Glomerulomegaly as an Early Marker of Obesity-Related Glomerulopathy in the Diet-Induced Obese Experimental Model and Use of Alpha-Linolenic Acid Rich Dietary Oils for the Treatment of Disease and Alteration of Oxylipin Profiles

by

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I. ABSTRACT

Obesity-related glomerulopathy (ORG) is an emerging epidemic for which an established model, diagnostic guidelines, and dietary treatments are absent. Oxylipins influence inflammation and hemodynamics, yet the renal oxylipin profile or the influence of dietary linoleic acid (LA) and α-linolenic acid (ALA) on their formation has yet to be examined. Therefore, obese-prone rats were provided high fat lard/soy diets to induce obesity and subsequently divided among 7 diets with varying LA and ALA levels. The diet-induced obese experimental model developed characteristics of ORG; morphology and histology revealed glomerulomegaly as an early diagnostic marker as it was the first pathological change and indicated further renal damage. Liquid chromatography-tandem mass spectrometry detected 30 oxylipins. Higher dietary ALA resulted in greater n-3 oxylipin levels and resulted in reduced progression of glomerulomegaly and glomerular damage. To conclude, ORG may be diagnosed earlier with glomerulomegaly and treated with dietary oils rich in ALA which alter the oxylipin profile.

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V. LIST OF ABREVIATIONS

Adiponectin knockout (APN-KO), AIN (American Institute of Nutrition), α-linolenic acid (ALA), arachidonic acid (ARA), body mass index (BMI), butylated hydroxytoluene (BHT), collision induced dissociation (CID), cyclic adenosine monophosphate (cAMP), cyclooxygenase (COX), diet-induced obese (DIO), dihomo-gamma-linolenic acid (DGLA), dihydroxydocosapentanoic acid (DiHDPA), dihydroxyeicosatrienoic acid (DHET), dihydroxyoctadecenoic acid (DiHOME), docosahexanoic acid (DHA), end stage renal disease (ESRD), eicosapentanoic acid (EPA), epoxyeicosatrienoic acid (EET), focal segmental glomerulosclerosis (FSGS), high fat (HF), high performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS), cytochrome P450 2c23 (CYP2c23), hydroxydocosahexanoic acid (HDoHE), hydroxyeicosapentanoic acid (HEPE), hydroxyeicosatetraenoic acid (HETE), hydroxyeicosatrienoic acid (HETrE), hydroxyheptadecatrienoic acid (HHTrE), hydroxyoctadecadienoic acid (HODE), interleukin (IL), linoleic acid (LA), lipoxygenase (LOX), low fat (LF), matrix metalloproteinase (MMP), obesity-related glomerulopathy (ORG), oxooctadecadienoic acid (OXoODE), prostaglandin (PG), saturated fatty acids (SFA), soluble epoxide hydrolase (sEH), thromboxane (TX), hydroxyoctadecatrienoic acid (HOTrE), transforming growth factor-ß (TGF-ß), tumor necrosis factor (TNF)

VI. CHAPTER 1 - LITERATURE REVIEW

Introduction

Obesity is a global epidemic in which chronic inflammation, oxidative stress, and vascular resistance are implicated in the development of chronic disease. For example, a condition referred to as obesity-related glomerulopathy (ORG) is newly emerging in obese individuals where oxidative stress and increased hydrostatic pressure within the glomerulus is implicated in its onset. Due to its recent discovery, established guidelines have yet to be created for diagnosis and treatment of patients with ORG. Oxylipins, metabolites of fatty acids, alter renal hemodynamics and inflammation and therefore offer an area for potential investigation in renal disease. It is unknown how levels of α-linolenic acid (ALA), linoleic acid (LA), and dietary n-6:n-3 fatty acid ratios influence the renal oxylipin profile or the production of the novel ALA-derived hydroxyoctadecatrienoic acids (HOTrEs) and LA-derived hydroxyoctadecadienoic acids (HODEs) in a state of obesity; the use of dietary fatty acids to combat the onset and progression of ORG may provide a treatment option for obese individuals. Thus an opportunity exists for therapeutic investigation.

Obesity

The prevalence of obesity (body mass index (BMI) $\geq$ 30 kg/m^2) continues to climb; 17% of Canadian adults were classified as obese in 2008, which was an increase from 15% in 2003. Additionally, 34% of Canadian adults were deemed overweight or pre-obese (BMI $\geq$ 25 kg/m^2) in 2008 (Statistics Canada, 2010).

Obesity rates have risen over the last several decades for a multitude of reasons that include both nutritional and physical activity influences. The transition from hunting

and gathering to wide access to a variety of foods in the grocery store and fast food chains is called the nutrition transition; this transition has allowed North Americans to consume an extensive variety of foods, including unhealthy options high in fat and sugar, that were not accessible in the past (Popkin & Gordon-Larsen, 2004). Approximately one quarter of Canadians consumed something from a fast food outlet the day prior according to the Canadian Community Health Survey (2006). The current intake from fat is 31% of calories among Canadians; although more than a quarter of individuals aged 31-50 years consume over the recommended 35% of calories from fat per day (Canadian Community Health Survey, 2006). Physical activity levels have decreased as a result of transitioning from farming to a service sector industry and advanced modes of transportation that require little energy expenditure. The nutrition transition has resulted in an increased consumption of calories and decreased energy expenditure, which has led us to the current obesity epidemic (Popkin & Gordon-Larsen, 2004).

The obesity epidemic is of significant concern because of its association with the metabolic syndrome and chronic disease. The metabolic syndrome is a predominant condition among North Americans and is characterized by hypertension, increased blood triglycerides, decreased high density lipoprotein cholesterol, large waist circumference, and insulin resistance (Davy & Melby, 2003). Men and women with waist circumferences greater than 102 cm and 88 cm, respectively, are at high risk for type 2 diabetes, hypertension, cardiovascular disease, and dyslipidemia (National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III), 2002). Additionally, obesity is linked to a decreased life expectancy; the Framingham Cohort study observed a 7.1 year and 5.8 year decrease in the life span of obese female and male non-smokers respectively (Peeters

et al., 2003). More specifically in relation to renal disease, waist to hip ratio is an independent risk factor for chronic kidney disease (Elsayed et al., 2008). Therefore, it is evidently crucial to study preventive and treatment approaches to lower the prevalence of obesity and the associated chronic diseases.

Obesity & Renal Health

Obesity has a significant impact on renal health and function. In 2001, 406,801 Americans were living with End Stage Renal Disease (ESRD) and the prevalence is expected to climb; by 2030 it is estimated that the United States will have 2.2 million ESRD patients (Collins et al., 2003). This rise in ESRD is in part due to the prevalence of obesity and perhaps the rise of ORG; between the years of 1986 and 2000, the incidence of biopsy for ORG increased by ten-fold (Kambham, Markowitz, Valeri, Lin, & D'Agati, 2001). ORG has potential to lead to renal insufficiency and failure; once ORG has progressed to glomerular damage, the renal survival percentage is 77 and 51% at 5 and 10 years (Praga et al., 2001).

ORG is characterized by an indolent course of onset, proteinuria, glomerulomegaly with or without focal segmental glomerulosclerosis, and milder foot process fusion which suggests a different mechanism of podocyte injury compared to idiopathic focal segmental glomerulosclerosis. In obese patients, hyperfiltration and low grade chronic inflammation are prevalent, and both contribute to renal injury and reduced function (Kambham et al., 2001).

ORG patients often experience insulin resistance (Chen et al., 2011) but do not display characteristics of diabetic nephropathy which is quite distinct from ORG. Even despite an obese individual living with diabetes, they may not present with diabetic

nephropathy and their renopathophysiology may be characteristic of ORG and require further clinical investigation (Sellars, Blydt-Hansen, Dean, Gibson, Birk, & Ogborn, 2009). Insulin resistance itself can further exacerbate renal injury and contribute to the onset of ORG. Hyperglycemia can result in an increase of reactive oxygen species (ROS) which can further damage the renal system. A proposed mechanism of action is through mitochondrial production of ROS in response to chronic elevated blood glucose levels (Forbes, Coughlan, & Cooper, 2008). TGF-ß is elevated in the kidney in the presence of diabetes and proposed to be a major mediator of diabetic renal injury. Neutralization of TGF-ß in diabetic rats can prevent renal hypertrophy, mesangial matrix expansion, and renal insufficiency (Ziyadeh, 2004). Therefore, chronic insulin resistance and hyperglycemia may result in chronic renal inflammation which perhaps can result in renal hypertrophy in ORG.

The onset of ORG is associated with increased hydrostatic pressure within the glomerulus and the associated increase in albuminuria which may be due to hypertension and increased renal plasma flow (Chagnac, Weinstein, Herman, Hirsh, Gafter, & Ori, 2003; Ribstein, du Cailar, & Mimran, 1995). Elevated hydrostatic pressure can result in increased glomerular volume, mesangial matrix expansion, stretching of the glomerular elements, and eventually glomerulosclerosis (Fogo & Ichikawa, 1991).

Glomerular inflammation and oxidative stress is also associated with the onset of ORG (Wu, Liu, Xiang, Zeng, Chen, Ma, & Li, 2006b). Dysregulation of adipokines such as leptin and adiponectin are implicated in obesity and associated with chronic inflammation. For example, serum leptin concentrations are positively correlated to body fat percentage, higher levels are observed in the obese and type 2 diabetic population

(Considine et al., 1996; Wolf et al., 1999), and renal leptin receptors are elevated in patients with ORG (Wu et al., 2006). Leptin is important to consider because it acts as a pro-fibrogenic factor on the renal system by upregulating collagen type IV production, transforming growth factor ß-1 (TGFß-1), and pro-inflammatory T-helper 1 immune responses (Tang, Yan, & Zhuang, 2012; Wolf et al., 1999; Wolf, Chen, Han, & Ziyadeh, 2002). Naïve rats infused with leptin develop glomerulosclerosis and proteinuria (Wolf, Hamann, Cheol, et al., 1999). Therefore, the obesity-associated elevation in leptin may further exacerbate glomerular injury.

Adiponectin is an anti-inflammatory adipocyte specific protein that is decreased in obese individuals (Weiss, et al., 2003). Low levels of adiponectin induce an inflammatory state as shown in an adiponectin knockout (APN-KO) mouse model. In comparison to the wild-type model, the APN-KO mice had significantly higher intraglomerular macrophage infiltration, monocyte chemotactic protein-1, tumor necrosis factor-, albumin excretion, glomerulomegaly, and tubulointerstitial fibrosis (Ohashi et al., 2007). As a result, the absence of normal levels of adiponectin as seen in obesity may be causally related to chronic inflammation and the development of glomerulomegaly and glomerular damage in ORG.

Animal Model of ORG

Despite the prevalence and severity of ORG, an accurate animal model has not been established in order to further the knowledge on the pathophysiology or potential treatments. The establishment of an accurate animal model can aid in the investigation of an earlier biomarker in order to facilitate earlier diagnosis and commencement of treatment to create the best survival outcomes for patients. Current models may not mimic

the development of obesity or progression of ORG accurately. For example, the importance of leptin in ORG's progression was discussed earlier and therefore leptin should be present in the animal model. However, models of obesity such as the *ob/ob* mice are leptin deficient (Kumpers et al., 2007), *db/db* mice are leptin receptor deficient (Kumpers et al., 2007) and Zucker *fa/fa* rats have a leptin receptor defect (Phillips et al., 1996). In addition, the Zucker *fa/fa* rat and *db/db* mouse may develop ORG but also potential confounding conditions such as progressive tubulopathy, a common finding in patients with type 2 diabetes mellitus (Hayden & Sowers, 2011).

In contrast, the diet-induced obese (DIO) rat model mimics the onset of human obesity rather than developing obesity through a genetic mutation uncharacteristic of humans. The DIO animal model develops visceral obesity, hyperleptinemia, hyperinsulinemia, and dyslipidema (Madsen et al., 2010) which are similar to that of humans with ORG (Chen et al., 2011) (Wu et al., 2006). Even though it is well accepted that the DIO model mimics human obesity, the investigation of whether or not the DIO model can replicate ORG is unknown.

Clinical Diagnosis of ORG

Despite the increasing prevalence of ORG, recent studies have only reported clinical characteristics but diagnostic guidelines have yet to be established and approved. In addition, an early marker that can be reproducibly associated with an endpoint such as glomerular damage or renal failure is needed in order to facilitate early diagnosis and treatment. ORG is unique from other renal diseases as it progresses with a more indolent course and can appear as nephrotic range proteinuria without histological damage (Kambham et al., 2001) (Wesson, Kurtzman, & Frommer, 1985). Glomerulomegaly

(enlargement of the glomeruli) presents in patients with ORG due to hyperfiltration (Chagnac, et al., 2003; Ribstein, et al., 1995) and may act as a sufficient diagnostic marker in patients with renal biopsies. Glomerulomegaly has been implicated as the stage prior to glomerulosclerosis (Fogo, et al., 1991); however this is controversial and remains to be confirmed (Hughson, Hoy, Douglas-Denton, Zimanvi, & Bertram, 2011). The establishment of an animal model can facilitate the investigation of glomerulomegaly onset and its association to the further potential of renal injury. The identification of an early diagnostic marker can facilitate treatment investigations in the early stages of ORG where the potential to slow disease progression is superior in comparison to later stages. Potential treatment investigations for ORG can include dietary interventions with varying fatty acid compositions which have shown to influence renal injury in the later stages of other renal diseases. Table 1.1 below summarizes the findings of select studies on ORG.

Table 1.1– Summary of Select Studies on Obesity-Related Glomerulopathy

Subjects	**Methods**	**Results**
ORG Patients (n=71) (Kambham et al., 2001)	Biopsy analysis with morphometry and assessment of clinical markers such as proteinuria	ORG can present as glomerulomegaly alone or with focal segmental glomerulosclerosis (FSGS). 62% of patients had hypertension. Proteinuria was present in the non-nephrotic and nephrotic range. Mild tubular atrophy and interstitial fibrosis was occasionally present.
Obese adolescents (n=7) (Adelman, Restaino, Alon, & Blowey, 2001)	12 year follow-up on histological, morphological, serum, and urinary markers	Patients presented with obesity, mild hypertension, elevated 24 hour protein excretion, normal creatinine clearance, glomerulomegaly, some with FSGS, and increased mesangial matrix. After 12 years of follow-up, one patient developed ESRD, and one improved proteinuria levels with weight loss.
Obese Adults (n=8) Normal Weight Healthy Controls (n=9)	Weight loss intervention via gastroplasty Assessed	The obese individuals had GFR and RPF values 61% and 32% greater, respectively, than normal weight controls. Gastroplasty resulted in a

(Chagnac et al., 2003)	glomerular filtration rate (GFR), renal plasma flow (RPF), and albuminuria	decrease of BMI by ~32%. Weight loss resulted in a decrease of GFR, RPF, and albuminuria.
13-year old girl with ORG, severe proteinuria, and insulin resistance (case study) (Georgaki-Angelaki et al., 2010)	Weight loss and ACE inhibitors Renal biopsy, eGFR, and proteinuria assessment 2 year follow-up	1 year follow-up: -30% weight reduction -No proteinuria present 2 year follow-up: -38.5% weight loss total - mild proteinuria returned and an increase in glomerulosclerosis, glomerulomegaly, and mesangial matrix expansion were observed.
Obese patients with obesity-focal segmental glomerulosclerosis (n=15) Non-obese patients with idiopathic focal	Renal biopsies, proteinuria, and Kaplan-Meier renal survival assessment (duration; 82±57	ORG patients reached nephrotic range proteinuria at follow-up without nephrotic syndrome, and presented with glomerulomegaly and glomerulosclerosis, 46% patients experienced progressive renal

segmental glomerulosclerosis (n=15) (Praga et al., 2001)	months)	insufficiency and 5 began dialysis. Renal survival after 5 and 10 years were 77 and 51%, respectively. Risk of renal failure was positively correlated to serum creatinine and negatively associated to creatinine clearance.
ORG patients (n=6) Control patients (n=2) (Wu et al., 2006)	Gene expression profiles of glomeruli from renal biopsies	Lipid metabolism, inflammatory cytokine, and insulin resistance related genes were significantly different compared to controls. Inflammatory cytokines such as tumor necros factor (TNF)-, interleukin (IL)-6 signal transducer, and interferon-γ were increased in ORG glomeruli versus control.

Dietary ALA Rich Oils

Potential treatments for ORG may include dietary oils rich in the omega 3 fatty acid, ALA, such as canola and flaxseed oils. Canola oil has approximately 11% ALA, 21% LA, 59% oleic acid, and 3.8% palmitic acid (Ohara et al., 2008). Canola oil as a result has a 2:1 ratio of n-6 fatty acids to n-3s; a ratio of 2:1 is proposed to be optimal for conversion of ALA to eicosapentanoic acid (EPA) and docosahexanoic acid (DHA) (Davis & Kris-Etherton, 2003). Dietary n-6:n-3 ratios between 2-5:1 have reduced mortality risk and the severity of various chronic diseases such as cardiovascular disease, asthma, rheumatoid arthritis, and colorectal cancer (Simopoulos, 2002). However, specific dietary recommendations for obese individuals at risk for renal injury have yet to be investigated. Dietary recommendations may include fatty acid compositions that can be achieved with the addition of specific oils such as canola and flax into the diet. Researchers have shown the renal protective effects of canola oil in Sprague-Dawley rats with streptozotocin-induced diabetes. The rats were 10 weeks old upon commencement of dietary intervention which lasted for 30 weeks. The canola oil enriched diet reduced albuminuria and tubulointerstitial fibrosis in comparison to the corn oil diet (high n-6 diet) (Garman, Mulroney, Manigrasso, Flynn, & Maric, 2009).

Flaxseed oil contains 9% saturated fat, 18% monounsaturated fat, 57% ALA and 16% LA (Flax Council of Canada, 2012). A diet supplemented with 5% flax oil to a polycystic kidney disease model resulted in lower levels of serum creatinine, fibrosis, macrophage infiltration, and mean cystic change (Ogborn, Nitschmann, Bankovic-Calic, Weiler, & Aukema, 2002). Additional studies have shown similar protective effects of flax oil in other models of polycystic kidney disease (Sankaran, Lu, Bankovic-Calic,

Ogborn, & Aukema, 2004). It is unknown how dietary ALA influences glomerular hydrostatic pressure or glomerulomegaly. However, ALA may provide renoprotection through hindering inflammation through prevention of lipotoxicity and generation of indicators of endoplasmic reticulum stress as shown in renal proximal tubular cells (Katsoulieris, Mabley, Samai, Green, & Chatterjee, 2009). Table 1.2 summarizes the findings of select studies on dietary fatty acids and renal disease.

Other Dietary Oils

Canadians consume on average 31% of their energy from fat; however a substantial proportion of the population consumes beyond the recommended 35% of energy from fat. Approximately 16% of fat is consumed by Canadians from foods like pizza, sandwiches, hamburgers, and hot dogs; additionally 8.5% is consumed from baked goods such as doughnuts and cookies. Therefore, approximately 24.5% of a Canadian's fat intake tends to come from unhealthy food choices which are very high in saturated fatty acids (SFA) and low in n-3 fatty acids (Canadian Community Health Survey: Overview of Canadians' eating habits, 2006).

High n-6 fatty acid intake is associated with the production of oxylipins from arachidonic acid which overall have a higher potential for inflammation and vasoconstriction than the n-3 derived oxylipins (Calder, 2006b). Therefore, consumption of dietary oils rich in n-6 fatty acids such as safflower, corn, and soybean oil may exacerbate renal injury and reduce function through the influx of pro-inflammatory and vasoconstrictive metabolites. Specifically in relation to factors that are associated with the onset of ORG, high intakes of n-6 fatty acids have been associated with increased glomerular capillary pressure and enlargement (Brown, et al., 2000). The role of oxylipins in renal health is discussed in more detail below (Calder, 2006b).

Table 1.2 – Summary of the Effects of Dietary Fatty Acids on Renal Health

Animal Model/Subjects	Dietary Intervention	Results
Sprague Dawley rats (Wang et al., 2003)	High Fat (36% fat) Control (4.4% fat)	Renal ω-hydroxylase & epoxygenase were down-regulated in the cortex, medulla, & papilla but not in renal microvessels of high fat (HF) rats
Han:SPRD-cy heterozygotes (PKD) (Ogborn et al., 2002)	Flax Oil (5%) Corn Oil (5%)	Flax oil consumption resulted in lower serum creatinine, fibrosis, and macrophage infiltration compared to corn oil diet
Humans with lupus nephritis (n= 8) (Clark et al., 1995)	15, 30, and 45 g flaxseed/every day	30 g dose of flaxseed increased serum ALA and decreased serum creatinine.
11/12 nephrectomy in dogs (n=6/group). (Brown, et al. 2000)	Menhaden fish oil, safflower oil, or beef tallow supplemented diet	Safflower group had significantly greater glomerular capillary pressure and glomerular enlargement than the beef tallow group.
Hemodialysis patients (n=145) (Noori et al., 2011)	3 day food record, C-reactive protein (CRP), and 6-year survival rates	For every 1 unit increase in dietary n-6:n-3, CRP increased by 0.55 mg/L on average. Lower n-6:n-3 ratios had better outcomes for inflammation change scores and survival than those with higher n-6:n-3 ratios.
Humans (n=19,256) (Lin et al., 2010)	Cross sectional analysis. SFA intake & albuminuria	~13% of energy from SFA (35 g/day) had a 69% chance of developing high albuminuria levels

Arachidonic Acid Metabolism to Oxylipins and Effects on Renal Health

Oxylipins are metabolites of fatty acids which include octadecanoids, eicosanoids, and docosanoids which are strongly involved in renal hemodynamics and injury. The n-6 fatty acid, linoleic acid, is converted to arachidonic acid (ARA) in the body and overall the metabolites of ARA have higher pro-inflammatory potential than oxylipins produced from n-3 fatty acids. ARA can be metabolized via three enzymatic pathways: cyclo-oxygenase (COX), cytochrome P450 (CYP450), and lipoxygenase (LOX). Some of these oxylipins are protective of the kidney and some are detrimental (Imig, 2006). Below is a brief description of the ARA oxylipin pathway and associated effects on renal health. Figure 1.1 summarizes the following description below, based on the in-text references, in a diagram and depicts the metabolism of several oxylipins from n-6 fatty acids.

COX produces 2 series eicosanoids from prostaglandin (PG) H_2 which is further metabolized to PGI_2, D_2, $F_{2\alpha}$, E_2, and thromboxane (TX) A_2. PGI_2 performs as a vasodilatory oxylipin by influencing adenylate cyclise activity and therefore perhaps cyclic adenosine monophosphate (cAMP) levels (Chaudhari, Gupta, Kirschenbaium, 1990). As a result, PGI_2 may promote homestatic renal blood flow; on the contrary, prostaglandin $F_{2\alpha}$ can constrict the renal arteries and therefore have a negative impact on renal hemodynamics (Goldberg & Toda, 1975). PGE_2 can act either as a vascoconstrictor or vasodilator depending which receptor it binds to. Activation of EP1 and EP3 (PGE_2 receptors) causes vasconstriction whereas activation of EP2 and EP4 results in vasodilation (Audoly et al., 2001; Tang, Loutzenhiser, & Loutzenhiser, 2000; Imig, Breyer, & Breyer, 2002). PGE_2 may facilitate its influence on renal hemodynamics by altering the levels of cyclic adenosine monophosphate (cAMP) (Tang et al. 2000). TXA_2

has been implicated as a powerful renal vasoconstrictor (Golino et al., 1989) and is associated with renal vascular resistance and damage (Uriu, Kaizu, Hashimoto, Komine & Etoh, 1994). PGD_2 synthase has been positively associated with the presence of renal failure (Melegos, Grass, Pierratos, Diamandis, 1999). Overall, the majority of the metabolites produced from ARA by COX have the potential to be vasoconstrictive and therefore perhaps exacerbate glomerular capillary pressure and glomerulomegaly.

The second enzymatic pathway is CYP450 which produces epoxyeicosatrienoic acids, hydroxyeicosatetraenoic acids, and dihydroxyeicosatrienoic acids (EETs, HETEs, and DHETs, respectively). CYP4A produces primarily 20-HETE (Bell et al. 1993) which has demonstrated the ability to constrict the afferent arteriole in the presence of increased arteriole tone (Arima, Omata, Ito, Tsunoda, Abe, 1996); this may result in altered renal hemodynamics, however, this has yet to be evidenced. Nitric oxide (NO) can impair the formation of 20-HETE through binding to CYP4A which contributes to NO's vasodilatory role (Alonso-Galicia, Drummond, Reddy, Falck, & Roman, 1997). As a result, 20-HETE may have detrimental effects on hemodynamics (reviewed by Roman (2002)). EET's produced by CYP2C (Imig, 2006) act against the HETE's detrimental effects because they are anti-inflammatory and pro-fibrinolytic vasodilators in renal afferent arterioles as shown in hypertensive rats (Zhao et al., 2004). DHETs are produced from EETs by the enzyme soluble epoxide hydrolase (sEH). Conversion of EETs to DHETs can cause a decrease in the biological activity of the metabolite and result in a decreased beneficial effect on the kidney in comparison to EETs (Imig, Navar, Roman, Reddy, Falck, 1996).

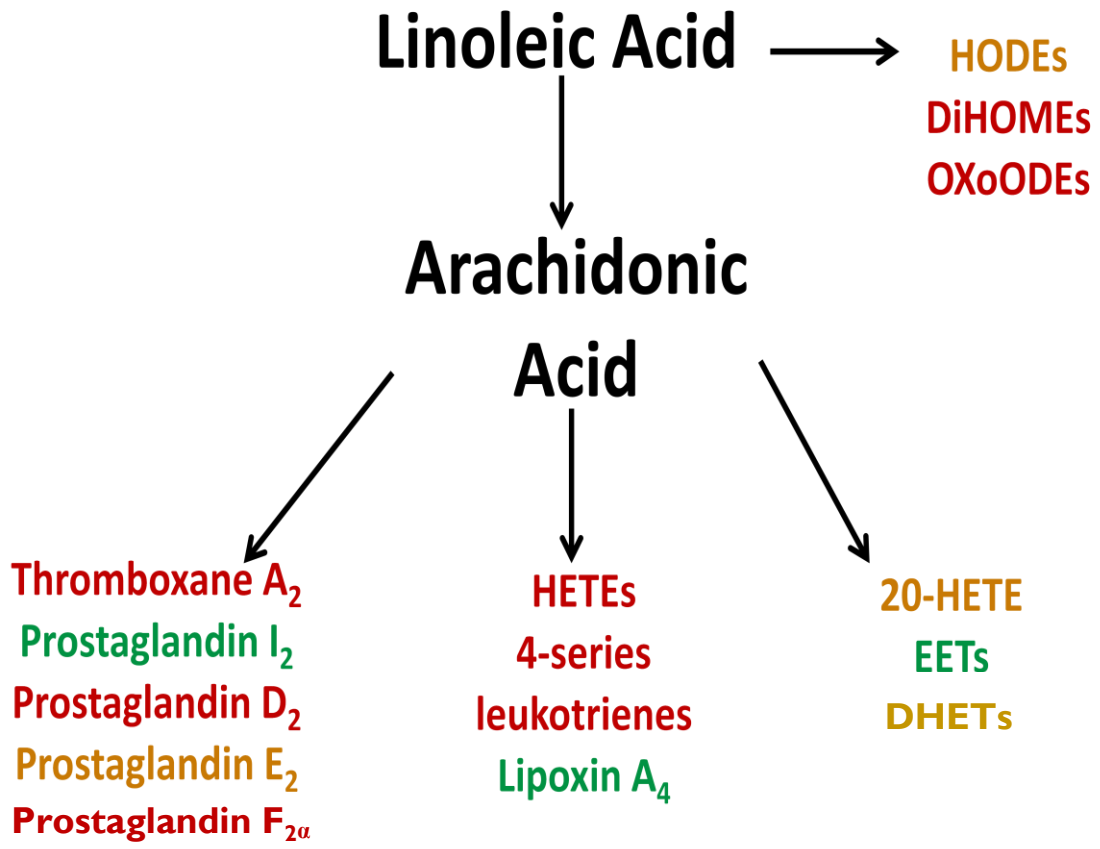

Figure 1.1: Metabolism of Fatty Acids into N-6 Oxylipins. Green text indicates oxylipins which promote vasodilation or have anti-inflammatory properties. Red text indicates oxylipins that are vasoconstrictive or pro-inflammatory. Orange text indicates oxylipins that have a bi-directional role and display both beneficial and detrimental effects. Abbreviations used: Epoxyeicosatrienoic acids (EETs), dihydroxyeicosatrienoic acid (DHETs), hydroxyoctadecadienoic acid (HODEs), dihydroxyoctadecenoic acid (DiHOMEs), oxooctadecadienoic acid (OXoODEs).

The third enzymatic pathway is LOX which metabolizes arachidonic acid (ARA) into HETEs, 4-series leukotrienes (LTs) and lipoxins (LXs) (Imig, 2006). The oxylipins produced from ARA by LOX may be beneficial or detrimental to health. LTA_4, 12-HETE, and 15-HETE display afferent arteriole vasoconstrictive properties ((Imig, 2006; Yiu, Zhao, Inscho, & Imig, 2003) and leukotrienes have been implicated in post-ischemic renal injury and reduced renal blood flow (Klausner, et al., 1989). In contrast, lipoxins display anti-inflammatory characteristics by inhibiting monocyte recruitment and promoting inflammation resolution (Petasis et al., 2005).

LOX can also metabolize the n-6 fatty acid, linoleic acid, into hydroxyoctadecadienoic acids (HODEs). HODEs have been implicated as both detrimental and beneficial oxylipins. They have been used as markers of oxidative stress (Horie et al., 2012; Morita et al., 2012; D. Wang et al., 2008), are implicated in atherosclerosis progression (Ku, Thomas, Akeson, & Jackson, 1992), and are positively associated with the progression of Alzheimer's disease (Yoshida et al., 2009). In contrast, 13-HODE can decrease tumor cell adhesion and lung colonization by high metastatic cells (Honn et al., 1992) and may prevent epidermal hyperproliferation (Ziboh, Miller, & Cho, 2000). Yet, the influence of HODEs specific to the kidney has yet to be investigated. Table 1.3 below summarizes the current knowledge on the physiological influence of n-6 derived oxylipins.

Table 1.3 – Summary of the Physiological Influence of n-6 Oxylipins

N-6 oxylipin	Physiological Influence
Linoleic Acid Derived Oxylipins	
9-HODE	Possesses ability to decrease tumor cell adhesion in B16 amelanotic melanoma (B16a) cells (Honn et al., 1992); marker of oxidative stress (Horie et al., 2012); positively associated with Alzheimer's disease (Yoshida et al., 2009).
13-HODE	Marker of oxidative stress (Horie et al., 2012); positively associated with Alzheimer's disease (Yoshida et al., 2009). May modulate protein kinase C and suppress epidermal hyperproliferation and influence differentiation (Ziboh, Miller, & Cho, 2000).
9,10-DiHOME	Metabolized from the epoxygenase product of linoleic acid, EpOME by sEH (Greene, Williamson, Newman, Morisseau, Hammock, 2000). Induces chemotaxis (Totani et al., 2000) and is cytotoxic to renal proximal tubular cells (Moran, Weise,

	Schnellmann, Freeman, & Grant, 1997).
12,13-DiHOME	Metabolized from the epoxygenase product of linoleic acid, EpOME by sEH (Greene, Williamson, Newman, Morisseau, Hammock, 2000). Induces chemotaxis (Totani et al., 2000) and is cytotoxic to renal proximal tubular cells (Moran, Weise, Schnellmann, Freeman, & Grant, 1997).
9-OXoODE	Produced from the lipoxygenase product of linoleic acid, 9-HODE. Marker of lipid oxidation (Feldstein et al., 2010).
13-OXoODE	Produced from the lipoxygenase product of linoleic acid, 13-HODE. Marker of lipid oxidation (Feldstein et al., 2010).
Dihomo-Gamma-Linolenic Acid Derived Oxylipins	
15-HETrE	Produced by LOX and can inhibit 5-LOX activity at 4.6 μM in human polymorphonuclear neutrophils (Petrich, Ludwig, Kühn, & Schewe, 1996).
Arachidonic Acid Derived Oxylipins	

5,6-DHET	Mild vasodilator produced from 5,6-EET by sEH with a significant loss of biological activity (Imig, Navar, Roman, Reddy, Falck, 1996).
11,12-DHET	Mild vasodilator produced from 11,12-EET by sEH with a significant loss of biological activity (Imig, Navar, Roman, Reddy, Falck, 1996).
14,15-DHET	Mild vasodilator produced from 14,15-EET by sEH with a significant loss of biological activity (Imig, Navar, Roman, Reddy, Falck, 1996).
5-HETE	Metabolized via LOX (Roman, 2002). Stimulates the pro-inflammatory response by aggregating human neutrophils (O'Flaherty, Thomas, Lees, McCall, 1981). Is the most chemotactic of the HETEs (Goetzl, Brash, Tauber, Oates, Hubbard, 1980).
8-HETE	Metabolized via LOX (Roman, 2002). Levels increase in the skin of mice under

	tumor promoting conditions (Gschwendt et al., 1986).
9-HETE	Metabolized via CYP450 (Roman, 2002). Possesses chemotactic properties in human neutrophils similar to the same capacity of 8-HETE and greater than 11-HETE (Goetzl, et al., 1980).
11-HETE	Metabolized via CYP450 (Roman, 2002). Possesses chemotactic properties in human neutrophils similar to the same capacity of 12-HETE (Goetzl, et al., 1980).
12-HETE	Metabolized via LOX (Roman, 2002). Stimulates the pro-inflammatory response by aggregating human neutrophils (O'Flaherty, Thomas, Lees, McCall, 1981). Vasoconstrictor of renal arcuate arteries (Ma, Harder, Clark, & Roman, 1991).
15-HETE	Metabolized via LOX (Roman, 2002). Possesses anti-inflammatory characteristics and can prevent formation of LTB_4 and polymorphonuclear infiltration in rat glomeruli (Fischer, Christman, & Badr,

	1992).
16-HETE	Metabolized via CYP450 (Roman, 2002). Vasodilatory characteristics as seen in the renal arteries of rabbits (Carroll, Balazy, Margiotta, Huang, Falck, & McGiff, 1996).
15d PGD_2	An inflammatory response was created after ending a course of statins and a decrease in 15d PGD_2 was observed (Zahuranec & Majersik, 2006).
PGD_2	Metabolized via COX and possesses anti-neoplastic properties in mouse L1210 leukemia cells (Fukushima, Kato, Ueda, Ota, Narumiyat, & Hayaishif, 1982). Stimulates the innate immune response and inflammation (Serhan & Petastasis, 2011) and its synthase is positively associated with failure failure (Melegos et al., 1999).
PGE_2	Metabolized via COX (Roman, 2002). Promotes natriuresis (Guan et al., 1998) but can increase renal vascular resistance in the renal arteries of rats (Baer & McGiff, 1979)

6-keto $PGF_{1\alpha}$	Synthesized from and is a marker of PGI_2. PGI_2 can reduce renal vascular resistance in the renal arteries of rats (Baer, et al., 1979). Significantly higher levels are observed in newborns than adults and decreases after the first week of life (Scherer, Fischer, Siess, & Weber, 1982).
$PGF_{2\alpha}$	Metabolized via COX (Roman, 2002). Possesses vasoconstrictive properties in bovine, canine, and human coronary arteries (Kulkarni, Roberts, & Needleman, 1976).
TXB2	Metabolized from and is a marker of TXA_2 (Patrono et al., 1983) which is a renal vasoconstrictor (Ogletree, 1987).
12-HHTrE	Metabolized via COX (Roman, 2002). 12-HHTrE is easily influence (decreased) by albumin in human platelet suspensions (Broekman, Eiroa, Marcus, 1989).

Abbreviations: hydroxyheptadecatrienoic acid (HHTrE), hydroxyeicosatrienoic acid (HETrE)

ARA derived oxylipins have a significant influence on renal hemodynamics and can be bi-directional and either ameliorate or exacerbate renal disease. The balance of oxylipin production is crucial for the maintenance of renal health. Unfortunately, obesity may change the oxylipin profile and produce damaging results.

The Effect of Obesity on Oxylipin Producing Enzymes

Obesity may alter the protein levels of enzymes responsible for producing oxylipins and therefore potentially alter oxylipin profiles and may lead to deleterious effects. For example, obese Zucker rats have elevated protein levels of CYP4A and COX-2 in addition to, decreased CYP2C11 and CYP2C23 in the renal microvasculature when compared to lean Zucker rats. The obese rats also had elevated urinary albumin levels indicating glomerular damage (Dey et al., 2004).

Because COX-2 produces some vasoconstrictive oxylipins (PGE_2 (Baer et al., 1979), $PGF_{2\alpha}$ (Kulkarni, et al., 1976), TXB_2 (Ogletree, 1987)) and CYP4A primarily produces 20-HETE which is vasoconstrictive (Arima, et al., 1996), increased protein expression of CYP4A and COX-2 may result in greater quantities of vasoconstrictive oxylipins and result in detrimental effects on renal hemodynamics in obesity. Increased protein expression of COX-2 has also been associated with progressive polycystic renal disease (Wakefield, Ogborn, Ibrahim, & Aukema, 2011; Sankaran, Bankovic-Calic, Ogborn, Crow, & Aukema, 2007). In addition, CYP2C enzymes produce the EETs which have potential for vasodilation (Zhao, et al., 2004); therefore a decrease in CYP2C enzymes may result in a reduction of EETs and the ability to dilate renal vasculature and therefore may further progress constricted renal blood flow.

Additionally, provision of a high fat diet to Sprague-Dawley rats resulted in significantly lower omega-hydroxylase and epoxygenase activity in the cortex, medulla,

and papilla of the kidneys. The rate of formation of 20-HETE and EETs were positively correlated with the reduced expression of CYP4A and CYP2C23. Rats provided a high fat diet also had significantly elevated systolic blood pressure, body fat, and body weight (Wang et al., 2003). Since 20-HETE and EETs play a significant role in natriuresis and blood pressure (Imig et al., 2005), it can be concluded that increased and decreased levels of 20-HETE and EETs can modulate blood pressure and therefore renal hemodynamics and health.

The enzymes that metabolize fatty acids into oxylipins can either increase or decrease in obesity, renal disease, or with the provision of a high fat diet which may have detrimental effects on health. However, it is unknown if varying levels of ALA and LA with a high fat diet can alter the protein levels of oxylipin enzymes. Research into the effect of dietary ALA and LA on enzymes will provide information on how to promote homeostatic renal hemodynamics. N-3 fatty acids may offer a way to balance oxylipin levels in favour of maintaining renal health.

N-3 Fatty Acid Metabolism to Oxylipins and the Effect on Renal Health

Dietary n-3 fatty acids are incorporated into membrane phospholipids as are n-6 fatty acids. Greater consumption of dietary ALA can result in higher levels of ALA and longer chain derivatives in the kidney. For example, ALA and docosahexanoic acid (DHA) were higher in the renal tissue of normal rats and rats with polycystic kidney disease fed only ALA as the source of n-3 fatty acids (flax oil) in comparison to those provided corn oil diets (Ogborn, et al., 2002).

Phospholipases cleave fatty acids from the membranes subsequently metabolized into oxylipins. Therefore, if there is a greater consumption of n-3 fatty acids, there may be

a greater proportion of n-3 fatty acids in the phospholipids, and a larger quantity of n-3 oxylipins. This is evidenced by humans who consumed 4 g/day of n-3 ethyl ester fatty acids and increased their omega 3 oxylipin levels by 2-5 times and reduced serum n-6 oxylipin levels by 20% (Shearer, Harris, Pedersen, & Newman, 2010). Figure 1.2 below summarizes the following descriptions and depicts the production of several n-3 derived oxylipins

N-3 fatty acids ALA, DHA, and eicosapentanoic acid (EPA) may compete with ARA for metabolism to oxylipins as they employ the same enzymes. Therefore, n-3 fatty acids may be able to inhibit the synthesis of high potential pro-inflammatory n-6 derived oxylipins. This can be evidenced by the reduction of PGE_2, TXB_2, or HETEs with n-3 supplementation through fish or flax supplementation in the presence of induced inflammation in mononuculear cells of humans or colon cells of mice (Trebble et al., 2003; Caughey, Mantzioris, Gibson, Cleland, & James, 1996; Neilson et al., 2012).

COX and LOX enzymes produce low pro-inflammatory potential oxylipins from EPA; COX metabolizes EPA to 3 series prostaglandins and 5-LOX produces 5 series leukotrienes (Calder, 2010). The three series produced from n-3 fatty acids are non-mitogenic whereas the two series prostaglandins from ARA are. Furthermore, PGE_3 for example is much less inflammatory than PGE_2 because PGE_3 induces COX-2 and interleukin-6 (IL-6) to a much lesser degree (Bagga, Wang, Farias-Eisner, Glaspy, & Reddy, 2003). As a result, oxylipins derived from n-3 fatty acids have less inflammatory potential and therefore may reduce inflammation in the body by competing with n-6 fatty acids for metabolism. However, it is unknown how 3-series prostaglandins and 5-series leukotrienes influence glomerulomegaly or renal hemodyanimcs.

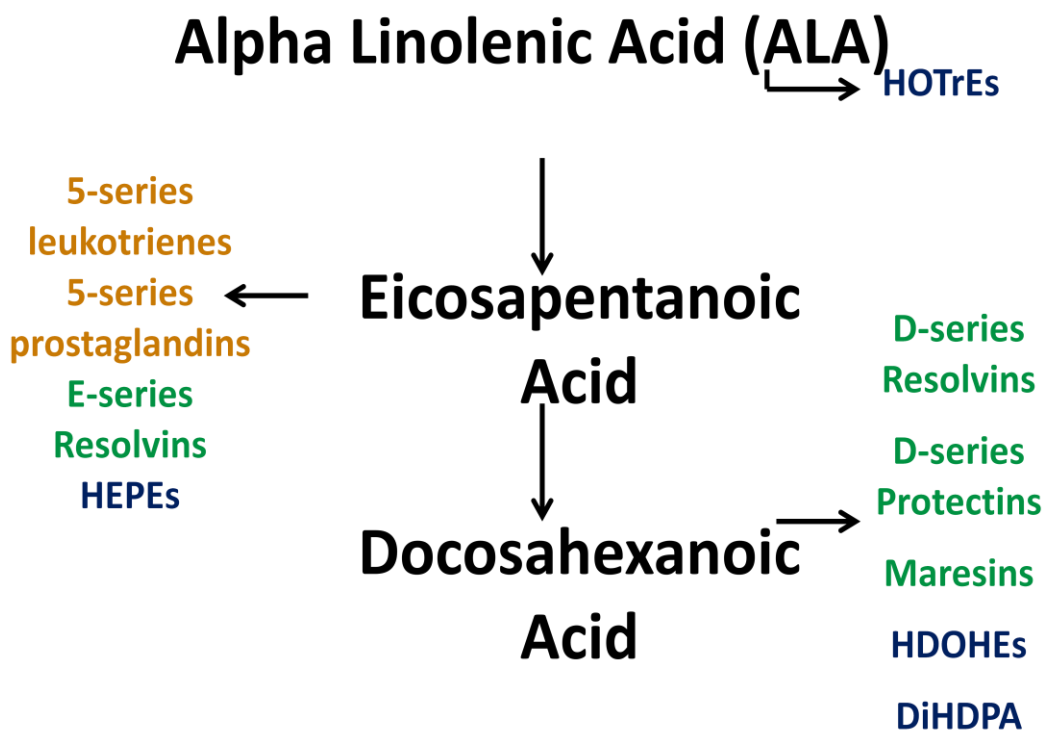

Figure 1.2: Metabolism of Fatty Acids into N-3 Oxylipins. Green text indicates oxylipins which promote vasodilation or have anti-inflammatory properties within the kidney. Red text indicates oxylipins that are vasoconstrictive or pro-inflammatory. Orange text indicates oxylipins that have a bi-directional role and display both beneficial and detrimental effects. Blue text indicates that the influence of the oxylipin on the kidney is unknown. Abbreviations: hydroxyoctadecatrienoic acid (HOTrEs), hydroxyeicosapentanoic acid (HEPEs), hydroxydocosahexanoic acid (HDoHEs), dihydroxydocosapentanoic acid (DiHDPA)

COX and LOX also produce E-series resolvins from EPA and D-series resolvins, protectins, and maresins from DHA which have anti-inflammatory potential and are inflammation resolving (Serhan & Petasis, 2011; Serhan et al., 2011). Oxylipins derived from DHA and EPA may be able to shift the oxylipin balance to maintain renal health and function in a state of inflammation.

Conversion of ALA to EPA and DHA in animal models is efficient in tissues such as the kidney (Ogborn, et al. 2002). Therefore, the production of EPA and DHA oxylipins should be seen in animals fed ALA. Even though benefits of EPA and DHA oxylipins have been researched, the formation of oxylipins from ALA has yet to be investigated.

LOX enzymes can oxidize ALA to produce hydroxyoctadecatrienoic acids (HOTrEs) (Belkner, Wiesner, Kuhn, & Lankin, 1991). HOTrEs have exhibited anti-inflammatory characteristics *in vitro.* Schulze-Tanzil et al. reported that 13-HOTrE suppressed the expression of IL-1ß release from matrix metalloproteinases (MMPs) -1, -3, and -9 in human chondrocytes *in vitro* (Schulze-Tanzil et al., 2002). The suppression of MMPs may be essential in ORG because dysregulation (increase and decrease) of MMPs have been implicated in the progression of diabetic nephropathy by influencing mesangial matrix expansion and basement membrane thickening (Thrailkill, Clay Bunn, & Fowlkes, 2009) (Lelongt, Legallicier, Piedagnel, & Ronco, 2001) and may therefore influence renal pathology in ORG.

Despite the importance of n-6 and n-3 oxylipins on inflammation and hemodynamics, it is unknown how varying dietary LA and ALA levels and ratios influence the oxylipin profile. The ability to alter the renal oxylipin profile to be more favourable in terms of reducing inflammation, vasoconstriction, and therefore renal injury

may offer a potential treatment route. Table 1.4 below summarizes the current literature on the physiological influence of n-3 oxylipins detected in the current study. Table 1.5 below summarizes the influence of n-3 supplementation on oxylipin levels.

Conclusion

Obesity has a significant influence on renal health and may lead to ORG and subsequently ESRD. Examination of a potential animal model will facilitate research on the renopathophysiology in order to improve the current foundation of knowledge on the causes of ORG and guidelines for diagnosis. An animal model can also facilitate the investigation of potential treatments such as through dietary modification in order to prevent renal injury. Dietary oils rich in ALA have provided renoprotection in models of other renal diseases but have yet to be investigated for the influence on ORG. In addition, the effect of varying levels of ALA and LA on renal pathology has yet to be examined. In addition, oxylipins are known to significantly influence renal hemodynamics and inflammation and therefore renal disease progression. Due to past methodological limitations, only a few oxylipins were detected at a time in dietary interventions or in the presence of renal disease. Fortunately, due to advances in technology, many oxylipins can be detected at one time; yet analysis of several renal oxylipins has yet to be performed. It is unknown how varying dietary ALA and LA levels influence oxylipins or their relationship to renal pathology in obesity. The thesis herein will provide information on all the gaps in knowledge mentioned here in relation to ORG, dietary treatments, and the renal oxylipin profile.

Table 1.4 – Summary of N-3 Oxlipins and Physiological Influences

N-3 oxylipin	Physiological Influence
	ALA Derived Oxylipins
9-HOTrE	9-HOTrE is produced by LOX (Galliard & Phillips, 1971) and serum levels are unaffected by long chain n-3 ethyl ester supplementation in humans (Keenan, et al., 2012).
13-HOTrE	13-HOTrE is produced by LOX (Collins et al., 2008) and serum levels are unaffected by long chain n-3 ethyl ester supplementation in humans (Keenan, et al., 2012). HOX-α and 13-HOTrE can suppress IL-1ß expression of MMPs in human chondrocytes (Schulze-Tanzil, et al. 2002)
	EPA Derived Oxylipins
5-HEPE	A metabolite of 5-HEPE, 5-OXO-EPE, is 10 times less active than 5-OXO-ETE for the recruitment of human neutrophils and eosinophils (Powell, Gravel, Gravelle, 1995). May be beneficial in skin inflammatory conditions such as psoriasis (Grimminger et al., 1993).
18-HEPE	18-HEPE is a pre-cursor to the E-series resolvins. Levels are increased in the presence of aspirin in human leukocytes. The 18-E-series resolvins can reduce neutrophil infiltration and stimulate phagocytosis in acute murine peritonitis (Oh, Pillai, Recchiuti, Yang, & Serhan,

2011).

DHA Derived Oxylipins	
19,20 DiHDPA	Metabolized by CYP450 enzymes (VanRollins, Baker, Sprecher, & Murphy, 1984). 13,14-DiHDPA possesses potent vasodilatory properties in porcine coronary microvessels and can activate calcium activated potassium channels (Ye, Zhang, Oltman, Dellsperger, Lee, & VanRollins, 2002).
4-HDoHE	DHA, 14- and 17-HDoHE can be converted to the novel maresins which possess anti-inflammatory and resolving properties in a murine model of acute inflammation (Serhan, et al., 2012).

Abbreviations used: hydroxyeicosapentanoic acid (HEPE), dihydroxydocosapentanoic acid (DiHDPA), hydroxydocahexanoic acid (HDoHE)

Table 1.5 – Summary of Findings on N-3 Oxylipins

Animal Model/Subjects	Methods	Results
Healthy humans (n=30) (Keenan et al., 2012)	11±2 mg/kg/d n-3 ethyl ester supplementation HPLC-MS/MS	Supplementation increased serum n-3 fatty acids, decreased serum n-6 fatty acids, and increased n-3 oxylipins with no influence on n-6 oxylipins.
Healthy humans (n=10) (Shearer, Harris, Pedersen, & Newman, 2010)	4 g/day n-3 ethyl esters	N-3 supplementation resulted in a 2-5 time increase in n-3 oxylipins and a 20% decrease in n-6 oxylipins.
In Vitro structural chemistry (Serhan & Petasis, 2011)	Review	N-3 oxylipins including resolvins, protectins, and maresins can resolve inflammation by removal of microbes and apoptotic cells, and prevention of the infiltration and migration of polymorphonuclear neutrophils
Polycystic Kidney Disease Rat model (Ogborn,	7% of diet as corn or flax oil by weight	Rats provided flax oil resulted in lower levels of renal PGE_2, renal

Nitschmann, Bankovic-Calic, Weiler, & Aukema, 2006).		inflammation, and cystic change.
Healthy males aged 26-36 years (n=6). Von Schacky, C. (1985).	Cod liver oil supplementation ranging from 10-40 mL/day over 40 weeks. Assessment of TXB_2 and B_3 as an indication of TXA levels and urinary $PGI_{2\text{-}M}$ and I_3-M as an indication of their endogenous levels.	Formation of TXA_2 was reduced by 50% in blood, TXA_3 levels increased slightly, and urinary PGI_3-M increased significantly. The greatest changes were seen at the 40 mL of cod liver oil supplementation.

VII. STUDY RATIONALE

The studies herein aim to establish an accurate animal model of ORG. The establishment of an animal model will facilitate the investigation of finding an earlier biomarker that is associated with endpoints such as glomerular damage and renal insufficiency. The animal model also will allow for the investigation of a potential dietary treatment for ORG with the use of various dietary oils with varying fatty acid compositions. The varying levels of dietary ALA and LA will allow for the novel investigation of how dietary LA:ALA ratios can influence the renal oxylipin profile. Examination of the renal oxylipin profile will provide insight into the etiology and progression of renal disease and therefore targets for future treatment investigation.

Hypothesis

The DIO experimental model will provide an adequate representation of ORG in which an early marker will be examined for the potential as a diagnostic marker. Diets rich in ALA will result in greater renal ALA levels, greater protein levels of enzymes producing oxylipins from ALA, higher n-3 oxylipins, lower n-6 oxylipins, and slow the progression of ORG in comparison to rats provided lower ALA diets.

Objectives 1-4 will be covered in Chapter 2- Diet-Induced Obese Rats Provide Evidence for the Use of Glomerulomegaly as an Early Diagnostic Marker and ALA Rich Oils in the Treatment of Obesity-Related Glomerulopathy. Objectives 4-8 will be detailed in Chapter 3 - Dietary Linoleic Acid and Alpha-Linolenic Acid Alter Renal Oxylipins in Diet-Induced Obesity.

Objectives

1) To examine the pathophysiology of renal disease in the DIO model and determine

if it is characteristic of ORG

2) To examine the potential of an early diagnostic marker of ORG
3) To determine how varying dietary LA, ALA levels, and LA:ALA ratios influence the progression of ORG in the DIO model
4) To understand if dietary LA, ALA levels, and LA:ALA ratios influence fatty acid deposition in the kidneys
5) To examine the influence of varying levels of ALA, LA and LA:ALA ratios on the renal oxylipin profile
6) To examine if novel renal oxylipins in particular, ALA and LA derived oxylipins, are associated with renal pathology in obesity
7) To clarify if the novel ALA derived HOTrEs and LA derived HODEs are present in the kidney and observe their quantities
8) To assess whether obesity, dietary ALA, and LA levels influence enzyme levels responsible for oxylipin formation

In order to assess the objectives, an animal study was designed with 104 obese-prone rats that were fed a high fat diet (55% of energy) rich in saturated and monounsaturated fatty acids (lard/soy) which has previously been reported to effectively induce obesity (de Wit et al., 2012). A control group was provided a low fat (LF) soy oil diet to serve as a LF control. Subsequently, two baseline groups were sacrificed in order to provide baseline renal tissue for histological analysis and to assess change in renopathophysiology.

The remaining rats provided the high fat diet were randomized to seven treatment groups with varying levels and ratios of LA and ALA and a LF soy group remained until the end of the study. This allowed for the analysis of several situations:

1) The same dietary ratio with different quantities of LA and ALA
2) Elevated levels of ALA with the same levels of LA
3) Elevated levels of LA with the same levels of ALA
4) Elevated ALA with less LA and vice versa
5) The same fatty acid composition at different quantities of dietary fat by energy

Renal histology and morphology provided insight into the early renal injury process of the DIO animals and the potential for an early diagnostic marker. Assessment of proteinuria and serum creatinine provided insight into renal injury and function, respectively. The assessment of renal fatty acids determined the influence of diet on the renal fatty acid composition which constituted pre-cursors to renal oxylipins. Assessment of the renal oxylipin profile provided insight into the influence of dietary fatty acid compositions on oxylipins that may have a role in renal pathology. Lastly, Western immunoblotting provided knowledge on the effects of varying dietary ALA and LA levels on the protein quantitites of enzymes responsible for producing oxylipins.

VIII. CHAPTER 2

Diet-Induced Obese Rats Provide Evidence for the Use of Glomerulomegaly as an Early Diagnostic Marker and ALA Rich Oils in the Treatment of Obesity-Related Glomerulopathy

Contributions of Co-authors to Chapter 2

Stephanie P.B. Caligiuri, Karin Dunthorne, Mélanie Grégoire, Tom Blydt-Hansen, Carla Taylor, Peter Zahradka, and Harold Aukema

Stephanie P.B. Caligiuri – performed all histological and morphological analyses of renal tissue, in part created the methods for histological assessment, lyophilized and pulverized all renal tissue for fatty acid analysis, analyzed the chromatograms and quantified the renal fatty acid composition, performed all proteinuria, serum, and urine creatinine assays, performed all statistical analysis, wrote the chapter and created all tables and figures.

Karin Dunthorne – Assisted with diet design, diet preparation, animal care, provision of diet to animals, body weight recording, urine collection, termination, tissue weighing and collection.

Mélanie Grégoire – homogenized renal tissue, extracted and methylated fatty acids from renal tissue.

Tom Blydt-Hansen – provided education on histological techniques and guidance throughout histological analysis, and provided direction and feedback on Chapter 2.

Carla Taylor – wrote the grant application and allocated funds for the investigation. Designed the study and provided supervision for Karin Dunthorne throughout the animal trial. Provided feedback on Chapter 2.

Peter Zahradka – was a co-applicant on the grant application, assisted with the study design, and co-supervised Karin Dunthorne throughout the animal trial

Harold Aukema – was a co-applicant on the grant application, provided direction and helpful insights for the experimental analysis, provided revisions and editing.

Abstract

ORG is an emerging condition which can lead to renal failure. To improve patient outcomes, early detection would be aided by more definitive diagnostic criteria, which can be facilitated by an accurate model. Such a model and early diagnostic criteria would be useful to examine interventions such as dietary fatty acids, which influence renal diseases in later stages. Therefore, obese-prone rats were provided high fat (55% energy) diets for 12 weeks to induce obesity and subsequently provided dietary oils with varying levels of ALA and LA for eight weeks. The model developed obesity, glomerulomegaly, proteinuria, and scarce glomerular damage with an indolent course. Morphometry and histology revealed glomerulomegaly as the first structural alteration. Its utility as a predictor for the presence of ORG and potential of renal injury was evidenced by its relationship to visceral adiposity ($p<0.0001$, $r=0.44$), proteinuria ($p<0.0001$, $r=0.55$), change in proteinuria ($p=0.0092$, $r=0.42$) and glomerular damage ($p<0.0001$, $r=0.46$). Diets high in ALA resulted in greater renal ALA which was associated with the prevention of glomerulomegaly and glomerular damage. To conclude, the DIO model provided an accurate representation of ORG which implicated glomerulomegaly as a surrogate diagnostic marker. This enables early intervention such as with ALA rich dietary oils to slow ORG progression.

Introduction

ORG is an emerging epidemic due to the rising global prevalence of obesity. It has been estimated that from the years 1986-2000 the incidence of ORG biopsies has increased by 10% (Kambham, Markowitz, Valeri, Lin, & D'Agati, 2001). This finding is likely due to the fact that obesity (body mass index ≥ 30 kg/m^2), has more than doubled globally since the year 1980 (Anonymous, 2011).

ORG has been primarily diagnosed in individuals without diabetes but often in the presence of insulin resistance (Chen et al., 2011). The most common clinical and histological characteristics of ORG are: hyperfiltration, proteinuria, glomerulomegaly, and sometimes the presence of focal segmental glomerulosclerosis; these obesity associated alterations in renal structure and function can lead to renal insufficiency (Praga et al., 2001) (Kambham et al., 2001) (Adelman, Restaino, Alon, & Blowey, 2001).

The identification of an animal model for ORG would benefit the advancement in knowledge on the pathophysiology and potential treatments. Common models of obesity such as the Zucker *fa/fa* rat and *db/db* mouse may develop ORG but can also develop potential confounding conditions such as progressive tubulopathy, a common finding in diabetic nephropathy (Hayden & Sowers, 2011). Characteristics distinct of diabetic nephropathy such as severe visceral epithelial cell foot process fusion, presence of glomerular epithelia hyperplasia, glomerular basement membrane thickening with mesangial sclerosis, nephrotic syndrome, and a quicker progression to renal failure should be avoided because they are not indicative of ORG (Tervaert et al., 2010) (Darouich et al., 2011) (Adelman et al., 2001) (Verani, 1992) (Kambham et al., 2001).

The etiology of the diet-induced obese (DIO) model mimics the development of

obesity in the human, as it develops visceral obesity, hyperleptinemia, hyperinsulinemia, and dyslipidema (Madsen et al., 2010) which are characteristics similar to those of humans with ORG (Chen et al., 2011) (Wu, Liu, Xiang, Zeng, Chen, Ma, & Li, 2006a). Despite the well established ability of the DIO model to mimic human obesity, the ability of the model to mimic ORG has yet to be determined. The DIO model may have an advantage over genetic models of obesity due to its presence of intact leptin and its receptor. Leptin or its receptor is defective or deficient in many genetic models such as the *ob/ob* mice which are leptin deficient (Kumpers et al., 2007), *db/db* mice which are leptin receptor deficient (Kumpers et al., 2007) and Zucker *fa/fa* rats which have a leptin receptor defect (Phillips et al., 1996). Not only is leptin positively correlated to body fat (Considine et al., 1996) but it also is associated with the presence of ORG (Wu, Liu, Xiang, Zeng, Chen, Ma, & Li, 2006a) and induces inflammation, oxidative stress, TGF-ß production, modulation of growth, and increased hydrostatic pressure within the kidney (Tang, Yan, & Zhuang, 2012; Wolf et al., 1999; Wolf, Chen, Han, & Ziyadeh, 2002), which are stimulators of ORG onset (Weinberg, 2006; Chagnac et al., 2003).

Establishment of an accurate animal model may facilitate the investigation of early diagnostic markers. Currently diagnosis and treatment of renal disease does not occur until clear histological damage or clinical biomarkers of renal insufficiency are evident (Tervaert et al., 2010) (Praga et al., 2001), when the timeframe to prevent progression is not optimal. In the clinical setting, renal biopsies may be issued for patients with persistently moderate or increasing proteinuria (Fuiano et al., 2000) and may only show glomerulomegaly without glomerular damage in patients with ORG (Kambham et al., 2001) (Wesson, Kurtzman, & Frommer, 1985) (Serra et al., 2008). The sole presence of glomerulomegaly does not provide sufficient information for nephrologists to begin

treatment and to date is not used as a diagnostic endpoint for renal diseases such as diabetic nephropathy (Tervaert et al., 2010), despite its potential to lead to glomerular damage (Fogo, et al., 1991). The distinctness of ORG, its associated risk for renal failure, and absence of established diagnostic guidelines necessitates the investigation of early markers. Establishment of an early marker would facilitate the potential for treatment investigations, early diagnosis, and early management of ORG.

Dietary intervention for ORG with the n-3 fatty acid, C18:3n3, ALA may be a viable treatment option as evidenced by its influence on disease progression in later stages of other renal diseases. Dietary oils and seeds rich in ALA have reduced serum creatinine, fibrosis, and cystic change in models of polycystic kidney disease (Sankaran, Lu, Bankovic-Calic, Ogborn, & Aukema, 2004; Ogborn, et al., 2002; Ogborn, et al., 1999; Ogborn, et al., 2006) and improved serum creatinine in humans with lupus nephritis (Clark et al., 1995). Renoprotection by dietary sources of ALA may be through a reduction in inflammation, cytokine production, macrophage infiltration (Ogborn et al., 2002; Ogborn et al., 1999), and/or reduction of some pro-inflammatory n-6 derived eicosanoids and an increase in less inflammatory or pro-resolving n-3 resolving mediators as evidenced by long chain n-3 intake (Shearer, et al., 2010; Serhan, et al., 2011). Dietary n-6:n-3 ratios of 2-5:1 have been proposed to reduce the risk of mortality and severity of conditions in other chronic diseases such as cardiovascular disease, asthma, rheumatoid arthritis, and colorectal cancer (Simopoulos, 2002). The importance of dietary fatty acids is further evidenced by the beneficial effect of low dietary n-6:n-3 fatty acid ratios on inflammatory scores and survival rates in hemodialysis patients (Noori et al., 2011).

Therefore, the purpose of the current study was to investigate the DIO rat as a

model of ORG, to confirm glomerulomegaly as an indicator of ORG and potential for renal injury, and to examine the influence of diets containing varying levels of ALA, LA, and LA:ALA ratios on ORG progression.

Methods

Animals and Diet

All research procedures were performed in accordance with the National Institute of Health's Guide for the Care and Use of Laboratory Animals and approved by the University of Manitoba Animal Care Committee. The findings related to renal health presented herein are part of a larger study that includes dietary fat effects on obesity, hepatic health, and insulin resistance (Dunthorne, 2011).

Male Obese-Prone Sprague-Dawley rats at 7 weeks of age were purchased from Charles River Laboratories (St. Constant, PQ), acclimatized for approximately 14 days and housed individually. Eighty-two rats were provided a high fat (HF, 55% of calories) lard/soy diet for 12 weeks to induce obesity. A diet formulation high in saturated fat has been used to effectively induce obesity in this rat model (de Wit et al., 2012). Twenty-two rats were provided a low fat (LF, 25% of calories) soy diet to serve as a control. After 12 weeks of feeding, 12 rats in each of the HF lard/soy and LF soy groups were sacrificed as baseline controls. The remaining HF rats were divided among 7 HF treatment groups (n=10/group) for 8 more weeks, while the remaining LF soy group remained on the LF soy diet. The eights diets contained varying levels of ALA and LA as shown in Table 2.1. The percent energy breakdown for the HF groups was 55% from fat, 15% from protein, and 30% from carbohydrate; the LF soy group provided 25% of energy from fat, 15% from protein, and 60% from carbohydrate. Rats had free access to food and water*;* feed

intake did not differ across groups during the entire treatment phase. The average feed intake across dietary groups in the last week of the intervention ranged from 133-150 g/week (Karin Dunthorne, 2011).

Urine was collected at the end of week 12 (baseline period) and again at week 19 with the use of metabolic cages and stored at -80°C until proteinuria analysis. The rats were terminated via carbon dioxide asphyxiation and cervical dislocation. Trunk blood was collected in 15 mL centrifuge tubes and centrifuged at 700 g for 15 minutes at 4°C to separate the serum. The serum was removed and stored at -80°C until serum creatinine analysis. Upon termination, the right kidneys were rapidly removed, frozen in liquid nitrogen and later analyzed for renal fatty acid content. Left kidneys used for histological analyses were bisected longitudinally across the hilum and fixed in 10% formalin for 48 hours followed by short term storage in phosphate buffered saline (0.01 M, pH 7.6) at 4˚C before being embedded in paraffin wax and sectioned at 5 µm.

Table 2.1 – Diet Ingredients

Diet Ingredient (g/kg)	LF[1] Soy[3]	HF[2] Lard/Soy	HF Canola/Flax	HF Soy	HF Canola	HF High Oleic Canola/Canola	HF High Oleic Canola	HF Safflower
Corn Starch	347	209	209	209	209	209	209	209
Maltodextrin	115	69	69	69	69	69	69	69
Sucrose	166	100	100	100	100	100	100	100
Cellulose	50	64	64	64	64	64	64	64
Casein	156	186	186	186	186	186	186	186
Lard	0	280	0	0	0	0	0	0
High Oleic Canola Oil	0	0	0	0	0	154	308	0
Canola Oil	0	0	231	0	308	154	0	0

Diet Ingredient (g/kg)	LF[1] Soy[3]	HF[2] Lard/Soy	HF Canola/Flax	HF Soy	HF Canola	HF High Oleic Canola/Canola	HF High Oleic Canola	HF Safflower
Flaxseed Oil	0	0	77	0	0	0	0	0
Safflower Oil	0	0	0	0	0	0	0	308
Soybean Oil	117	29	0	308	0	0	0	0
Mineral Mix AIN-93G	35	45	45	45	45	45	45	45
Vitamin Mix AIN-93G	10	13	13	13	13	13	13	13
L-cysteine	2.3	3	3	3	3	3	3	3
Choline Bitartrate	2.5	3	3	3	3	3	3	3
BHT	0.014	0.037	0.037	0.037	0.037	0.037	0.037	0.037
Fatty Acid (g/100 g fatty acids								
Total Saturated Fat	17	49	8	16	8	8	7	11

Diet Ingredient (g/kg)	LF[1]	Soy[3]	HF[2] Lard/Soy	HF Canola/Flax	HF Soy	HF Canola	HF High Oleic Canola/Canola	HF High Oleic Canola	HF Safflower
C16:0 (palmitic acid)	10	24	4	10	4	4	4	7	
C18:0 (stearic acid)	4	20	2	4	2	2	2	2	
Total	21	42	54	22	65	71	76	16	
Monounsaturated Fat									
C18:1 (oleic acid)	19	37	50	20	60	66	71	15	
Total Polyunsaturated	62	9	38	62	27	22	16	73	
Fat									
C18:2n6 (LA)	53	8	18	53	18	16	15	73	
C18:3n3 (ALA)	9	1	20	9	8	5	2	0.2	
LA:ALA	6:1	8:1	1:1	6:1	2:1	3:1	8:1	365:1	

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[1]LF = low fat, [2]HF = high fat
[3]The diets are ordered by control groups first (LF then HF lard/soy) followed by the treatment diets ranging in highest ALA to lowest.
AIN-93 G vitamin mix, AIN-93 G mineral mix, choline bitartrate, cellulose fibre, cornstarch, L-cysteine, maltose dextrin (fine), sucrose, and Teklad vitamin free casein were purchased from Dyets Inc. - Animal Test Diets for Science and Industry, Bethlehem, PN
Butylated hydroxytoluene (BHT) was purchased from Sigma-Aldrich, St. Louis, MO
Capri canola oil, Capri, vegetable (soy) Oil, Nutra-Clear™ high-stability canola oil, and Jubilee deep fry beef fat (Lard) were purchased from Bunge Canada, Oakville, ON
Certified organic flax oil was purchased from Omega Nutrition, Vancouver, BC
Refined High Linoleic Safflower Oil was purchased from Alnoroil Company, Inc., Valley Stream, NY
The diet ingredients were previously published by Karin Dunthorne, 2011 of which copyright permission was obtained

Image Analysis for Histology and Morphology

Tissue preparation and image collection were performed as described previously with minor alterations (Sankaran, et al., 2006; Sankaran, Bankovic-Calic, Ogborn, Crow, & Aukema, 2007; Ogborn et al., 1999). Briefly, tissue sections were stained with Masson's Trichrome to evaluate mean glomerular volume, glomerulosclerosis, glomerular damage, and interstitial fibrosis. All histological analyses were performed blinded. For fibrosis and tubular atrophy analysis, thirteen images each of the cortex and medulla under 10x magnification were captured with random stage movement using an Olympus BX60 Microscope and SPOT Advanced Software. Images for glomerulosclerosis and mean glomerular volume were captured through random stage movement under 20x magnification until images of 30 glomeruli were captured. Step by step details for the methodology of histology and morphology are located in the thesis appendicies (Protocol 1).

Interstitial Fibrosis and Tubular Atrophy

Interstitial fibrosis was assessed morphometrically by the presence of blue stain in the interstitium accompanied by tubular atrophy or damaged glomeruli as defined by Farris & Colvin (2012). Tubular atrophy was identified by thickening of the tubular membrane and enlargement of the lumen space. The area of the blue pigmentation in the presence of an atrophied tubule or damaged glomerulus was quantified using a morphometric colour standard using Image-Pro Plus Software 6.0.

Glomerular Damage

Glomerulosclerosis was defined by score 3 and 4 below while glomerular damage included scores 2-6 as follows: 1-(normal): cellular elements intact, uniform staining, open capillary loops, absence of mesangial matrix expansion, 2-(early lesion): segmental mesangial matrix expansion extending to outer membrane, open capillary loops, and intact cellular elements, 3-(segmental glomerulosclerosis): segmental accumulation of blue stain, obliteration of capillary loops in sclerosed area starting from the periphery, while capillary loops and tuft structure were preserved in a segment of the glomerulus, 4-(global glomerulosclerosis): complete obliteration of capillary loops over entire glomerulus, accumulation of blue stain, organized sclerotic changes in all glomerular tuft segments, 5-(periglomeular fibrosis and ischemic tuft contraction): severe tuft contraction with multiple layers of fibrotic tissue encircling the basement membrane. 6-(atubular glomeruli): severe tuft contraction, Bowman's space filled with protein cast, calcification, and free-floating podocytes. The number of damaged glomeruli was divided by the total number of glomeruli analyzed (30) to create a percentage of glomerular damage.

Mean Glomerular Volume

The maximum diameter of each glomerular tuft was measured using Image-Pro Plus Software 6.0 measurement tools on the same 30 glomeruli analyzed for glomerular damage. Using standard stereological techniques, the diameters (μm) were converted to mean glomerular volume (MGV) using the following equations: Mean Glomerular Area (MGA)=$\prod r^2$ and MGV=$1.25(MGA)^{3/2}$, as developed by Weibel (1980) and described previously (Sankaran et al., 2006).

Proteinuria

Urinary protein was assessed as a measurement of renal injury. Proteinuria concentrations were examined using the Bradford colorimetric method for total protein determination (Bradford, 1976). Proteinuria was normalized to urinary creatinine levels and represented as mg protein/mg creatinine. Details of the proteinuria methodology can be found in the thesis appendices (Protocol 2).

Serum and Urine Creatinine

Serum creatinine was assessed as a marker of renal insufficiency. Serum and urine creatinine were measured using a modified Jaffe method based on the creatinine-picrate reaction (Heinegard & Tiderstrom, 1973). Serum creatinine was expressed as mg/mL. Details of the creatinine methodology can be found in the thesis appendices (Protocol 3).

Fatty Acid Analysis

Lyophilized kidney tissue was homogenized in Tyrodes solution (pH 7.6). An antioxidant cocktail (0.2 mg/mL BHT, 0.2 mg/mL ethylenediaminetetraacetic acid, 2 mg/mL triphenylphosphine, and 2 mg/mL indomethacin in a solution of 2:1:1 methanol:ethanol:water) was added as 3% of total volume to prevent fatty acid oxidation. Lipids were extracted from the kidney homogenate by solvent extraction as described (Sankaran et al., 2004). After separation of renal triglycerides with thin layer chromatography (heptane/isopropyl/acetic acid, 60/40/3 v/v/v), fatty acids were transmethylated and methyl esters were quantified by gas chromatography as described (Sankaran et al., 2004). Fatty acids greater than 0.05 ng/mg were reported; values below

this threshold were labelled as trace. Step by step details of the fatty acid methodology are located in the thesis appendices (Protocol 4).

Statistical Analysis

Statistical analyses were carried out using SAS version 9.2 (SAS Institute Inc, Cary, NC). Data were tested for normality and homogeneity of variance using Shapiro-Wilk and Levene's test respectively. Data were analyzed using one-way ANOVA followed by a post-hoc comparison using Duncan's multiple range test. Non-Gaussian data were analyzed via Kruskal-Wallis followed by Tukey's Studentized Range Test. Correlation analyses were carried out using Pearson or Spearman correlation dependent upon normality. All tests were set at a significance level of 0.05. Data are represented as mean ± standard error, with the exception of correlation analyses which are represented as means only. Data analyses included the total sample size of 104 unless otherwise indicated.

Results

The baseline groups (LF soy and HF lard/soy) did not differ in body or adipose mass. At the end of the study, the LF soy group gained significant body (+90 g), peri-renal (+0.9 g/100 g body mass), and mesenteric adipose mass (+0.5 g/100 g body mass) compared to its matched baseline group. The HF soy group had significantly more peri-renal and epididymal adipose mass than the LF soy group. All high fat end point groups gained significant amounts of body, peri-renal adipose, and mesenteric adipose mass over 8 weeks in comparison to the HF lard baseline. At the end of the study, rats provided HF diets weighed approximately 643 ± 6 g. These HF rats gained 10-12 g/week, which represented a 2-3% increase in body mass per week (Table 2.2).

Table 2.2: Body, Kidney, and Adipose Mass at Termination across Dietary Groups

	Low Fat or Baseline Groups			High Fat Groups						
	LF Soy Baseline	**LF Soy**	**Lard/Soy Baseline**	**Lard/Soy**	**Canola/Flax**	**Soy**	**Canola**	**High Oleic Canola/ Canola**	**High Oleic Canola**	**Safflower**
Body (g)	543 ± 13^{B}	633 ± 20^{A}	543 ± 12^{B}	655 ± 22^{A}	624 ± 13^{A}	643 ± 10^{A}	639 ± 14^{A}	663 ± 11^{A}	639 ± 19^{A}	647 ± 14^{A}
Kidney (g)	3.7±0.1	3.7±0.1	3.6±0.1	3.9±0.1	3.9±0.1	3.8±0.1	3.8±0.1	3.9±0.1	4.0±0.1	3.9±0.1
Peri-renal Adipose (g/100 g body mass)	3.7 ± 0.2^{C}	4.6 ± 0.2^{B}	3.9 ± 0.2^{BC}	5.9 ± 0.3^{A}	5.6 ± 0.2^{A}	5.4 ± 0.2^{A}	6.0 ± 0.2^{A}	6.0 ± 0.2^{A}	5.8 ± 0.3^{A}	6.0 ± 0.2^{A}
Epididymal Adipose (g/100 g body mass)	2.8 ± 0.2^{C}	2.8 ± 0.1^{C}	2.8 ± 0.2^{C}	3.1 ± 0.1^{BC}	3.3 ± 0.1^{AB}	3.3 ± 0.1^{AB}	3.6 ± 0.1^{A}	3.5 ± 0.1^{A}	3.4 ± 0.1^{AB}	3.4 ± 0.9^{A}
Mesenteric Adipose (g/100 g body mass)	2.0 ± 0.2^{B}	2.5 ± 0.2^{A}	1.6 ± 0.1^{B}	2.4 ± 0.1^{A}	2.6 ± 0.2^{A}	2.8 ± 0.2^{A}	2.9 ± 0.2^{A}	2.9 ± 0.2^{A}	2.6 ± 0.1^{A}	2.9 ± 0.2^{A}

Values in the same row with different superscripts differ significantly ($p<0.05$)
Groups are ordered as controls first (LF then HF lard/soy groups) followed by the HF groups ranging in highest dietary ALA to lowest (Dataset was previously produced by Karin Dunthorne, 2011 of which copyright permission was obtained).

Glomerulomegaly

At the end of the study, glomerulomegaly with scarce glomerular damage was observed. The mean glomerular volumes (MGVs) were approximately 17% larger in the HF lard/soy group and 29% in the LF soy group compared to their respective baseline controls (Figure 2.1). The LF soy and HF soy groups did not differ in mean glomerular volume. The average increase in glomerular volume across the HF treatment groups was 16% (range: 5-25%) compared to the HF lard/soy baseline.

The MGVs of the rats in the HF canola/flax, HF canola, and HF soy groups did not differ significantly compared to the HF lard/soy baseline group, whereas the high oleic canola/canola, high oleic canola, safflower, and lard/soy groups had significantly greater (17-25% greater) MGVs. In addition, rats provided the HF canola diet (11% of dietary fat as ALA) had significantly lower MGVs compared to the safflower and lard/soy fed rats and experienced only a 5% increase in MGV.

Figure 2.2 depicts the positive relationship between visceral adiposity and the progression of ORG; visceral adipose mass (sum of peri-renal, epididymal, and mesenteric adipose depots) was positively correlated to MGV (Figure 2.2). Additionally, all three adipose depots were individually correlated to MGV ($r = 0.4$).

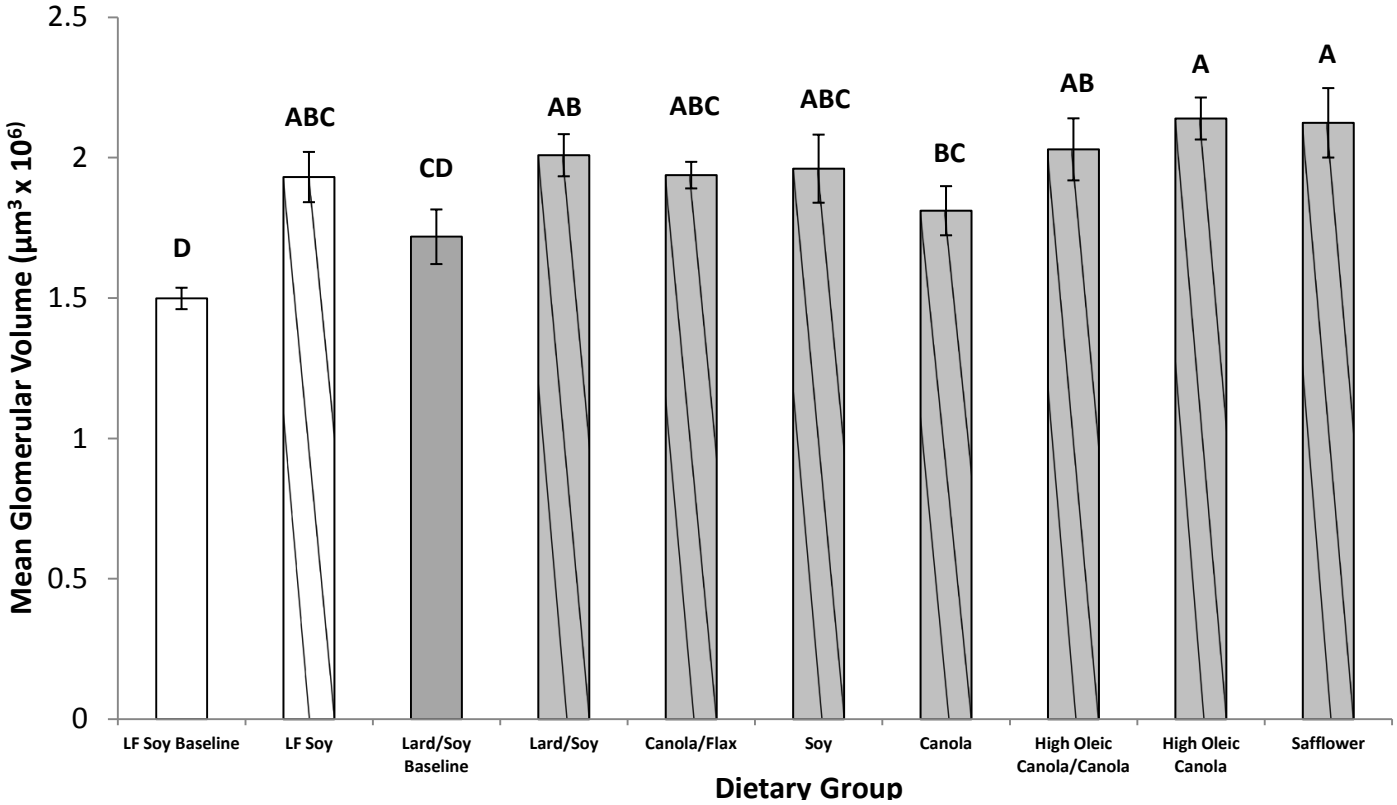

Figure 2.1: Mean Glomerular Volume by Dietary Group. Columns (mean ± standard error) without a common letter differ significantly ($p < 0.05$). Data were analyzed with one-way ANOVA and Duncan's Multiple Range test

Rats were terminated at the 12 week baseline (non-striped bars) or 20 week time point (striped bars) and provided with low fat (white bars) or high fat (grey bars) diets. Groups are ordered by controls first (LF then HF lard/soy groups) followed by the HF groups providing the highest dietary ALA to the lowest.

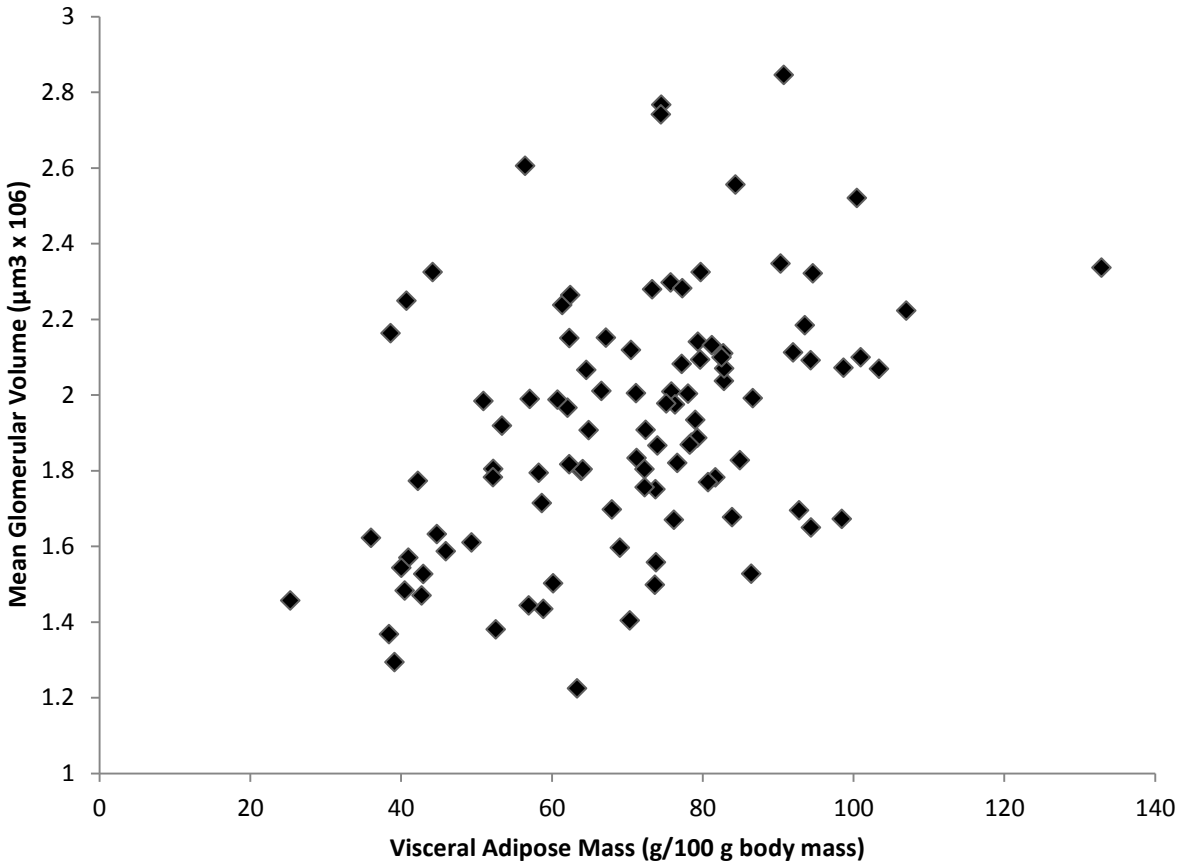

Figure 2.2: Correlation of Percent Visceral Adipose Mass by MGV. Visceral adipose was the sum of peri-renal, epididymal, and mesenteric adipose depots. Data was analyzed with Pearson's correlation. p-value <0.0001, r = 0.44 (n=100).

Glomerular Damage

Scarce glomerular damage was observed; more than 90% of the glomeruli were classified as normal across the dietary groups and there was no detection of global glomerulosclerosis. Approximately 72% of the glomerular damage consisted of segmental glomerulosclerosis (range: 62-83%); early lesions comprised the majority of the remaining proportion of glomerular damage with only 5 glomeruli displaying periglomerular fibrosis with ischemic tuft contraction and 2 atubular glomeruli in all glomeruli analyzed. Representative images of the glomerular damage observed are depicted in Figure 2.3. Some of the glomeruli presented with the beginnings of mesangial matrix expansion in a small segment of the tuft that did not extend to the periphery and therefore were classified as normal rather than early lesions; there was also an absence of mesangial hypercellularity. The interstitium appeared normal as evidenced by the extreme scarce presence of interstital fibrosis, interstital infiltration, and tubular atrophy; only 0.96% of slide images contained interstitial fibrosis or tubular atrophy.

The LF soy baseline and HF lard/soy baseline groups had 0 and 0.28% of glomerulosclerosis, respectively. Rats provided the LF soy diet experienced a non-significant increase to 3% of glomeruli as damaged at the end of the study period, whereas the HF lard/soy group exhibited a significant elevation in glomerular damage to 8.3% of total glomeruli. There was no significant difference in glomerular damage between the LF soy and HF soy groups. The HF safflower group also experienced significantly greater glomerular damage (8% damaged glomeruli) compared to the HF lard/soy baseline (Figure 2.4). In the DIO model, the percentage of damaged glomeruli was positively correlated with MGV (Figure 2.5). This positive relationship existed regardless of diet.

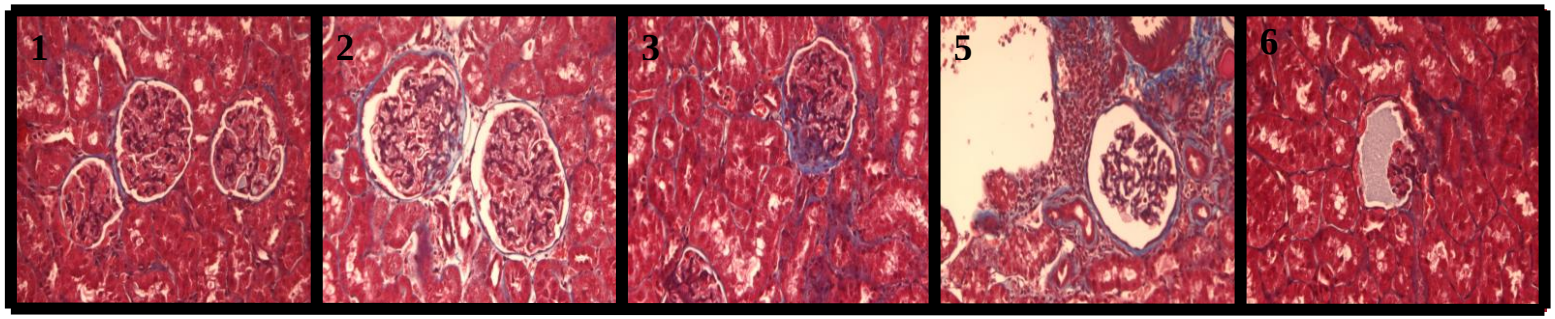

Figure 2.3: Histological Images of Normal and Damaged Glomeruli. Slides were stained with Masson's Trichrome and analyzed under 20x magnification. Glomerular damage scores and corresponding images are as follows:

1 – Normal

2 – Early Lesion (left) & Normal Glomerulus (right)

3 – Segmental Glomerulosclerosis

5 – Periglomerular Fibrosis with Ischemic Tuft Contraction

6 – Atubular

**Global glomerulosclerosis (score 4) was not observed.

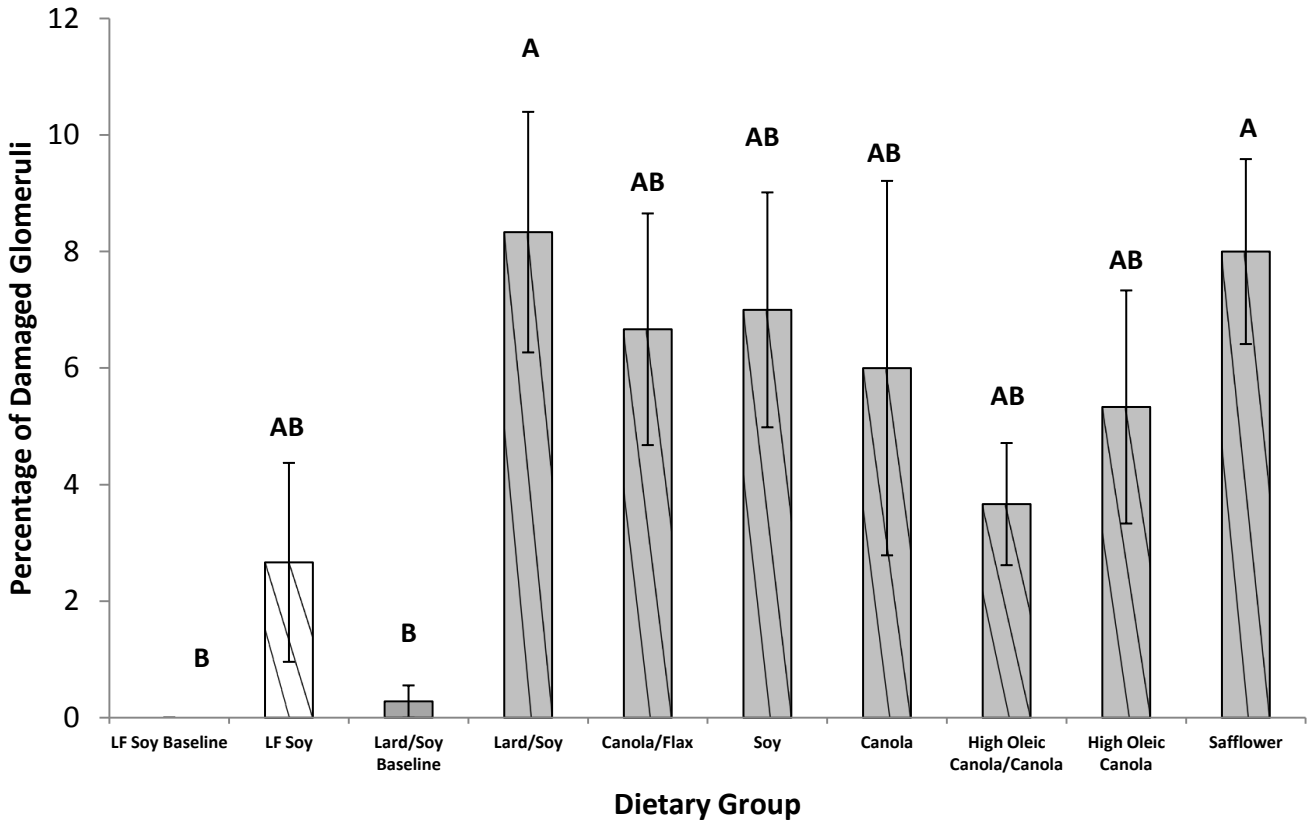

Figure 2.4: Percentage of Damaged Glomeruli by Dietary Group. Data points (mean±standard error) without a common letter differ significantly ($p < 0.05$). Data were analyzed with Kruskal-Wallis and Tukey's Studentized Range Test. Rats were terminated at the 12 week baseline (non-striped bars) or 20 week time point (striped bars) and provided with low fat (white bars) or high fat (grey bars) diets. Groups are ordered by controls first (LF then HF lard groups) followed by the HF groups providing the highest dietary ALA to the lowest.

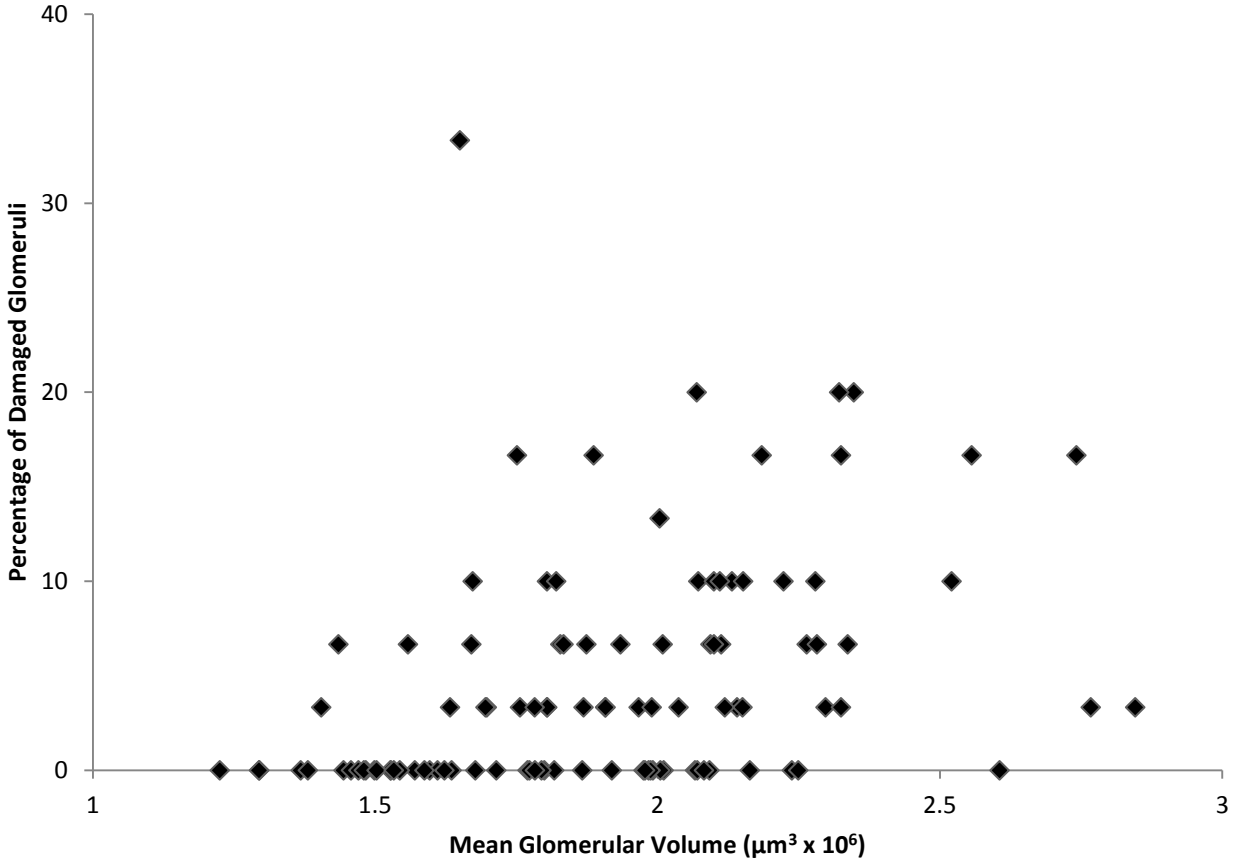

Figure 2.5: Correlation between Mean Glomerular Volume and Glomerular Damage
Spearman's Correlation: p-value < 0.0001, r = 0.46

Proteinuria

Proteinuria increased over the treatment phase in all groups. However, the differences between groups for change in proteinuria did not vary significantly regardless of dietary fatty acid composition or level of dietary fat (Figure 2.6).

At the end of the study, proteinuria values were positively correlated with mean glomerular volume (Figure 2.7) regardless of diet, as was the change in proteinuria and change in glomerular volume ($p = 0.009$, $r = 0.42$).

Serum Creatinine

Serum creatinine values did not differ significantly between the baseline groups, the LF soy baseline and LF soy endpoint, the LF soy versus the HF soy, or the HF lard/soy baseline versus the remaining HF groups. The serum creatinine values ranged from 0.0043-0.0050 mg/mL. The correlation between serum creatinine and MGV was not significant ($p=0.90$).

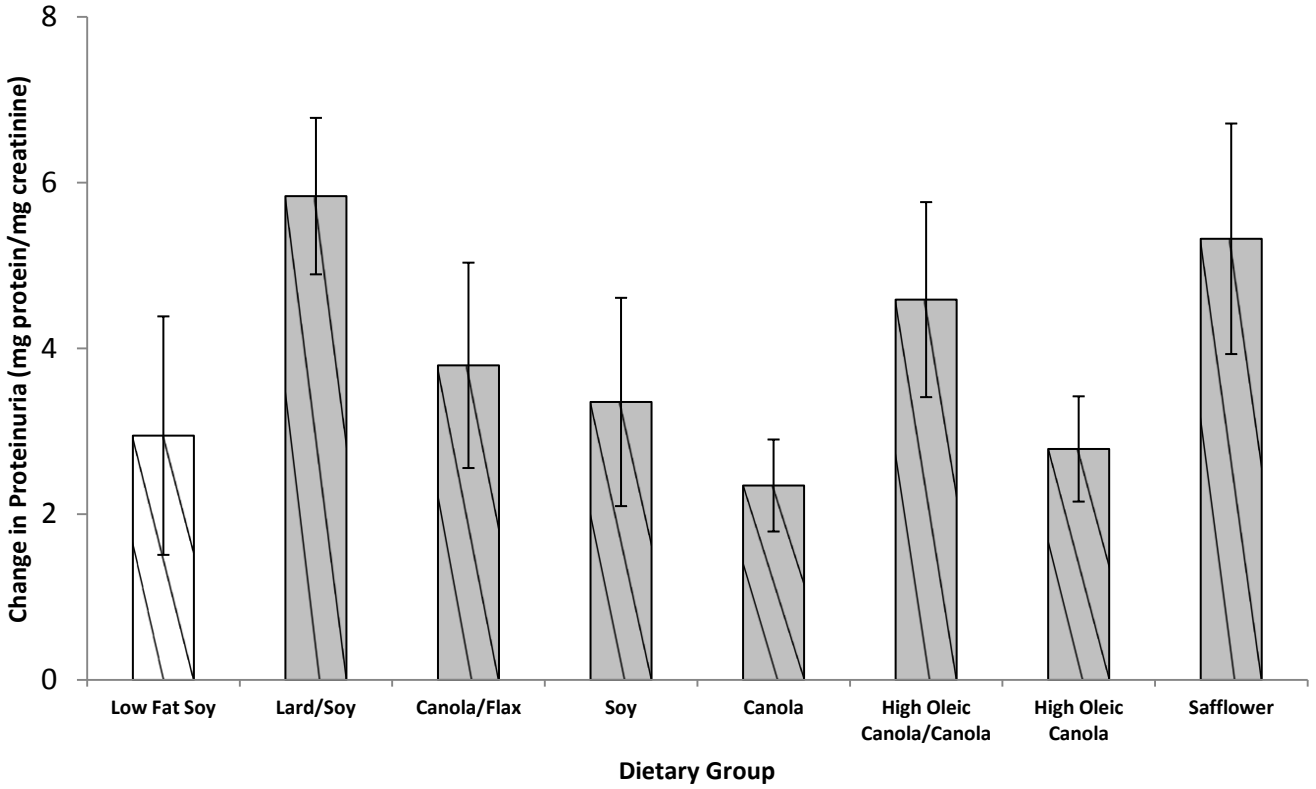

Figure 2.6: Change in Proteinuia during the Treatment Phase by Dietary Group. Data are represented as mean ± standard error (n= 4-6/group). The data were analyzed using Kruskal-Wallis (p-value = 0.40). The baseline proteinuria values were subtracted from the endpoint values.
Rats were provided low fat (white bars) or high fat (grey bars) diets. The groups are ordered as controls first (LF soy and HF lard followed by the HF groups ranging in highest dietary ALA to lowest.

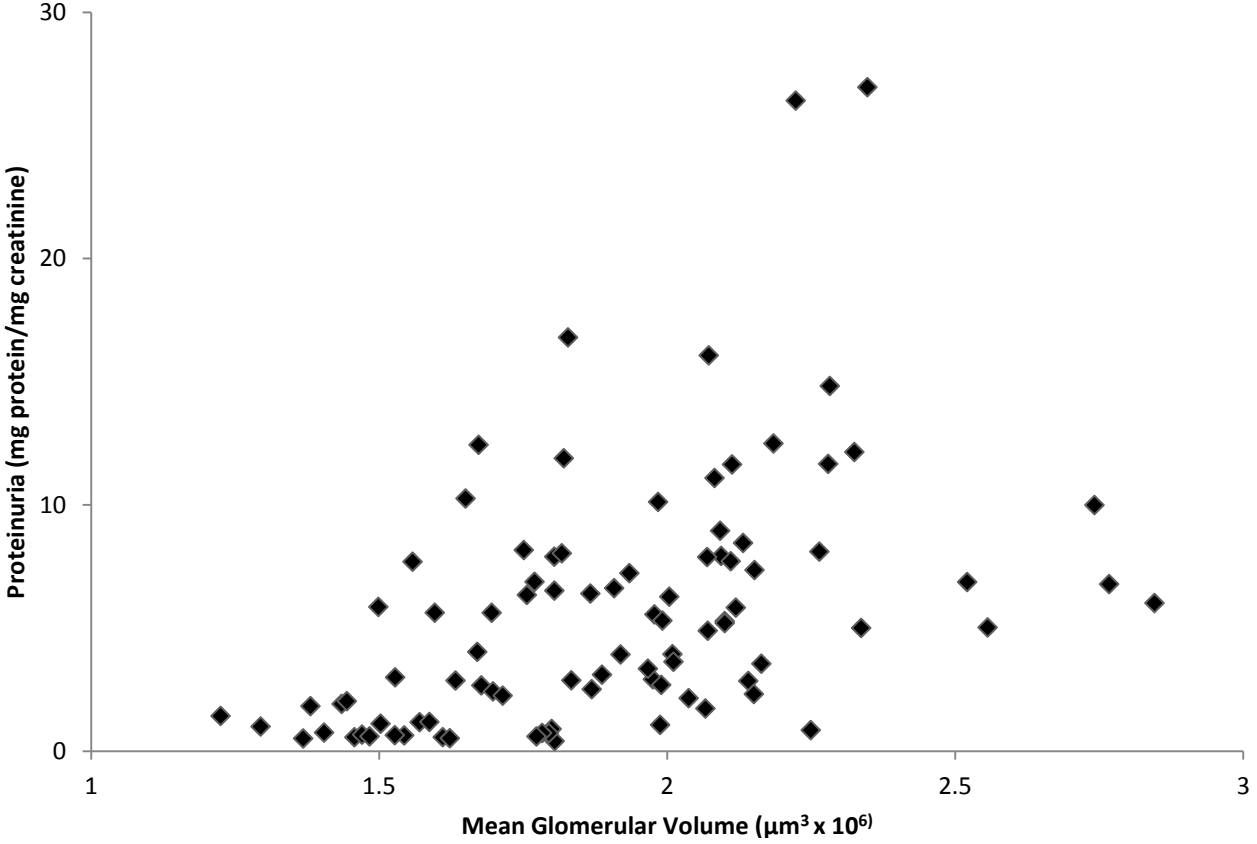

Figure 2.7: Correlation of Mean Glomerular Volume to Proteinuria. Spearman's Correlation: p-value <0.0001, r = 0.55 (n=91)

Fatty Acid Compositions

The renal triglyceride fatty acids illustrated the well established effect of dietary fatty acids on tissue fatty acid composition (Table 2.3). Diets high in LA resulted in greater renal n-6 fatty acids and likewise diets providing greater quantities of ALA resulted in greater n-3 fatty acids in the kidneys. The HF lard/soy diet which supplied the greatest amount of saturated and monounsaturated fatty acids resulted in the HF lard/soy baseline rats to have more saturated and monounsaturated fatty acids and less polyunsaturated fatty acids than the LF soy baseline group. Age appeared to influence renal triglyceride fatty acid deposition in the LF soy but not HF lard/soy groups; the LF soy baseline group had significantly more C16:1, C18:3n3, and less C20:3n6 than the LF soy endpoint group. The LF soy and HF soy groups differed in their quantities of C16:0, C16:1, C18:0, C18:1, and C18:3n3.

In relation to renal pathology, the groups that did not experience a significant increase in MGV (HF canola/flax, HF soy, and HF canola) had the highest levels of dietary ALA (8-20% of fat as ALA) and renal ALA (2.3-5.5 nmol%). Furthermore, the 4 groups of rats that experienced a significant increase in MGV were provided diets lower in ALA which resulted in lower renal ALA than the HF canola/flax, HF soy, and HF canola groups. The two diets providing the lowest quantities of ALA and the highest n-6 (HF safflower) or saturated fatty acids (HF lard/soy) resulted in a significant increase in glomerular damage.

Table 2.3 – Renal Triglyceride Fatty Acid Composition (nmol%) by Dietary Group

	Low Fat or Baseline Groups			High Fat Groups						
Fatty Acid	**LF Soy Baseline**	**LF Soy**	**Lard/Soy Baseline**	**HF Lard/Soy**	**HF Canola/Flax**	**HF Soy**	**HF Canola**	**HF High Oleic Canola/ Canola**	**HF High Oleic Canola**	**HF Safflower**
C14:0	1.1 ± 0.19^{BC}	0.90 ± 0.04^{C}	2.0 ± 0.27^{A}	1.6 ± 0.15^{AB}	0.73 ± 0.06^{C}	0.82 ± 0.06^{C}	0.78 ± 0.06^{C}	0.64 ± 0.07^{C}	0.68 ± 0.03^{C}	0.72 ± 0.06^{C}
C14:1	0.12 ± 0.08^{BC}	0.16 ± 0.07^{ABC}	0.20 ± 0.02^{ABC}	0.13 ± 0.05^{BC}	0.19 ± 0.07^{ABC}	0.33 ± 0.06^{A}	0.28 ± 0.03^{AB}	0.08 ± 0.05^{C}	0.18 ± 0.06^{ABC}	0.22 ± 0.04^{ABC}
C15:0	0.08 ± 0.05^{C}	0.16 ± 0.03^{BC}	0.38 ± 0.02^{A}	0.38 ± 0.01^{A}	0.16 ± 0.05^{BC}	0.24 ± 0.01^{AB}	0.22 ± 0.02^{BC}	0.15 ± 0.05^{BC}	0.20 ± 0.01^{BC}	0.18 ± 0.01^{BC}
C16:0	23.8 ± 1.2^{A}	22.7 ± 0.64^{A}	24.7 ± 0.15^{A}	24.5 ± 0.48^{A}	16.9 ± 0.95^{C}	19.5 ± 0.49^{B}	16.5 ± 0.39^{C}	17.2 ± 0.66^{C}	16.0 ± 1.3^{C}	16.4 ± 0.71^{C}
C16:1	4.1 ± 0.51^{A}	2.7 ± 0.34^{B}	2.6 ± 0.23^{B}	2.1 ± 0.22^{BC}	1.2 ± 0.21^{D}	1.05 ± 0.04^{D}	1.6 ± 0.30^{CD}	0.86 ± 0.08^{D}	0.95 ± 0.06^{D}	0.75 ± 0.04^{D}
C17:1	0.08 ± 0.05^{C}	0.22 ± 0.08^{BC}	0.62 ± 0.02^{A}	0.61 ± 0.01^{A}	0.29 ± 0.01^{B}	0.30 ± 0.01^{B}	0.29 ± 0.01^{B}	0.29 ± 0.01^{B}	0.31 ± 0.02^{B}	0.20 ± 0.01^{BC}
C18:0	4.1 ± 0.26^{E}	4.4 ± 0.52^{DE}	10.1 ± 0.44^{A}	10.8 ± 0.56^{A}	5.5 ± 0.16^{BC}	6.44 ± 0.15^{B}	4.9 ± 0.11^{CDE}	5.3 ± 0.07^{CD}	4.9 ± 0.16^{CDE}	5.3 ± 0.07^{CD}
C18:1	26.3 ± 1.05^{F}	25.9 ± 0.48^{F}	48.6 ± 0.45^{D}	49.4 ± 0.36^{D}	54.7 ± 0.82^{C}	32.9 ± 0.52^{E}	58.6 ± 0.36^{B}	60.5 ± 0.50^{B}	64.1 ± 1.4^{A}	26.4 ± 0.68^{F}
C18:2	35.7 ± 1.7^{B}	38.2 ± 1.5^{B}	9.6 ± 0.14^{CD}	9.3 ± 0.48^{D}	13.8 ± 0.17^{C}	34.3 ± 0.47^{B}	13.5 ± 0.23^{CD}	12.3 ± 0.34^{CD}	11.0 ± 0.29^{CD}	48.0 ± 1.4^{A}
C18:3n3	3.6 ± 0.19^{B}	3.2 ± 0.12^{C}	0.58 ± 0.02^{G}	0.49 ± 0.03^{GH}	5.5 ± 0.21^{A}	2.9 ± 0.08^{D}	2.3 ± 0.11^{E}	1.4 ± 0.06^{F}	0.55 ± 0.03^{G}	0.19 ± 0.02^{H}
C20:1	0.10 ± 0.04^{DE}	0.13 ± 0.01^{CDE}	0.20 ± 0.01^{C}	0.21 ± 0.02^{C}	0.50 ± 0.03^{AB}	0.09 ± 0.06^{E}	0.47 ± 0.01^{B}	0.55 ± 0.05^{AB}	0.58 ± 0.02^{A}	0.19 ± 0.01^{CD}
C20:2	0.22 ± 0.02^{A}	0.19 ± 0.03^{A}	trace^{B}	trace^{B}	trace^{B}	0.20 ± 0.03^{A}	trace^{B}	trace^{B}	trace^{B}	trace^{B}
C20:3n6	0.11 ± 0.04^{B}	0.18 ± 0.02^{A}	trace^{C}	trace^{C}	trace^{C}	0.19 ± 0.02^{A}	trace^{C}	trace^{C}	trace^{C}	0.22 ± 0.01^{A}

	Low Fat or Baseline Groups			High Fat Groups						
Fatty Acid	**LF Soy Baseline**	**LF Soy**	**Lard/Soy Baseline**	**HF Lard/Soy**	**HF Canola/Flax**	**HF Soy**	**HF Canola**	**HF High Oleic Canola/ Canola**	**HF High Oleic Canola**	**HF Safflower**
C22:4	trace^{B}	0.08±0.03^{B}	trace^{B}	0.05±0.03^{B}	trace^{B}	trace^{B}	trace^{B}	0.08±0.05^{B}	0.05±0.05^{B}	0.24±0.01^{A}
C22:6n3	0.02±0.02^{CD}	0.15±0.03^{A}	trace^{D}	0.05±0.03^{ABCD}	0.13±0.05^{AB}	0.14±0.05^{AB}	0.10±0.01^{ABC}	0.06±0.04^{ABCD}	0.04±0.04^{BCD}	trace^{D}
n-6:n3	**10:1^{B}**	**12:1^{B}**	**17:1^{B}**	**18.5:1^{B}**	**2.5:1^{B}**	**12:1^{B}**	**6:1^{B}**	**9:1^{B}**	**20:1^{B}**	**270:1^{A}**

Mean ± standard error within the same row without a common letter differ significantly ($p<0.05$) (n=4/group). Groups are ordered as controls first (LF followed by HF lard/soy groups) then the HF groups ranging in highest dietary ALA to lowest.

Discussion

The data herein indicate that the DIO animal model presents with characteristics of ORG as seen in humans (Kambham et al., 2001) (Adelman et al., 2001) (Praga et al., 2001). The DIO animal model developed obesity, proteinuria, glomerulomegaly, scarce glomerulosclerosis, and had an absence of increased serum creatinine, moderate interstital fibrosis or tubular defects, all with an indolent course of progression. Other animal models tend to progress to moderate or severe renal disease within a short time span which is not characteristic of ORG. For example, the *fa/fa* Zucker rat at 20 weeks of age progresses to moderate glomerulosclerosis and severe interstitial fibrosis which is more characteristic of diabetic nephropathy (Slyvka et al., 2009). Similarly, Sprague-Dawley rats fed high fat diets experience progressive renal necrosis, glomerulosclerosis, and tubular defects within 3 months (Altunkaynak et al., 2008). The DIO model is preferred by many due to the induction of obesity through diet which is more representative of the onset of human obesity rather than a genetic mutation model. Investigators have additionally confirmed that the adipose tissue of DIO models has genetic similarities to humans for immune response and angiogenesis related genes (Li et al., 2008).

As obesity progressed in the DIO model, glomerulomegaly increased and appeared as the first indication of renal structural change; this is likely due to increased renal plasma flow and glomerular filtration rate commonly seen in the early stages of ORG (Chagnac et al., 2003). In the current study, MGV in the DIO model was positively correlated to proteinuria and glomerular damage; additionally, the change in glomerulomegaly was positively correlated to the change in

proteinuria. This provides evidence that glomerulomegaly is an early indication of ORG and its pathological process. Glomerulomegaly may be associated with progressive renal injury overtime and may identify individuals at risk for renal failure. Use of glomerulomegaly as an earlier diagnostic marker may prevent the risk of renal failure, as ORG patients who already present with focal segmental glomerulosclerosis can progress to renal failure with a predicted 5 and 10 year renal survival percentage of 77 and 51%, respectively (Praga et al., 2001).

Clinical guidelines for the diagnosis of ORG have yet to be established and approved; pathologists may not identify the presence of renal disease in ORG patients until clear endpoint markers such as glomerulosclerosis or clinically high serum creatinine appears in the later stages where treatment time is not optimal. Earlier signs of ORG such as glomerulomegaly are currently not used as diagnostic markers for renal disease such as in diabetic nephropathy (Tervaert et al., 2010). However, the current study provides evidence for the use of glomerulomegaly as a diagnostic marker for ORG in individuals and as a surrogate marker for the indication of glomerular and renal damage.

Using the DIO rat as a model of early ORG and glomerulomegaly as an indicator of the potential for further disease progression, the current study provided evidence that dietary oils high in ALA may provide a protective effect when initiated in the early stages of ORG. Development of glomerulomegaly and glomerular damage from baseline was prevented in rats provided the 3 diets highest in ALA (HF canola/flax, HF canola, and HF soy). Furthermore, glomerular damage was highest in the diets with the lowest ALA (HF lard/soy and HF safflower). The

level of dietary fat (25 versus 55%) did not appear to significantly influence renal pathology but did significantly influence adipose depot mass (peri-renal and epididymal) and renal fatty acid deposition for several fatty acids. However, the differences in renal fatty acid composition could be due to the HF soy group first being supplied the HF lard/soy diet in the obesity-induction phase whereas the LF soy group remained on the LF soy diet throughout the entirety of the study.

Dietary recommendations often are made based on n-6:n-3 ratios in conjunction with fatty acid levels; however, this study provides evidence that the quantity of fatty acids may be the more important component. For example, the HF soy diet provided a higher dietary n-6:n-3 ratio of 6:1 compared to the HF high oleic canola/canola diet which supplied a ratio of 3:1. This resulted in a 12:1 n-6:n-3 ratio in the renal tissue of the HF soy group compared to the HF high oleic canola/canola group ratio of 9:1. Despite the higher n-6:n-3 ratio, the actual quantity of ALA in the renal tissue was significantly higher (by 2 times) in the HF soy group. Notably, the rats in the HF soy group did not develop significant glomerulomegaly whereas the HF high oleic canola/canola group did. Additionally, the HF soy and HF high oleic canola diets supplied similar dietary n-6:n-3 ratios; however, the HF soy diet supplied almost 5 times more ALA and LA which resulted in greater renal ALA levels (> 5 times more) and prevention of glomerulomegaly by comparison. Therefore, the quantity of dietary ALA rather than the n-6:n-3 ratio may be more important for the amelioration of renal pathology in obesity.

The Institute of Medicine has set a recommendation of at least 1.1 g/day and

1.6 g/day of ALA for women and men, respectively, which constitutes approximately 0.6-1.2% of total energy intake (Institute of Medicine of the National Academies, 2002). The percentage of energy as ALA in the current study ranged from approximately 4.4-12% in the three diets supplying the most ALA. Thus, the levels that are protective in ORG would be considerably higher than the current recommended intakes, but achievable with the use of dietary flax and canola oils.

The recommendation of increasing the consumption of dietary oils rich in ALA is also supported by other findings in the later stages of renal disease. For example, canola oil supplementation in a model of diabetes prevented glomerular injury in comparison to a corn oil supplementation (high n-6 fatty acid content). (Garman, Mulroney, Manigrasso, Flynn, & Maric, 2009). Sources of ALA such as flaxseed have provided renoprotection in humans living with lupus nephritis (Clark et al., 1995), an animal model of renal failure (Ingram et al., 1995) and animal models of polycystic kidney disease (Ogborn et al., 1999; Ogborn et al., 2002; Ogborn et al., 2006; Sankaran et al., 2004; Sankaran et al., 2006).

Increased hydrostatic pressure, glomerular filtration rate, and renal plasma flow have been implicated in the onset and progression of glomerulomegaly, glomerular damage (Fogo, et al., 1991), and ORG (Ribstein, et al., 1995; Chagnac et al., 2003). The renoprotective effects of dietary ALA in ORG may be due to a decrease in glomerular hydrostatic pressure which has been previously evidenced by fish oil supplementation (EPA and DHA) resulting in a significantly lower percentage filtration fraction, glomerular capillary pressure, and glomerular volume in a model of renal ablation compared to a safflower oil diet (Brown et al., 2000). A

gap in knowledge exists for the influence of ALA on renal hemodynamics; however, the influence of ALA on renal inflammation has been documented.

Inflammation and oxidative stress within the glomerulus are also implicated in the onset and progression of ORG (Wu, Liu, Xiang, Zeng, Chen, Ma, & Li, 2006b). ALA may have provided protection from lipotoxicity; lipotoxicity is present in obesity and associated with the increased production of reactive oxygen species, necrosis, apoptosis, and inflammation (Weinberg, 2006). ALA in the renal proximal tubular cell line, NRK-52E, reduces detriments of lipotoxicity such as cell death and increases in endoplasmic reticulum stress markers, phosphorylated eukaryotic initiation factor 2α, transcription factor C/EBP homologous protein (CHOP), and glucose regulated protein 78 (Katsoulieris, Mabley, Samai, Green, & Chatterjee, 2009). Additionally, some oxylipins produced from the n-3 fatty acids may provide protection against inflammation and tissue damage. Three-series prostaglandins and 5-series leukotrienes produced from EPA have less potential to be pro-inflammatory than their 2- and 4-series counterparts, respectively, produced from arachidonic acid (Bagga et al., 2003). In addition, resolvins, protectins, and maresins produced from EPA or DHA can reduce and resolve inflammation by preventing polymorphonuclear neutrophil infiltration and transmigration (Serhan & Petasis, 2011).

Dietary ALA may also slow the progression of fibrosis and glomerulosclerosis. This can be evidenced by the work performed by Garman et al. (2009) who showed that canola oil supplementation in a model of diabetes reduced glomerulosclerosis and fibrosis through the reduction of collagen type I and IV

proteins, maintenance of podocyte integrity, and reduction of TGF-ß.

In conclusion, the current study provides evidence that further investigation of ORG can be facilitated with the use of the DIO model. Glomerulomegaly has the potential to be an early diagnostic biomarker for the presence of ORG and potential for renal damage in early ORG. Use of glomerulomegaly in the clinical setting can facilitate earlier diagnosis leading to further investigation or initiation of treatments such as with sources of dietary ALA (canola and flaxseed oils) in order to provide the best outcomes for patients with ORG.

Chapter 2 References

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IX. CHAPTER 3

Dietary Linoleic Acid and Alpha-Linolenic Acid Alter Renal Oxylipins in Diet-Induced Obesity

Contribution of Co-Authors

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Carla Taylor – wrote the grant application and allocated funds for the investigation. Designed the study and provided supervision for Karin Dunthorne throughout the animal

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Tom Bldyt-Hansen – provided insight on mean glomerular volume assessment which was used as a marker of early ORG in correlation to omega 3 oxylipins. Provided feedback, suggested ideas for statistical analysis, and revisions on the chapter..

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Abstract

Advances in metabolomics allows for the analysis of a wide range of metabolites including oxylipins; however assessment of varying dietary LA and ALA levels or ratios on oxylipins or assessment of the renal oxylipin profile has yet to be examined. Previously, dietary ALA prevented glomerulomegaly in ORG perhaps through an alteration of oxylipins; yet this remains to be investigated. Therefore, to assess the above, 104 diet-induced obese rats were provided eight diets with varying levels of ALA and LA for eight weeks. Of the 64 oxylipins scanned with HPLC-MS/MS, 30 were quantified, and 18 were influenced by diet. Higher dietary ALA resulted in greater 13-HOTrE, 18-HEPE, and total HEPEs, with no effect on n-6 oxylipins. Higher dietary LA resulted in more 9-HODE, total HODEs, total DiHOMEs 12,13-DiHOME, total OXoODEs, and 9-OXoODE with no effect on n-3 oxylipins. The renoprotective effect of ALA could in part be explained by an inverse relationship of n-3 oxylipins with pathology ($p<0.05$, $r= -0.3$). To conclude, dietary LA and ALA influenced renal oxylipins of which there appeared to be an absence of competition between n-3 and n-6 fatty acids.

Introduction

Fatty acids play a significant role in the promotion of health and progression of disease. The physiological influence of fatty acids can be direct by preventing or promoting inflammation; for example fatty acids can either reduce (Katsoulieris, Mabley, Samai, Green, & Chatterjee, 2009) or promote (Zhang, Xue, Zhang, Yang, & Shi, 2012) apoptotic cell death and endoplasmic reticulum stress. However, perhaps the physiological influence of fatty acids is even more significant through their oxygenated metabolites referred to as oxylipins. Oxylipins include metabolites such as the eicosanoids, octadecanoids, and docosanoids. Eicosanoids have been the class of oxylipins most investigated, but recently docosanoids including the resolvins, protectins, and maresins have been of key interest (Serhan, Krishnamoorthy, Recchiuti, & Chiang, 2011). However, little research has focused on the octadecanoids including the direct metabolites of linoleic acid, HODEs and more so the direct metabolites of ALA, HOTrEs.

The unique ALA-derived octadecanoids, 9- and 13-HOTrE have been identified in human serum at combined concentrations ranging from 0.004-3.9 μM in normal adults (Psychogios et al., 2011) and 0.02-70 μM for 9- and 13-HODE (Psychogios et al., 2011). The serum concentrations of these 4 octadecanoids are significantly greater than the concentrations of EPA and DHA derived resolvins (0.001-0.521 μM) (Psychogios et al., 2011) and may therefore play a very influential role in health. 13-HOTrE *in vitro*, has in fact displayed anti-inflammatory characteristics and can reduce the production of matrix metalloproteinases (MMPs) in human chondrocytes (Schulze-Tanzil et al., 2002). However, it is unknown where HOTrEs are present in the body, their influence on health,

or how diet can alter their quantity or production.

In contrast, HODEs have been examined for their potential as markers of oxidative stress (Horie et al., 2012; Morita et al., 2012; Wang et al., 2008). HODEs may also display differential characteristics; 13(S)-HODE decreased tumor cell adhesion and prevented lung colonization by high metastatic cells (Honn et al., 1992) while 9-HODE was associated with atherosclerosis progression by stimulating the release of IL-1 in oxidized LDL particles (Ku, Thomas, Akeson, & Jackson, 1992).

Dietary n-6 and n-3 fatty acids may influence the production of HOTrEs and HODEs; for example, a diet high in n-6 fatty acids can result in a greater production of oxylipins such as leukotriene B_4, prostaglandins E_2 and D_2 which are proinflammatory mediators (Lawrence, Willoughby, & Gilroy, 2002) (Calder, 2006). In contrast, a diet high in n-3 fatty acids may be able to increase n-3 derived oxylipins and alter the balance to be more anti-inflammatory (Keenan et al., 2012) (Calder, 2010). Resolving mediators such as resolvins, protectins, and maresins produced from the long chain n-3 fatty acids can resolve the inflammatory process and prevent further tissue damage (Serhan & Petasis, 2011b) through the removal of microbes and apoptotic cells, and prevention of the infiltration and migration of polymorphonuclear neutrophils (Serhan, Krishnamoorthy, Recchiuti, & Chiang, 2011).

It is well understood that dietary n-6:n-3 ratios influence the deposition of fatty acids *in vivo* (Dyer & Greenwood, 1991); however, the influence of varying levels of ALA, LA, and LA:ALA on a wide range of oxylipins has yet to be investigated. Due to recent advancements in metabolomics, detection of hundreds of metabolites including oxylipins derived from fatty acids is now possible and have been isolated in human serum

(Psychogios et al., 2011) (Keenan et al., 2012). However, the levels of the LA and ALA derived oxylipins have yet to be assessed in tissue or whether they along with the enzymes that produce them are influenced by varying dietary LA and ALA levels or ratios. Changes in the levels of COX, LOX, and CYP450 can significantly influence the production of oxylipins (Sankaran, et al., 2007; Wang et al., 2003) and therefore should be investigated in order to understand if dietary fatty acid compositions can influence the oxylipin levels through alteration of enzyme levels.

There is a current debate about the optimal dietary n-6:n-3 ratio and levels; previous investigations have promoted ratios ranging from 2-5:1 for the promotion of health in various chronic diseases (Simopoulos, 2002), which is in line with the recommendation from the National Institutes of Health of 2-3:1(Simopoulos, Leaf, & Salem, 1999). In addition to ratios, consumption of fatty acids at certain quantities is also recommended; the Institute of Medicine has recommended ALA consumption at 1.1-1.6 g/day for women and men, respectively (Institute of Medicine of the National Academies, 2002). Emphasis has been placed on the optimal ratio for ALA conversion to EPA and DHA which some researchers have proposed to be 2-4:1 (Davis & Kris-Etherton, 2003). Perhaps an optimal dietary LA and ALA levels or ratios can be determined based on the respective oxylipin profile produced.

The ability to alter the oxylipin profile through diet may be particularly important for obese individuals. Obesity is associated with an increased protein level of enzymes that can produce pro-inflammatory and vasoconstrictive oxylipins and a decrease in the anti-inflammatory protective mediators (Zhao & Imig, 2003) (Imig, Zhao, Dey, & Shaw, 2005) (Zhao et al., 2005). Oxidative stress, inflammation, (Wu, et al., 2006) and altered

hemodynamics (Ribstein, et al., 1995; Chagnac, et al., 2003) in the kidneys of obese individuals is associated with the onset of an emerging condition referred to as ORG. ORG is characterized by hyperfiltration, glomerulomegaly, and in the later stages, glomerulosclerosis and nephrotic range proteinuria without nephrotic syndrome that can lead to renal failure (Kambham, et al., 2001; Darouich et al., 2011; Adelman, et al., 2001; Verani, 1992). It is anticipated that the incidence of ORG will climb along with the increasing rates of obesity (Kambham et al., 2001). Despite the clinical significance and increasing prevalence of ORG, there is a lack of information regarding treatment. Previous investigations have concluded that diets higher in ALA may be renoprotective in an animal model of ORG, perhaps in part due to the influence on the oxylipin profile (As seen in Chapter 2).

Therefore, the purpose of the current investigation was to assess the quantities of renal oxylipins, the influence of varying dietary LA and ALA levels and ratios on the *in vivo* renal oxylipin profile and select enzyme levels, and the potential correlations between oxylipins and renal pathology using a diet-induced obese experimental model with ORG.

Methods

Animals and Diet

The research investigation was conducted in conformity with the Public Health Service Policy, Canadian Council on Animal Care and approved by the University of Manitoba Animal Care Committee. The findings reported in this paper are part of a larger study that includes the investigation of dietary fat influences on obesity, hepatic health,

muscle fatty acid compositions, insulin resistance (Dunthorne, 2011), and renal health (Chapter 2). Male Obese-Prone Sprague-Dawley rats (Charles River Laboratories St. Constant, PQ) were 9 weeks old when provided either a HF lard/soy diet (55% energy) to induce obesity or a LF soy oil diet (25% energy) as a control for 12 weeks. Subsequently, a HF lard/soy and LF soy group were sacrificed to serve as baseline controls. An additional LF soy group continued throughout the treatment phase and the remaining DIO rats were provided 7 treatment diets for 8 weeks containing dietary oils with various levels of ALA and LA. The nutrient requirements for the rats were based on the American Institute of Nutrition (AIN)-93G diet formulation with casein as the source of protein (Reeves, 1997); the macronutrient distribution for the HF diets by energy was: 30% carbohydrate, 15% protein, 55% fat; the LF soy diet's macronutrients distribution was 60% carbohydrates, 15% protein, and 25% fat. The dietary fatty acid analysis data is shown in Table 3.1; additional diet details are provided in Table 2.1.

The DIO rats presented with early stage ORG as indicated by glomerulomegaly, increased proteinuria, and scarce focal segmental glomerulosclerosis. The three diets highest in ALA, canola/flax, soy, and canola prevented glomerulomegaly while the rats on the remaining diets lower in ALA, experienced a 17-25% increase in glomerular volume from baseline.

The animals were terminated via carbon dioxide asphyxiation, cervical dislocation, and trunk blood collection. The right kidneys were removed, frozen in liquid nitrogen, and stored at -80°C; the renal tissue was later lyophilized, pulverized, and stored at -80°C until homogenization.

Table 3.1: Diet Fatty Acid Composition (g/100 g of fatty acids)

Fatty Acid	Canola/Flax	Soy	Canola	High Oleic Canola/Canola	High Oleic Canola	Lard/Soy	Safflower	Low Fat/Soy
Total Saturated Fat (%)	**8**	**16**	**8**	**8**	**7**	**49**	**11**	**17**
C16:0 (palmitic acid)	4	10	4	4	4	24	7	10
C18:0 (stearic acid)	2	4	2	2	2	20	2	4
Total Monounsaturated Fat (%)	**54**	**22**	**65**	**71**	**76**	**42**	**16**	**21**
C18:1 (oleic acid)	50	20	60	66	71	37	15	19
Total Polyunsaturated Fat (%)	**38**	**62**	**27**	**22**	**16**	**9**	**73**	**62**
C18:2n6 (LA)	18	53	18	16	15	8	73	53
C18:3n3 (ALA)	20	9	8	5	2	1	0.2	9
LA:ALA	**1:1**	**6:1**	**2:1**	**3:1**	**8:1**	**8:1**	**365:1**	**6:1**

Data are analyzed values and expressed as g/100 g of fatty acids (n=1/diet). Diets are ordered by highest to lowest dietary ALA followed by the LF soy control. The fatty acid composition of the diet was previously published by Karin Dunthorne, 2011 of which copyright permission was obtained.

Renal Pathology

In this investigation, MGV was used as an early marker of ORG. Step by step details of methodology concerning histology are located in the thesis appendices, Protocol 1.

Homogenization of Tissue for Fatty Acid, Eicosanoid, and Western Analysis

Lyophilized whole kidney tissue was homogenized in Tyrodes salt solution (pH 7.6) in a 1:28 w/v ratio and aliquoted for fatty acid, eicosanoid, and Western analysis. The aliquots were further processed as described under each analysis.

Fatty Acid Analysis

To prevent fatty acid oxidation, an antioxidant cocktail (0.2 mg/mL BHT, 0.2 mg/mL ethylenediaminetetraacetic acid, 2 mg/mL triphenylphosphine, and 2 mg/mL indomethacin in a solution of 2:1:1 methanol:ethanol:water) was added to the homogenate as 3% of total volume. The aliquots were flushed with nitrogen and stored at -80°C until further analysis. Diet samples were homogenized in a 1:20 w/v ratio of 2:1 chloroform:methanol with 0.01% BHT. Fatty acids were extracted by solvent extraction as described (Sankaran, Lu, Bankovic-Calic, Ogborn, & Aukema, 2004). The renal fatty acids were further separated by thin layer chromatography to isolate the phospholipid fraction (heptane/isopropyl/acetic acid, 60/40/3 v/v/v), transmethylated, and quantified by gas chromatography (Sankaran et al., 2004). Values are expressed as ng/mg dry tissue with values <10 ng/mg dry tissue labelled as trace. Details of the fatty acid analysis are located in the thesis appendices, Protocol 4.

Eicosanoid Extraction and Isolation

A 500 µL aliquot of homogenate was further prepared by adding 5 µL of 1% Triton and vortexed every 10 minutes for a total of 30 minutes on ice. To a 200 µL aliquot of homogenate, 500 µL of 100:1 methanol:formic acid, 800 µL of pH 3 purified water, 90 µL of 100% ethanol, and 10 µL of the antioxidant cocktail were added and vortexed. The samples were flushed with nitrogen gas and stored at -80°C until further analysis.

Later, aliquots were thawed and deuterated internal standards, purchased from Cayman Chemical were added before extraction. Extraction and isolation of the eicosanoids were performed as described (Deems, Buczynski, Bowers-Gentry, Harkewicz, & Dennis, 2007). Briefly, samples were acidified to a pH of 3 and centrifuged for 5 minutes at 3000 x g. Strata-X, 33 µm, polymeric reversed phase columns (Phenomenex, product no. 8B-S100-UBL) were conditioned with 2 mL of methanol followed by 2 mL of pH 3 water. The sample was applied to the column followed by a 10% methanol in pH 3 water wash that was rinsed from the tube previously holding the sample. To elute, 2 mL of methanol was added to the column. This eluate was then dried under a nitrogen water bath set to 37°C and reconstituted with 100 µL of solvent A (water:acetonitrile:formic acid (70:30:0.02 v/v/v LC-MS grade). Further details are available in thesis appendices, Protocol 5.

The analytes were separated with high performance liquid chromatography and tandem mass spectrometry (HPLC-MS/MS) using a Luna 5 µm C18 (2) (Phenomenex – product number: 00G-4252-B0), 250 x 2.0mm column. The solvent gradient duration was 35 minutes of which details are listed in Chapter 2 Supplemental Data (Table S3.4). The

HPLC was an Agilent 1100 series (pump: Agilent 1100 G1312A, Binary pump; Auto sampler: Agilent 1100 G1367A). The MS was an API 2000 with triple quadrupole and turbospray, model # 029345-K. Collision induced dissociation (CID) mass transitions for all deuterated standards and analytes are listed in the supplemental data following this chapter (Tables S3.1-S3.3). Detection and quantification limits were set at 3 and 5 levels above the background, respectively. Detector response factors were calculated for all analytes and applied to results during quantification (Listed in Table S3.3). All values for the oxylipins are expressed as ng/mg of dry tissue.

Western Immunoblotting

The tissue homogenate was diluted using 1.4x whole cell buffer (50 mM Tris in H_2O, 250 mM sucrose, 2 mM ethylene diamine tetraacetic acid tetra sodium salt dihydrate, 1 mM of ethylene glycol-bis (2-aminoethylether) tetraacetic acid, 50 mM sodium fluoride, 4.37 mL of purified H_2O, 0.5% Triton, 280 µL of 100 µM sodium orthovanadate, 25 µg/mL of aprotinin, 25 µg/mL leupeptin, 25 µg/mL pepstatin, 1 µg/mL soy trypsin inhibitor, 144 µM 4-(2-aminoethyl benzene-sulfonyl fluoride), and 10 mM of ß-mercaptoethanol – also listed in Thesis Appendices Table 5.5) to reach a final protein concentration ranging from 3-5 µg/µL. Renal whole cell fractions were used for the detection of COX-1 and 2, 12/15-LOX, 15-LOX 2, 5-LOX, CYP2c23, sEH, and ß-actin as the house keeping protein. The protein concentrations of the homogenates were quantified using the Bradford method for total protein determination (Bradford, 1976).

For the detection of COX-1, 12/15-LOX, 15-LOX 2, and CYP2c23, 14 µg of protein was applied to the gel for each sample. To detect COX-2, sEH, and 5-LOX, 28 µg of protein was applied to the gel. A reference sample was loaded in duplicate on each gel

and served as the standard by which samples could be compared across different membranes.

The primary antibodies were incubated on the membrane at 4°C overnight at the following concentrations: COX-1 (1:8000; Abcam ab109025), COX-2 (1:2000; Cayman 160106), 12/15-LOX (1:8000; Santa Cruz sc32940), 15-LOX 2 (1:2000; Santa Cruz sc46048) 5-LOX (1:500; Cell Signaling 3289), CYP2c23 (1:16000; Abcam ab53944), sEH (1:100; Cayman 10010146) and ß-actin (1:16000; Cell Signaling 4970).

The membranes were incubated with an anti-rabbit or anti-goat secondary antibody for 1 hour at room temperature. Chemiluminescent peroxidase (Sigma, catalogue no. cps1120-1kt) was applied to the membranes and detected using Cell Biosciences Flurochem HD2 with chemidisplay. The bands were analyzed using densitometry and corrected for individual background. All samples were divided by ß-actin and the reference sample. Details of the methodology and optimization for antibodies and protein loading amounts are located in Protocol 6 of the Appendices and Figure 5.1.

Conversion and Product/Precursor Ratios

The quantity of oxylipins produced by 15-LOX and 5-LOX were grouped and divided by the quantity of the respective fatty acid substrate to infer the amount of product produced per amount of substrate available by the respective enzyme.

Statistical Analysis

All statistical analyses were carried out using SAS version 9.2 (SAS Institute Inc. Cary, NC). All data were tested for normality and homogeneity of variance using

Shapiro-Wilk and Levene's test respectively. The oxylipin profile and enzyme selectivity data were analyzed using analysis of variance (ANOVA) or Kruskal-Wallis dependent upon normality and followed by a post hoc test with either Tukey's Studentized Range Test (non-Gaussian data) or Duncan's Multiple Range Test (Gaussian distribution). The correlation analyses were carried out with Pearson's correlation test for groups with detectable renal ALA. Logistic regression analysis was used to predict renal HOTrE levels by renal ALA. All analyses were set at a significance level of 0.05. The data is presented as mean ± standard error; in the case of scatterplots, the data points are means.

Results

During the baseline period, the HF lard/soy and LF soy groups gained approximately 361 ± 9 and 367 ± 12 grams respectively. At the end of the 20 week study, there were no significant differences in body weight across the dietary groups with an average body mass of 644 ± 6 g overall. Despite similar body masses, the LF soy group had significantly lower percentages of peri-renal and epididymal adipose mass than the treatment groups. The kidney mass did not change across dietary groups (baseline or treatment) with an average of 3.8 g for both kidneys combined (Chapter 2).

Renal Oxylipin Profile

The primary objective of the study was to analyze the renal oxylipin profile under the influence of several dietary LA and ALA levels and ratios, as this has yet to be reported. In order to investigate this objective, 7 treatment diets were provided to DIO rats and a LF soy group was used as the reference as soy oil is the lipid constituent of the AIN93 diet for laboratory rodents and provides adequate amounts of LA and ALA (Reeves, 1997). Sixty-four oxylipins were scanned in the lipidomics analysis, of which

30 oxylipins were detected from LA, DGLA, ARA, ALA, EPA, and DHA (Table 3.2 and 3.3). In the LF soy group, LA oxylipins constituted the largest group with HODEs, OXoODEs, and DiHOMEs comprising on average 60.7 ± 0.02% of total oxylipins. ARA derived oxylipins consisting of prostanoids, HETEs, and DHETs comprised the second largest proportion of oxylipins at 31.4 ± 0.01%. For the remainder of the n-6 oxylipins, the DGLA metabolite, 15-HETrE, constituted 0.2 ± 0.0% of oxylipins. For the n-3 oxylipins, ALA, EPA, and DHA comprised 4.9 ± 0.0%, 1.3 ± 0.0%, and 1.5 ± 0.0% of total oxylipins, respectively.

Altering the quantity of dietary fat, while keeping the proportion of dietary fatty acids the same, did not modify the oxylipin profile as seen with insignificant differences between the HF soy and LF soy groups. The oxylipin profile also did not change significantly with age; only one difference in a minor oxylipin (15-HETrE) was observed between the LF soy baseline and LF soy groups. No differences were noted between the HF lard/soy baseline and HF lard/soy groups.

Table 3.2- N-3 Derived Oxylipins by Dietary Group (ng/mg dry tissue)

Oxylipin	Canola/Flax	Soy	Canola	High Oleic Canola/ Canola	High Oleic Canola	Lard/Soy	Safflower	Lard/Soy Baseline	Low Fat Soy	Low Fat Soy Baseline
-Linolenic Acid Derived Oxylipins										
HOTrEs										
9-HOTrE	257±25^{AB}	171±21^{ABC}	136±21^{BCD}	58±11^{CDE}	25±8^{ED}	14±4^{E}	11±5^{E}	29±2^{ED}	256±58^{AB}	284±32^{A}
13-HOTrE	139±21^{AB}	115±21^{BC}	61±8^{CD}	42±8^{ED}	9±4^{ED}	6±3^{ED}	trace^{E}	13±5^{ED}	120±16^{ABC}	178±19^{A}
Total	**396±61^{ABC}**	**286±40^{BC}**	**197±22^{CD}**	**99±14DE**	**35±11DE**	**20±6^{E}**	**11±5^{E}**	**42±5DE**	**376±69^{AB}**	**461±42^{A}**
Eicosapentanoic Acid Derived Oxylipins										
HEPEs										
5-HEPE	87±16^{A}	50±10^{ABC}	42±16^{ABCD}	36±9^{BCD}	16±9^{CD}	12±5^{CD}	trace^{D}	15±7^{CD}	60±11^{ABC}	75±12^{AB}
18-HEPE	85±22^{A}	39±12^{AB}	25±13^{B}	16±8^{B}	4±3^{B}	2±2^{B}	trace^{B}	trace^{B}	37±16^{AB}	52±17^{AB}
Total	**172±34^{A}**	**89±17^{BCD}**	**66±20^{BCDE}**	**52±12^{BCDE}**	**21±10^{CDE}**	**14±5DE**	**trace^{E}**	**15±7DE**	**98±21^{ABC}**	**127±23^{AB}**
Docosahexanoic Acid Derived Oxylipins										
HDoHE										
4-HDoHE	105±12^{AB}	104±8^{AB}	107±14^{AB}	105±11^{AB}	82±11^{ABC}	72±7^{BC}	32±4^{C}	96±10^{AB}	114±19^{AB}	140±19^{A}
DiHDPA										

Oxylipin	Canola/Flax	Soy	Canola	High Oleic Canola/ Canola	High Oleic Canola	Lard/Soy	Safflower	Lard/Soy Baseline	Low Fat Soy	Low Fat Soy Baseline
19,20-DiHDPA	1.5±0.42^{AB}	1.2±0.3^{AB}	1.7±0.29^{A}	0.94±0.3^{AB}	1.2±0.37^{AB}	0.73±0.25^{AB}	0.21±0.14^{B}	0.79±0.24^{AB}	1.4±0.26^{AB}	1.7±0.24 ^{A}
Total Omega 3 Derived Oxylipins										
Total	**675±107^{AB}**	**480±54^{ABC}**	**372±51^{BCD}**	**257±29^{CDE}**	**139±24DE**	**107±14DE**	**43±8^{E}**	**153±17DE**	**589±103^{AB}**	**730±62^{A}**

Data is presented as mean ± standard error. Abbreviations used: Hydroxyoctadecatrienoic acid (HOTrE), hydroxyoctadecadienoic acid (HODE), hydroxyeicosapentanoic acid (HEPE), hydroxydocosahexanoic acid (HDoHE) and dihydroxydocosapentanoic acid (DiHDPA). Trace was defined as < 0.2 ng/mg dry tissue.

Table 3.3 – N-6 Derived Oxylipins by Dietary Group (ng/mg dry tissue)

Oxylipin	Canola/Flax	Soy	Canola	High Oleic Canola/ Canola	High Oleic Canola	Lard/Soy	Safflower	Lard/Soy Baseline	Low Fat Soy	Low Fat Soy Baseline
Linoleic Acid Derived Oxylipins										
HODEs										
9-HODE	861±274^{BC}	1615±174^{AB}	586±56^{C}	514±42^{C}	596±60^{C}	355±32^{C}	2149±207^{A}	473±42^{C}	1808±261^{A}	2083±213^{A}
13-HODE	891±214^{B}	1605±139^{B}	667±61^{B}	515±45^{B}	587±53^{B}	406±36^{B}	2210±253^{A}	513±37^{B}	1789±190^{B}	2113±187^{B}
Total	**1751±485^{B}**	**3220±310^{A}**	**1253±109^{B}**	**1029±85^{B}**	**1183±109^{B}**	**761±67^{B}**	**4359±456^{A}**	**987±76^{B}**	**3596±444^{A}**	**4195±398^{A}**
DiHOMEs										
9,10-DiHOME	49±16^{BCD}	68±7^{BC}	33±5^{CD}	28±3^{D}	32±2^{CD}	16±2^{D}	131±14^{A}	16±1^{D}	69±7^{BC}	72±8^{BC}
12,13-DiHOME	19±7^{CD}	34±3^{BC}	14±1^{D}	10±1^{D}	13±2^{D}	8±1^{D}	54±7^{A}	9±1^{D}	39±4^{AB}	38±5^{AB}
Total	**68±23^{BC}**	**102±10^{B}**	**47±5^{C}**	**38±4^{C}**	**45±3^{C}**	**24±3^{C}**	**184±20^{A}**	**25±1^{C}**	**108±10^{B}**	**110±13^{B}**
OXoODEs										
9-OXoODE	166±59^{BCD}	425±124^{ABC}	90±22^{D}	121±19^{CD}	81±17^{D}	65±11^{D}	569±114^{A}	62±12^{D}	488±93^{A}	443±45^{AB}
13-OXoODE	189±58^{BCD}	379±94^{ABC}	105±21^{CD}	126±22^{CD}	105±16^{CD}	85±11^{D}	483±102^{A}	78±9^{D}	443±105^{AB}	424±45^{AB}
Total	**356±117^{BCD}**	**804±217^{ABC}**	**194±43^{D}**	**247±41^{CD}**	**186±32^{D}**	**150±21^{D}**	**1052±194^{A}**	**141±19^{D}**	**931±193^{A}**	**866±88^{AB}**
Dihomo-Gamma-Linolenic Acid Derived Oxylipins										
HETrE										
15-HETrE	13±2^{BC}	19±1^{B}	13±2^{BC}	10±2^{C}	13±2^{BC}	12±2^{BC}	33±4^{A}	15±2^{BC}	18±4^{BC}	33±4^{A}
Arachidonic Acid Derived Oxylipins										

Oxylipin	Canola/Flax	Soy	Canola	High Oleic Canola/ Canola	High Oleic Canola	Lard/Soy	Safflower	Lard/Soy Baseline	Low Fat Soy	Low Fat Soy Baseline
					DHETs					
5,6-DHET	8±2	10±2	6±2	8±1	8±1	6±1	12±2	8±3	9±2	11±3
11,12-DHET	14±2^{B}	13±1^{B}	14±2^{B}	14±1^{B}	22±3^{A}	10±2^{B}	17±1^{AB}	14±2^{B}	13±2^{B}	16±2^{AB}
14,15-DHET	11±2	12±1	11±1	11±1	13±1	10±1	16±2	13±1	10±1	13±1
Total	**33±5^{AB}**	**35±3^{AB}**	**31± 3^{AB}**	**33±4^{AB}**	**43±4^{AB}**	**26±3^{B}**	**46±5^{A}**	**35±4^{AB}**	**34±4^{AB}**	**43±5^{AB}**
					HETEs					
5-HETE	541±74^{B}	676±35^{AB}	549±45^{B}	609±60^{AB}	755±75^{AB}	521±63^{B}	878±100^{A}	803±67^{AB}	699±64^{AB}	893±81^{A}
8-HETE	80±19	93±24	75±11	95±13	88±18	79±13	143±17	113±11	103±25	138±15
9-HETE	232±37^{AB}	304±29^{AB}	202±34^{B}	224±36^{B}	292±49^{AB}	262±37^{AB}	393±64^{AB}	307±38^{AB}	231±52^{AB}	426±38^{A}
11-HETE	134±16	155±15	138±16	155±11	163±24	145±26	221±38	162±12	156±20	182±16
12-HETE	140±30^{D}	206±32^{ABCD}	147±26^{CD}	162±17^{BCD}	191±29^{ABCD}	224±28^{ABCD}	325±42^{A}	307±48^{AB}	190±34^{ABCD}	296±33^{ABC}
15-HETE	490±85^{B}	547±52^{AB}	549±75^{AB}	513±65^{AB}	593±94^{AB}	622±90^{AB}	773±81^{AB}	731±86^{AB}	581±93^{AB}	905±115^{A}
16-HETE	5±5	7±4	trace	7±4	11±5	2±2	17±6	16±5	7± 5	16±5
Total	**1621±236^{D}**	**1989±159^{CD}**	**1659±178^{D}**	**1765±172^{CD}**	**2093±250^{BCD}**	**1854±237^{CD}**	**2749±307^{AB}**	**2439±198^{ABC}**	**1967±250^{CD}**	**2859±265^{A}**
					Prostanoids					
15d PGD_2	6±2	5±1	3±1	3±1	7±2	6±2	9±3	4±1	5±1	6±2

Oxylipin	Canola/Flax	Soy	Canola	High Oleic Canola/ Canola	High Oleic Canola	Lard/Soy	Safflower	Lard/Soy Baseline	Low Fat Soy	Low Fat Soy Baseline
PGD_2	95±15	104±13	81±14	71±12	74±9	77±12	96±27	90±13	81±10	101±13
PGE_2	37±12	30±4	21±5	29±4	17±2	22±3	3±1	18±4	18±2	24±2
6-k-$PGF_{1\alpha}$	36±8	37±5	33±7	32±4	34±5	44±8	46±7	25±2	29±6	27±3
$PGF_{2\alpha}$	43±15	26±5	28±5	27±3	28±3	33±6	42±9	32±5	25±5	29±3
TXB_2	21±7	21±3	19±4	17±3	25±3	22±5	27±6	16±5	19±4	20±4
12-HHTrE	112±55	272±69	377±108	204±77	160±89	258±80	275±106	216±73	220±103	438±93
Total	**350±61**	**496±71**	**563±127**	**381±93**	**346±104**	**461±95**	**538±140**	**402±82**	**397±118**	**644±97**
Total Omega 6 Derived Oxylipins										
Total	**4193±853^{BC}**	**6664±684^{AB}**	**3760±387^{C}**	**3503±345^{C}**	**3908±441^{CD}**	**3288±381^{C}**	**8961±900^{A}**	**4043±352^{CD}**	**7051±806^{A}**	**8750±725^{A}**
n6:n3 Oxylipin Ratio										
Oxylipin Ratio	**6±0^{B}**	**14±1^{B}**	**10±1^{B}**	**14±1^{B}**	**28±4^{B}**	**31±3^{B}**	**210±28^{A}**	**26±3^{B}**	**12±1^{B}**	**12±1^{B}**

Data are expressed as mean ± standard error. Abbreviations used: hydroxyeicosatetraenoic acid (HETE), dihydroxyoctadecenoic acid (DiHOME), dihydroxyeicosatrienoic acid (DHET), hydroxyeicosatrienoic acid (HETrE), oxooctadecadienoic acid (OXoODE), hydroxyheptadecatrienoic acid (HHTrE), prostaglandin (PG), and thromboxane (TX). Trace was defined as < 0.2 ng/mg dry tissue.

Altering the LA or ALA content of the diets resulted in marked changes in oxylipins derived directly from these fatty acids. This is illustrated most clearly when comparing kidneys from rats provided HF diets with the greatest differences in LA or ALA. When kidneys from rats provided the safflower oil diet with 73% LA were compared to kidneys from rats given the lard/soy diet with 8% LA, HODE levels were six times higher, and the HODE metabolites (OXoODEs) followed a similar pattern, being seven times higher. In addition, metabolites of the LA epoxygenase pathway (DiHOMEs) were almost eight times higher in kidneys of rats given safflower compared to the lard/soy group. Similarly, ALA metabolites were higher when more dietary ALA was provided in the diet, as shown in kidneys from rats provided the canola/flax diet with 20% ALA compared to the safflower diet with 0.2% ALA. Kidneys from rats given the canola/flax diet had 36 times higher levels of HOTrEs. Lack of standards for HOTrE metabolites for epoxygenase metabolites of ALA (analagous to OXoODEs and DiHOMEs produced from LA, respectively) precluded analysis of these ALA derived metabolites.

The effect of dietary LA and ALA levels on their respective oxylipins did not appear to be inhibited by higher dietary levels of their n-6 or n-3 counterparts. For example, when comparing kidneys from the soy and canola diets, both ALA and resulting renal HOTrE levels were similar, despite the level of dietary LA and renal HODEs being approximately three times higher in the soy group. In the reverse situation comparing canola/flax, canola, high oleic canola/canola and high oleic canola diets, the dietary levels of LA and resulting renal HODE levels were similar, despite the range of dietary ALA and renal HOTrEs differing by tenfold between the highest and lowest ALA groups.

Diets higher in ALA also positively influenced the production of oxylipins from its

longer chain derivatives. When kidneys from rats provided the canola/flax diet with 20% ALA were compared to those of the canola diet with 8% dietary ALA, 18-HEPE levels were 3.5-fold higher with no influence on n-6 oxylipin levels. The increase in 18-HEPE could be due to the fact that there was 2.5-fold more EPA in the renal tissue of the canola/flax group. In contrast, DHA oxylipins (4-HDoHE and 19,20-DiHDPA) did not change with more dietary ALA, likely due to the similar quantities of DHA in the renal tissue between groups. In the opposite situation, greater quantities of LA while ALA remained constant had no influence on EPA or DHA derived oxylipin levels in the renal tissue. In contrast, the longer chain derivative of LA, ARA, appeared to not be influenced by a significant increase in dietary LA, and only a significant change in a couple of ARA derived oxylipins was observed; greater levels of LA (lard/soy with 8% LA and safflower with 73% LA) only resulted in more of total DHETs and 5-HETE.

Oxylipins and Renal Pathology

A rationale for examining the kidneys in these obese rats with early stage ORG was the prior observation that diets higher in ALA appeared to provide a renoprotective effect (Chapter 2). To examine potential relationships between oxylipins and the progression of renal injury in this model, correlation analyses of renal oxylipin levels with glomerular volume, a marker of early ORG was performed. This analysis revealed associations between only four oxylipins and MGV (all with $r = -0.3$, $p < 0.015$) (Figure 3.1). Notably, all four oxylipins were formed from ALA, either directly (to form 9- and 13-HOTrE) or indirectly via desaturation/elongation to EPA and DHA (to form 5-HEPE and 4-HDoHE, respectively). All other oxylipins including the LA, ARA, and DGLA derived oxylipins were not significantly correlated to MGV.

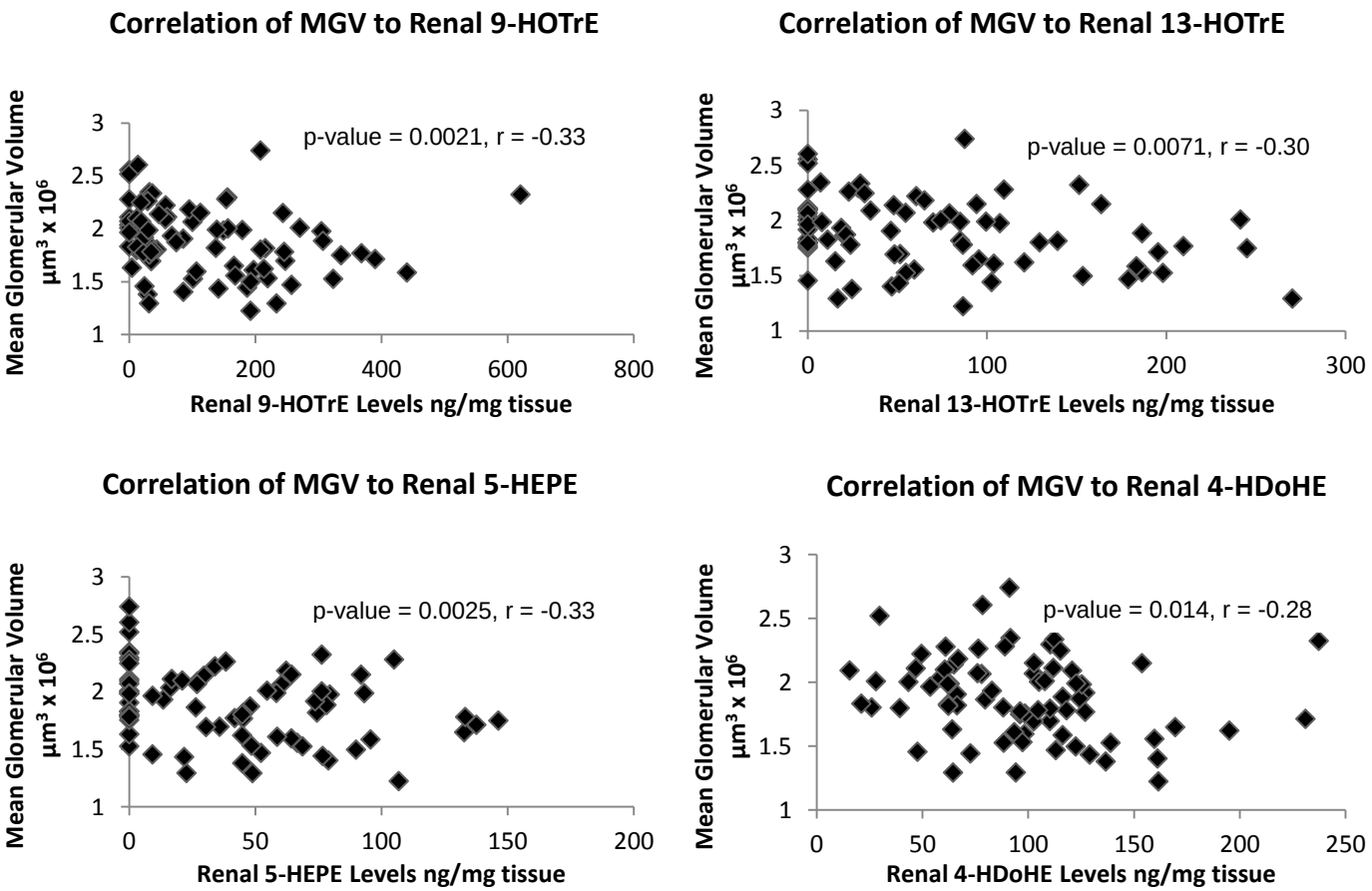

Figure 3.1: Correlation between Omega 3 Derived Metabolites and Mean Glomerular Volume. Data points are represented as means (n=80, 8/group). The correlation analysis was carried out using Spearman's correlation.

Renal Phospholipid Fatty Acids

The renal phospholipid fatty acid composition was examined to determine the effect of diet on the composition of oxylipin precursors. ALA, LA, and EPA were influenced significantly by dietary fatty acid compositions, whereas ARA did not change across dietary groups and DHA was only lower in the safflower group (Table 3.4). The safflower group which provided the most LA, had greater renal LA than the canola, high oleic canola/canola, high oleic canola, and lard/soy groups. The rats provided ALA rich diets had greater levels of n-3 fatty acids; the canola/flax group had the greatest renal levels of ALA and EPA. The renal phospholipid fatty acid data is also available in nmol% in the Appendices section, Table 5.7.

Renal HOTrE and 5-HEPE levels were correlated to renal ALA (Figure 3.2 and 3.3); however, renal 4-HDoHE was not significantly correlated to renal ALA (p-value = 0.06). Renal HOTrEs could be estimated by renal ALA using the following logistic regression equation: Renal HOTrEs (ng/mg tissue) = -10.5 + 0.4 (renal ALA (ng/mg dry tissue)). Renal 5-HEPE could be estimated by renal ALA using the following logistic regression equation: Renal 5-HEPE (ng/mg tissue) = -1.9 + 0.02 (renal ALA ng/mg dry tissue). Interestingly, renal LA did not appear to compete with ALA for metabolism to their respective oxylipins. In fact, in a logistic regression equation with ALA, LA had a trend for a positive relationship with renal HOTrEs, albeit not significant (p = 0.27).

Table 3.4- Renal Phospholipid Fatty Acid Composition (ng/mg dry tissue) by Diet Group

Fatty Acid	Canola/Flax	Soy	Canola	High Oleic Canola/ Canola	High Oleic Canola	Lard/Soy	Safflower	Lard/Soy Baseline	Low Fat/Soy	Low Fat/Soy Baseline
C14:0	75±13	66±2	67±6	73±7	74±6	87±11	62±4	79±4	91±25	78±3
C14:1	73±18	72±7	55±4	68±1	67±15	83±2	58±1	61±6	82±9	87±12
C16:0	11394±1129	11776±114	12105±331	12368±313	11734±508	10849±709	10730±717	10244±717	12452±812	12901±642
C16:1	215±63	145±8	298±73	308±73	231±58	275±50	114 ±8	215±17	313±57	330±19
C17:0	212±18^{BCD}	233±5^{BC}	237±18^{BC}	259±6^{B}	24±10^{BC}	342±28^{A}	199±6^{CD}	319±25^{A}	159±19^{D}	160±5^{D}
C17:1	26±15^{D}	32±16^{CD}	51±4^{BC}	60±4^{AB}	61±4^{AB}	78±5^{A}	Trace^{E}	78±6.6^{A}	Trace^{E}	Trace^{E}
C18:0	10741±1101	11562±190	10898±434	11379±413	10781±251	10974±1015	10950±842	10779±727	11204±901	11331±707
C18:1	6055±551^{AB}	4240±32^{CDE}	6470±205^{A}	6613±170^{A}	6468±222^{A}	5205±463^{BC}	3428±157^{E}	4649±255^{CD}	3610±314^{E}	3687±267DE
C18:2n6	6849±698^{AB}	7810±171^{A}	5840±284^{BC}	5519±358^{BC}	4530±73^{C}	4871±496^{C}	7421±595^{A}	4881±497^{C}	8134±673^{A}	8116±484^{A}
C18:3n3	366±36^{A}	177±11^{B}	171±5^{B}	105±6^{C}	Trace^{D}	Trace^{D}	Trace^{D}	Trace^{D}	190±9^{B}	196±16^{B}
C20:0	142±13	153±7	148±8	150±5	147±3	142±13	143±10	134±4	137±17	135±9
C20:1	105±5^{A}	Trace ^{C}	129±5^{A}	140±15^{A}	140±8^{A}	56±20^{B}	Trace^{C}	53±19^{B}	Trace^{C}	43±15^{B}
C20:2	87±9^{C}	202±6^{B}	89±5^{C}	94±7^{C}	84±4^{C}	67±6^{C}	302±32^{A}	80±8^{C}	200±16^{B}	199±17^{B}
C20:3n6	467±45	397±11	394±25	425±34	347±12	397±33	337±32	383±37	435±64	467±19
C20:4n6	11935±1306	12708±206	13510±427	13987±890	14413±686	14541±1388	12920±1231	13054±1137	13589±1497	13575±959
C20:3n3	103±12^{A}	71±3^{AB}	55±18^{AB}	66±5^{AB}	45±16^{AB}	38±22^{AB}	14±14^{B}	56±19^{AB}	75±4^{AB}	86±7^{AB}
C20:5n3	381±27^{A}	98±3^{C}	153±13^{B}	112±13^{BC}	Trace^{D}	44±15^{D}	Trace^{D}	31±19^{D}	110±18^{BC}	109±8^{BC}

Fatty Acid	Canola/Flax	Soy	Canola	High Oleic Canola/ Canola	High Oleic Canola	Lard/Soy	Safflower	Lard/Soy Baseline	Low Fat/Soy	Low Fat/Soy Baseline
C22:0	234±24	290±24	242±15	252±8	243±9	299±28	270±18	288±14	262±22	244±15
C22:4n6	154±18^{E}	281±12^{BCD}	236±14^{D}	266±13^{CD}	323±17^{BC}	341±30^{B}	444±36^{A}	282±18^{BCD}	288±32^{BCD}	277±21^{BCD}
C22:5n3	278±24^{A}	171±4^{CD}	188±10^{BCD}	154±10^{D}	85±5^{E}	98±9^{E}	Trace^{F}	95±10^{E}	217±25^{B}	211±15^{BC}
C22:6n3	860±97^{A}	761±41^{A}	938±48^{A}	937±61^{A}	862±62^{A}	919±90^{A}	285±16^{B}	747±86^{A}	822±110^{A}	784±59^{A}
C24:0	1456±170	1701±145	1384±100	1417±64	1311±47	1607±179	1562±135	1599±66	1783±119	1663±72
C24:1	761±85^{AB}	375±16^{C}	842±48^{B}	940±76^{A}	988±48^{A}	393±42^{C}	327±24^{C}	356±13^{C}	383±27^{C}	373±21^{C}
Total n-6	**19491±2073**	**21397±389**	**20069±673**	**20292±1188**	**19698±717**	**20216±1872**	**21424±1922**	**18679±1644**	**18265±1184**	**22637±1483**
Total n-3	**1990±193^{A}**	**1278±59^{ABC}**	**1506±84^{ABC}**	**1374±86^{ABC}**	**992±55^{BCD}**	**1099±110^{BCD}**	**300±31^{D}**	**931±113^{BCD}**	**1867±417^{AB}**	**1385±101^{ABC}**
n-6:n-3	**10:1^{F}**	**17:1^{CD}**	**13:1^{E}**	**15:1DE**	**20:1^{B}**	**18:1^{BC}**	**71:1^{A}**	**20:1^{B}**	**10:1^{CDE}**	**16:1^{CD}**

Mean ± standard error without a common letter differ significantly ($p<0.05$). Trace was defined as < 10 ng/mg dry tissue.
Data is also expressed as nmol% in the Thesis Appendices Table 6.8.

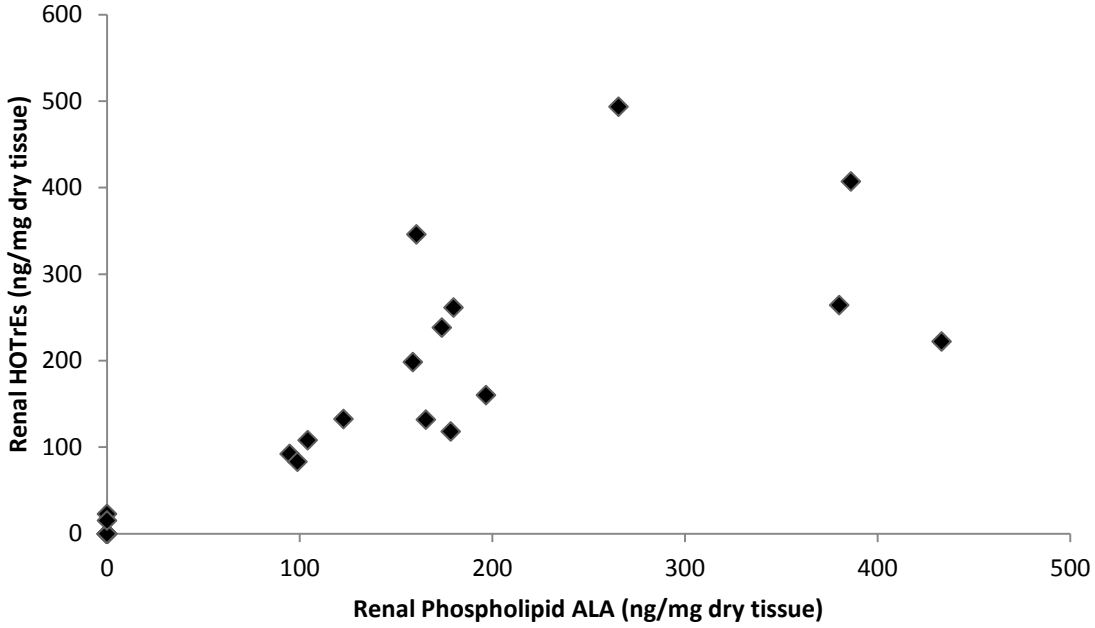

Figure 3.2- Correlation between Renal ALA and HOTrE Levels. Data points are represented as means and include all end point HF groups (n=27, (3-4/group)). The correlation analyses were carried out using Pearson's correlation (p-value < 0.0001, r = 0.93).

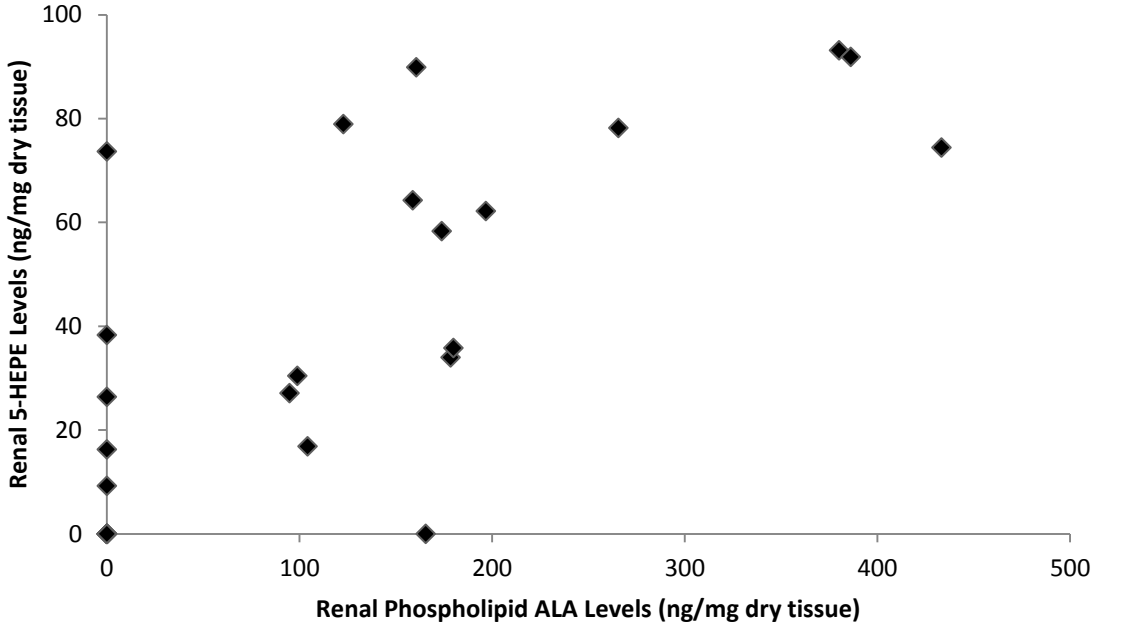

Figure 3.3: Correlation between Renal ALA and 5-HEPE. Data points are represented as means and include all end point HF groups (n=27, (3-4/group)). The correlation analyses were carried out using Pearson's correlation (p-value <0.0001, r = 0.72).

Conversion and Product/Precursor Ratios

Oxylipin quantities could be influenced by the levels of their respective enzymes that metabolize them from fatty acids. However, upon examination of the protein levels of select enzymes involved in oxylipin formation, there were no significant differences observed between groups for COX-1, COX-2, 5-LOX, 12/15-LOX, 15-LOX 2, sEH, or cytochrome P450 2c23 (available in supplemental data – figure S1 and table S5). Yet, there were differences in total enzyme activity as indicated by *in vitro* production (Appendices Table 5.7). The canola/flax group experienced the greatest production of 9- and 13-HOTrE while the safflower group experienced the largest production of 9-HODE, 9,10- and 12,13-DiHOME.

In addition, the amount of oxylipin produced per ng of fatty acid by LOX was different across various substrates and influenced the *in vivo* levels of oxylipins. LOXs were of key interest due to the significant influence of the dietary fatty acid composition on LOX products. The products of LOX were compared to one another; it is understood that 15-LOX can metabolize ALA into 13-HOTrE, LA into 13-HODE, (Collins et al., 2008), ARA into 15-HETE (Fogh & Kragballe, 2000), and DGLA into 15-HETrE (Ziboh et al., 2000). 5-LOX can metabolize ALA into 9-HOTrE (Galliard & Phillips, 1971), LA into 9-HODE (Galliard & Phillips, 1971) (Lopez-Nicolas, Perez-Gilabert, & Garcia-Carmona, 1999), ARA into 5-HETE (Ghosh & Myers, 1997), EPA into 5-HEPE (Laegreid, et al., 1988), and DHA into 4-HDoHE (Lee et al., 1984). Therefore, the products of 15-LOX and 5-LOX were grouped and the oxylipins were divided by the fatty acid substrate to calculate the relative amount of oxylipin produced by the respective enzyme.

The product to precursor ratios revealed that ALA and EPA were converted to their respective metabolites more than LA, AA or DHA by LOXs. For the 15-LOX reaction, 13-HOTrE was formed from ALA 4 times as much as 13-HODE from LA and 13 times as much as DGLA and ARA to their respective oxylipins (Figure 3.4). For the 5-LOX reaction, 9-HOTrE was produced from ALA approximately 2 times more than 5-HEPE from EPA, on average 6 times more than LA and DHA, and approximately 16 times more than ARA to their respective oxylipins (Figure 3.5).

Differences in product to precursor ratios were also observed between dietary groups; more HODEs from LA were produced in the safflower group compared to the other dietary groups for both 5-LOX and 15-LOX products. In addition, the HF soy group produced 3 and 2 times more 5-HEPE per ng of EPA than the HF canola/flax and HF high oleic canola/canola groups respectively for 5-LOX (Figure 3.5).

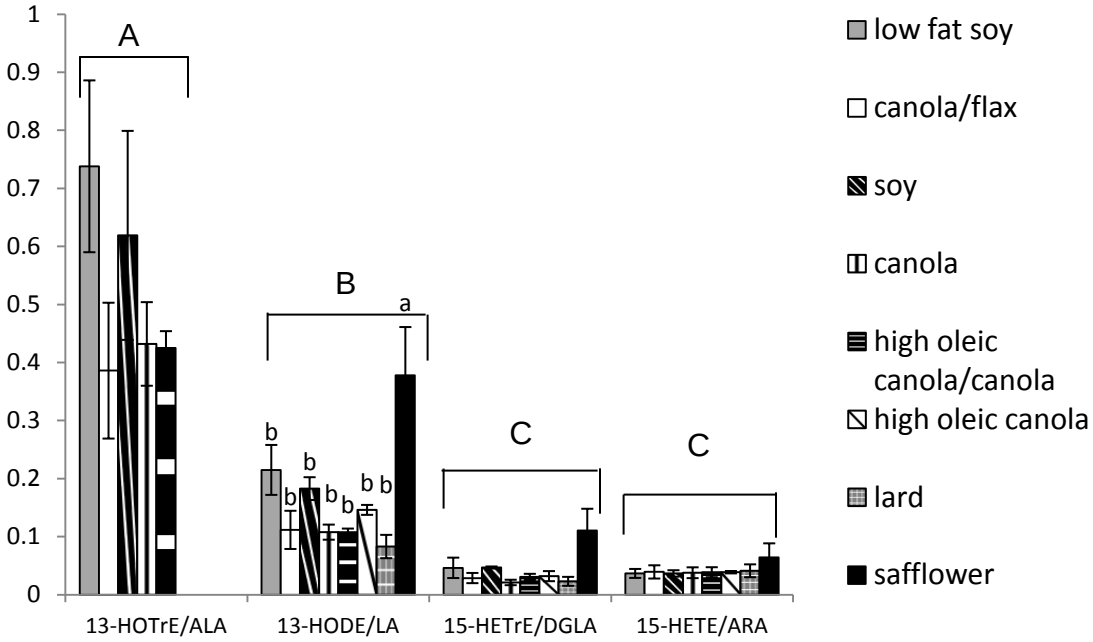

Figure 3.4 – Product to Precursor Ratios of 15-Lipoxygenase Products by Substrate and Dietary Group. The graph represents mean ± standard error (n=4/diet group). ANOVA followed by Duncan's Multiple Range test assessed differences across substrates. A separate ANOVA was run for groups within each substrate classification followed by Duncan's Multiple Range test. Differing uppercase letters indicate differences for the enzyme selectivity of 15-lipoxygenase for substrates ($p<0.0001$). Differing lower case letters indicates the differences between dietary groups for oxylipin production ($p<0.001$).

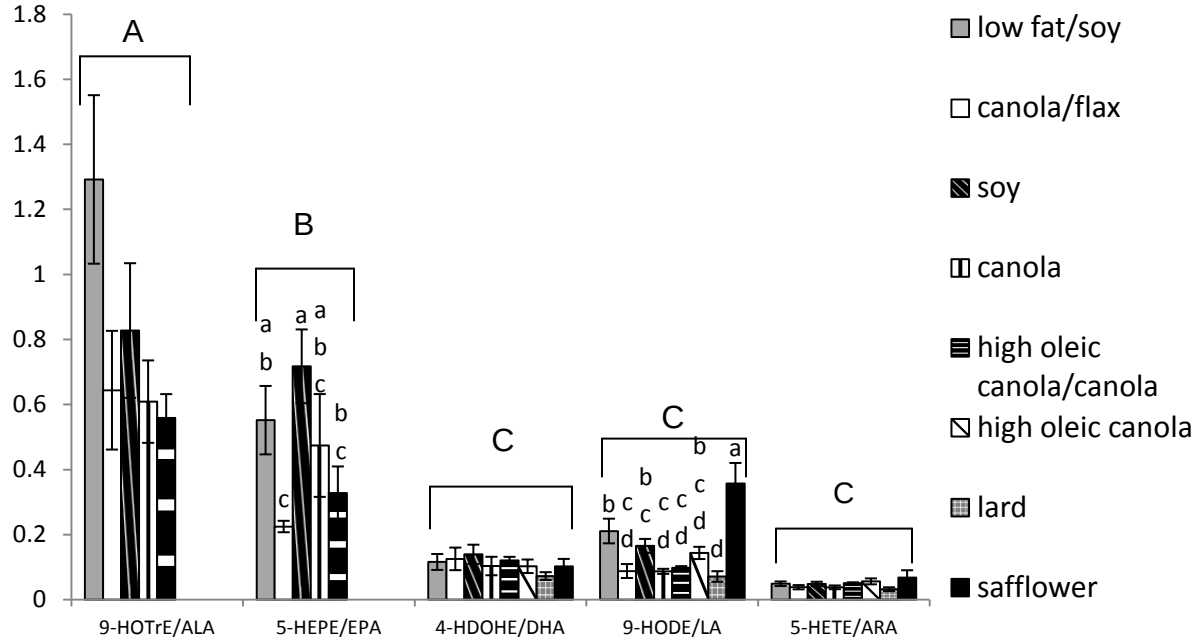

Figure 3.5: Product to Precursor Ratios of 5-Lipoxygenase Products by Substrate and Dietary Group. The graph represents mean ± standard error (n=4/diet group). ANOVA followed by Duncan's Multiple Range test assessed differences across substrates and dietary groups. A separate ANOVA was run for groups within each substrate classification followed by Duncan's Multiple Range test.

Differing uppercase letters indicate differences for the enzyme selectivity of 5-lipoxygenase for substrates ($p<0.0001$). Differing lower case letters indicate the differences between dietary groups for oxylipin production ($p<0.05$).

Discussion

Oxylipins are large contributors to the influence of fatty acids in health and disease progression. The current study examined the renal oxylipin profile and the influence of varying dietary LA and ALA levels and ratios by scanning for 64 oxylipins under the influence of eight different dietary treatments. Of the 64 oxylipins scanned, 30 renal oxylipins were quantified of which 18 were influenced by diet; in particular, the canola/flax diet resulted in the lowest n-6:n-3 oxylipin ratio of 6:1. N-3 oxylipins such as the HOTrEs, 4-HDoHE, 5-HEPE, and 18-HEPE were highest in the HF canola/flax group of which may promote health; 18-HEPE is a pre-cursor to both resolvin E_1 and resolvin E_2 which play an important role in the resolution of inflammation (Serhan & Petasis, 2011a). HOTrEs, 4-HDoHE, and 5-HEPE may be protective due to their inverse relationship with glomerular volume as seen in the current study; diets high in ALA such as canola/flax, canola, and soy enriched diets have been able to slow the progression of glomerulomegaly in ORG and this may be due to the influence of ALA on the oxylipin profile (Chapter 2).

Despite high levels of n-3 oxylipins in the HF canola/flax group, the n-6 oxylipins were not lower when ALA levels were doubled and LA was the same. Even when dietary LA levels were relatively similar and ALA was greater by 10 times (HF canola/flax versus HF high oleic canola diet) there was no significant decrease in n-6 oxylipins. Therefore, in order to decrease the quantity of renal n-6 oxylipins, a decrease in n-6 fatty acid intake would have to occur. A similar finding was found by Keenan et al.; male and female adults consumed n-3 ethyl ester supplements (900 mg total of n-3s) and were able to increase serum n-3 oxylipins but not decrease the n-6 oxylipins (Keenan et al., 2012).

But this is contrary to previous findings that report *ex vivo* production of eicosanoids in other tissues in the presence of inflammation. For example, supplementation with EPA and DHA reduced *ex vivo* synthesis of PGE_2 in peripheral blood mononuclear cells in humans (Trebble et al., 2003), flaxseed supplementation with and without fish oil supplementation reduced *ex vivo* production of PGE_2 and TXB_2 in mononuculear cells (Caughey, Mantzioris, Gibson, Cleland, & James, 1996), and EPA and DHA supplementation in mice reduced HETE levels in the colon (Neilson et al., 2012).

The current study supports the theory that a high level of dietary LA results in high levels of n-6 oxylipins; the HF canola diet provided 18% of fat as LA and the HF soy group provided 53% as LA with similar levels of ALA, and significantly more 9-HODE, total HODEs, total DiHOMEs, 12,13-DiHOME, total OXoODEs, and 9-OXoODE was observed. The metabolites of linoleic acid, HODEs, constituted a significant proportion of the renal oxylipins and may therefore play a very crucial role in renal health. HODEs have been implicated in oxidative stress (Horie et al., 2012; Morita et al., 2012; Wang et al., 2008) but may also be protective against tumor cell adhesion (Honn et al., 1992). In the present study, there were no correlations between 9- or 13-HODE and pathology; perhaps in further stages of renal disease, HODEs may play a more significant and clear role. Other n-6 oxylipins such as DiHOMEs are implicated in the inflammatory process by inducing chemotaxis (Totani et al., 2000) and creating a cytotoxic effect in renal proximal tubular cells (Moran, Weise, Schnellmann, Freeman, & Grant, 1997). In addition, OXoODEs have been implicated in oxidative stress by being products of lipid oxidation (Feldstein et al., 2010). Therefore, a higher consumption of LA may result in greater levels of harmful oxylipins. The current findings suggest that

perhaps dietary recommendations should be placed on dietary levels of ALA and LA rather than ratios, as the renal oxylipin profile was significantly influenced by the individual levels of ALA and LA as a proportion of total fat.

Despite the influence of varying ALA and LA levels on renal oxylipins, differing levels of fat by percentage of energy did not influence oxylipins; the LF and HF soy diets (25% versus 55%) did not differ in renal pathology (Chapter 2), *in vivo* oxylipin levels nor the amount of oxylipin produced by LOXs. This is contrary to previous findings where mice underwent *in vivo* peritoneal induced oxidative stress and experienced lower levels of PGE_2, 5-series leukotrienes, and 4-series leukotrienes at differing n-6:n-3 dietary ratios when provided high fat diets compared to rats provided low fat diets (Broughton & Wade, 2002b).

In addition to the influence of dietary fatty acids, the enzymes that metabolize the substrates into their respective metabolites can also influence the quantities of oxylipins (Sankaran et al., 2007) (Wang et al., 2003). Yet, no significant differences were observed between groups for protein levels of select enzymes responsible for oxylipin production; however, differences in product to precursor ratios were observed which can in part infer the selectivity of LOXs. For example, despite the canola/flax group having greater levels of total HEPEs, both the LF and HF soy groups produced more 5-HEPE per ng of renal phospholipid EPA. It can be hypothesized that perhaps high renal ALA and EPA results in product inhibition (HEPE) at a certain concentration. Or perhaps, high renal levels of ALA and EPA in the canola/flax group down-regulated the efficiency of LOX to produce HEPE. This finding is similar to the phenomenon that the elongase enzymes prefers to metabolize $C_{18}>C_{20}>C_{22}$ fatty acids (Hastings et al., 2004); therefore, with very high

intakes of ALA a lower production of EPA and DHA may be observed in the same way that may be seen for competition over LOX and the production of EPA and DHA derived oxylipins.

In addition, LOXs appeared to preferentially metabolize the n-3 fatty acids more than the n-6 fatty acids with the exception of DHA. Similar findings have been seen for other enzymes involved in fatty acid metabolism such as CYP450 and its preference for EPA and DHA over ARA (Konkel & Schunck, 2011). Toledo et al. (2011) indicated that LOX may have better specificity for LA over arachidonic acid due to LA's flexibility for the LOX structure (Toledo, Masgrau, Lluch, & Gonzalez-Lafont, 2011). The current study observed a similar finding when 2 15-LOX metabolites were compared; more 13-HODE, the metabolite of LA, was produced per ng of LA than 15-HETE produce per ng of ARA.

Once the influence of diet on renal oxylipins was assessed, the examination of oxylipins and renal pathology was evaluated. In this early stage of ORG, intriguingly, the prostaglandins and thromboxane did not change across dietary groups and were not correlated to pathology. Typically, higher levels of prostaglandins and thromboxanes are observed in the kidneys of progressive disease compared to normal animal models (Wakefield et al., 2011; Sankaran et al., 2007) most likely due to their role in the innate immune response and induction of inflammation (Serhan & Petasis, 2011b). The lack of correlation to pathology in the current study could be due to the fact that ORG is not characteristic of progressive inflammation in addition to this model being in the early stages of renal injury. In fact in this early stage of ORG, four ALA derived oxylipins (9-HOTrE, 13-HOTrE, 5-HEPE, and 4-HDoHE) were inversely correlated to MGV and may

be earlier indicators of renal health.

The causes of ORG include increased glomerular hydrostatic pressure, hyperfiltration, (Chagnac et al., 2003; Ribstein, et al., 1995) and systemic inflammation which creates chronic mild inflammation within the glomerulus (Wu, et al., 2006b). The HEPEs and HDoHEs are pre-cursors to the resolvins which possess inflammation resolving properties and therefore could contribute to renoprotection (Serhan et al., 2011). To date, no data exists on HOTrEs', HEPEs', or HDoHEs' ability to alter renal hemodynamics; but perhaps these oxylipins have the ability to dilate vessels like 6-keto $PGF_{1\alpha}$ (Baer, et al., 1979) and 16-HETE in order to prevent increased glomerular hydrostatic pressure (Carroll, et al., 1996).

What is known, is that HOTrEs can influence matrix metalloproteinases (MMPs) which play a role in diabetic nephropathy, a renal disease that has similarities to ORG. Schulze-Tanzil et al. reported that 13-HOTrE suppressed the expression of MMPs -1, -3, and -9 in human chondrocytes *in vitro* (Schulze-Tanzil et al., 2002). MMPs are critically important in the progression of renal disease because dysregulation (increase and decrease) of MMPs have been implicated in the progression of diabetic nephropathy by influencing mesangial matrix expansion and basement membrane thickening (Thrailkill, Clay Bunn, & Fowlkes, 2009; Lelongt, Legallicier, Piedagnel, & Ronco, 2001) and mesangial matrix expansion may occur in ORG (Kambham et al., 2001; Adelman et al., 2001). Significantly more messenger RNA and mean positive area of MMP-9 in the glomeruli was observed in the type 2 diabetic mice with diabetic nephropathy compared to the wild type mice (Qing-Hua et al., 2008). In humans, an increase in urinary MMP-9 was identified with diabetic nephropathy and increased with further progression of

disease (Tashiro et al., 2004). Furthermore, MMPs, more specifically gelatinases, can increase fibrin, a mediator of glomerular injury (Lelongt et al., 2001) and induce a state of inflammation in mesangial cells (Turck, Pollock, Lee, Marti, & Lovett, 1996). To further clarify the role of MMPs in renal disease, Zeisburg et al. (2006) inhibited MMPs early in Alport syndrome, a hereditary progressive renal disease, which caused a delay in proteinuria and preserved glomerular integrity, while inhibition in the later stages was associated with progressive glomerular and interstitial fibrosis (Zeisberg et al., 2006). Therefore, in this early stage of ORG, HOTrEs, which are capable of inhibiting MMPs, may have prevented dysregulation of MMPs, thereby preserving glomerular integrity and prevention of glomerulomegaly.

To conclude, the current study has provided foundational knowledge that dietary levels of ALA and LA can influence the renal oxylipin profile in an experimental model of obesity. We have also been the first to examine the renal oxylipin profile to this extent, identify the absence of competition between ALA and LA for metabolism into oxylipins, assess product to precursor ratios as an insight into LOX selectivity, and identify HOTrEs, HEPEs, and HDoHE in the kidney and their association in the prevention of glomerulomegaly. Lastly, we identified the potential of dietary ALA to tip the balance of the oxylipin profile to potentially be more renoprotective in a model of obesity.

Chapter 3 References

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Supplementary Data for Chapter 3

Table S3.1 – Collision-Induced Dissociation (CID) Mass Transitions and Catalogue Numbers for Deuterated Internal Standards

Oxylipin Internal Standard	CID Mass Transition (Da)	Catalogue Number
6-k-$PGF_{1\alpha}$-d4	373.3>211.1	315210
TXB_2-d4	273.2>173.1	319030
$PGF_{2\alpha}$-d4	357.3>197.1	10007275
PGE_2-d4	355.2>275.2	10007273
PGD_2-d4	355.2>193.1	312010
LTB_4-d4	335.0>195.0	320110
20-HETE-d6	325.0>295.0	390030
15-HETE-d8	327.0>226.0	334720
5-HETE-d8	327.0>116.0	334230
13-HODE-d4	299.0>198.0	338610
9-HODE-d4	299.0>172.0	338410
12,13-DiHOME-d4	317.0>185.0	10009994
14,15-DHET-d11	348.0>206.6	10008040
9,10-DiHOME-d4	317.0>203.0	10009993
11,12-DHET-d11	348.0>167.0	10007975
15d-PGJ_2-d4	319.0>275.0	318570
8,9-DHET-d11	348.0>127.0	10009998
5-OxoETE-d7	323.0>209.0	334250

Table S3.2 – CID Mass Transitions, Surrogate Deuterated Internal Standards, and Detector Response Factors

Oxylipin	CID Mass Transition (Da)	Internal Standard	Detector Response Factor
	HOTEs		
9-HOTrE	293.0>171.0	9-HODE-d4	0.67
13-HOTrE	293.0>195.0	13-HODE-d4	0.39
	HEPEs		
5-HEPE	317.0>115.0	5-HETE-d8	0.88
18-HEPE	317.0>215.0	15-HETE-d8	0.43
	HDoHEs		
4-HDoHE	343.2>101.0	5-HETE-d8	3.86
	HODEs		
9-HODE	295.0>171.0	9-HODE-d4	1.73
13-HODE	295.0>195.0	13-HODE-d4	1.62
	DHETs		
5,6-DHET	337.0>145.0	11,12-DHET-d11	0.85
11,12 DHET	337.0>167.0	11,12-DHET-d11	2.12
14,15-DHET	337.0>207.0	14,15-DHET-d11	1.36
	DiHDPAs		
19,20-DiHDPA	361.5>228.7	14,15-DHET-d11	0.15
	DiHOMEs		
9,10-DIHOME	313.0>201.0	9,10-DiHOME-d4	3.94
12,13-DIHOME	313.0>183.0	12,13-DiHOME-d4	2.99
	HETEs		
5-HETE	319.0>115.0	5-HETE-d8	1.43
8-HETE	319.0>155.0	5-HETE-d8	1.16
9-HETE	319.0>151.0	5-HETE-d8	0.45
11-HETE	319.0>167.0	5-HETE-d8	5.18
12-HETE	319.0>179.0	15-HETE-d8	2.58
15-HETE	319.0>219.0	15-HETE-d8	1.17
16-HETE	319.0> 232.8	15-HETE-d8	0.99

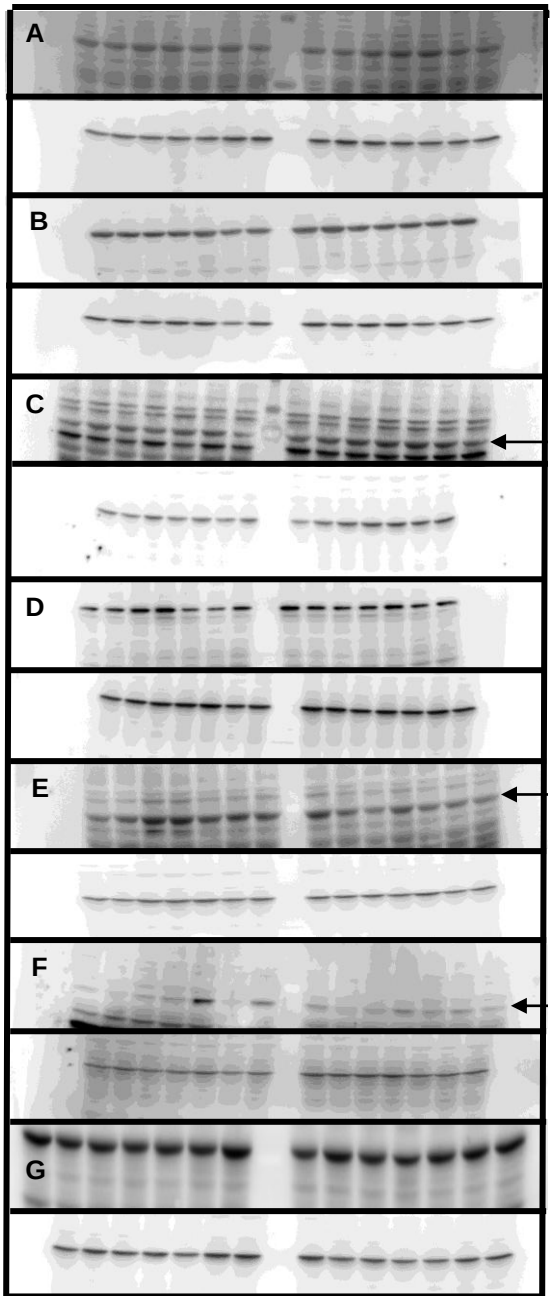
Oxylipin	CID Mass Transition (Da)	Internal Standard	Detector Response Factor
HHTres			
12-HHTre	279.2>217.0	15-HETE-d8	0.16
HETrEs			
15-HETrE	321.2>221.0	13-HODE-d4	3.74
OXoODEs			
9-OXoODE	293.0>185.0	5-OxoETE-d7	2.97
13 –OXoODE	293.0>113.0	5-OXoETE-d7	7.52
Prostanoids			
15d PGD_2	333.0>271.0	15-d-PGJ_2-d4	8.24
PGD_2	351.2>189.1	PGD_2-d4	1.03
PGE_2	351.2>271.2	PGE_2-d4	3.77
6-keto-$PGF_{1\alpha}$	369.2>163.0	6-keto-$PGF_{1\alpha}$-d4	1.64
$PGF_{2\alpha}$	353.2>193.1	$PGF_{2\alpha}$-d4	3.37
TXB_2	369.2>169.1	TXB_2-d4	1.38

Table S3.3 – Oxylipins and CID Mass Transitions Scanned but Undetected/Not Quantified

Oxylipin	CID Mass Transition
TxB_3	367.0>169.0
$PGF_{3\alpha}$	351.0>193.0
8-iso $PGF_{2\alpha}$	353.0>193.0
PGE_3	349.0>269.0
11-dehydro TXB_2	367.2>161.0
11-dehydro-TXB_3	365.2>303.0
PGD_3	349.0>269.0
13,14-dehydro-15-keto-$PGF_{2\alpha}$	353.0>291.0
17(R)-Resolvin D_1	375.5>121.2
Resolvin D_1	375.0>215.0
LTB_4	335.0>195.0
LTC_4	624.6>272.0
LTD_4	495.0>177.0
LTE_4	438.0>333.0
15-HEPE	317.0>175.0
11-HEPE	317.0>195.0
12-HEPE	317.0>179.0
8-HEPE	317.0>155.0
9-HEPE	317.0>123.0
8,9-DHET	337.0>155.0
15d-PGJ_2	315.0>271.0
20-HETE	319.0>289.0
19-HETE	319.0>275.0
18-HETE	319.0>261.0
17-HETE	319.0>247.0
5,6-LXA_4	351.2>115.0
14,15-EET	319.0>219.0
11,12-EET	319.0>167.0
8,9-EET	319.0>127.0
5,6-EET	319.0>191.0
15(R)-LXA_4	351.5>217.5
14-HDoHE	343.3>161.0
5(S),15(S)-DiHETE	335.2>201.0
13(S)-HOTE γ	293.2>193.0

Table S3.4 – HPLC Solvent Gradient

Total Time (min)	Solvent A (%)
0.0	100.0
0.5	100.0
2.0	75.0
9.0	55.0
10.0	40.0
14.0	25.0
14.5	10.0
15.0	0.0
17.0	0.0
19.0	100.0
35.0	100.0



Western blots are denoted by the following letters and are always followed by the corresponding western blot of ß-actin (45 kDa). A: 15-lipoxygenase 2 (75 kDa), B: 12/15-lipoxygenase (75 kDa), C: 5-lipoxygenase(78 kDa), D: Cyclooxygenase 1 (69 kDa), E: Cyclooxygenase 2 (70 kDa), F: Soluble epoxide hydrolase (62.5 kDa), G: CYP2c23 (56 kDa). Western blots contain bands from all dietary groups (n=8/group). All statistical tests (Kruskal-Wallis) for the enzymes had a p-value >0.05 and were not statistically different across the dietary groups (n=6/group).

Figure S3.1: Western Immunoblotting Images of Select Enzymes and the Corresponding ß-actin

Table S3.5-Western Immunoblotting Protein Levels of Select Enzymes (Arbitrary Units) (n=6/group).

Enzyme	Canola/Flax	Canola	Soy	High Oleic Canola/Canola	High Oleic Canola	Lard/Soy	Safflower	Lard/Soy Baseline	Low Fat/Soy	Low Fat/Soy Baseline	p-value
5-LOX	1.8±0.4	2.0±0.5	1.3 ±0.3	1.7±0.4	1.9±0.5	1.5±0.3	1.8±0.2	1.5±0.3	1.1±0.3	1.3±0.2	0.80
12/15-LOX	0.9±0.2	0.8±0.1	0.8±0.2	0.9±0.1	1.0±0.2	0.8±0.1	0.9±0.2	1.0±0.2	0.6±0.1	0.7±0.1	0.50
15-LOX 2	1.0±0.2	1.1±0.2	0.9±0.1	1.2±0.2	1.2±0.2	1.1±0.2	1.1±0.2	1.2±0.2	1.0±0.2	0.8±0.1	0.84
COX-1	1.0±0.2	1.5±0.4	1.4±0.3	1.9±0.3	2.0±0.5	1.8±0.4	2.0±0.5	1.8±0.4	1.3±0.2	0.9±0.2	0.27
COX-2	1.2±0.4	1.5±0.5	1.2±0.3	1.4±0.3	1.3±0.3	1.3±0.2	1.6±0.4	1.4±0.3	1.1±0.2	1.1±0.2	0.98
sEH	1.2±0.2	1.0±0.4	1.3±0.4	1.0±0.1	0.9±0.3	2.8±1.4	1.5±0.4	1.1±0.3	2.8±2.2	1.0±0.3	0.79
CYP2c23	1.2±0.2	1.1±0.1	1.0±0.1	1.2±0.1	1.1±0.2	1.0±0.1	1.1±0.1	0.9±0.1	0.8±0.1	0.9±0.05	0.26

X. CHAPTER 4 - THESIS DISCUSSION

The current study utilized the DIO animal model which presented with ORG similar to that of humans by developing obesity, proteinuria, glomerulomegaly, scarce glomerular damage with no increase in serum creatinine, little to no interstitial fibrosis, or tubular atrophy (Kambham et al., 2001) (Adelman et al., 2001) (Praga et al., 2001). The establishment of an accurate animal model led to the finding of glomerulomegaly as the first structural change of ORG. Diagnostic guidelines for ORG have yet to be approved and glomerulomegaly currently utilized as a diagnostic marker for renal diseases such as diabetic nephropathy (Tervaert et al., 2010). The current study provided evidence that glomerulomegaly can act as a surrogate indicator for the presence of glomerular damage and potential of further renal damage; therefore, glomerulomegaly may be used as an early diagnostic marker for ORG which can facilitate earlier commencement of treatment and hopefully the improvement of quality of life and survival outcomes for patients.

The finding of an early diagnostic marker facilitated the investigation of a potential early dietary intervention with fatty acids. The DIO animals were provided diets with varying levels of ALA and LA; it was concluded that the diets supplying the greatest levels of ALA (HF canola/flax, HF soy, and HF canola) resulted in the greatest renal ALA and was associated with a slowed progression of glomerulomegaly and glomerular damage. The potential mechanism of action could be the direct anti-inflammatory effects of ALA by reducing renal cell apoptosis and endoplasmic reticulum stress (Katsoulieris et al., 2009) or a reduction in glomerular hydrostatic pressure (Brown, et al., 2000).

The renoprotective effect of ALA rich diets was further investigated by examining the influence of varying dietary LA and ALA levels on the renal oxylipin

profile. This analysis revealed that a high intake of ALA (8-20% of dietary fat as ALA) resulted in an n-6:n-3 oxylipin ratio of 6-14:1 and an increase in n-3 oxylipins without altering n-6 oxylipins. Other studies have reported similar findings that supplementation with n-3 fatty acids can increase n-3 oxylipins without altering n-6 oxylipins in human serum (Keenan et al., 2012).

The renoprotective role of the ALA rich diets in ORG could in part be explained by the novel n-3 oxylipins, HOTrEs, 4-HDoHE, and 5-HEPE which were inversely correlated to MGV ($p<0.05$, $r= -0.3$). The HOTrEs may have exerted their renoprotective effects through regulating MMPs which maintain glomerular integrity and prevention of renal damage such as in the early stages of Alport's syndrome, a genetic renal disease (Zeisberg et al., 2006).

The formation of oxylipins from the n-3 fatty acids was greater than the production of n-6 oxylipins by lipoxygenases. Other investigators have reported similar findings for cytochromeP450 which prefers to metabolize EPA and DHA over ARA (Konkel & Schunk, 2011). This is important to note because the inhibition of enzymes involved in fatty acid metabolism such as cyclooxygenase is common to slow the progression of renal disease (Sankaran et al. 2007). Similar inhibition of lipoxygenase may create a detrimental balance for renal oxylipins because several protective metabolites such as lipoxins, HEPEs, HDoHEs, resolvins, protectins, and maresins (Serhan, et al., 2011) are produced by lipoxygenation and therefore lipoxygenase inhibition should potentially be avoided.

Limitations

The limitations of the current study include the absence of a dietary group with the long chain fatty acids EPA and DHA. However, the objective of the study was to investigate sources of ALA on the renal oxylipin profile; therefore, the study design fulfilled the objective. Another potential limitation is the absence of a lean control group. The low fat group was assumed to gain less weight than the high fat diet groups; however, this was not the case. Although, the low fat group did gain less adipose mass and therefore could still be considered a lean control. In terms of the product/pre-cursor data, these results provide an insight into the enzyme selectivity of 5- and 15-LOX, however, we did not directly measure the specificity of the enzymes. Lastly, due to the indolent nature of ORG, a longer treatment phase may have facilitated larger differences between groups particularly in the pathological assessments such as glomerular damage and proteinuria.

Strengths

The animal model mimicked the onset of human obesity which is superior to genetic models of obesity which does not represent the natural progression of obesity in humans. The high fat diets provided to the DIO rats resulted in the development of renal disease that was characteristic of ORG. This imitation of ORG allowed for investigation of potential diagnostic measures and dietary treatments. Other methods of inducing obesity through diet in animal models such as a high carbohydrate diet could have potentially resulted in pathological characteristics similar to that of diabetic nephropathy in which animal models already exist. Additionally, the presence of baseline groups was a strength of the study because it allowed for histological comparison between baseline

and endpoint groups to understand the renopathophysiology of ORG in the DIO animal model.

The methods of the current study allowed for analysis of the renal oxylipin profile to an extent never performed before with greater than 60 oxylipins scanned. This study was also the first to identify HOTrEs in the kidney, their quantities, and how diet influenced their levels.

The varying levels of dietary ALA and LA among the groups provided excellent comparisons to allow for the understanding of how varying fatty acid compositions can affect the renal oxylipin profile. More specifically, the diets allowed us to make the comparison of when LA was held constant and ALA increased and vice versa in order to carefully understand the influence of only one polyunsaturated fatty acid. Additionally, the diet composition included plant sources of ALA which are often overlooked in research investigations due to the focus on the long chain n-3 fatty acids. The current study provides evidence for the functional properties of dietary oils rich in ALA and therefore support for the promotion of Canadian agricultural products.

Implications

The current study has laid the foundation for future clinical trials to investigate the efficacy of the proposed diagnostic marker and dietary interventions. For example, clinical trials can investigate the use of glomerulomegaly as an early diagnostic marker and oils rich in ALA as early treatments either alone or in conjunction with other treatment methods. If these findings are found to be efficacious in humans, earlier diagnosis and treatment may be able to occur for patients with ORG and potentially

reduce the number of individuals progressing to renal failure. The reduction of patients progressing to renal failure will not only improve the quality of life for patients but also the burden on the healthcare system. Patients who progress to end stage renal disease may use dialysis as a treatment method to maintain health and Health Canada estimates a cost of $220 per dialysis treatment, which is equivalent to $33,540 every year per patient (Health Canada, 2006).

The use of dietary oils rich in ALA as a potential treatment will help add value to Canadian crops and advance the Canadian agricultural sector by promoting the production of ALA sources such as canola and flax. This not only will increase demand and revenue but potentially jobs for Canadians as well.

The identification of the renal oxylipin profile and the influence of dietary LA and ALA levels advances the knowledge on lipid biochemistry and potential dietary fatty acid recommendations in order to promote renal health. Examination of the role of oxylipins in renal health can facilitate the pharmaceutical use of beneficial mediators in chronic diseases such as the DHA derived resolvins in the treatment of bacterial sepsis (Spite, Norling, Summers, Yang, Cooper, Petasis, et al. 2009). These findings also facilitate the understanding of how the fatty acids we consume can influence renal disease progression, ie: through alteration of oxylipins.

Future Research Directions

Clinical trials can utilize the foundational knowledge created here to assess whether glomerulomegaly can act as an effective early marker for diagnosis of ORG, or a marker of improvement or decline in treatment investigations. Clinical trials can also

investigate the efficacy of high dietary ALA oils such as canola and flaxseed oil in the treatment of ORG. The current study lays the foundation for further investigations of HOTrEs and their influence on renal health through *in vitro* assessments with mesangial or glomerular cell lines. Animal studies can also be performed in which rats are injected with specific oxylipins to determine the influence on renal health. Further investigation on how the whole diet rather than n-3 supplementation alone can influence the oxylipin profile will provide evidence for better dietary recommendations for fatty acids in order to promote renal health in obesity.

Chapter 1 and 4 References

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X. CHAPTER 5 - APPENDICES

Protocol 1 – Histology and Morphology

Authored by Stephanie Caligiuri and Tom Blydt-Hansen

Use of Microscope

- To begin, you must take photos of your slides using the Olympus BX60 Microscope and the attached computer
- Open SPOT advanced software.
- Turn on microscope (small black switch on right back side of microscope)
- Turn on very left black box labelled SPOT
- Place a slide under the microscope lens using the 10x objective lens if analyzing fibrosis or the 20 x objective lens if you are analyzing glomerulosclerosis or mean glomerular volume.
- Make sure you have the same light settings every time you take photographs
    - The 3 small black dials labelled LBD, ND6, and ND25 on the microscope need to be the same every time. (LBD and ND6 need to have the white dots facing left and downward. ND25 needs to have the white dot facing upward on the left side)
    - The lightness of your photograph is controlled by the black dial on the microscope with the light bulb image above it. Make sure this is the same all the time.
    - Note: if the colour of your photos appear different (ex: purple when they should be red) re-calculate the white balance values and follow directions accordingly. To do this: press the button on the right hand side in the

SPOT software (when mouse is over button it says “compute white balance values”

- Take representative photos of your slides, choose randomly and systematically the photo without bias. For example, move the slide downward and to the right a small amount every time starting from the bottom corner of the cortex.
- If taking photographs for fibrosis: take 13 photos of the cortex and 13 of the medulla
- If taking photographs for glomerulosclerosis or MGV take enough photos to capture 30 glomeruli.
- Focus the photo by clicking the focus button on the right hand side of the computer screen and use the black large dial on the right hand side of the microscope. Once focused, click the top right button of the computer screen to capture the image.
- Then move your slide to take a new photo, focus if you need to, then click the 2nd button on the right of the computer screen to continually take photos.

Glomerulosclerosis Analysis

*Glomerulosclerosis will be represented by a percentage of glomeruli that are segmentally or globally sclerosed over the total number of glomeruli analyzed (30). Glomerular damage will consist of categories 2-6 and likewise divided by the total number of glomeruli.

1. Normal Glomeruli
    - Uniform, light staining of mesangial structures without segmental accumulation of blue stain.
    - Cellular elements are still intact (presence of nuclei)
    - Open capillary loops present and mesangial matrix area not expanded/thickened.
2. Early Lesion (segmental mesangial matrix expansion)
    - Segmental tuft area accumulation of blue stained matrix, obviously more dense than adjacent glomerular tuft area.
    - Segmental thickening of mesangial matrix (connective tissue) extending to the outer margin of the glomerular tuft. Segmental distortion of tuft architecture is present in the affected area compared with adjacent normal tuft area.
    - Absence of “organized” sclerosis - Open capillary loops are present throughout the entire tuft, and cellularity of mesangial, capillary and epithelial cell elements is preserved.
    - Absence of tuft-capsule adhesion (bridge of connective tissue connecting glomerular tuft with Bowman’s capsule)
3. Segmental Glomerulosclerosis
    - Segmental tuft area accumulation of blue stained matrix, obviously more dense than adjacent glomerular tuft area.
    - Organization of sclerotic changes; extending out to the periphery of the glomerular tuft. Confluence of matrix deposition with reduction of normal

cellular elements in the affected segment.

- Obliteration of capillary loops in the affected area (Starting from the periphery; does not include the area around the hilum and not starting from the centre of the glomeruli that includes the mesangial matrix)
- Open capillary loops and tuft architecture are preserved in at least one glomerular tuft segment.
- While not required, the finding of tuft-capsule adhesions strongly suggests an underlying area of segmental sclerosis (bridge of connective tissue connecting glomerular tuft with Bowman's capsule in direct contact with underlying sclerotic segment as defined above)

4. Global Glomerulosclerosis
    - Segmental tuft area accumulation of blue stained matrix, obviously more dense than adjacent glomerular tuft area.
    - Organized, sclerotic changes in all glomerular tuft segments.
    - Obliteration of capillary loops over the entire tuft area.

5. Periglomerular fibrosis and ischemic tuft contraction
    - Requires finding of both ischemic contraction AND periglomerular fibrosis
    - Ischemic contraction is noted when the glomerular tuft occupies only a small proportion of Bowman Space; capillary loops are seen but are collapsed onto one another. Glomerular tuft has a shrivelled appearance. Think of it as a bag of balloons with all of the air let out.

- Periglomerular fibrosis is noted when you see “multiple layers” of fibrous tissue encircling the glomerulus outside and associated with Bowman’s capsule. The capsule basement membrane itself looks thick, but also fibrous changes extend to adjacent tissue encircling the glomerulus.

6. Atubular glomeruli (glomeruli where the attached tubule has died)
    - Severe tuft contraction
    - Variable detachment of podocytes which float free within the cystic expansion of Bowman’s space
    - Bowman’s space is also filled with protein cast and sometimes calcification.

Mean Glomerular Volume (MGV)

- Using the same glomeruli that you analyzed for glomerulosclerosis, now analyze the MGV using Image-Pro Plus Software.
- Open Image-Pro Plus Software 6.0
- Select Complete
- Select Done
- Open your photographs one sample at a time (for example, all photos for rat #1)
- Click the measure tab, then click Measure distances
    - Click the measure tab again, and select Data Collector. In the window that appears select the layout tab. Under image select name, and under Distances select Distance and in the drop

down box below that select values.

- In the measure distance window, select the line button that looks like this |--|
- Draw the **longest diameter** of the glomeruli using this tool.
- In the data collector window, under the data list tab, select collect now.
- After analyzing your data you can export it using the export tab on the data collector window, select export now. It will be exported to an active excel sheet. (if you don't have an excel spreadsheet open, open a new one)
- The values from the diameter measurement will be in pixels.
- In the excel document indicate the rat ID and save

Fibrosis

- Open Image-Pro Plus Software 6.0
- Select Complete
- Select Done
- 10x magnification photographs will be used to analyse fibrosis in the cortex and medulla.
- First you must create a colour standard. To do this open a kidney photograph that has a lot of fibrosis.
- Click the measure tab and then click "count/size"
- Click "select colours"
- Under the colour cube based tab click the left button that looks like a pen.
- Now put the mouse over your kidney photograph and select the truest

colours of blue you see that represent fibrosis. Look at the colour representation cube on the count/size window to better tell the difference between colours.

- Once you're happy with your colour selection, click File.
- Click Save file and save your colour standard under a recognizable name.
- You must load this standard for every picture you analyze for fibrosis.
- Now open your cortex photos for one rat ID.
- Under the measure tab, click the data collector.
    - Under the layout tab, select "image" in the first drop down box. Then select name and the click the right arrow to add it to the right.
    - Where it says "image" in the drop down box, select "count/size" then select "area" from the list below.
    - The dropdown box below select sum, then click the arrow to add it to the list to the right.
    - Click the top tab labelled "Data list". This is where your data will be placed.
    - Now, close the "segmentation – image x" window. In the count/size window click "select colours"
    - Click "File"
    - Click "Load File"
    - Select the colour standard that you just created
    - Click "yes" to replace the ranges.

- Close the segmentation window
- One the count/size window, select “Count”
- In the data collector window, select “collect now”.
- The area sum of the fibrosis from your photo should appear in the data list.
- Once you have finished analyzing your cortex photos for that one rat, click the “export” tab in the data collector window.
- Select export target
- Select export now. Your data will be exported to the active excel sheet. To change the export options, click “export options”.
- You can then proceed with your medulla pictures for the rat in the same way.
- If you wish to exclude certain elements of your photograph such as veins (which appear stained blue but are not fibrosis) you can simply crop it out using paint and open the modified picture in the Image pro plus software.

** True fibrosis however appears in the presence of tubular atrophy (thickening of tubular membrane and enlargement of lumen space) or a damaged glomerulus. If there is no tubular atrophy or a damaged glomerulus present, then I would not count the blue as true fibrosis. The accurate analysis of fibrosis is reviewed by Farris et al. (2012)(Farris & Colvin, 2012).

**If analyzing cystic area in polycystic kidneys, you can do it in a similar way. Instead of selecting the blue colours, you can select the white colours that represent the cysts.

Protocol 2 - Proteinuria Quantification

The protein concentration of both urine samples and Western kidney homogenate samples were performed using the Bradford method for total protein determination (Bradford, 1976). The urine samples were thawed and diluted by 10X with deionized water into 1.5 mL microcentrifuge tubes. Kidney homogenates were diluted with 1.4 x whole cell buffer; details of the whole cell buffer for Western immunoblot analysis are listed in Table 5.2.

Standard solutions were created from stock bovine serum albumin (BSA – 2mg/mL). The standards were diluted to create 1, 0.75, 0.625, 0.50, 0.375, 0.25, 0.125, and 0.0625 mg/mL and vortexed thoroughly. Using a 96-well clear plate (Costar 3368), 10 µL each of blanks (deionized water), standards, and diluted samples were plated in triplicate using a multi-channel pipette. Subsquently, 200 µL of Bradford Reagent (Sigma B6916) was pipetted into each well. The 96-well plate was then covered with a clear plastic plate cover and covered with a cardboard box to ensure darkness. The plate was placed on a rocker for 15 minutes at room temperature.

Subsequently, the plate was read at 595 nm using the SpectraMax 340 and Softmax Pro Software. The absorbance values of the blanks were subtracted from the sample absorbances. A standard curve was created based on the absorbance values from the plated standards and a linear regression equation was created. The R^2 values of the regression equation must have been greater than 0.98 otherwise the plate was re-done. This equation was used to determine the protein concentrations from the absorbance values of the samples. Based on the $y = mx + b$ equation, the y value represented the absorbance reading and the x value represented the protein concentration. The equation

was changed around to be x = (y-b)/m. The product of this equation was multiplied by the dilution factor (10). Proteinuria values were normalized to urinary creatinine and expressed as mg protein/mg creatinine.

Protocol 3 - Serum and Urine Creatinine

Serum and urine creatinine was analyzed by the Heinegard and Tiderstrom modified Jaffe Method (Heinegard & Tiderstrom, 1973). Serum samples remained undiluted and urine samples were diluted 20X with deionized water. Creatinine standards (Sigma C3613) were diluted from the stock solutions (0.01 mg/mL, 0.03 mg/mL and 0.10 mg/mL) to 0.1, 0.08, 0.06, 0.05, 0.03, 0.02, 0.01, and 0.005 mg/mL.

Solutions to be made:

- 0.05 M Sodium Phosphate and 0.05 M Sodium Borate Solution is required and can be made as described below:
  - Add 13.4 g of $Na_2HPO_4$7H2O (sodium phosphate) and 6.9 g of BNaO2.4H20 (sodium borate) to 900 mL of distilled water. (Sodium phosphate = 268.07 g = 1 mole; therefore 13.4035 g = 0.05 mole. Sodium borate = 137.9 g = 1 mole; therefore 6.895 g = 0.05 mole)
  - The solution was brought to a pH of 12.7 with 10 M sodium hydroxide. The volume can be brought up to 1 L with deionized water.
- 4% aqueous Sodium Dodecyl Sulfate **(**SDS) was made by mixing 40 g in 1 L of deionized water and kept at room temperature.
- 60% acetic acid solution was prepared fresh every day by mixing 60 mL of concentrated acetic acid with 40 mL of deionized water.

- Picric Acid Solution was made by combining the 0.05 M sodium phosphate + 0.05 M borate solution, 4% aqueous SDS, and picric acid (1.3% Sigma P6744-1GA) with a 2:2:1 v/v/v ratio. Special precautions were taken with picric acid in order to prevent it from drying out and being an explosive hazard. For example, all tips were placed in a bucket with water.
- Lastly a 15% acetic acid solution was prepared fresh by mixing 60% acetic acid with the picric acid solution in a 1:4 v/v dilution (for example, 1 mL of 60% acetic acid and 3 mL of picric acid solution).

A 96-well clear plate (Costar 3368) was plated with 20 µL of deionized water as the blank, standards, and samples in triplicate. Subsequently, 200 µL of picric acid solution was added to each well and mixed once by expelling and drawing up and expelling again with a multi-channel pipette. The plate was covered with a plastic sheet cover and cardboard box and put on a rocker for 40 minutes at room temperature. Next, remove the cover was removed and any remaining bubbles were popped with a needle. The plate was read at 500 nm using SpectraMax 340 and Softmax Pro Software and the data was saved.

Next 20 µL of the 15% acetic acid solution was added to each well using a multi-channel pipette and mixed as the same way before. The plate sat for a few minutes and bubbles were popped with a needle again. The plate was read at 500 nm and the data was saved. Next, all sample absorbances were subtracted from the blanks. Then, the pre-acid absorbance values were subtracted from the post-acid absorbance value from the average of the triplicates. A standard curve was created in the same way as with the Bradford protein assay in order to create a linear regression equation. The equation was used to

assess creatinine values. X = (y-b)/m and this product was multiplied by the dilution factor (20).

Tissue Homogenization

The whole kidney was lyophilized with a freeze drier, pulverized, and stored at -80°C. Forty-five mg of the renal tissue was homogenized in 1250 µL of Tyrodes Salt Solution (pH 7.6 – Sigma #T2145). The Tyrodes solution was prepared by dissolving the Tyrodes salts into 1 L of purified water. Next, 100 mg of sodium bicarbonate was dissolved into 80 mL of Tyrodes solution and pH'd to 7.6 with HCl or NaOH. The final volume was brought up to 100 mL with purified water.

Protocol 4 - Fatty Acid Analysis

The fatty acid extraction and isolation was based on the methods described by Ogborn et al. (Ogborn, Nitschmann, Weiler, & Bankovic-Calic, 2000) with the addition of thin layer chromatography (TLC). Lipids were extracted, triglyceride and phospholipid fractions isolated, and fatty acid methyl esters analyzed by gas chromatography (GC).

To the 250 µL aliquot of tyrodes and kidney homogenate, 8.34 µL of antioxidant cocktail was added. Glass tubes were soaked in Contrad solution and rinsed thoroughly. The TLC plates (Analtech, Inc. silicia gel G 20 cm x 20 cm 250 µm, catalogue no. 01011) were prepared by incubating them in a 110 °C oven for 1 hour and subsequently cooled in a dessicator.

Next, 2.5 mL of a 2:1 chloroform:methanol with 0.01% BHT (0.03 g BHT, 200 mL of chloroform, and 100 mL of methanol) was added to the homogenate and vortexed. Ten µL of heptadecanoic acid [(16 mg/mL in chloroform) Nu Check Prep Inc., catalogue

no. N-17-A] as the standard for the triglyceride fraction of fatty acids was added to the sample. In addition, 100 µL of the 1,2-dipentadecanoyl-s*n*-glycero-3-phosphocholine [(1.4 mg/mL in chloroform) Avanti Polar Lipids, catalogue no. 850350] was added to the sample as the standard for the phospholipid fraction of the fatty acids. These quantities of standards were tested to be adequate for the kidney samples by prior testing. The quantity of standard can vary dependent upon quantity of fat present in tissue.

Next, 2.25 mL of 2:1 chloroform was added, vortexed, and 0.95 mL of 0.73% sodium chloride was added. Next, the samples were centrifuged for 10 minutes at 800 rcf. Next, the lower phase of the biphasic solution was removed with a Pasteur pipette and place into a clean 4 mL glass tube. The samples were then dried down in a 37°C nitrogen water bath and re-constituted with 100 µL of 2:1 chloroform:methanol.

Next, the TLC tank was equilibrated by placing in chromatography paper and filling the tank with 60 mL of heptane, 40 mL of isopropyl either, and 3 mL of acetic acid. Subsequently, vertical lanes were indented on the previously heated TLC plates using the dull side of a blade. The samples were spotted one by one in each lane and the plate was placed in the tank until the solvent line reached 1 centimetre from the top. The plate was then dried in the fume hood and subsequently sprayed with 0.01% 1-Anilinonaphthalene-8-Sulfonic Acid (ANS) in water. A UV light was held above the plate to mark the phospholipid and triglyceride bands with a pencil. The retention factor for the phospholipid was 0, and for the triglycerides was 0.61; meaning that the phospholipid fraction stayed at the spot where the sample was spotted and the triglycerides moved 60% to the solvent front. Next the fractions were scraped into a labelled tube and 1.5 mL of toluene and 1.2 mL of methanolic HCl were added and the

tube was placed in an 80°C oven for 1.5 hours.

Next, 1 mL of deionized water was added, vortexed, and centrifuged at 800 rcf. The top layer was removed from the biphasic mixture with a Pasteur pipette and put into a clean tube. The sample was then dried under a nitrogen water bath and 200 µL of hexane re-constituted the sample. The samples were stored at -20°C until analyzed on the GC.

The fatty acids were separated on a DB225MS column (30 m X 0.25 mm diameter and 0.25 µm film thickness; Agilent Technologies Canada Inc., Mississauga, Ontario) using a Varian 450 GC with a flame ionization detector and hydrogen used as the carrier gas. The temperature program was 70°C for 2 min, raised to 180°C at 30°C/min, held for 1 min, raised to 200°C at 10°C/min, held for 2 min, raised to 220°C at 2°C/min and held for 10 min. Finally the temperature was raised to 240°C at 20°C/min and held for 5 min. Total run time was 36.67 min and samples were run with a 10:1 split ratio and a 1.3ml/min column flow. An external standard containing standards for more than 30 fatty acids was run just before and after the samples in order to determine the retention times of the analytes. The peaks were analyzed accordingly to the internal standard (C15:0 for phospholipids and C17:0 for triglycerides) and divided by the mg of dry tissue used.

Protocol 5 - Oxylipin Extraction and Analysis

Practice samples were processed in order to run the Eicosanoid 12 scan which searched for 64 oxylipins. Subsequently, the scan was narrowed down to approximately 44 oxylipins which created the Stephanie 2 method. A list of all analytes scanned for with

the Collision Induced Dissociation (CID) Mass Transitions are listed in Chapter 3 – Tables S3.1-S3.2.

The method for oxylipin extraction was based on the Deems (2007) method (Deems, Buczynski, Bowers-Gentry, Harkewicz, & Dennis, 2007). The kidney homogenates were further processed after homogenization with tyrodes by adding 5 µL of 1% Triton to the 500 µL aliquot and vortexed several times while sitting on ice for 30 minutes. In order to assess exogenous production of oxylipins, a 200 µL aliquot of the homogenate was incubated for 5 minutes in a 37°C water bath which has shown to result in significant exogenous production previously. The other 200 µL aliquot remained on ice. To the samples, 500 µL of 100:1 methanol:formic acid, 800 µL of pH 3 deionized water, 90 µL of 100% ethanol, and 10 µL of the antioxidant cocktail were added and vortexed. The samples were stored at -80°C freezer and later thawed for further analysis.

Once thawed, deuterated standards were added according to the quantities listed in Table 5.1 below (catalogue numbers are listed in the supplemental section of Chapter 3 – Table S3.1). Strata-X solid reverse phase columns (phenomenex 8B-S100-UBL) were pre-conditioned with 2 mL of methanol and 2 mL of pH 3 deionized water. Subsequently, the sample was applied to the column, followed by 1 mL of a 10% methanol in pH 3 deionized water that washed the tube previously holding the sample. Lastly, 1 mL of methanol was added to elute the sample and it was subsequently dried down using a 37°C nitrogen water bath. The sample was re-constituted using 100 µL of solvent A (water-acetonitrile-formic acid – 70:30:0.02 v/v/v LC-MS grade). The samples were stored at -20°C for a maximum of 4 days and analyzed on the liquid chromatography mass spectrometer (LC-MS/MS). Further details of the LC-MS/MS procedure, model, and

parameters are listed in Chapter 3, methods section. Once the chromatograms were produced, the peak area of the analyte and surrogate standard were quantified using Analyst Version 1.5 (Applied Biosystems Concord, ON). The peak areas were converted to nanograms; the analytes were divided by the surrogate internal standard quantity, normalized to the detector response factors, and divided by the mg of dry tissue used. The detector response factors are provided in Chapter 3, table S3.2.

Table 5.1 – Volume and Concentration of Deuterated Internal Standards

Internal Standard	Volume Added (µl)	Stock Concentration (ng/µl)	Final Concentration (ng/µl)
6-k-$PGF_{1\alpha}$-d4	60.0	25	1.00
TXB_2-d4	30.0	25	0.50
$PGF_{2\alpha}$-d4	15.0	50	0.50
PGE_2-d4	22.5	50	0.75
PGD_2-d4	60.0	25	1.00
LTB_4-d4	60.0	25	1.00
20-HETE-d6	180.0	25	3.00
15-HETE-d8	60.0	25	1.00
5-HETE-d8	120.0	25	2.00
13-HODE-d4	120.0	25	2.00
9-HODE-d4	120.0	25	2.00
12,13-DiHOME-d4	60.0	25	1.00
14,15-DHET-d11	60.0	25	1.00
9,10-DiHOME-d4	60.0	25	1.00
11,12-DHET-d11	60.0	25	1.00
15d-PGJ_2-d4	60.0	25	1.00
8,9-DHET-d11	60.0	25	1.00
5-OxoETE-d7	150.0	25	2.50
Total	1357.5		
Solvent (Ethanol) Added	142.5		
Final Total	1500.0		

Protocol 6 - Western Immunoblotting

Western Immunoblotting was used to detect the protein levels of cyclooxyenase 1 and 2, 12/15-lipoxygenase, 15-lipoxygenase 2, 5-lipoxygenase, cytochrome P450 2c23 (CYP2c23), soluble epoxide hydrolase (sEH) and ß-actin.

The kidney homogenate in Tyrodes was further processed by adding 1.4 x whole cell buffer in order to reach a final protein concentration of 3-5 µg/µL. The ingredients of the 1.4x WCB are listed below.

Dose responses and primary antibody optimization were tested on all enzymes. The samples were prepared to ensure either 14 or 28 µg was present in the sample. A reference sample was created by pooling 10 µL of homogenate from one sample per dietary group. The reference sample was loaded as the first and last lane on every gel; this was done in order to normalize all bands and establish comparison on different membranes. Sample buffer was added to the sample, heated at 98°C for 5 minutes, cooled, and centrifuged at 3000 g for 1 minute. The samples and a protein ladder (ThermoScientific 26634) were then applied to an 8-16% tris-glycine pre-cast gel (Novex EC60485BOX) and separated based on electrophoresis. The gels were then transferred onto a polyvinyl difluoride membrane (GE Healthcare Amersham Hybond-P Cat No. RPN2020F) and blocked with 5% skim milk solution at room temperature for 1 hour. Subsequently, the primary antibodies were applied to the membranes overnight (16-18 hours) at 4°C. The next morning the membranes were washed with Tris Buffered Saline with Tween (TBS-T) three times for ten minutes each and the secondary anti-body (anti-rabbit or anti-goat dependent upon primary antibody) was applied to the membrane for one hour at room temperature. The membranes were again washed three times for a total

of 30 minutes with TBS-T. Chemiluminescent peroxidase (Sigma, catalogue no. cps1120-1kt) was applied to the membranes and detected using Cell Biosciences Flurochem HD2 with chemidisplay. The bands were analyzed using densitometry and corrected for individual background. All samples were divided by ß-actin and the reference sample.

Often the membranes were stripped and re-probed. To strip the membranes, immediately after imaging, the membranes were washed with TBS-T 3 times for 10 minutes each, placed in Restore Plus Western Blotting Stripping Buffer (ThermoScientific #46430) for 15 minutes x 2, washed again with TBS-T in the same way as before, and blocked with 5% skim milk solution for 60 minutes. The membrane was then re-probed overnight with another primary antibody.

Table 5.2 – Ingredients of 1x and 1.4 x Whole Cell Buffer

Component	**1 X WCB (whole cell buffer) 20mL**	**Final concentration of 1X WCB**	**1.4 X WCB 20mL**
500 mM Tris-in water Fisher BP 154-1 (6.1 g/ 100 mL)	2 mL	50 mM	2 mL
0.5 M sucrose (17.2 g/ 100 mL) Sigma, S9378	10 mL	250 mM	10 mL
200 mM EDTA (pH = 7.6) Sigma, ED4SS, (0.76 g/10 mL)	200µl	2 mM	280 µl
100 mM EGTA (pH = 7.5) Sigma, E4378 (0.38 g/10 mL) Add NaOH to get it to dissolve and bring pH to 7.5	200 µl	1 mM	280 µl
0.4 M NaF (0.168 g/10 mL) Sigma, S6521	2.5 µl	50 µM	3.5 µl
ddH_2O Water	5405.9 µl		4368.26 µl
10 % Triton X-100 Sigma T8787	1 mL	0.5%	1.4 mL

Component	**1 X WCB (whole cell buffer) 20mL**	**Final concentration of 1X WCB**	**1.4 X WCB 20mL**
10 mM Na orthovanadate Sigma, S6508 (1.839 mg/mL)	200 µl	100 µM	280 µl
2.5 mg/mL aprotinin Sigma, A1153	200 µl	25 µg/mL	280 µl
1 mg/mL leupeptin Sigma, L2884	500 µl	25 µg/mL	700 µl
2.5 mg/mL pepstatin (in 90:10 methanol : glacial acetic acid) Sigma, P5318	200 µl	25 µg/mL	280 µl
1 mg/mL STI Sigma, T9003	20 µl	1 µg/mL	28 µl
50mM ABSF (12 mg/mL) 4-(2-aminoethyl) benzene-sulfonyl fluoride, Sigma, A8456	57.6 µl	144 µM	80.64 µl
ß-mercaptoethanol	14 µl	10 mM	19.6 µl

Figure 5.1 – Dose Responses and Antibody Optimization

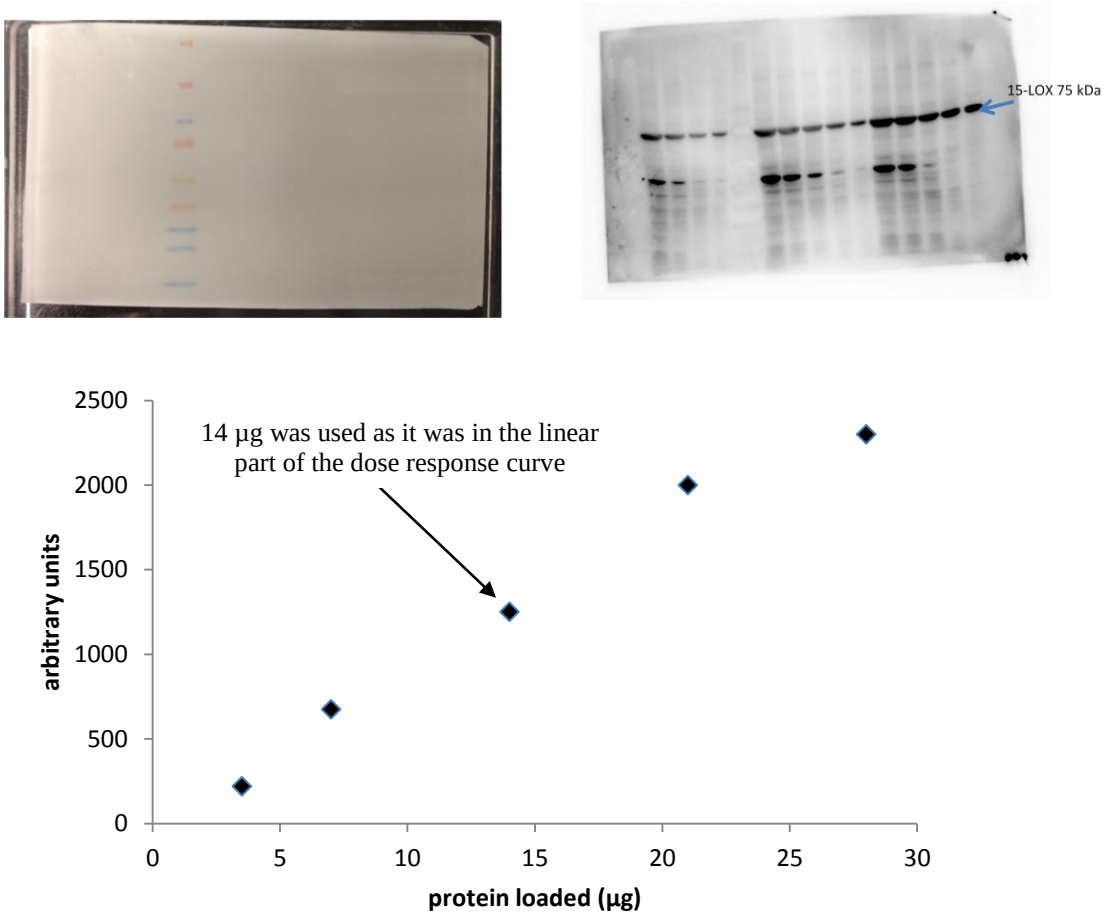

Figure 5.1a: 12/15-LOX Dose response. 3.5, 7, 14, 21, and 28 ug from 3 dietary groups (canola/flax, high oleic canola, and lard). Protein ladder marker on membrane (left) and imaged bands (right) with the dose response curve

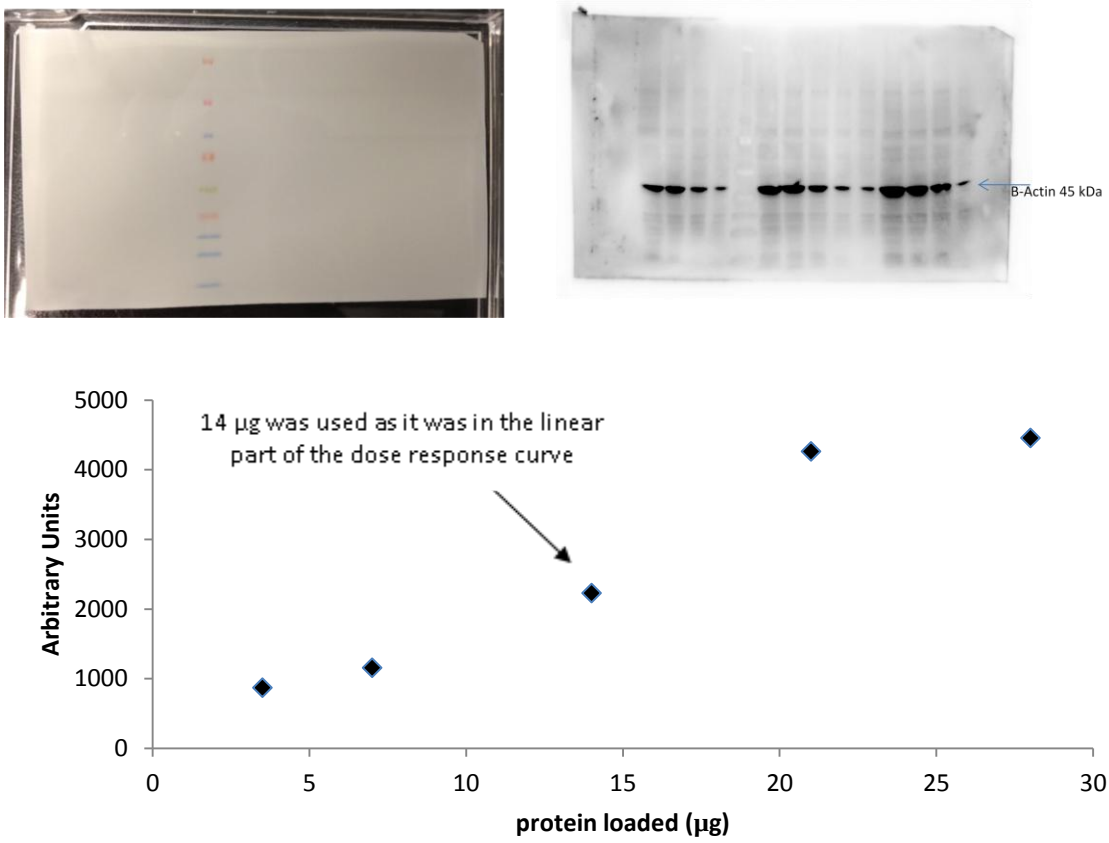

Figure 5.1b: ß-actin Dose Response. 3.5, 7, 14, 21, and 28 ug from 3 dietary groups (canola/flax, high oleic canola, and lard). Protein ladder marker on membrane (left) and imaged bands (right) with the dose response curve.

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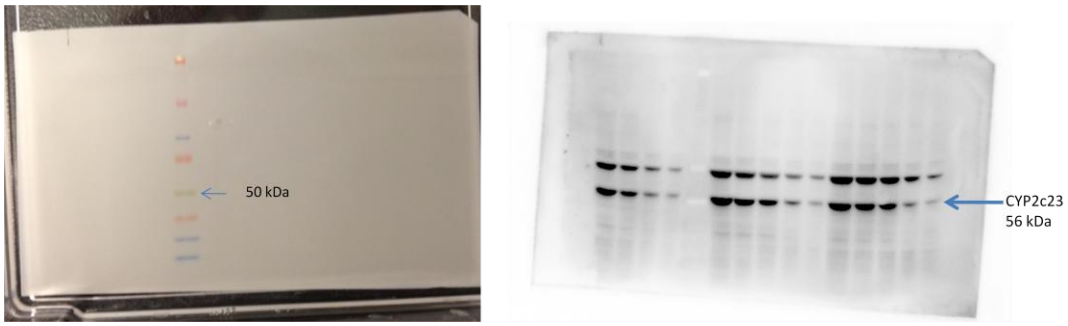


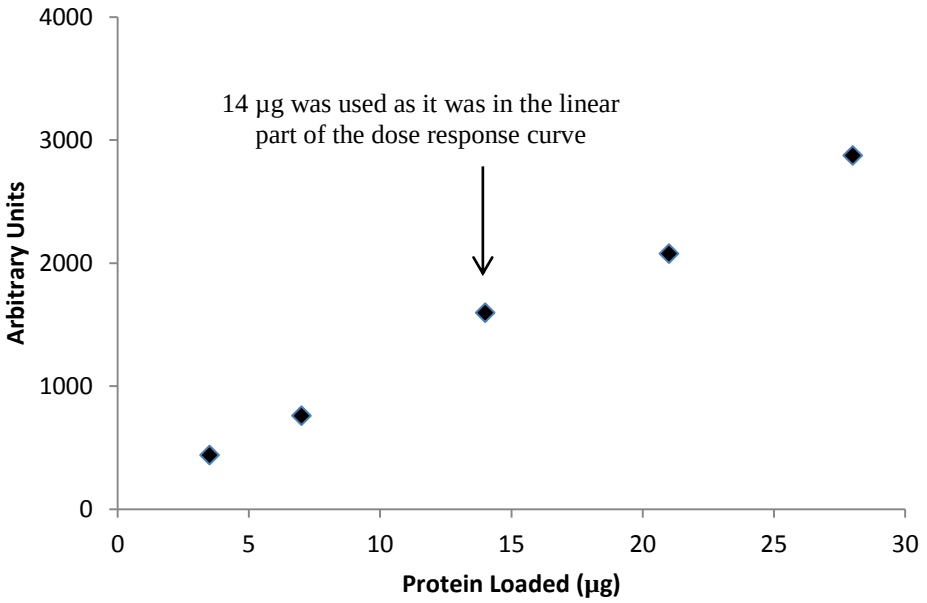
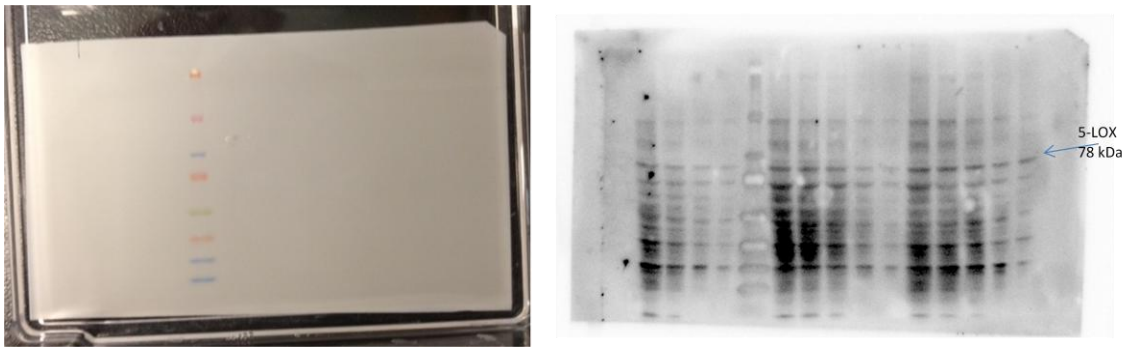


Figure 5.1c: CYP2c23 Dose Response. 3.5, 7, 14, 21, and 28 ug from 3 dietary groups (canola/flax, high oleic canola, and lard). Protein ladder marker on membrane (left) and imaged bands (right) with the dose response curve



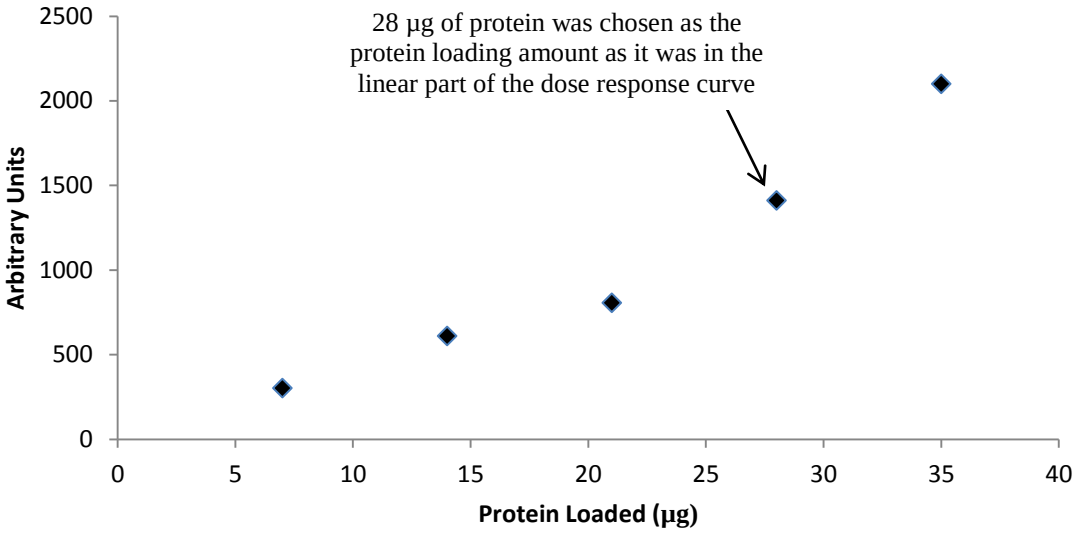


Figure 5.1d: 5-LOX Dose Response. 7, 14, 21, 28, and 35 ug from 3 dietary groups (canola/flax, high oleic canola, and lard). Protein ladder marker on membrane (left) and imaged bands (right) with the dose response curve

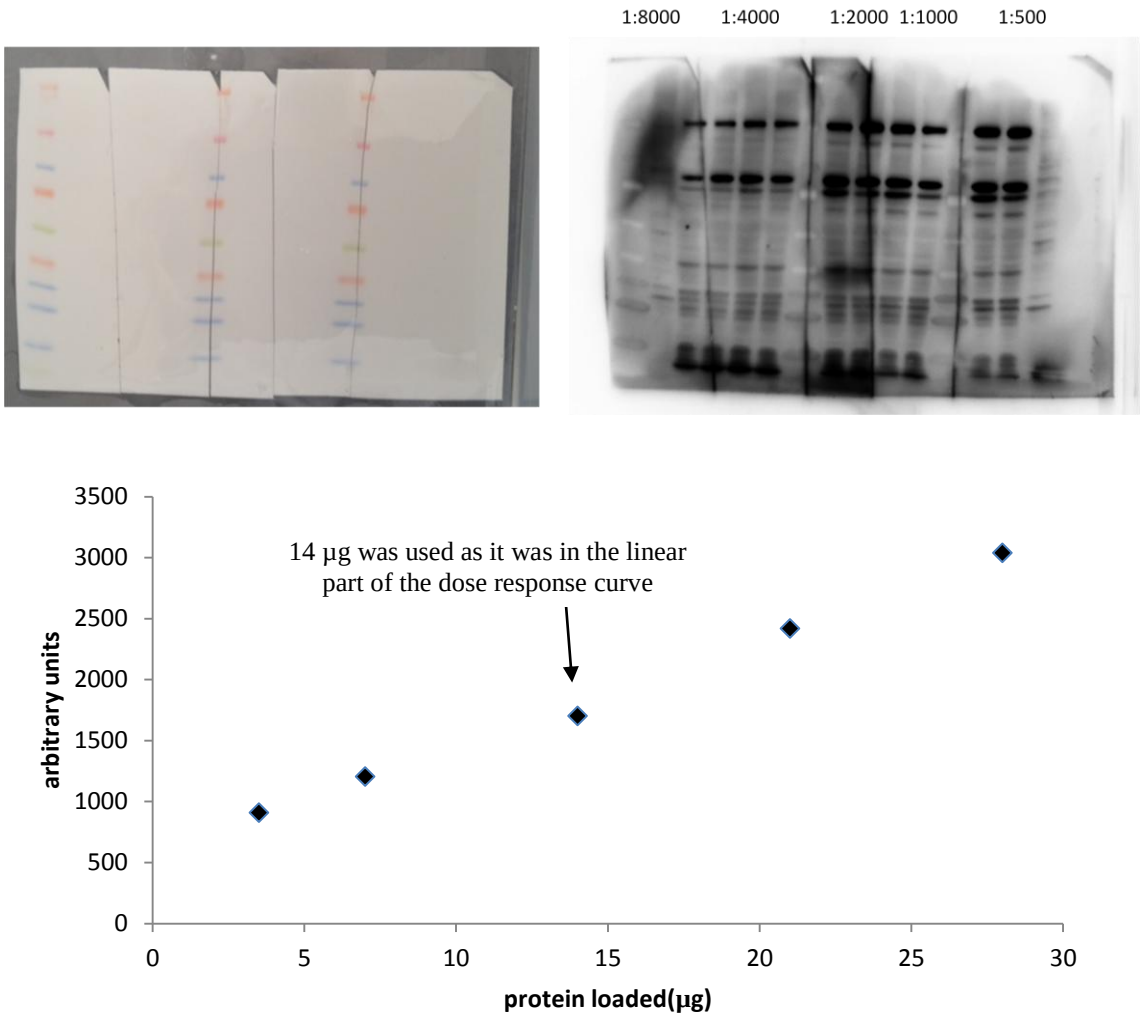
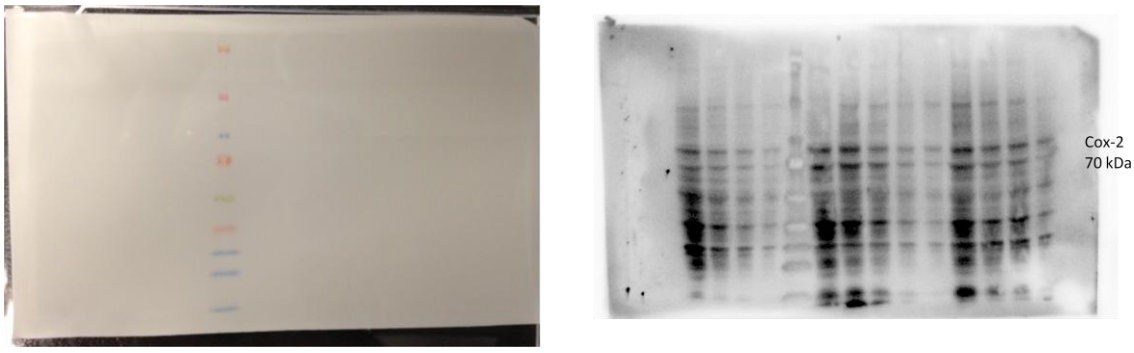


Figure 5.1e: COX-1 Dose Response. Various primary antibody concentrations at 14 µg of protein (canola/flax, high oleic canola, and lard). Protein ladder marker on membrane (left) and imaged bands (right) with the dose response curve



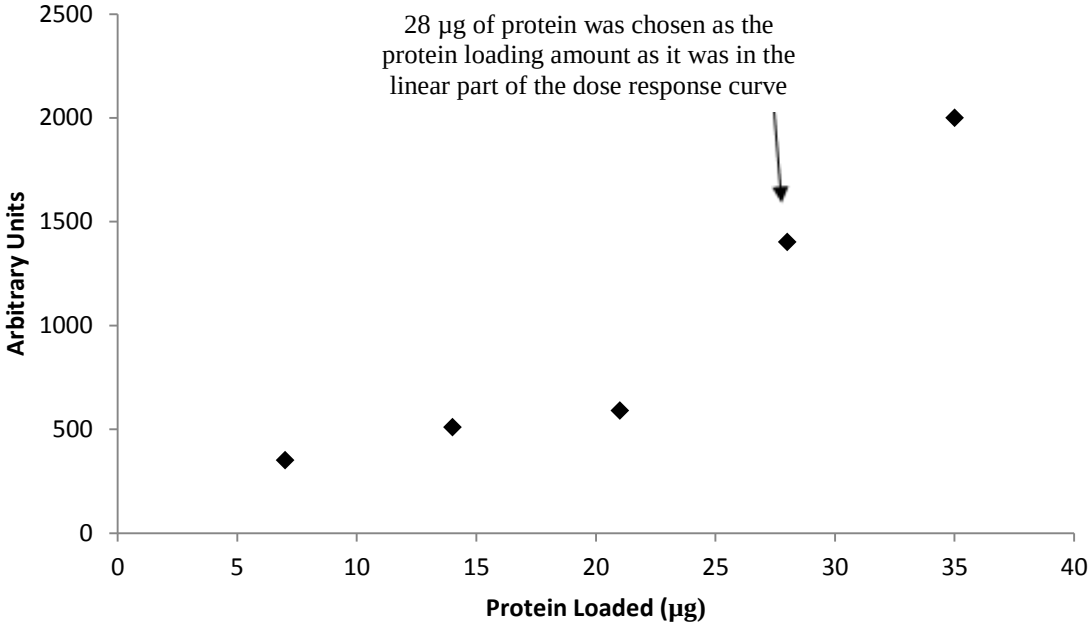


Figure 5.1f: COX-2 Dose Response. 7, 14, 21, 28, and 35 µg of protein from 3 dietary groups (canola/flax, high oleic canola, and lard). Protein ladder marker on membrane (left) and imaged bands (right) with the dose response curve

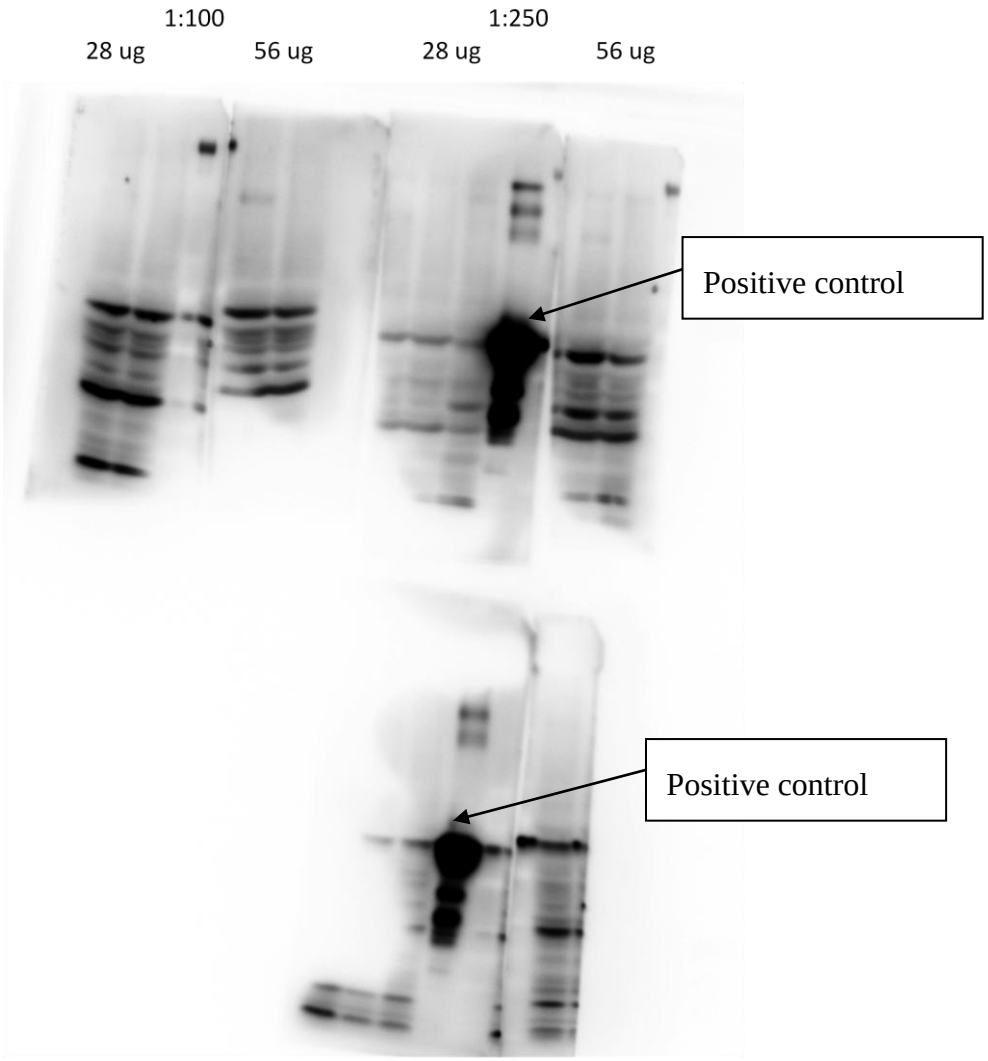
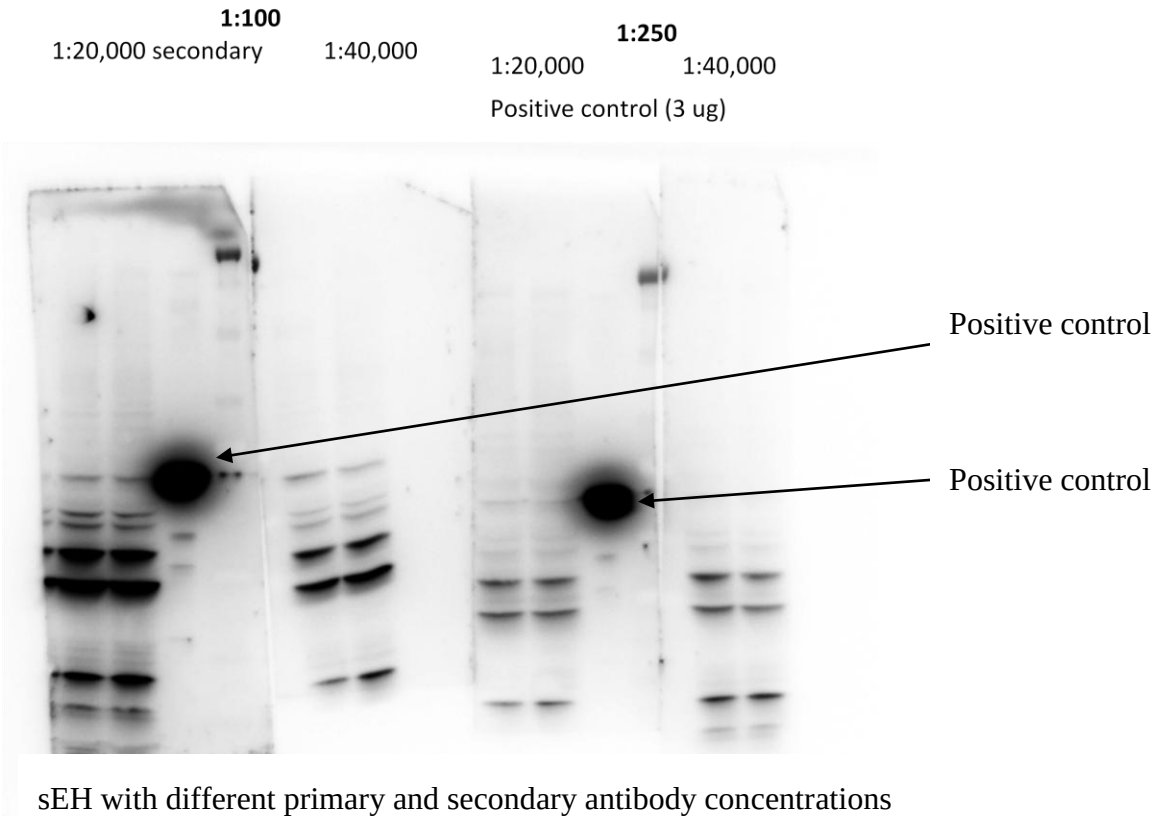


Figure 5.1g: **sEH Dose Response.** sEH at varying primary antibody concentrations and protein loading amounts with 2 dietary groups (high oleic canola and lard). sEH was not detectable at 14 µg and therefore a membrane with 28 and 35 µg was run. Due to the 35 µg band being significantly darker than 28 µg, 28 µg was chosen as the loading amount



sEH with different primary and secondary antibody concentrations with the 28 µg of protein loaded

Table 5.6 - Conditions for Detection of Select Enzymes for Western Immunblotting

Enzyme	Quantity of Protein Loaded (µg)	Dilution of Primary Antibody	Supply Company Catalogue Number
Cyclooxygenase 1	14	1:8000	Abcam ab109025
Cyclooxygenase 2	28	1:2000	Cayman 160106
12/15 Lipoxygenase	14	1:8000	Santa Cruz sc32940
15 Lipoxygenase 2	14	1:2000	Santa Cruz sc46048
5 Lipoxygenase	28	1:500	Cell Signaling 3289
Soluble Epoxide Hydrolase	28	1:100	Cayman 10010146
CYP2c23	14	1:16000	Abcam ab53944
ß-actin	14 or 28 dependent upon first antibody	1:16000	Cell Signaling 4970

Additional Results

Table 5.7 - Enzyme Activity (ng/mg dry tissue/minute)

Oxylipin	Canola/Flax	Canola	Soy	High Oleic Canola/Canola	High Oleic Canola	Lard	Safflower	Lard Baseline	Low Fat/Soy	Low Fat/Soy Baseline
9-HOTrE	43±19^{A}	4±8^{B}	3±3^{B}	8±2^{B}	0.02±1^{B}	1±2^{B}	-1±2^{B}	1±1^{B}	-0.5±7^{B}	14±5^{AB}
13-HOTrE	21±10^{A}	6±1^{AB}	2±3^{B}	-0.5±1^{B}	3±1^{AB}	0.3±1^{B}	0.4±0.4^{B}	1±2^{B}	9±2^{AB}	8±6^{AB}
5-HEPE	8±6	5±3	1±4	1±2	-2±2	0.4±2	trace	-0.4±2	2±4	5±2
18-HEPE	10±7	7±5	-4±3	0.1±2	-0.5±1	trace	0.0±0.0	0.5±0.4	3.1±3.1	3.2±2.2
4-HDoHE	4.2±6.4	5.2±6.4	-2.6±7.7	-3.9±8.8	5.8±7.7	3±4.1	1±4	5±3	10±6	11±7
9-HODE	33±31^{B}	49±10^{B}	49±31^{B}	50±13^{B}	34±9^{B}	45±4^{B}	145±24^{A}	45±4^{B}	98±25^{AB}	145±23^{A}
13-HODE	30±23^{B}	54±12^{AB}	55±21^{AB}	54±8^{AB}	37±8^{AB}	34±7^{AB}	115±36^{AB}	49±8^{AB}	117±20^{A}	109±20^{AB}
5,6-DHET	1±1	1±0	1±1	1±0	1±0	1±0	1±0	1±1	0±1	1±0
11,12-DHET	1±0	3±1	2±0	1±1	1±1	1±1	2±0	2±1	2±0	3±0
14,15-DHET	2±0	2±0	2±0	2±0	2±0	2±0	2±0	2±0	2±0	3±0
19,20-DiHDPA	trace	trace	trace	trace	0.1±0	0.1±0.1	trace	0.1±0	0.1±0	trace

Oxylipin	Canola/Flax	Canola	Soy	High Oleic Canola/Canola	High Oleic Canola	Lard	Safflower	Lard Baseline	Low Fat/Soy	Low Fat/Soy Baseline
9,10-DIHOME	2±2^{D}	4±1^{CD}	7±2^{BCD}	4±1^{CD}	4±1^{CD}	2±1^{D}	17±3^{A}	3±1^{CD}	11±2^{ABC}	14±3^{AB}
12,13-DIHOME	2±1^{C}	2±0^{BC}	3±1^{BC}	2±0^{BC}	2±0^{BC}	1±0^{C}	6±1^{A}	2±0^{BC}	3±1^{BC}	5±1^{AB}
5-HETE	11±12	8±20	29±17	34±16	20±11	34±8	29±23	44±8	21±9	31±12
8-HETE	-1±4	1±3	5±7	8±4	6±2	1±5	7±5	7±6	3±7	5±6
9-HETE	-2±8	12±9	-10±12	15±13	10±11	13±10	7±11	36±7	14±12	5±6
11-HETE	7±3	14±3	24±9	20±9	15±5	23±3	20±5	27±3	18±4	18±4
12-HETE	13±8	10±4	11±4	18±6	21±6	7±3	18±7	18±5	16±4	15±3
15-HETE	34±13	28±13	32±14	54±16	77±21	25±10	56±13	39±12	42±9	32±12
16-HETE	trace	1±1	5±1	2±2	5±2	5±2	1±3	5±2	3±1	3±2
12-HHTrE	14±7	14±7	13±6	9±3	12±4	12±4	19±5	11±3	16±7	18±4
15-HETrE	trace	4±1	trace	5±1	3±1	2±2	7±2	4±1	7±2	5±1
9-OXoODE	4±5	8±8	-6±26	3±4	12±5	1±3	-5±30	10±6	14±25	9±18
13 - OXoODE	5±7	12±6	11±22	5±4	13±5	3±3	18±28	12±6	27±25	19±21

Oxylipin	Canola/Flax	Canola	Soy	High Oleic Canola/Canola	High Oleic Canola	Lard	Safflower	Lard Baseline	Low Fat/Soy	Low Fat/Soy Baseline
PGD_2	14±5	26±5	19±4	30±3	24±2	32±5	29±4	22±6	14±1	17±3
PGE_2	3±2	7±2	8±4	5±1	6±1	7±1	6±1	7±1	3±0	5±1
6-k-$PGF_{1\alpha}$	$12±3^{B}$	$24±6^{AB}$	$15±3^{AB}$	$20±3^{AB}$	$24±4^{AB}$	$32±7^{A}$	$24±5^{AB}$	$18±2^{AB}$	$14±4^{AB}$	$12±1^{B}$
$PGF_{2\alpha}$	10±3	23±6	18±5	18±2	22±2	29±7	22±5	24±3	12±3	18±2
TXB_2	4±1	8±2	6±1	8±1	7±1	11±2	9±2	7±1	4±1	5±1

Values are mean ± standard error. Values without a common letter differ significantly ($p<0.05$).

Table 5.8: Renal Phospholipid Fatty Acid Composition (nmol%) by Diet Group

Fatty Acid	Canola/Flax	Canola	Soy	High Oleic Canola/ Canola	High Oleic Canola	Safflower	Lard/Soy	Lard/Soy Baseline	Low Fat/Soy	Low Fat/Soy Baseline
C14:0	0.1±0.0	0.2±0.0	0.2±0.0	0.2±0.0	0.2±0.0	0.2±0.0	0.2±0.1	0.2±0.0	0.2±0.0	0.2±0.0
C14:1	0.1±0.0	trace	0.2±0.0	0.1±0.1	0.1±0.1	0.1±0.1	0.1±0.1	0.1±0.0	0.2±0.0	0.2±0.0
C16:0	24±0.3	25±0.2	25±0.1	25±0.9	25±1	25±0.7	24±0.6	24±0.4	26±0.5	26±0.4
C16:1	0.5 ± 0.1^{abc}	0.6 ± 0.1^{a}	0.3 ± 0.0^{bc}	0.6 ± 0.1^{a}	0.5 ± 0.1^{abc}	0.3 ± 0.0^{c}	0.6 ± 0.1^{ab}	0.5 ± 0.0^{abc}	0.6 ± 0.1^{a}	0.7 ± 0.1^{a}
C17:0	0.4 ± 0.0^{c}	0.5 ± 0.0^{bc}	0.5 ± 0.0^{bc}	0.5 ± 0.0^{b}	0.5 ± 0.0^{b}	0.4 ± 0.0^{c}	0.7 ± 0.0^{a}	0.7 ± 0.0^{a}	0.3 ± 0.0^{d}	0.3 ± 0.0^{d}
C17:1	0.1 ± 0.0^{bc}	0.1 ± 0.0^{abc}	0.1 ± 0.0^{abc}	0.1 ± 0.0^{ab}	0.1 ± 0.0^{abc}	0.0 ± 0.0^{c}	0.2 ± 0.0^{a}	0.1 ± 0.0^{ab}	0.0 ± 0.0^{c}	0.0 ± 0.0^{c}
C18:0	21 ± 0.3^{cd}	20 ± 0.3^{d}	22 ± 0.1^{ab}	21 ± 0.2^{cd}	20 ± 0.2^{d}	22 ± 0.1^{ab}	21 ± 0.3^{bc}	23 ± 0.3^{a}	21 ± 0.4^{cd}	21 ± 0.2^{cd}
C18:1	12 ± 0.2^{b}	12 ± 0.2^{ab}	8 ± 0.1^{d}	12 ± 0.2^{ab}	12 ± 0.3^{a}	7 ± 0.2^{e}	10 ± 0.1^{c}	10 ± 0.2^{c}	7 ± 0.1^{e}	7 ± 0.2^{e}
C18:2	13 ± 0.1^{b}	11 ± 0.3^{c}	15 ± 0.1^{a}	10 ± 0.1^{cd}	9 ± 0.2^{e}	15 ± 0.4^{a}	10 ± 0.4^{d}	10 ± 0.4^{cd}	15 ± 0.3^{a}	15 ± 0.3^{a}
C18:3n3	0.7 ± 0.0^{a}	0.3 ± 0.0^{b}	0.3 ± 0.0^{b}	0.2 ± 0.0^{c}	$trace^{d}$	$trace^{d}$	$trace^{d}$	$trace^{d}$	0.4 ± 0.0^{b}	0.4 ± 0.0^{b}
C20:0	0.25 ± 0.0^{abc}	0.25 ± 0.0^{abc}	0.26 ± 0.0^{a}	0.25 ± 0.0^{abc}	0.25 ± 0.0^{ab}	0.26 ± 0.0^{a}	0.25 ± 0.0^{ab}	0.26 ± 0.0^{a}	0.23 ± 0.0^{bc}	0.22 ± 0.0^{c}
C20:1	0.2 ± 0.0^{a}	0.2 ± 0.0^{a}	$trace^{b}$	0.2 ± 0.0^{a}	0.2 ± 0.0^{a}	$trace^{b}$	0.1 ± 0.0^{b}	0.1 ± 0.0^{b}	$trace^{b}$	$trace^{b}$

Fatty Acid	Canola/Flax	Canola	Soy	High Oleic Canola/ Canola	High Oleic Canola	Safflower	Lard/Soy	Lard/Soy Baseline	Low Fat/Soy	Low Fat/Soy Baseline
C20:2	0.2±0.0^{c}	0.2±0.0^{c}	0.4±0.0^{b}	0.2±0.0^{c}	0.2±0.0^{c}	0.5±0.0^{a}	0.1±0.0^{c}	0.2±0.0^{c}	0.3±0.0^{b}	0.3±0.0^{b}
C20:3n6	0.83±0.0^{a}	0.68±0.0^{cd}	0.70±0.0^{cd}	0.71±0.0^{c}	0.61±0.0^{e}	0.63±0.0^{de}	0.72±0.0^{bc}	0.74±0.0^{bc}	0.74±0.0^{bc}	0.80±0.0^{ab}
C20:4n6	21.3±0.2^{e}	23.4±0.5^{d}	22.4±0.1^{de}	23.6±0.7^{cd}	25.5±1.2^{ab}	24.2±0.5^{bcd}	26.5±0.6^{a}	25.3±0.5^{abc}	23.3±0.7^{d}	23.1±0.3^{de}
C20:5n3	0.07±0.0^{a}	0.03±0.0^{b}	0.02±0.0^{b}	0.02±0.0^{b}	trace^{b}	trace^{b}	trace^{b}	trace^{b}	0.02±0.0^{b}	0.02±0.0^{b}
C22:0	0.38±0.0^{c}	0.37±0.0^{c}	0.46±0.0^{abc}	0.38±0.0^{c}	0.39±0.0^{c}	0.45±0.0^{abc}	0.49±0.0^{ab}	0.51±0.0^{a}	0.40±0.0^{bc}	0.37±0.0^{c}
C22:4n6	0.25±0.0^{g}	0.37±0.0^{f}	0.45±0.0^{de}	0.41±0.0^{ef}	0.52±0.0^{bc}	0.77±0.0^{a}	0.57±0.0^{b}	0.51±0.0^{cd}	0.45±0.0^{de}	0.43±0.0^{ef}
C22:6n3	1.4±0.02^{abcd}	1.5±0.07^{ab}	1.3±0.04^{cd}	1.5±0.05^{abc}	1.4±0.10^{abcd}	0.50±0.02^{e}	1.6±0.07^{a}	1.3±0.10^{abcd}	1.3±0.09^{bcd}	1.2±0.03^{d}
C24:0	2.1±0.07^{bc}	2.0±0.11^{c}	2.5±0.12^{a}	2.0±0.07^{c}	1.9±0.06^{c}	2.4±0.08^{ab}	2.4±0.12^{ab}	2.6±0.12^{a}	2.6±0.08^{a}	2.4±0.06^{ab}
C24:1	1.1±0.03^{c}	1.2±0.06^{bc}	0.6±0.01^{d}	1.3±0.08^{b}	1.5±0.07^{a}	0.5±0.02^{d}	0.6±0.03^{d}	0.6±0.04^{d}	0.6±0.01^{d}	0.5±0.01^{d}

Mean ± standard error without a common letter differ significantly ($p<0.05$).