrile – Isopropyl Alcohol [50:50; v/v]

*MS Grade

To make 1000mL:

500mL acetonitrile

500mL isopropyl alcohol

Vacuum filter through Whatman #4 filter paper

H. Oxylipin Analysis

Step 1: Measuring Tissue and Tyrode's

Use Tyrode's salt solution (pH 7.6) to homogenize the wet liver and heart tissue and the dry kidney tissue.

Step 2: Homogenizing

4.5.4.1 Ensure there is enough prepared of:

- Tyrode's salt solution (pH 7.6) – Check for deterioration. See Solutions Preparation below
- 1% Triton solution – See Solutions Preparation
- Bead-Homogenizer tubes with lids that have been soaked overnight in Contrad solution, rinsed and dried.
- 100:1 Methanol and Formic Acid
- pH 3 water – water where the pH has been adjusted to 3.0 using 1M HCL

4.5.4.2 Obtain large container of ice

4.5.4.3 Remove the required tissue samples from the -80C freezer and keep on ice

4.5.4.4 Label 2mL tubes with sample IDs

4.5.4.5 Kidney samples will appear to be a fine powder (lyophilized) while the other samples will be snap-frozen. Proceed to weighing samples.

4.5.4.6 Divide weighed sample by 0.36 (if dry tissue) or 1.75 (if wet tissue) to determine your amount of Tyrode's

1. Sample Calculation:

   0.0446g (weighed dry sample) → 446mg

   446/0.36 = x

   1239 = **x (Amount of Tyrode's in uL)**

Divide X by 2 because using ½ now and the other ½ for the washes (further divided by 3 for the 3 washes)

x/2 = 1239/2 = 620

[(x/2)/3] = 620/3 = 206

206uL is the amount added to the washes, 620*3+620 = 1239 (Total Tyrode's)

7. In your beaded tubes (Bead Homogenizer for extraction), place your weighed sample and measured x/2 Tyrode's.
8. Using the Bead Homogenizer (in the cold room), set to Program #1 and run for one cycle.
9. Remove samples, vortex for 15s to remove any bubbles, and pipette as much solution as possible into a 5mL vial
10. Add your [(x/2)/3] value of Tyrode's to your tissue sample and place in the bead homogenizer
11. Repeat steps 8 – 10 3x.

Step 3: Aliquoting homogenate

1. Remove an aliquot of 200uL from each sample for the oxylipin analysis and place it in a labeled microcentrifuge tube.

Step 4: Preparation for Solid Phase Extraction of Oxylipins

1. Add 1uL of 1% Triton solution for every 100uL aliquot for oxylipin analysis (final concentration of Triton in homogenate should be 0.01%: 0.01 x 100 = 1uL).
2. Vortex for 10 seconds.
3. Incubate each tube on ice for 10 minutes.
4. Repeat steps 2 & 3 3x/sample until all samples are completed.
5. For the oxylipin extraction, vortex and aliquot 200uL of Tyrode's homogenate into two labeled 2mL microtubes.

6. Vortex each 2mL microtube for 10 seconds.
7. Working quickly, add (in the order listed) to the samples:
    - 500uL of 100:1 methanol formic acid
    - 800uL of pH3 water
    - 90uL of 100% ethanol
    - 10uL of antioxidant cocktail
8. Vortex for 5 seconds
9. If not undergoing extraction right away, store samples in -80°C freezer.

Step 5: Solid Phase Extraction

Before you begin, ensure there is enough of the following:

- Internal standard
- Strata-X SPE columns
- pH3 water
- methanol (water)
- 10% methanol in pH3 water
- Solvent A

NOTE: Samples should be kept on ice whenever possible. DO NOT allows the columns to run dry unless stated in the instructions.

1. Remove homogenized samples from the -80°C freezer and defrost on ice.
2. As the samples defrost, turn on the centrifuge and cool to 4°C. Turn on nitrogen evaporator water bath to 37°C – if the evaporator doesn't have enough water in it, add distilled water.
3. Add 1mL of pH3 water into corresponding empty 2mL microtubes labelled to samples. Once thawed, vortex and add 1mL of the sample into its corresponding 2mL microtube.

4. Add 10uL of internal standard (kept in -20°C freezer) to each 2mL microtube. Vortex. Run nitrogen over internal standard before capping and storing in the -20°C freezer.
5. Vortex and test the pH of the mixture using appropriate pH strips (pH of 3 is required). If necessary, use 10M HCl to reach pH3.
6. In the cooled 4°C centrifuge, insert the microtubes for 5 minutes at 3000rpm to remove debris.
7. Set up enough columns for each sample in the fume hood. Use the Strata-X SPE columns.
8. Place an empty waste vial under each column.
9. Pre-condition the column with 1mL methanol. Allow the methanol to drip through for 1 minute, then gentle apply pressure with a BD 10mL syringe to increase flow. Don't allow dropping to be lower than 1.5 – 2 seconds apart. Do not allow column to dry.
10. Add 2000uL of pH3 water to each column. Push through the same way.
11. Using a "kim-wipe collar" to avoid water from the outside of the tube to drip, apply sample to column. Let gravity allow the sample to drip.
12. Add 1000uL of 10% methanol in pH3 water to each sample test tube. Vortex and centrifuge as in step 6.
13. While centrifuging, label the needed amount of 1.5mL microtubes for each sample.
14. Using gentle pressure, push the samples through the columns (not allowing it to dry). Apply the new mixture from step 12 to the column. Push through to dry using BD 10mL syringe full of air.
15. Set up microtubes underneath the columns corresponding to the correct samples.
16. Add 1000uL of 100% methanol to each column. Allow dripping through for at least 1 min, and then push through with gentle pressure. Make column dry by pushing through with syringe a few times.

17. If samples not being run on the same day, cap with nitrogen and store at -80ºC (dry down later).
18. Ensure the nitrogen evaporator is at 37ºC and clean needle with 100% chloroform.
19. Set up microtubes in the evaporator and dry down the samples (~1.5 hours), checking on them every 15 minutes.
20. Once samples have dried down completely, add 100uL of Solvent A to each of the microtubes. Run nitrogen over tubes before capping and then vortex.
21. Samples can be stored in the -20ºC freezer for up to 1 week, if needed.
22. Label GC/LC vials and add a 200uL target propylene conical insert into each one.
23. Ensuring samples are thawed, vortex them.
24. Centrifuge samples at 4ºC for 1 min @ 7000rpm.
25. Transfer each sample into its appropriate vial and store in the -20ºC freezer and run them within 1 week.

I. Analysis of LC-MS Data Protocol (Multi-Quant)

General Rules and Tips for selecting peaks

1. Click on small icon with graphs to view the data in graph form. Click on the magnifying glass icon to zoom in.
2. The data is now set up for easy viewing between the table and graph form.
3. The Internal Standards are listed first – usually the correct peaks are selected but go over the selection and correct any errors.
4. Below the Internal Standards list are the analytes. When you select an analyte, all the samples for that one analyte will be shown on the graph view.
5. If the analyte has a deuterated internal standard, select the peak according to the peak in the internal standard.
6. You should have the printed primary list next to you. If the analyte does not have a deuterated internal standard, consult the primary list to determine the retention time for the sample.
7. If there is no primary for the analyte, consult the table that lists all the analytes with their expected retention times. NOTE: these retention times are an approximation and your sample will occur with a slight deviation to the times.
8. The peaks should be >5x the baseline. If not, use the "select peak to not found" icon.
9. The peaks should include an area $\geq$ 3.000e3. If not, use the "select peak to not found" icon.
10. If peaks are hard to identify, change the "Gaussian Smooth Width" value and "Apply". This should make the peaks clearer and allow you to select the correct peak.
11. Don't be misled by small peaks. If the baseline is very low, the peak could still be 5x above the baseline. Zoom in for a more accurate view.

J. Known Oxylipins in Order of Detection

6k $PGF_{1\alpha}$; TXB_2; $PGF_{2\alpha}$; PGE_2; PGD_2; 11β $PGF_{2\alpha}$; TXB_1; $PGF_{1\alpha}$; PGE_1; PGD_1; d17 6k $PGF_{1\alpha}$; TXB_3; $PGF_{3\alpha}$; PGE_3; PGD_3; dihomo $PGF_{2\alpha}$; dihomo PGE_2; dihomo PGD_2; dihomo PGJ_2; dihomo 15d PGD_2; 6k PGE_1; 6,15-dk,dh-$PGF_{1\alpha}$; 15k $PGF_{1\alpha}$; 15k $PGF_{2\alpha}$; 15k PGE_1; 15k PGE_2; 15k PGD_2; dh $PGF_{2\alpha}$; dhk $PGF_{2\alpha}$; dhk PGE_2; dhk PGD_2; bicyclo PGE_2; 11bdhk $PGF_{2\alpha}$; 2,3-dinor 11β $PGF_{2\alpha}$; 5-iso $PGF_{2\alpha VI}$; 8-iso $PGF_{2\alpha III}$; tetranor 12-HETE; 11βPGE_2; PGA_2; PGB_2; PGK_2; PGJ_2; PGK_1; 15d PGA_2; 15d PGD_2; 15d PGJ_2; 12-HHTrE; 5-HETE; 8-HETE; 9-HETE; 11-HETE; 12-HETE; 15-HETE; 16-HETE; 17-HETE; 18-HETE; 19-HETE; 20-HETE; 5,6 diHETE; 5,15 diHETE; 8,15 diHETE; 14,15 diHETE; 17,18 diHETE; 5-HEPE; 8-HEPE; 9-HEPE; 11-HEPE; 12-HEPE; 15-HEPE; 18-HEPE; 4-HDoHE; 7-HDoHE; 8-HDoHE; 10-HDoHE; 11-HDoHE; 13-HDoHE; 14-HDoHE; 16-HDoHE; 17-HDoHE; 20-HDoHE; LTB_4; 20oh LTB_4; 20cooh LTB_4; 6t LTB_4; 12epi LTB_4; 6t,12epi LTB_4; 12oxo LTB_4; LTC_4; LTD_4; LTE_4; 14,15-LTC_4 (EXC_4); 14,15-LTD_4 (EXD_4); 14,15-LTE_4 (EXE_4); 9-HODE; 13-HODE; 9-HOTrE; 13-HOTrE; 13-HOTrE-γ; 5-HETrE; 8-HETrE; 15-HETrE; 6R-LXA_4; 6S-LXA_4; 15R-LXA_4; LXA_5; LXB_4; RvE_1; RvD_1; PD_1; 15t PD_1; 10S, 17S-DiHDoHE (PDX); RvD_2; RvD_5; 17k DPA; 4k DHA; 17k DHA; 7R Maresin-1; HXA_3; HXB_3; 5-oxoETE; 12-oxoETE; 15-oxoETE; 15-oxoEDE; 9-oxoODE; 13-oxoODE; 9 oxoOTrE; 13 oxoOTrE; 5,6 EpETrE; 8,9 EpETrE; 11,12 EpETrE; 14,15 EpETrE; 5,6 DiHETrE; 8,9 DiHETrE; 11,12 DiHETrE; 14,15 DiHETrE; 14,15 EpETE; 17,18 EpETE; 16,17 EpDPE; 19,20 EpDPE; 16,17 DiHDoPE; 19, 20 DiHDoPE; 9,10 EpODE; 12,13 EpODE; 15,16 EpODE; 9,10 diHODE; 12,13 diHODE; 15,16 diHODE; 9,10 EpOME; 12,13 EpOME; 9,10 diHOME; 12,13 diHOME; 9,10,13 triHOME; 9,12,13 triHOME; ARA, ADA, EPA, DHA, ALA, 20cooh AA; 2,3-dinor TXB_2; 11d-TXB_2; 2,3-dinor 8-iso $PGF_{2\alpha}$; 2,3-dinor-6k $PGF_{1\alpha}$; 8-iso $PGF_{3\alpha}$; 8-iso 15k PGF_{2b}; 9-Nitrooleate; 10-Nitrooleate

SUPPLEMENTARY MATERIAL

Table 6: Serum oxylipin profiles in healthy and diseased mice. Significantly difference profiles denoted by * on those which had the lower oxylipin concentrations. Significance at *<0.05 **<0.001 ***<0.0001. βdhk = beta-dihydro-keto, dhk = dihydro-keto.

Pathway	*Fatty Acid*	*Oxylipin*	*Serum (ng/mL)*	
			Healthy	***Diseased***
COX	AA	**PGD_2**	1.56 ± 0.728	1.23 ± 0.868
		dhk PGD_2	0.468 ± 0.280	0.446 ± 0.317
		11β $PGF_{2\alpha}$	-	2.61 ± 1.01
		11bdhk $PGF_{2\alpha}$	1.48 ± 0.621	1.43 ± 1.19
		PGE_2	0.623 ± 0.289	0.548 ± 0.343
		dhk PGE_2	1.23 ± 0.883	0.775 ± 0.516
		11βPGE_2	0.401 ± 0.227	0.288 ± 0.197
		15k PGE_2	0.961 ± 0.407	1.18 ± 0.919
		$PGF_{2\alpha}$	0.167 ± 0.104	-
		15k $PGF_{2\alpha}$	0.498 ± 0.00	0.163 ± 0.136
		TXB_2	1.07 ± 56.0	0.769 ± 0.372
		2,3-dinor TXB_2	5.76 ± 4.07	4.87 ± 4.80
		12-HHTrE	11.6 ± 5.60	15.8 ± 18.7
	DGLA	PGK_1	0.070 ± 0.007***	-
		15k PGE_1	0.128 ± 0.078	0.216 ± 0.177
		TXB_1	0.200 ± 0.831	0.290 ± 0.378
	EPA	**PGE_3**	0.404 ± 0.135	0.387 ± 0.145
LOX	LA	**9-HODE**	242 ± 135	341 ± 528
		9-oxoODE	289 ± 210	693 ± 1320
		13-HODE	231 ± 114	460 ± 869
		13-oxoODE	214 ± 169	478 ± 889
		9,10,13 triHOME	154 ± 86.5	126 ± 161
		9,12,13 triHOME	61.1 ± 32.5	48.0 ± 55.0
	GLA	**13-HOTrE-γ**	3.48 ± 2.02	3.39 ± 1.70
	DGLA	**8-HETrE**	2.54 ± 1.23	1.81 ± 1.33
		15-HETrE	3.36 ± 1.91	3.50 ± 3.09
	AA	**5-HETE**	19.3 ± 9.32	18.4 ± 9.38
		5-oxoETE	11.4 ± 7.56	9.70 ± 9.82
		5,15 diHETE	0.547 ± 0.327	0.412 ± 0.318
		8-HETE	9.91 ± 7.43	9.18 ± 7.66
		9-HETE	7.01 ± 3.88	5.34 ± 4.67
		11-HETE	9.45 ± 4.55	9.21 ± 6.68
		12-HETE	43.3 ± 34.3	49.3 ± 33.2
		12-oxoETE	23.8 ± 18.4	24.5 ± 24.1
		tetranor 12-HETE	0.502 ± 0.299	1.84 ± 3.36
		15-HETE	23.1 ± 11.6	20.6 ± 12.2
		15-oxoETE	9.01 ± 5.54	8.23 ± 7.60
		8,15 diHETE	5.44 ± 4.45	3.30 ± 3.06
		6t LTB_4	1.41 ± 1.16	1.54 ± 1.28
		6t, 12epi LTB_4	1.81 ± 1.56	2.02 ± 1.48
		LTB_4	0.595 ± 0.374	0.426 ± 0.345
		12epi LTB_4	0.525 ± 0.359	0.294 ± 0.248

		6S-LXA$_4$	4.96 ± 3.07	3.17 ± 1.82
	ALA	**9-HOTrE**	38.2 ± 20.0	27.7 ± 17.2
		9 oxoOTrE	20.3 ± 14.4	13.6 ± 9.84
		13-HOTrE	22.0 ± 15.7	21.4 ± 21.3
	EPA	**5-HEPE**	2.89 ± 1.31	2.80 ± 1.58
		8-HEPE	3.75 ± 2.10	2.78 ± 2.85
		9-HEPE	3.01 ± 2.01	2.11 ± 1.64
		11-HEPE	1.98 ± 1.17	1.91 ± 1.66
		12-HEPE	7.00 ± 4.33	8.59 ± 6.55
		15-HEPE	3.36 ± 2.34	2.67 ± 2.36
	DHA	**4-HDoHE**	21.7 ± 7.26	27.3 ± 18.6
		7-HDoHE	5.57 ± 3.11	5.33 ± 4.57
		8-HDoHE	5.77 ± 3.09	5.96 ± 4.85
		10-HDoHE	4.23 ± 2.14	4.36 ± 2.97
		11-HDoHE	2.46 ± 1.13	3.14 ± 2.28
		13-HDoHE	8.39 ± 3.98	8.29 ± 6.03
		14-HDoHE	11.1 ± 5.75	13.5 ± 8.58
		16-HDoHE	7.95 ± 4.47	6.93 ± 5.41
		17-HDoHE	37.6 ± 23.0	26.2 ± 21.4
		4k DHA	4.24 ± 1.55	5.22 ± 3.97
CYP-e	LA	**9,10 EpOME**	12.6 ± 6.59**	2.68 ± 1.93
		9,10 diHOME	4.96 ± 2.06*	2.88 ± 1.88
		12,13 EpOME	25.7 ± 12.0**	9.85 ± 7.07
		12,13 diHOME	6.28 ± 2.65	4.43 ± 2.85
	AA	**5,6 EpETrE**	1.08 ± 0.552*	0.271 ± 0.215
		5,6 DiHETrE	0.543 ± 0.230	0.376 ± 0.279
		8,9 EpETrE	2.04 ± 0.836**	0.703 ± 0.258
		8,9 DiHETrE	0.241 ± 0.096	0.192 ± 0.135
		11,12 EpETrE	2.01 ± 1.04**	0.352 ± 0.259
		11,12 DiHETrE	0.164 ± 0.052	0.145 ± 0.105
		14,15 DiHETrE	0.359 ± 0.121	0.294 ± 0.150
	ALA	**12,13 EpODE**	1.20 ± 0.634*	0.536 ± 0.431
		12,13 diHODE	0.159 ± 0.0756	0.122 ± 0.0934
	EPA	**14,15 EpETE**	0.177 ± 0.170	0.102 ± 0.0833
		14,15 diHETE	1.44 ± 0.771	1.15 ± 0.738
		17,18 EpETE	0.324 ± 0.152*	0.152 ± 0.121
		17,18 diHETE	3.22 ± 1.45	2.51 ± 1.07
	DHA	**16,17 EpDPE**	9.44 ± 3.62***	2.75 ± 1.66
		19, 20 EpDPE	1.22 ± 0.332**	0.537 ± 0.366
		19, 20 DiHDoPE	0.794 ± 0.202	0.860 ± 0.491
CYP-h	AA	**16-HETE**	1.38 ± 0.711	1.52 ± 0.741
		17-HETE	0.238 ± 0.139	0.255 ± 0.149
		20cooh AA	7.85 ± 5.48	4.28 ± 3.72
	EPA	**18-HEPE**	5.36 ± 3.05	4.92 ± 2.79
	DHA	**20-HDoHE**	10.4 ± 3.61	10.9 ± 5.89
Non-enz	AA	**5-iso PGF$_{2\alpha VI}$**	0.080 ± 0.034	0.066 ± 0.018
Synthetic	AA	**15d PGA$_2$**	65.7 ± 28.5	48.0 ± 30.0

Table 7: Heart oxylipin profiles in healthy and diseased mice. Significantly difference profiles denoted by * on those which had the lower oxylipin concentrations. Significance at *<0.05 **<0.001 ***<0.0001. βdhk = beta-dihydro-keto, dhk = dihydro-keto.

Pathway	*Fatty Acid*	*Oxylipin*	*Heart (ng/g)*	
			Healthy	***Diseased***
COX	AA	**PGD_2**	1600 ± 1450	1410 ± 1450
		dhk PGD_2	904 ± 385*	481 ± 546
		PGE_2	543 ± 527	277 ± 254
		dhk PGE_2	5530 ± 3050	2480 ± 3170
		15k PGE_2	594 ± 307	366 ± 386
		6k $PGF_{1\alpha}$	1330 ± 423	1300 ± 413
		$PGF_{2\alpha}$	937 ± 321*	530 ± 404
		TXB_2	194 ± 49.1	158 ± 70.3
		12-HHTrE	2950 ± 1000	2690 ± 1410
	DGLA	**PGD_1**	-	20800 ± 26200*
		PGE_1	0.706 ± 0.331	0.337 ± 0.388
		15k PGE_1	0.293 ± 0.120	0.171 ± 0.193
		$PGF_{1\alpha}$	-	0.0137 ± 0.0149*
	ADA	**dihomo $PGF_{2\alpha}$**	0.0802 ± 0.0975	0.0165 ± 0.0331
	EPA	**TXB_3**	0.129 ± 0.0626**	0.0433 ± 0.0358
LOX	LA	**9-HODE**	266 ± 155	168 ± 147
		13-HODE	218 ± 122	142 ± 130
		9,10,13 triHOME	202 ± 93.8	105 ± 110
		9,12,13 triHOME	69.1 ± 30.5	42.4 ± 41.9
	GLA	**13-HOTrE-γ**	0.567 ± 0.381*	0.226 ± 0.197
	DGLA	**8-HETrE**	1.55 ± 0.799	0.962 ± 0.601
		15-HETrE	1.15 ± 0.653	0.693 ± 0.548
	AA	**5-HETE**	6.82 ± 3.61	4.48 ± 2.13
		5,15 diHETE	0.511 ± 0.391*	0.128 ± 0.150
		8-HETE	6.44 ± 3.19**	2.78 ± 1.64
		9-HETE	4.72 ± 2.60*	2.20 ± 1.74
		11-HETE	2.92 ± 1.72*	1.28 ± 0.620
		12-HETE	11.8 ± 9.97	8.08 ± 6.35
		15-HETE	8.86 ± 5.37	4.65 ± 2.62
		8,15 diHETE	5.15 ± 3.34	2.50 ± 2.26
		LTB_4	0.363 ± 0.251*	0.121 ± 0.114
		12epi LTB_4	0.317 ± 0.222*	0.0884 ± 0.0761
		6S-LXA_4	9.72 ± 4.45	5.92 ± 6.63
	ALA	**9-HOTrE**	9.35 ± 6.35	4.86 ± 4.38
		13-HOTrE	9.08 ± 5.55*	4.21 ± 3.77
	EPA	**5-HEPE**	0.654 ± 0.333*	0.322 ± 0.170
		8-HEPE	0.526 ± 0.393	0.438 ± 0.270
		9-HEPE	0.587 ± 0.242***	0.127 ± 0.119
		11-HEPE	0.161 ± 0.105*	0.0551 ± 0.0417
		12-HEPE	1.43 ± 1.28	1.10 ± 1.01
		15-HEPE	0.195 ± 0.134*	0.0826 ± 0.0716
	DHA	**4-HDoHE**	4.72 ± 1.92	4.26 ± 1.39

		7-HDoHE	3.56 ± 1.10**	1.90 ± 1.01
		8-HDoHE	3.18 ± 1.07**	1.75 ± 1.03
		10-HDoHE	1.53 ± 0.599**	0.799 ± 0.402
		11-HDoHE	1.38 ± 0.685*	0.696 ± 0.457
		13-HDoHE	4.50 ± 2.25*	2.27 ± 1.08
		14-HDoHE	2.71 ± 1.15	1.86 ± 1.07
		16-HDoHE	2.23 ± 1.06	1.37 ± 0.894
		17-HDoHE	8.72 ± 4.64*	4.35 ± 2.80
CYP-e	LA	**9,10 EpOME**	0.517 ± 0.365	0.963 ± 0.537
		9,10 diHOME	9.26 ± 5.41	6.11 ± 6.54
		12,13 diHOME	5.76 ± 3.35*	2.90 ± 2.89
	AA	**5,6 EpETrE**	0.0991 ± 0.0842	0.0676 ± 0.0270
		5,6 DiHETrE	0.0910 ± 0.0377	0.127 ± 0.123
		8,9 EpETrE	0.0905 ± 0.0563	0.0857 ± 0.0495
		8,9 DiHETrE	0.457 ± 0.245	0.294 ± 0.321
		11,12 DiHETrE	0.297 ± 0.155	0.161 ± 0.168
		14,15 DiHETrE	0.238 ± 0.140*	0.109 ± 0.0974
	EPA	14,15 diHETE	0.405 ± 0.202*	0.184 ± 0.263
	DHA	19, 20 DiHDoPE	0.114 ± 0.0287	0.103 ± 0.0598
CYP-h	AA	**16-HETE**	0.405 ± 0.243	0.304 ± 0.269
		18-HETE	0.151 ± 0.0960*	0.0720 ± 0.0703
	EPA	**18-HEPE**	0.360 ± 0.248*	0.183 ± 0.168
	DHA	**20-HDoHE**	3.56 ± 1.99	1.92 ± 0.878

Table 8: Kidney oxylipin profiles in healthy and diseased mice. Significantly difference profiles denoted by * on those which had the lower oxylipin concentrations. Significance at *<0.05 **<0.001 ***<0.0001. βdhk = beta-dihydro-keto, dhk = dihydro-keto.

Pathway	*Fatty Acid*	*Oxylipin*	*Kidney (ng/mg) (10^2)*	
			Healthy	***Diseased***
COX	AA	**PGD_2**	10.5 ± 1.00	12.9 ± 5.86
		15d PGD_2	1.02 ± 0.208**	0.500 ± 0.251
		PGE_2	9.45 ± 1.97	7.46 ± 0.684
		dhk PGE_2	2.52 ± 0.886	1.25 ± 1.41
		15k PGE_2	0.407 ± 0.079	0.421 ± 0.233
		6k $PGF_{1\alpha}$	13.3 ± 2.30	19.1 ± 5.32
		$PGF_{2\alpha}$	6.93 ± 1.04	7.00 ± 1.63
		dhk $PGF_{2\alpha}$	0.951 ± 0.270	0.892 ± 0.587
		12-HHTrE	209 ± 4290	154 ± 67.7
	DGLA	**PGE_1**	1.91 ± 0.492	1.64 ± 0.699
		$PGF_{1\alpha}$	0.333 ± 0.106	0.381 ± 0.189
		d17 6k $PGF_{1\alpha}$	0.445 ± 0.074	0.432 ± 0.137
	EPA	**PGE_3**	1.20 ± 0.398	1.54 ± 2.10
		$PGF_{3\alpha}$	1.51 ± 0.398	1.99 ± 1.34
LOX	LA	**9-HODE**	514 ± 180	588 ± 114
		9-oxoODE	361 ± 120	348 ± 149
		13-HODE	376 ± 115	450 ± 146
		13-oxoODE	261 ± 86.8	223 ± 59.9
		9,10,13 triHOME	95.8 ± 24.6	92.0 ± 32.2
		9,12,13 triHOME	34.5 ± 10.0	36.2 ± 10.7
	GLA	**13-HOTrE-γ**	10.3 ± 2.66	8.00 ± 4.40
	DGLA	**8-HETrE**	9.58 ± 1.32**	5.73 ± 0.886
		15-HETrE	7.68 ± 2.66	6.09 ± 1.58
	AA	**5-HETE**	83.0 ± 17.3*	54.6 ± 10.9
		5-oxoETE	11.6 ± 5.12	10.7 ± 4.37
		5,15 diHETE	0.73 ± 0.085	0.930 ± 0.610
		8-HETE	23.7 ± 5.91	20.7 ± 7.05
		9-HETE	25.8 ± 4.64*	19.2 ± 2.68
		11-HETE	34.5 ± 4.72	31.7 ± 2.50
		12-HETE	107 ± 13.1	99.8 ± 21.2
		12-oxoETE	81.3 ± 14.8	64.1 ± 39.8
		tetranor 12-HETE	0.605 ± 0.110**	0.317 ± 0.126
		15-HETE	107 ± 18.5	93.2 ± 10.6
		15-oxoETE	58.1 ± 15.5*	38.8 ± 7.34
		8,15 diHETE	19.5 ± 3.46	14.3 ± 6.45
		6t, 12epi LTB_4	0.430 ± 0.150	0.490 ± 0.360
		LTB_4	0.420 ± 0.052	0.470 ± 0.180
	ALA	**9-HOTrE**	48.8 ± 19.8	38.5 ± 20.1
		9 oxoOTrE	7.07 ± 2.60	5.45 ± 1.54
		13-HOTrE	22.8 ± 10.7	21.1 ± 12.2
	EPA	**RvE_1**	0.230 ± 0.110	6.81 ± 14.5
		5-HEPE	3.87 ± 0.330*	1.28 ± 0.246

		11-HEPE	2.85 ± 0.55*	1.94 ± 0.411
		12-HEPE	27.0 ± 7.08*	16.0 ± 3.90
		15-HEPE	3.24 ± 0.563**	1.92 ± 0.194
	DHA	**4-HDoHE**	64.1 ± 21.3	44.2 ± 9.53
		7-HDoHE	19.0 ± 5.87	15.7 ± 4.13
		8-HDoHE	53.0 ± 7.68**	33.3 ± 6.92
		10-HDoHE	23.4 ± 3.15	20.6 ± 3.10
		11-HDoHE	23.4 ± 3.15***	13.7 ± 2.21
		13-HDoHE	83.3 ± 8.72*	68.3 ± 11.9
		14-HDoHE	43.4 ± 11.6	45.7 ± 6.27
		16-HDoHE	25.3 ± 9.26	22.6 ± 1.94
		17-HDoHE	84.6 ± 36.6	81.6 ± 7.89
CYP-e	LA	**9,10 EpOME**	2.48 ± 1.80	5.08 ± 5.16
		9,10 diHOME	3.35 ± 0.73	3.2 ± 0.921
		12,13 EpOME	3.16 ± 2.23	7.02 ± 6.63
		12,13 diHOME	4.77 ± 1.11	5.13 ± 1.19
	AA	**5,6 EpETrE**	0.91 ± 0.59	1.49 ± 1.33
		8,9 DiHETrE	1.00 ± 0.17*	0.760 ± 0.110
		11,12 EpETrE	0.900 ± 0.620	1.78 ± 1.51
		11,12 DiHETrE	0.71 ± 0.09	0.680 ± 0.058
		14,15 DiHETrE	1.35 ± 0.145*	1.14 ± 0.107
	ALA	**12,13 EpODE**	0.150 ± 0.093	0.3 ± 0.32
	EPA	17,18 diHETE	4.93 ± 0.990	5.64 ± 6.68
CYP-h	AA	**16-HETE**	6.94 ± 2.40	6.44 ± 1.46
	EPA	**18-HEPE**	6.56 ± 1.81*	3.95 ± 1.87
	DHA	**20-HDoHE**	49.7 ± 9.92	38.9 ± 6.94
Non-enz	AA	**5-iso $PGF_{2\alpha VI}$**	0.850 ± 0.110	1.34 ± 1.19
		8-iso $PGF_{2\alpha III}$	1.32 ± 0.13	1.33 ± 0.226

Table 9: Liver oxylipin profiles in healthy and diseased mice. Significantly difference profiles denoted by * on those which had the lower oxylipin concentrations. Significance at *<0.05 **<0.001 ***<0.0001. bdhk = beta-dihydro-keto, dhk = dihydro-keto.

Pathway	*Fatty Acid*	*Oxylipin*	*Liver (ng/mg)*	
			Healthy	***Diseased***
COX	AA	15d PGD_2	0.211 ± 0.472	0.320 ± 0.486
		PGJ_2	-	0.234 ± 0.400
		PGE_2	0.00241 ± 0.00414	0.00207 ± 0.00269
		15k PGE_2	0.732 ± 1.24	0.0310 ± 0.0183
		6k $PGF_{1\alpha}$	0.706 ± 0.267	0.976 ± 0.662
		$PGF_{2\alpha}$	1.70 ± 2.71	3.13 ± 3.79
		TXB_2	0.507 ± 0.293	0.389 ± 0.202
		12-HHTrE	29.9 ± 32.6	22.8 ± 16.1
	DGLA	**PGD_1**	-	0.0239 ± 0.0429
		PGE_1	0.0661 ± 0.0718	0.0588 ± 0.103
		15k PGE_1	0.179 ± 0.168	0.0239 ± 0.0136**
		$PGF_{1\alpha}$	0.968 ± 0.710	0.780 ± 0.443
	EPA	**$PGF_{3\alpha}$**	1.71 ± 0.762	1.67 ± 1.43
LOX	LA	**9-HODE**	286 ± 358	114 ± 70.6
		13-HODE	296 ± 296	85.4 ± 62.4**
		9,10,13 triHOME	90.6 ± 144	26.1 ± 16.1*
		9,12,13 triHOME	38.7 ± 54.1	12.7 ± 9.79
	GLA	**13-HOTrE-γ**	7.17 ± 7.61	2.74 ± 1.51
	DGLA	**8-HETrE**	6.75 ± 8.85	2.03 ± 0.974*
		15-HETrE	6.10 ± 6.80	1.34 ± 0.627**
	AA	**5-HETE**	39.0 ± 47.6	13.5 ± 4.24
		8-HETE	21.6 ± 31.6	9.55 ± 3.32
		9-HETE	19.7 ± 28.6	8.15 ± 4.80
		11-HETE	92.2 ± 265	4.01 ± 1.36
		12-HETE	32.4 ± 20.8	20.9 ± 8.49*
		tetranor 12-HETE	0.941 ± 0.704	1.47 ± 1.55
		15-HETE	40.8 ± 38.8	11.5 ± 2.91
	ALA	**9-HOTrE**	32.4 ± 40.7	8.55 ± 6.67
		13-HOTrE	11.7 ± 10.2	7.14 ± 3.18
	DHA	**4-HDoHE**	17.5 ± 10.9	7.26 ± 4.09
		7-HDoHE	5.64 ± 5.75	5.16 ± 2.83
		10-HDoHE	53.7 ± 147	2.84 ± 1.12
		11-HDoHE	52.7 ± 141	2.34 ± 1.46
		13-HDoHE	5.68 ± 7.71	6.20 ± 2.41
		14-HDoHE	6.46 ± 4.42	2.75 ± 1.20**
		16-HDoHE	5.07 ± 5.71	2.75 ± 0.802
		17-HDoHE	16.8 ± 18.8	10.5 ± 4.37
CYPe	LA	9,10 diHOME	2.36 ± 0.691	2.58 ± 0.889
		12,13 EpOME	5.66 ± 8.61	-***
		12,13 diHOME	11.5 ± 3.39	12.8 ± 5.29
	AA	**5,6 EpETrE**	1.30 ± 1.51	0.466 ± 0.679
		8,9 DiHETrE	0.654 ± 0.256	0.703 ± 0.228
		11,12 DiHETrE	-***	1.46 ± 0.541

		14,15 DiHETrE	2.95 ± 0.714	3.49 ± 0.978
	EPA	14,15 diHETE	22.1 ± 16.6	21.8 ± 4.57
		17,18 diHETE	9.90 ± 5.71	12.2 ± 5.25
	DHA	19, 20 DiHDoPE	5.13 ± 1.50	4.87 ± 2.11
CYPh	AA	**16-HETE**	4.01 ± 5.15	-***
		17-HETE	0.365 ± 0.312	0.281 ± 0.204
		18-HETE	-***	0.532 ± 0.735
	DHA	**20-HDoHE**	-***	4.64 ± 9.03

Table 10: Serum oxylipin profiles in healthy male and female mice. Significantly difference profiles denoted by * on those which had the higher oxylipin concentrations. Significance at *<0.05 **<0.001 ***<0.0001. bdhk = beta-dihydro-keto, dhk = dihydro-keto.

Pathway	*Fatty Acid*	*Oxylipin*	*Serum (ng/mL)*	
			Male	*Female*
COX	AA	**PGD_2**	1.56 ± 0.728	1.85 ± 1.09
		dhk PGD_2	0.468 ± 0.280	0.352 ± 0.239
		11β $PGF_{2\alpha}$	-	2.52 ± 0.991***
		11βdhk $PGF_{2\alpha}$	1.48 ± 0.621	4.45 ± 1.67***
		PGE_2	0.623 ± 0.289	0.558 ± 0.294
		dhk PGE_2	0.921 ± 0.938	1.25 ± 0.715
		11βPGE_2	0.401 ± 0.227	0.514 ± 0.334
		15k PGE_2	0.961 ± 0.407	2.36 ± 1.60*
		$PGF_{2\alpha}$	0.0626 ± 0.103	0.172 ± 0.206
		15k $PGF_{2\alpha}$	0.0623 ± 0.176	0.330 ± 0.177**
		TXB_2	1.07 ± 0.560	0.904 ± 0.741
		2,3-dinor TXB_2	5.76 ± 4.07	3.68 ± 2.34
		12-HHTrE	11.6 ± 5.60	20.7 ± 6.93**
	DGLA	PGK_1	0.070 ± 0.007***	-
		15k PGE_1	0.112 ± 0.085	0.091 ± 0.074
		TXB_1	0.200 ± 0.083***	-
	EPA	**PGE_3**	0.303 ± 0.219	1.26 ± 0.630**
LOX	LA	**9-HODE**	242 ± 135	306 ± 127
		9-oxoODE	289 ± 210	537 ± 875
		13-HODE	231 ± 114	260 ± 105
		13-oxoODE	214 ± 169	262 ± 395
		9,10,13 triHOME	154 ± 86.5	175 ± 73.7
		9,12,13 triHOME	61.1 ± 32.5	70.1 ± 31.6
	GLA	**13-HOTrE-γ**	3.48 ± 2.02	4.93 ± 2.36
	DGLA	**8-HETrE**	2.54 ± 1.23	3.84 ± 1.62
		15-HETrE	3.36 ± 1.91	4.46 ± 1.91
	AA	**5-HETE**	19.3 ± 9.32	48.6 ± 19.7**
		5-oxoETE	11.4 ± 7.56	24.4 ± 36.2
		5,15 diHETE	0.547 ± 0.327	1.76 ± 1.12**
		8-HETE	9.91 ± 7.43	34.9 ± 21.1**
		9-HETE	7.01 ± 3.88	21.5 ± 13.7**
		11-HETE	9.45 ± 4.55	30.4 ± 17.7**
		12-HETE	43.3 ± 34.3	40.2 ± 22.1
		12-oxoETE	23.8 ± 18.4	36.5 ± 49.3
		tetranor 12-HETE	0.502 ± 0.299	0.768 ± 0.561
		15-HETE*	23.1 ± 11.6	64.8 ± 29.0**
		15-oxoETE	9.01 ± 5.54	19.0 ± 27.4
		8,15 diHETE	5.44 ± 4.45	11.7 ± 6.49*
		6t LTB_4	1.41 ± 1.16	2.13 ± 1.61
		6t, 12epi LTB_4	1.81 ± 1.56	3.00 ± 2.15
		LTB_4	0.595 ± 0.374	1.86 ± 1.11**
		12oxo LTB_4	-	0.785 ± 0.486**

		12epi LTB_4	0.525 ± 0.359	1.54 ± 0.928*
		6S-LXA_4	4.96 ± 3.07	9.34 ± 5.46*
		LXA_5	-	2.76 ± 1.85***
	ALA	**9-HOTrE**	38.2 ± 20.0	31.3 ± 13.2
		9 oxoOTrE	20.3 ± 14.4	20.4 ± 34.1
		13-HOTrE	22.0 ± 15.7	21.0 ± 10.4
	EPA	**5-HEPE**	2.89 ± 1.31	3.93 ± 1.56
		8-HEPE	3.75 ± 2.10	4.98 ± 2.80
		9-HEPE	3.01 ± 2.01	3.36 ± 2.04
		11-HEPE	1.98 ± 1.17	2.30 ± 1.12
		12-HEPE	7.00 ± 4.33	4.07 ± 1.34
		15-HEPE	3.36 ± 2.34	4.24 ± 1.96
	DHA	**4-HDoHE**	21.7 ± 7.26	33.5 ± 10.2*
		7-HDoHE	5.57 ± 3.11	9.18 ± 4.70
		8-HDoHE	5.77 ± 3.09	11.1 ± 7.38
		10-HDoHE	4.23 ± 2.14	7.17 ± 3.36*
		11-HDoHE	2.46 ± 1.13	4.32 ± 2.52
		13-HDoHE	8.39 ± 3.98	14.4 ± 6.79*
		14-HDoHE	11.1 ± 5.75	13.8 ± 5.77
		16-HDoHE	7.95 ± 4.47	14.4 ± 6.41*
		17-HDoHE	37.6 ± 23.0	67.6 ± 26.4*
		4k DHA	4.24 ± 1.55	4.94 ± 7.22
CYP-e	LA	**9,10 EpOME**	12.6 ± 6.59	10.7 ± 2.96
		9,10 diHOME	4.96 ± 2.06	3.54 ± 1.34
		12,13 EpOME	25.7 ± 12.0	20.8 ± 7.32
		12,13 diHOME	6.28 ± 2.65	3.96 ± 1.59*
	AA	**5,6 EpETrE**	0.941 ± 0.637	1.96 ± 1.02*
		5,6 DiHETrE	0.543 ± 0.230	0.654 ± 0.232
		8,9 EpETrE	2.04 ± 0.836	3.01 ± 0.822*
		8,9 DiHETrE	0.241 ± 0.096	0.302 ± 0.075
		11,12 EpETrE	2.01 ± 1.04	2.78 ± 0.971
		11,12 DiHETrE	0.164 ± 0.052	0.193 ± 0.059
		14,15 DiHETrE	0.359 ± 0.121	0.363 ± 0.085
	ALA	**12,13 EpODE**	1.20 ± 0.634	0.791 ± 0.350
		12,13 diHODE	0.159 ± 0.076	0.226 ± 0.243
	EPA	**14,15 EpETE**	0.177 ± 0.170	0.141 ± 0.149
		14,15 diHETE	1.44 ± 0.771	0.785 ± 0.824
		17,18 EpETE	0.324 ± 0.152	0.241 ± 0.137
		17,18 diHETE	3.22 ± 1.45	3.77 ± 2.81
	DHA	**16,17 EpDPE**	9.44 ± 3.62	8.24 ± 2.43
		19, 20 EpDPE	1.22 ± 0.332	0.993 ± 0.409
		19, 20 DiHDoPE	0.794 ± 0.202*	0.550 ± 0.242
CYP-h	AA	**16-HETE**	1.38 ± 0.711	3.86 ± 1.64**
		17-HETE	0.238 ± 0.139	0.384 ± 0.174
		18-HETE	-	0.197 ± 0.0858***
		20cooh AA	7.85 ± 5.48	15.8 ± 12.5
	EPA	**18-HEPE**	5.36 ± 3.05	5.91 ± 2.19
	DHA	**20-HDoHE**	10.4 ± 36.1	12.2 ± 4.09
Non-enz	AA	**5-iso $PGF_{2\alpha VI}$**	0.080 ± 0.034	0.149 ± 0.095*

Synthetic	AA	**15d PGA_2**	65.7 $\pm$ 28.5	230 $\pm$ 150**

Table 11: Heart oxylipin profiles in healthy male and female mice. Significantly difference profiles denoted by * on those which had the higher oxylipin concentrations. Significance at *<0.05 **<0.001 ***<0.0001. βdhk = beta-dihydro-keto, dhk = dihydro-keto.

Pathway	*Fatty Acid*	*Oxylipin*	*Heart (ng/mg)*	
			Male	*Female*
COX	AA	**PGD_2**	1.78 ± 1.33*	0.464 ± 0.129
		dhk PGD_2	0.863 ± 0.386***	-
		PGE_2	0.486 ± 0.554	0.090 ± 0.022
		dhk PGE_2	5.62 ± 3.03***	0.152 ± 0.041
		15k PGE_2	0.569 ± 0.307***	-
		6k $PGF_{1\alpha}$	1.35 ± 0.423	1.08 ± 0.306
		$PGF_{2\alpha}$	0.889 ± 0.321***	0.255 ± 0.0761
		TXB_2	0.181 ± 0.059**	0.110 ± 0.0507
		12-HHTrE	2.86 ± 1.07	5.32 ± 2.48**
	DGLA	**PGD_1**	-	0.019 ± 0.0091***
		PGE_1	0.676 ± 0.326**	0.044 ± 0.010
		15k PGE_1	0.279 ± 0.117***	-
		$PGF_{1\alpha}$	-	0.033 ± 0.008***
	ADA	**dihomo $PGF_{2\alpha}$**	0.068 ± 0.099*	-
		TXB_3	0.122 ± 0.064***	-
LOX	LA	**9-HODE**	240 ± 162***	25.1 ± 10.7
		13-HODE	203 ± 127***	16.7 ± 5.18
		9,10,13 triHOME	193 ± 95.6***	3.85 ± 1.21
		9,12,13 triHOME	66.5 ± 30.7***	1.61 ± 0.533
	GLA	**13-HOTrE-γ**	0.540 ± 0.399***	-
	DGLA	**8-HETrE**	1.43 ± 0.875***	0.343 ± 0.167
		15-HETrE	1.03 ± 0.671***	0.186 ± 0.062
	AA	**5-HETE**	6.35 ± 3.87***	1.76 ± 0.653
		5,15 diHETE	0.472 ± 0.411***	-
		8-HETE	6.03 ± 3.44***	1.78 ± 0.476
		9-HETE	4.36 ± 2.80***	-
		11-HETE	2.69 ± 1.81	1.12 ± 0.335
		12-HETE	11.5 ± 10.3	11.5 ± 7.77
		15-HETE	8.09 ± 5.57***	1.82 ± 0.604
		8,15 diHETE	4.91 ± 3.50***	-
		LTB_4	0.327 ± 0.264***	-
		12epi LTB_4	0.289 ± 0.232***	-
		6S-LXA_4	9.17 ± 4.42***	-
	ALA	**9-HOTrE**	8.45 ± 6.68**	1.01 ± 0.489
		13-HOTrE	***	1.02 ± 0.428
	EPA	**5-HEPE**	0.608 ± 0.369***	-
		8-HEPE	0.524 ± 0.394***	-
		9-HEPE	0.564 ± 0.271***	-
		11-HEPE	0.152 ± 0.111***	-
		12-HEPE	1.41 ± 1.30	1.96 ± 1.40
		15-HEPE	0.182 ± 0.141***	-
	DHA	**4-HDoHE**	4.40 ± 2.17**	1.83 ± 0.836

		7-HDoHE	3.37 ± 1.29*	2.05 ± 0.952
		8-HDoHE	2.98 ± 1.27**	0.990 ± 0.941
		10-HDoHE	1.44 ± 0.661*	0.815 ± 0.314
		11-HDoHE	1.27 ± 0.733*	0.541 ± 0.220
		13-HDoHE	4.21 ± 2.44	2.13 ± 0.746
		14-HDoHE	2.54 ± 1.28	1.71 ± 0.693
		16-HDoHE	2.03 ± 1.13*	0.763 ± 0.307
		17-HDoHE	7.92 ± 4.86*	2.95 ± 0.992
CYP-e	LA	**9,10 EpOME**	0.481 ± 0.391	0.195 ± 0.125
		9,10 diHOME	8.66 ± 5.63***	0.215 ± 0.056
		12,13 EpOME	-	0.336 ± 0.239***
		12,13 diHOME	5.34 ± 3.49***	0.243 ± 0.061
	AA	**5,6 EpETrE**	0.087 ± 0.086***	-
		5,6 DiHETrE	0.085 ± 0.036***	-
		8,9 EpETrE	0.082 ± 0.057***	-
		8,9 DiHETrE	0.427 ± 0.247***	-
		11,12 EpETrE	-	-
		11,12 DiHETrE	0.271 ± 0.154***	0.015 ± 0.003
		14,15 DiHETrE	0.214 ± 0.139***	0.018 ± 0.004
	ALA	**12,13 EpODE**	-	0.018 ± 0.011***
	EPA	14,15 diHETE	0.374 ± 0.220***	-
	DHA	19, 20 DiHDoPE	0.109 ± 0.027***	0.057 ± 0.019
CYP-h	AA	**16-HETE**	0.371 ± 0.252***	-
		18-HETE	0.137 ± 0.102***	-
	EPA	**18-HEPE**	0.328 ± 0.260***	-
	DHA	**20-HDoHE**	3.24 ± 2.10**	0.910 ± 0.378
Non-enz	AA	**8-iso $PGF_{2\alpha III}$**	-	0.029 ± 0.014***

Table 12: Kidney oxylipin profiles in healthy male and female mice. Significantly difference profiles denoted by * on those which had the higher oxylipin concentrations. Significance at *<0.05 **<0.001 ***<0.0001. βdhk = beta-dihydro-keto, dhk = dihydro-keto.

Pathway	*Fatty Acid*	*Oxylipin*	*Kidney (ng/mg)*	
			Male	*Female*
COX	AA	**PGD_2**	0.456 ± 0.0593	0.867 ± 0.148***
		15d PGD_2	0.044 ± 0.0094	0.089 ± 0.043
		PGE_2	0.392 ± 0.113	0.773 ± 0.162**
		dhk PGE_2	0.103 ± 0.0445	0.267 ± 0.122*
		15k PGE_2	0.019 ± 0.003	0.070 ± 0.019***
		6k $PGF_{1\alpha}$	0.572 ± 0.111	1.39 ± 0.162**
		$PGF_{2\alpha}$	0.297 ± 0.055	0.590 ± 0.044***
		dhk $PGF_{2\alpha}$	0.043 ± 0.011	0.089 ± 0.027**
		12-HHTrE	8.91 ± 2.11	14.5 ± 1.41***
	DGLA	**PGE_1**	0.080 ± 0.025	0.115 ± 0.038
		$PGF_{1\alpha}$	0.014 ± 0.0057	0.022 ± 0.005*
		d17 6k $PGF_{1\alpha}$	0.019 ± 0.005	0.022 ± 0.006
	EPA	**PGE_3**	0.048 ± 0.017	0.053 ± 0.023
		$PGF_{3\alpha}$	0.064 ± 0.020	0.064 ± 0.015
LOX	LA	**9-HODE**	22.6 ± 7.36	20.3 ± 6.24
		9-oxoODE	15.8 ± 4.96**	7.96 ± 2.21
		13-HODE	17.5 ± 4.87	12.7 ± 2.22
		13-oxoODE	11.7 ± 3.50*	6.63 ± 2.66
		9,10,13 triHOME	4.33 ± 0.993*	3.18 ± 0.650
		9,12,13 triHOME	1.56 ± 0.403*	1.06 ± 0.254
	GLA	**13-HOTrE-γ**	0.465 ± 0.107	0.373 ± 0.067
	DGLA	**8-HETrE**	0.423 ± 0.056**	0.296 ± 0.028
		15-HETrE	0.353 ± 0.108*	0.207 ± 0.046
	AA	**5-HETE**	3.74 ± 0.695	4.72 ± 0.885*
		5-oxoETE	0.520 ± 0.206	0.383 ± 0.134
		5,15 diHETE	0.032 ± 0.0042	0.046 ± 0.017
		8-HETE	1.06 ± 0.238	1.06 ± 0.185
		9-HETE	1.15 ± 0.190	1.38 ± 0.347
		11-HETE	1.52 ± 0.206	1.89 ± 0.165**
		12-HETE	4.71 ± 0.609	4.79 ± 0.512
		12-oxoETE	3.56 ± 0.636	3.21 ± 1.65
		tetranor 12-HETE	0.026 ± 0.0049	0.026 ± 0.009
		15-HETE	4.85 ± 0.749	6.43 ± 1.24*
		15-oxoETE	2.60 ± 0.623	2.41 ± 0.649
		8,15 diHETE	0.857 ± 0.146	0.830 ± 0.151
		6t, 12epi LTB_4	0.018 ± 0.008	0.023 ± 0.011
		LTB_4	0.019 ± 0.002	0.018 ± 0.005
	ALA	**9-HOTrE**	2.12 ± 0.819*	1.10 ± 0.330
		9 oxoOTrE	0.297 ± 0.116**	0.117 ± 0.028
		13-HOTrE	1.02 ± 0.429*	0.541 ± 0.135
	EPA	**RvE_1**	0.010 ± 0.005	0.021 ± 0.010*
		5-HEPE	0.159 ± 0.039	0.113 ± 0.051
		11-HEPE	0.124 ± 0.025**	0.084 ± 0.014

		12-HEPE	1.16 ± 0.317**	0.509 ± 0.087
		15-HEPE	0.142 ± 0.024	0.118 ± 0.047
	DHA	**4-HDoHE**	3.07 ± 0.970	2.23 ± 0.562
		7-HDoHE	0.891 ± 0.251	0.663 ± 0.141
		8-HDoHE	2.41 ± 0.315	1.86 ± 0.591
		10-HDoHE	1.08 ± 0.144**	0.755 ± 0.158
		11-HDoHE	0.856 ± 0.180*	0.609 ± 0.197
		13-HDoHE	3.77 ± 0.354	3.08 ± 0.827
		14-HDoHE	2.04 ± 0.519*	1.46 ± 0.273
		16-HDoHE	1.16 ± 0.376	0.882 ± 0.187
		17-HDoHE	3.85 ± 1.48	2.56 ± 0.667
CYP-e	LA	**9,10 EpOME**	0.111 ± 0.072*	0.037 ± 0.017
		9,10 diHOME	0.145 ± 0.032*	0.097 ± 0.025
		12,13 EpOME	0.142 ± 0.090*	0.049 ± 0.017
		12,13 diHOME	0.210 ± 0.046**	0.125 ± 0.032
	AA	**5,6 EpETrE**	0.042 ± 0.024*	0.020 ± 0.015
		8,9 DiHETrE	0.044 ± 0.007	0.046 ± 0.009
		11,12 EpETrE	0.041 ± 0.025*	0.020 ± 0.013
		11,12 DiHETrE	0.032 ± 0.004	0.033 ± 0.007
		14,15 DiHETrE	0.060 ± 0.006**	0.045 ± 0.007
	ALA	**12,13 EpODE**	0.006 ± 0.004**	0.002 ± 0.001
	EPA	17,18 diHETE	0.212 ± 0.046**	0.106 ± 0.047
	DHA	16,17 DiHDoPE	0.081 ± 0.015**	0.016 ± 0.004
		19, 20 DiHDoPE	0.696 ± 0.203**	0.183 ± 0.029
CYP-h	AA	**16-HETE**	0.314 ± 0.097	0.357 ± 0.050
	EPA	**18-HEPE**	0.292 ± 0.073	0.197 ± 0.065
	DHA	**20-HDoHE**	2.24 ± 0.399	2.17 ± 1.23
Non-enz	AA	**5-iso $PGF_{2\alpha VI}$**	0.037 ± 0.005	0.072 ± 0.027**
		8-iso $PGF_{2\alpha III}$	0.058 ± 0.006	0.116 ± 0.015***

Table 13: Liver oxylipin profiles in healthy male and female mice. Significantly difference profiles denoted by * on those which had the higher oxylipin concentrations. Significance at *<0.05 **<0.001 ***<0.0001. βdhk = beta-dihydro-keto, dhk = dihydro-keto.

Pathway	*Fatty Acid*	*Oxylipin*	*Liver (ng/mg)*	
			Male	***Female***
COX	AA	15d PGD_2	0.332 ± 0.570***	-
		PGE_2	-	0.066 ± 0.018***
		dhk PGE_2	-	0.633 ± 0.147***
		15k PGE_2	0.472 ± 0.293***	-
		6k $PGF_{1\alpha}$	0.606 ± 0.161***	0.261 ± 0.106
		$PGF_{2\alpha}$	0.191 ± 0.122	0.353 ± 0.089*
		TXB_2	0.457 ± 0.254***	0.107 ± 0.025
		12-HHTrE	13.333 ± 3.082**	4.432 ± 0.745
	DGLA	**PGE_1**	0.121 ± 0.048***	-
		$PGF_{1\alpha}$	1.041 ± 0.428***	-
	EPA	**$PGF_{3\alpha}$**	1.697 ± 0.419***	-
LOX	LA	**9-HODE**	286 ± 358**	9.65 ± 1.45
		13-HODE	296 ± 296***	6.79 ± 1.55
		9,10,13 triHOME	90.6 ± 144***	1.33 ± 0.277
		9,12,13 triHOME	38.7 ± 54.1***	0.583 ± 0.124
	GLA	**13-HOTrE-γ**	7.17 ± 7.61***	-
	DGLA	**8-HETrE**	6.75 ± 8.85***	-
		15-HETrE	6.10 ± 6.80***	-
	AA	**5-HETE**	37.4 ± 12.1***	-
		8-HETE	8.06 ± 3.74***	-
		9-HETE	5.5 ± 2.3***	-
		11-HETE	4.1 ± 1.9***	0.503 ± 0.136
		12-HETE	21.6 ± 5.2***	-
		tetranor 12-HETE	1.04 ± 0.665***	-
		15-HETE	21.3 ± 10.9***	-
	ALA	**9-HOTrE**	32.4 ± 40.7**	0.423 ± 0.139
		13-HOTrE	11.7 ± 10.2***	0.668 ± 0.298
	EPA	**12-HEPE**	-	1.72 ± 0.56***
	DHA	**4-HDoHE**	22.42 ± 8.04***	-
		7-HDoHE	3.34 ± 1.40***	-
		10-HDoHE	2.05 ± 0.84***	-
		11-HDoHE	1.93 ± 1.19****	-
		13-HDoHE	3.38 ± 1.1***	-
		14-HDoHE	4.67 ± 2.13***	-
		16-HDoHE	2.64 ± 1.89***	0.22 ± 0.04
		17-HDoHE	7.49 ± 3.02***	-
CYP-e	LA	**9,10 EpOME**	-	0.055 ± 0.038***
		9,10 diHOME	2.36 ± 0.691***	0.45 ± 0.078
		12,13 EpOME	5.66 ± 8.61***	0.12 ± 0.094
		12,13 diHOME	11.5 ± 3.39***	1.64 ± 0.259
	AA	**5,6 EpETrE**	1.30 ± 1.51***	-
		8,9 DiHETrE	0.654 ± 0.256***	-

		11,12 DiHETrE	0.96 ± 0.40**	0.22 ± 0.043
		14,15 DiHETrE	2.95 ± 0.71***	0.38 ± 0.078
	ALA	12,13 diHODE	0.18 ± 0.085***	-
	EPA	14,15 diHETE	22.0 ± 14.7***	1.41 ± 0.324
		17,18 diHETE	10.9 ± 4.92***	1.27 ± 0.20
	DHA	19, 20 DiHDoPE	5.13 ± 1.50***	0.69 ± 0.12
CYP-h	AA	**16-HETE**	1.52 ± 0.72***	-
		17-HETE	0.26 ± 0.066***	-