Linoleic Acid Derived Oxylipins are Elevated in Kidney and Liver and Reduced in Serum in Rats Given a High Protein Diet

By

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Abstract

High protein (HP) diets are often used as a means to reduce obesity, but their long-term effects remain unclear. In vitro studies suggest the involvement of a subset of oxylipins in the tissue response to HP diets. To examine the role of these bioactive lipids in vivo, normal adult male Sprague Dawley rats were provided isocaloric diets with LP (low protein, 8% protein by weight), NP (normal protein, 14%) or HP (50%) diets for two weeks, and targeted lipidomic analysis of oxylipins in kidney, liver and serum was performed by HPLC-MS/MS. The main group of oxylipins affected by the HP diets was the oxylipins derived from linoleic acid (LA), many of which were elevated in kidney and liver, but reduced in serum of rats provided the HP compared to NP or LP diets. Further studies are needed to elucidate the physiological effects of the changes in these novel oxylipins in response to dietary HP.

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List of Abbreviations

Δ	Delta
AA	Arachidonic acid
AdA	Adrenic acid
ALA	α-linolenic acid
ALT	Alanine aminotransferase
AMDR	Acceptable Macronutrient Distribution Range
AST	Aspartate aminotransferase
BHT	Butylated hydroxy toluene
BMI	Body mass index
CKD	Chronic kidney disease
COX	Cyclooxygenase
CYP450	Cytochrome P450
20 COOH AA	20 carboxy arachidonic acid
DGLA	Dihomo-γ-linolenic acid
dh	Dehydro
DHA	Docosahexaenoic acid
dhk	Dihydro-keto
DiHDoHE	Dihydroxy-docosahexaenoic acid
DiHDoPE	Dihydroxy-docosapentaenoic acid
DiHETE	Dihydroxy-eicosatetraenoic acid
DiHETrE	Dihydroxy-eicosatrienoic acid
DiHODE	Dihydroxy-octadecadienoic acid

DiHOME	Dihydroxy-octadecenoic acid
DPA	Docosapentaenoic acid
EDTA	Ethylenediaminetetraacetic acid
EPA	Eicosapentaenoic acid
EpDPE	Epoxy-docosapentaenoic acid
EpETE	Epoxy-eicosatetraenoic acid
EpETrE	Epoxy-eicosatrienoic acid
EpODE	Epoxy-octadecadienoic acid
EpOME	Epoxy-octadecenoic acid
ELOVL	Elongation of very long-chain fatty acid
ERPF	Effective renal plasma flow
ESRD	End-stage renal disease
Ex	Eoxin
FA	Fatty acid
GLA	Gamma-linolenic acid
GFR	Glomerular filtration rate
HDoHE	Hydroxy-docosahexaenoic acid
HEPE	Hydroxy-eicosapentaenoic acid
HETE	Hydroxy-eicosatetraenoic acid
HETrE	Hydroxy-eicosatrienoic acid
HHTrE	Hydroxy-heptadecatrienoic acid
HEPE	Hydroxy-eicosapentanoic acid

HODE	Hydroxy-octadecadienoic acid
HOTrE	Hydroxy-octadecatrienoic acid
HP	High protein
HpDoHE	Hydroperoxy-docosahexaenoic acid
HpETE	Hydroperoxy-eicosatetraenoic acid
HPLC	High performance liquid chromatography
Hx	Hepoxilin
IOM	Institute of Medicine
k	Keto
KFC	Kidney Foundation of Canada
LA	Linoleic acid
LC/MS	Liquid chromatography/mass spectrometry
LOX	Lipoxygenase
LP	Low protein
MGV	Mean glomerular volume
NE	Nonenzymatic products
NAFLD	Non-alcoholic fatty liver disease
NHANES	National Health and Nutrition Examination Survey
NKF	National Kidney Foundation
NP	Normal protein
OH	Hydroxy
oxo-ETE	Oxo-eicosatetraenoic acid

oxo-ODE	Oxo-octadecadienoic acid
oxo-OTrE	Oxo-octadecatrienoic acid
PG	Prostaglandin
PD	Protectin
PGEM	Prostaglandin E metabolite
PGDM	Prostaglandin D metabolite
PUFA	Polyunsaturated fatty acid
PDX	10(S), 17(S)- dihydroxy-docosahexaenoic acid
Rv	Resolvin
SAS	Statistical analysis software
sEH	Soluble epoxide hydrolase
SPE	Solid phase extraction
TLC	Thin-layer chromatography
TriHOME	Trihydroxy-octadecenoic acid
TX	Thromboxane
WHO	World Health Organization

Chapter 1 Literature Review

1.1 Introduction

Dietary protein is a necessary constituent of healthy diets. According to U.S. and Canadian dietary reference intakes [1], the recommended allowance for protein for adults of 0.8 g/kg of body weight per day is "the average daily intake level that is sufficient to meet the nutrient requirement of nearly all (~98%) healthy individuals".

However, there is increasing interest in increasing protein consumption to reduce body weight. 54% of the Canadian population over the age of 18 self-reported as overweight or obese in 2014 [2]. In a list ranking countries with the highest obesity rates, Canada ranks 35th highest out of 194 countries in which its population has a Body Mass index (BMI) of at least 25 kg/m^2 [3]. In 2014, 38% of men and 40% of women were overweight, of which 13% were obese across the globe. From 1980 to 2016 the prevalence of obesity more than doubled worldwide [4].

As the incidence of obesity and overweight is becoming more common, high protein (HP) diets are gaining in popularity to improve weight loss. However, there are controversies surrounding HP diet consumption and insufficient data to support the long term safety of HP diets.

As the report on Dietary Reference Intakes for Macronutrients by The Institute of Medicine (IOM, 2005) states:

“There was insufficient evidence to suggest an upper limit for protein and insufficient data to suggest an upper limit for an Acceptable Macronutrient Distribution Ranges (AMDR) for protein. To complement the AMDRs for fat (20 to 35 percent energy) and carbohydrate (45 to 65

percent energy) for adults, protein intakes may range from 10 to 35 percent of energy intake to ensure a nutritionally adequate diet" (p 844).

By this definition a diet containing more than 35% of energy from protein is considered to be a HP diet. On the one hand, HP diets possess many reported beneficial effects such as improving weight loss, increasing satiety, and improving body composition, lipid-related risk factors and fasting insulin levels [5–7]. On the other hand, there have been reports of HP diets having detrimental effects on the kidney. For example in animal models it has been found that long term consumption of HP diets resulted in enlarged kidneys, increased glomerular filtration rates, renal and glomerular hypertrophy, fibrosis and higher renal glomerulosclerosis [8–12]. HP diets also cause glomerular hyperfiltration and hyperemia, acceleration of chronic kidney disease and increased risk for nephrolithiasis in individuals with chronic kidney disease or other susceptible groups such as those with diabetes [13,14].

Kidney health is influenced by oxylipins and kidney damage is associated with changes in prostanoids [15]. Prostanoids are prostaglandins (PG) and thromboxanes (TX) and belong to a group of bioactive lipids referred to as oxylipins. Oxylipins are oxidized metabolites formed from various polyunsaturated fatty acids (PUFA) and exhibit regulatory functions such as blood pressure regulation and immune function [16,17]. The rate of prostanoid production is directly related to the amount of protein in the diet [18]. In rats given a HP diet, the synthesis of major vasodilatory prostanoids PGE_2, $PGF_{2\alpha}$ and vasoconstrictory TXB_2 were increased compared to a low protein (LP) diet [18]. Renal blood flow and glomerular filtration rate can be increased with dietary HP because of increased renal prostanoid formation [19,20]. In the development of kidney disease in rats with renal ablation elevated thromboxane formation may be an important mediator [21]. These studies show that HP can change one class of oxylipin (prostaglandins) in

the kidney that could exert adverse effects on kidney. Liver is the key site of protein metabolism and liver and blood oxylipins have been associated with a number of diseased liver conditions and other disorders [22–28], but these also have not been examined in the context of HP diets. Serum oxylipins analysis will help to determine how well they reflect tissue oxylipin profiles.

Therefore, the purpose of this project is to examine the effect of dietary protein level on the kidney, liver and serum oxylipin profiles.

1.2 Dietary protein

1.2.1 Different levels of protein

Even though HP diets have been popular throughout the last century for weight management there is no uniform agreement on what constitutes a HP diet. According to many literature reports, a diet consisting of 25% or more calories from protein or intakes of daily protein >1.5 g/kg is considered to be a HP diet [13,29]. There are some common HP diets namely Atkins, South Beach, Zone, Protein Power and Stillman diets. Although differences are present among these popular HP diets, their main philosophy is that eating more carbohydrate leads to obesity and other complications. Protein and some of the fats can lead to weight loss and so they are considered healthy. The level of protein in theses diets varies from 71 to 162 g/d, or 26% to 64% of energy; on the other hand carbohydrate intake is 7 to 56 g/d, or 3% to 16% of total energy intake (reviewed by St Jeor et al.) [30]. Those diets recommend avoiding carbohydrates and eating lean meat, fish, eggs and cheese.

1.3 HP diets and health

HP diets have several beneficial and adverse effect on health. Although there are controversies, it has a beneficial role in weight management, in improving body composition as

well as diminishing cardiovascular disease risk [7,31]. HP diets exert their weight lowering effects by increasing satiety, therefore decreasing energy intake and postprandial insulin level and by eliciting a higher thermic effect. The thermal effect of protein is between 20–25% of energy intake [32], whereas the thermic effect of both carbohydrate and fat is between 5–15% [33,34]. The thermal effect of protein helps by promoting a negative energy balance by increasing energy expenditure [35]. Protein metabolism occurs immediately as the body has no storage capacity for protein. Expenditure of energy during protein synthesis, urea production and gluconeogenesis is higher than fat and carbohydrate. It is believed that all above mentioned mechanisms happen together and are interrelated [36–38]. However HP diets showed no beneficial effects on cardiovascular disease, glycemic control or obesity in a systematic review of long term randomized controlled trials [39].

Although there are several studies indicating that HP diets are good for bone health its beneficial effects still remains controversial [40]. Some authors claim that increasing insulin like growth factor -1 or higher intestinal calcium absorption with a HP diet may improve calcium absorption as well as bone health [41–43]. A meta-analysis of randomized controlled trials revealed a positive effect of protein supplementation on mineral density of spine bone [44]. In another study, a HP diet decreased the risk of fractures in post-menopausal women [45]. Many researchers conclude that HP diets do not lead to calcium bone loss and do not cause adverse effects on bone health [14,46]. In contrast, other researchers have observed that HP diets may be harmful for bone health. Moreover, inadequate calcium supply is considered responsible for the undesirable effect of HP diets on bone health, based on clinical data [46]. HP diets also may have an adverse effect on bone health by increasing calciuria [14]. A HP diet is responsible for a

higher acid load which cannot be neutralized by the kidneys, so as a result, the body collects calcium from bones to balance the pH, and the calcium is then excreted in the urine [14,46,47].

Research related to the effects of HP diets on hypertension is controversial, as both positive and no effects have been demonstrated so far. In a recent study overweight and obese females were fed a HP diet (30% energy as protein, 40% as carbohydrate) and results showed that diastolic blood pressure decreased more in this group compared to those that consumed a high carbohydrate diet containing 20% energy as protein and 50% as carbohydrate [6]. Meta-analysis of 40 trials showed that HP diets results in a decrease in blood pressure independent of weight change [48]. However, no significant differences were found for systolic or diastolic blood pressure in other meta-analyses comparing HP diets to LP [7,39]. It is thought that HP consumption is responsible for higher intake of sodium hence subsequent hypertension but there is no valid data to support this claim [49,50]. In the Omni Heart study a HP diet (25% energy as HP, 48% as carbohydrate) compared with a high carbohydrate diet (15% energy as protein, 58% as carbohydrate) reduced mean systolic blood pressure by 3.5 mm Hg among hypertensive individuals and 1.4 mm mercury among normal individuals [51].

Dietary protein is a very important macronutrient for athletes, particularly body builders, power lifters and other exercisers and should be consumed on a daily basis. Current Recommended Dietary Allowance seems to provide adequate protein for athletes but it may not provide an ideal dietary protein for high levels of exercise [52]. Athletes have been consuming HP diets for centuries. They mainly consume HP diets to increase muscle mass and for the recovery from intense exercise. Intense exercise, mostly exercise with a high-strength element, may lead to reduction in the ability to accomplish subsequent exercise sessions, muscle pain and loss of muscle proteins [53]. For improving these negative consequences HP intake during

recovery has been advocated. Although the recommendation for strength and power athletes are usually in the range of 1.5–2.0 g protein/kg/d, usually they consume over 2 g protein/kg/d and many consume over 3 g protein/kg/d [54]. The recommendations may vary based on training status: skilled athletes would require less amount of protein compared to non-experienced counterparts [55]. To stimulate muscle protein synthesis, leucine and other branched-chain amino acids, play a significant role. Depending on the caloric deficiency, protein consumption, ranging from 1.8–2.0 g/kg/day may be beneficial in preventing lean mass losses and promoting fat loss during energy restriction period [55].

Apart from several beneficial effects there are some negative effects of HP diets which mainly focus on kidney function but no definite conclusion have been reached yet [29]. Effect of HP diets on kidney and liver health are discussed in greater detail in section 1.5.

1.4 Protein sources

The above mentioned HP diets generally have used protein sources from animal origin because plants contain more carbohydrates. However, in numerous studies animal proteins have been replaced by plant proteins [56–60]. The plant protein source mostly used has been soy protein [50] while others used include wheat gluten, nuts/seeds.

Health outcomes vary depending on the protein source [61]. The beneficial effects of soy protein, dairy and whey protein, mostly in the normal range have been observed on several parameters such as weight loss, glucose homeostasis and hepatic steatosis in several studies [62–66].

A meta-analysis of 41 prospective, randomized controlled trials revealed that soy protein supplementation was beneficial including causing decreases in total cholesterol, low-density

lipoprotein cholesterol and triglycerides and increases in high-density lipoprotein cholesterol among adults with or without hypercholesterolemia [67]. In a study of 100 obese subjects, those consuming a soy-based meal replacement formula (240 g/day) compared to 90 g/day lost more weight (7.0 kg vs. 2.9 kg) and considerably more in body fat mass [68]. On the other hand other researchers have reported no effects of protein supplementation [69,70].

A recent study in overweight and obese humans consuming whey protein or casein protein (54g/day) or a control group (54 g glucose) for 12 weeks found no significant difference in body weight, BMI, waist circumference, total body fat, or lean body mass [69]. In obese rats a HP diet from animal and plant mixed protein sources (complete milk protein, egg white, soy protein isolate and wheat gluten) is more beneficial than a HP casein-based diet in reducing insulin resistance and hepatic steatosis [50].

1.5 HP diets and kidney health

1.5.1 Kidney disease

Chronic kidney disease (CKD) is a condition when the kidney loses its ability to keep healthy and results in kidney damage, impaired kidney function and renal injury. The National Kidney Foundation (NKF) definition of CKD includes: 1) "Kidney damage for 3 months, as defined by structural or functional abnormalities of the kidney, with or without decreased glomerular filtration rate (GFR) manifest by either: pathological abnormalities or markers of kidney damage, including abnormalities in the composition of blood or urine, or abnormalities in imaging tests 2) GFR<60 mL/min/1.73 m^2 for 3 or more months, with or without kidney damage" [71]. Diabetes, high blood pressure, family history, older age and ethnic group these are the risk factors for chronic kidney disease. According to the NKF there are five stages of CKD characterized by increasing degree of kidney impairment (Table 1.1).

Table 1.1: Stages of chronic kidney disease and protein recommendations [1]

Stage	Description	GFR (mL/min/1.73 m^2)	Protein recommendation (g/kg body weight/day)
1	Kidney damage with normal or elevated GFR	≥ 90	0.75
2	Kidney damage with normal with mild decrease GFR	60–89	0.75
3	Moderate decrease GFR	30–59	0.75
4	Severe decrease GFR	15-29	0.6-0.75
5	Kidney failure/ESRD[2]	<15 or dialysis	0.6 (non-dialyzed) 1.2-1.3 dialyzed

[1]Adapted from National Kidney Foundation, 2002 [72]

[2] ESRD=end stage renal disease

A recent report stated that 30 million American adults are suffering from CKD and millions of other Americans are at higher risk [73]. Around 2.6 million Canadians have kidney disease, or are at risk of this chronic disease. An average of 16 people's kidneys are failing every day. Over the past 20 years, the number of Canadians who received treatment for kidney failure has tripled. Mostly senior citizen 65 years of age or older are new renal failure patients and the failure rate is 53% [74]. In 2010, 39,352 people received treatment for kidney failure among them 59% (23,188) were on dialysis and 41% (16,164) had an operative transplant [74].

1.5.2 Effect of HP diets on kidney (human studies)

There is no clear evidence that HP diets have negative effects in healthy individuals with normal kidney function [13,46]. Early studies were mostly based on single-meal studies and found the effects of HP diets on kidney were mainly on kidney hemodynamic responses. In 12 healthy individuals, provided HP diets (1.5 g/kg) there was an increase in urine concentration and the peak of postmeal renal plasma flow (RPF) was raised by 13%. As well, postmeal GFR peak was 10% higher than baseline 3 hours after the meal [75]. Similar findings were found in other studies showed that single HP diets lead to an increase in RPF, proteinuria and GFR [76–78]. The eleven year prospective cohort Nurses' Health Study on women (42 to 68 years) with protein consumption (60 g to 93 g /day) found that HP consumption was not linked with GFR change in women with normal kidney function [79]. Another recent long term study (2 years) also found no detrimental effects of HP diets on glomerular filtration rate, albuminuria, or fluid and electrolyte balance in healthy, obese subjects [80]. Moreover, no noticeable effects of HP on markers for kidney health were seen in two recent meta-analyses which compared HP to LP on long term health outcomes [39,81]. HP intake alters kidney size and function in the short term

without any adverse long term effects; these alterations may be considered normal adaptive mechanisms in the healthy kidney [82,83].

In contrast to healthy individuals free of renal diseases, HP diets cause adverse effects such as glomerular hyperfiltration and hyperemia, acceleration of chronic kidney disease, increased proteinuria and increased risk for nephrolithiasis in individuals with chronic kidney disease or other susceptible groups such as those with diabetes [13,14,46]. There is a higher chance of glomerular hyperfiltration in obese individuals [84,85] which could play an important role in diet-associated hyperfiltration related kidney damage [80]. Some of these studies are summarized in Table 1.2.

Diet is the major determinant of acid load and HP diets play a crucial role in this case. Diets which have a high acid content may contribute to increasing the risk of developing kidney failure in patients suffering from chronic kidney disease. 1486 adults with chronic kidney disease in the National Health and Nutrition Examination Survey III (NHANES III) had a higher acid load which was strongly related with progression to kidney damage. High acid diet groups were three times more likely to progress to kidney damage compared to patients who had low acid diets [86]. Acidosis, mineral metabolism disorders, and oxidative stress which are related to the advancement of chronic kidney disease, are positively influenced by diets containing LP in CKD patients [87,88].

Effects of HP diets in human study is controversial. Studies providing a HP regimen have mainly been conducted on obese individuals, diabetes and other chronic disease patients. In a weight reduction study a total of 65 healthy, overweight and obese adults were randomly assigned to 3 different groups i.e. a high carbohydrate group (12% protein, 58% carbohydrate),

Table 1.2: Summary of effect of protein level on kidney (human studies)

Long term (Disease/obese)			
References	**Protein level**	**Duration**	**HP Effects**
Coppo et al. 1988	1.6 g/kg/d (meat derived protein)	4 weeks	Proteinuria increased by 200%
Bilo et al. 1989	80-90 g/d (soy, lactoprotein and beef)	4 weeks	50% rise in proteinuria Significant rise in GFR and renal plasma flow
Wetzels et al. 1988	139.6 g/d, 1.8 g/kg/d (HP), 0.6 g/kg/d LP (animal protein)	4 weeks	30% rise in proteinuria Glomerular filtration rate (inulin clearance) did not change significantly
Long term (Normal/healthy)			
Viberti et al. 1987	NP diet 75 g/d (animal and vegetable protein) After 3 weeks low-protein diet 43 g/d were given	3 weeks	GFR was lower on LP group than on NP as was renal plasma flow Amount of urinary albumin was also lower after LP than NP
Short term (Normal/healthy)			
Bergstrom et al. 1985	LP and HP diet (milk, cheese, meat and fish)	6 days	In HP diet group GFR was higher than LP diet After providing LP diet ERPF also increased significantly.
Frank et al. 2009	HP 2.4 g/kg/d NP diet g/kg/d (milk and milk products)	7 days	GFR and filtration fraction increased on HP diet. Renal plasma was not significantly different in HP and NP. In HP group blood urea nitrogen, serum uric acid, urinary albumin, and urea excretion rose significantly.
Verhoef et al. 2005	HP 21% of energy as protein (supplement, milk protein) LP 9% of energy as protein	8 days	Total homocysteine (tHcy) concentrations rose with HP diet throughout the day but fasting tHcy concentrations remain unchanged.

HP group (25% energy from protein, 45% as carbohydrate), and a control group. Diets were provided ad libitum. The greater weight (8.9 kg reduction) and fat loss (7.6 kg) was seen in subjects in the HP group after six months of study. Fasting plasma triglycerides and fasting free fatty acids were also decreased in the HP group [89]. Similar findings also have demonstrated greater weight and fat loss using HP (25–30% of energy) ad libitum diets compared to high carbohydrate diets [90–93]. One of the reasons may be that during consumption of HP diet (ad libitum), subjects usually eat a smaller amount [94].

However, some studies showed no significant difference in weight loss when comparing energy-restricted, HP diets to high carbohydrate diets [36,95,96]. Two other studies showed that subjects in the HP diet groups lost more weight compared to subjects in the high carbohydrate diet groups [6,97]. A meta-analysis of randomized controlled trials showed that HP diets provide more favorable changes on weight loss and body composition when compared to isocaloric, HP, low fat energy-restricted diets with standard protein [7].

HP diets showed no adverse effects on kidney in a very recent human study. In a randomized crossover trial, 12 healthy resistance-trained men were given 2.6 ± 0.8 and 3.3 ± 0.8 g/kg/day of HP, respectively for four months and found no changes on blood lipids or markers of renal and hepatic function. This study was the first crossover trial provided HP diets over 4 times the recommended dietary allowance, using resistance-trained subjects observed no harmful effects [98].

In contrast to effects on normal kidney, protein consumption more than the AMDR may adversely affect people with mild renal insufficiency. In a study, risk for microalbuminuria was gradually augmented with higher protein consumption, particularly in those whose daily protein intake was more than 1.5g/kg body weight, which is considered moderately high (~20% of daily

energy). This findings was reported by a Dutch survey among 680 healthy Caucasians who were 50 to 75 years old [99]. However, Skov et al. showed that protein level did not alter albumin excretion in overweight individuals in a 6 month study [100].

According to NHANES III, 23-32% of adults (>20 years of age) have mild renal insufficiency and 41-47% individuals >40 years of age have GFR between 60 and 89 [101]. These individuals are thought to be healthy and often not aware of their health condition. In a study, each 10g increase in protein, specifically nondairy animal protein was linked with a decrease in GFR by 7.72 ml/min per 1.73 m^2 of women with mild renal insufficiency whose GFR was between 55 and 80 ml/min [79]. Women in the highest quintile had a 3.51 times higher risk of at least a 15% reduction in GFR in comparison with the lowest quintile.

To sum up, HP diets have a role in increasing GFR, proteinuria, RPF, urinary albumin, serum uric acid, blood urea nitrogen and natriuresis mainly in obese/diseased individuals but to a lesser extent in healthy subjects also.

1.5.3 Effect of HP diet on kidney (animal studies)

Even though the effect of HP diet on kidney disease has not been thoroughly studied in humans, animal model data are available.

Dietary protein at upper limit of AMDR have potential negative effects on healthy pigs as well as rats including enlarged kidneys, proteinuria and, glomerulosclerosis. Pigs provided HP (35% energy as protein) compared to normal protein (15% energy as protein) diet had larger kidneys at both 4 and 8 months. The HP group had 55% more renal fibrosis and 30% more glomerulosclerosis compared to the normal protein group. Moreover, the HP group also had higher renal and glomerular volumes, renal monocyte chemoattractant protein-1 levels and

plasma homocysteine levels in comparison with the normal protein diet group [8]. In rats, higher kidney weights, proteinuria and creatinine clearance, larger glomeruli and more glomerulosclerosis were found in rats who received a HP diet (35% energy as protein) for 17 months compared with rats who received a normal (15% energy as protein) protein diet [12]. Animal studies related to HP diets and kidney function are summarized in table 1.3.

Animals on HP diets also have higher rates of protein excretion, kidney weight and size and proteinuria [102–105]. A strong correlation between renal lesions and proteinuria was found in normal Sprague-Dawley rats provided 7, 23 and 42% protein as energy in over 20 months starting at 66-70 days [102]. Average proteinuria was greater in rats provided 23% protein and also rats on the highest protein diet had proteinuria. Serum creatinine was higher in comparison with other two diets that remained unchanged over time by the end of the study. Tubulo-interstitial damage and focal segmental glomerular sclerosis was found on the highest protein diet. On the other hand 6 out of the 10 rats on the 23% diet and 3 out of 10 on the 7% diet had any histological damage.

HP diets are responsible for increasing the kidney load therefore affecting filtration rates. To explain permselectivity of the glomerulus, rats were given 8 and 22% protein before being administered different sized polyethylene glycols intravenously. In the normal rats there was an increase in the amounts of polyethylene glycols recovered in the urine on the higher protein diet, meaning that normal filtration process was hampered [106]. Another study found renal hypertrophy occurred in the HP fed animals (32% casein) for 5 to 6 weeks and also GFR in the HP rats was almost double than LP rats provided a low protein diet that contained 10% casein [9].

In a long term study the rats fed 23% protein, renal pathology scores were 40% higher compared to the 14-15% diet group [107–109]. Similar results also were found in several studies

Table 1.3: Summary of effect of protein level on kidney (animal studies)

Long term (Disease)				
References	**Protein level**	**Duration**	**Species**	**HP Effects**
Hostetter et al. 1986	6% and 40% (Teklad rodent diet, casein)	4 and 8 months	135 Male Munich-Wistar rats, 12 weeks of age at start of study Reduced renal mass	Higher GFR at both time Higher protein excretion Greater prevalence of sclerotic glomeruli were seen in animals with loss of renal mass after 8 months. Higher prevalence of sclerotic glomeruli at each level of initial renal mass
Long term (Normal/healthy)				
Spector et al. 2012	40, 18, 6, and 2% (Casein)	3 weeks	Female Sprague Dawley rat Weighing 200-240 g	HP groups gained more weight Mean serum urea nitrogen (UN) concentration and 24-h urine UN excretion were highest HP groups had the largest quantity of urinary urea reabsorption
Ichikawa et al. 1980	40% casein 6% casein	4 months	28 Munich-Wistar rats Weighing 90g	HP increased body weight and kidney weight and GFR
Aparicio et al. 2013	45% HP 10% NP (soy protein supplement)	12 weeks	20 Wistar rats Young, weighing 148±6g	Kidney weight 22% higher Glomerular size 30% higher and glomerular area was 13% higher.
Klahr et al. 1992	40% casein HP 6% casein LP	8 weeks	Rat	Increased synthesis of eicosanoid in medullary or papillary tubules but not in cortex tubules
Yanagisawa et al. 1998	40% casein HP 6% casein LP	8 weeks	Male Lewis rats Weighing 250g	Medullary tubules produced higher PGE_2, 6-keto $PGF_{1\alpha}$, and TxB_2 eicosanoids (1.5–

				2.0 times) compared to LP group No significant difference in cortical tubules from HP or LP
Wakefield et al. 2011	35% as HP and 15 % NP (plant and animal whole protein)	4, 8, 12 and 17 months	70-d-old female Sprague-Dawley rats	17 % higher kidney weights, 3 times higher proteinuria and 27 % higher creatinine clearance were found in HP diet group than NP diet. Rats provided HP diets had enlarged glomeruli and more glomerulosclerosis than NP-fed rats.
Jia et al. 2010	35% as HP and 15 % NP (whole plant and animal protein)	Either 4 or 8 months	Pigs	Kidney enlarged with HP diet compared with NP diet at both 4 and 8 mo. Renal and glomerular volumes were found 60-70% higher. 55% more fibrosis and 30% more glomerulosclerosis were also observed by the end of the study.
Short term (Disease)				
Stahl et al. 1987	510 g/kg (HP) and 87.6 g/kg (LP)	2 weeks	White male Wistar rats (4 groups, 15 each) Weighing 155-165g Remnant kidney (70% reduction)	Formation of PGE_2, 6-keto $PGF_{1\alpha}$, and TxB_2 in isolated rat glomeruli were increased in HP group Higher remnant kidney weight on HP
Don et al. 1989	High (40%) or low (8.5%) casein isocaloric diet	10 to 14 days	Male Sprague Dawley rats Weighing 155-240g Diabetes mellitus, Heymann nephritis, and partial renal ablation	Body weight and renal weight, urinary albumin excretion were greater with HP. PGE_2, $PGF_{2\alpha}$, and TxB_2 was higher in the rats that fed the HP regimen. No major differences were found between the two dietary protein groups in the production of

				eicosanoids by preparations of renal papillae
Chen et al. 2003	Casein or soybean protein (200 or 100 g/kg diet)	1 week	Male Sprague–Dawley rats Weighing 200–250g Chronic renal failure, five-sixths nephrectomy	Chronic renal failure was accelerated in rats consuming 200 g casein/kg diet.
Short term (Normal/healthy)				
Don et al. 1989	High (40%) or low (8.5%) casein isocaloric diet	10 to 14 days	Male Sprague Dawley rats Weighing 155-240g	Body weight and renal weight, urinary albumin excretion were greater with HP. PGE_2, $PGF_{2\alpha}$, and TxB_2 was higher in the rats that fed the HP regimen. No major difference in the production of eicosanoids by renal papillae
Hammond et al. 1998	7 %, 15 % or 46 % casein by mass	2 weeks	Male and female mice (30 each) 70-90 days old	As protein intake rate increased liver, kidney and stomach mass also increased Kidney wet mass increased by 26-32% with acclimation to HP, dry mass also increased but changes were slightly smaller. Nitrogen filtration rate increased on HP diet Male GFR 69% higher in HP

that examined the effect of protein restriction on nephropathy and lifespan in Fisher rats [110–112]. However, in another study healthy Wistar male rats were fed 52% protein as energy for 6 months, no significant negative effects on renal function and pathology were found [113].

Glomerular and serum IGF levels were increased in rats fed HP diets and exhibited renal hypertrophy [114–116]. Compared to LP diet, rats and mice provided HP diets (23.2% to 44%) have a higher chance of forming nephropathy. As a result, glomerular sclerosis, hypercellular glomeruli, chronic inflammatory cell infiltration, atrophy, tubular protein casts and tubulointerstitial fibrosis, tubule regeneration were observed in the HP groups [102,104,108].

In summary, similar to human studies, HP effects on rats involve increasing body weight, kidney weight, GFR, proteinuria, creatinine clearance, albuminuria both in long and short term studies. Kidney weights are elevated after as little as 1-2 week of HP feeding, indicating that HP diet effects begin to occur very shortly after onset of HP diets. HP diets are also responsible for increasing some vasoactive compounds such as PGs which will be discussed next.

1.5.4 Effect of HP diet on liver

There are few studies on HP diet effects on liver disease and most of these found beneficial effects of HP diets on the liver. For example, a HP diet (35% energy as protein) prevented and reversed the first stage of non-alcoholic fatty liver disease (NAFLD), which is known as hepatic steatosis in male mice independently of carbohydrate and fat intake and more efficiently than a 20% decrease in energy consumption [117]. Intrahepatic fat deposition induced by a hypercaloric, high fat diet was considerably prevented by a HP intake (25% energy as protein) in a human study [118]. A few other short-term studies also showed anti-steatotic effects on the liver after consumption of HP diets independent of reductions in weight [50,119].

In another study 100 obese men and women having BMI 27 to 40 were provided two isocaloric weight loss meals containing HP 2.2 g/kg LBM and SP 1.1 g/kg LBM. At baseline levels of AST, ALT, bilirubin, and alkaline phosphatase were normal for all subjects. There were no significant changes observed in liver function as all markers (AST, bilirubin, alkaline phosphatase) remained in the normal range throughout the study period [120].

Szepesi et al. studied the effects of a HP diet (90% casein and CHO free) for 6 to 8 weeks on rat livers and found that all the enzymes (glucose-6-phosphatase, fructose-1, 6-diphosphatase, L-α glycerophosphate dehydrogenase, glucose-6-phosphate dehydrogenase, malic enzyme and pyruvate kinase) studied, were increased by the HP diet except the enzyme phosphorylase. The HP regimen noticeably decreased liver glycogen values [121]. Overall, protein-enriched meals in comparison with normal protein meals have no potential negative effects on liver function.

1.6 Oxylipins

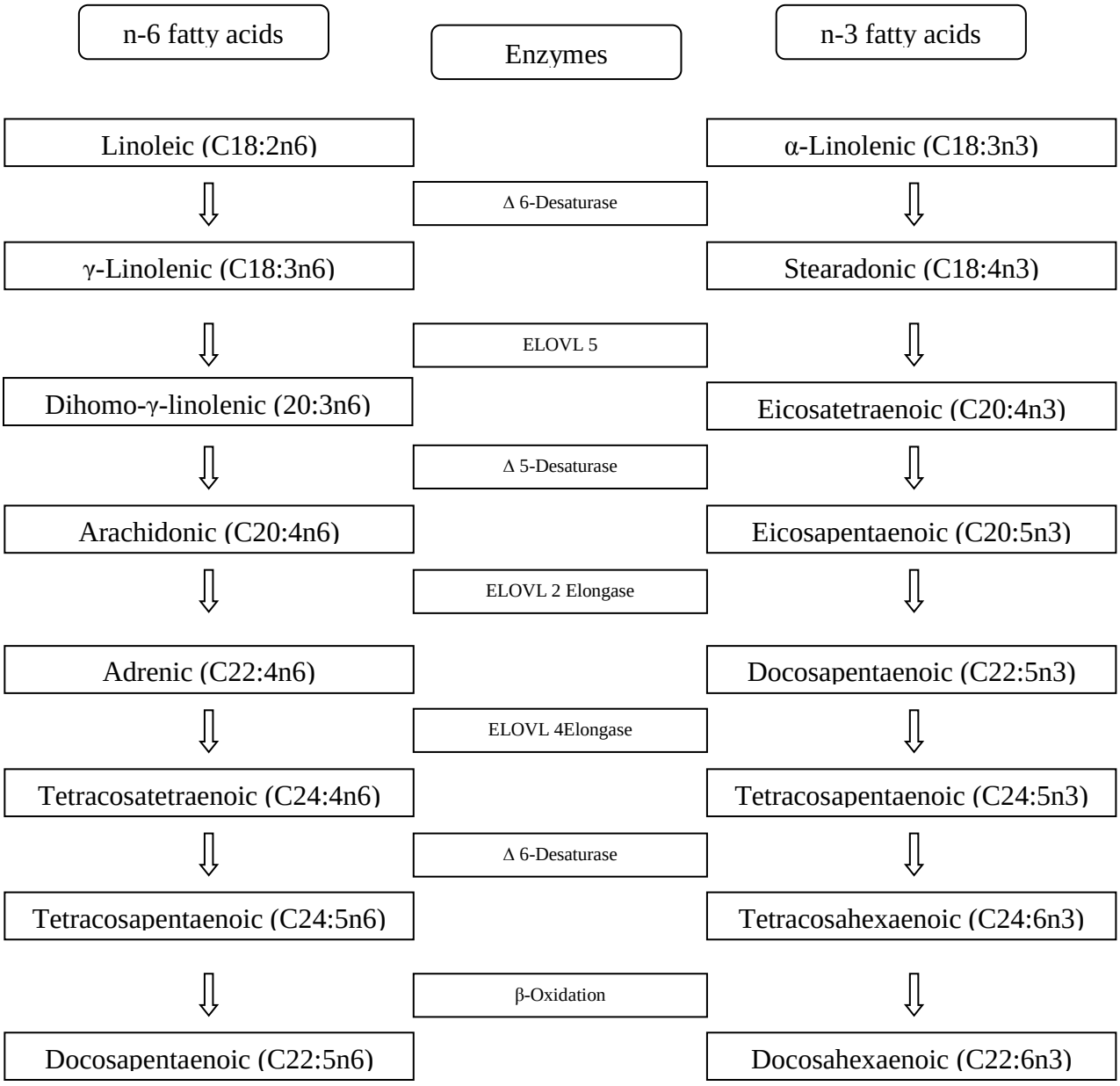
1.6.1 An overview of oxylipins

Oxylipins are a group of oxidized metabolites which have many important physiological effects and are formed from PUFA by pathways involving at least one step of mono or dioxygen-dependent oxidation [17]. They are found widespread in all tissues, urine and blood. Many oxylipins have a short half-life, so they are not stored, but synthesized when needed in a tightly regulated way [17]. The half-life of many oxylipins, however, is not known. By the action of cytosolic phospholipase A_2 ($cPLA_2$) they are released from membrane phospholipids to form eicosanoids, octadecanoids, and docosanoids from 20, 18 and 22 carbon fatty acids, respectively [122,123].

1.6.2 Fatty acid metabolism and oxylipin formation

The essential fatty acids are converted by desaturase and elongase enzymes to form longer chain PUFA that have significant physiological functions in our body. Linoleic acid (LA) is desaturated to form gamma-linolenic acid (GLA), followed by elongation to dihomo-gamma-linolenic acid (DGLA), and then again desaturated to arachidonic acid (AA). Further elongation results in the formation of adrenic acid (n-6). Finally, after elongation, desaturation and β-oxidation, adrenic acid is converted to docosapentaenic acid (n-6). The same enzymes catalyze the conversion of alpha-linolenic acid into eicosapentaenoic acid (EPA), docosapentaenoic (DPA) (n-3), and docosahexaenoic acid (DHA). This is the pathway in the rats but other mammals utilize a different pathway [124]. Fatty acid metabolism is shown in figure 1.1.

Figure 1.1: Fatty acid metabolism



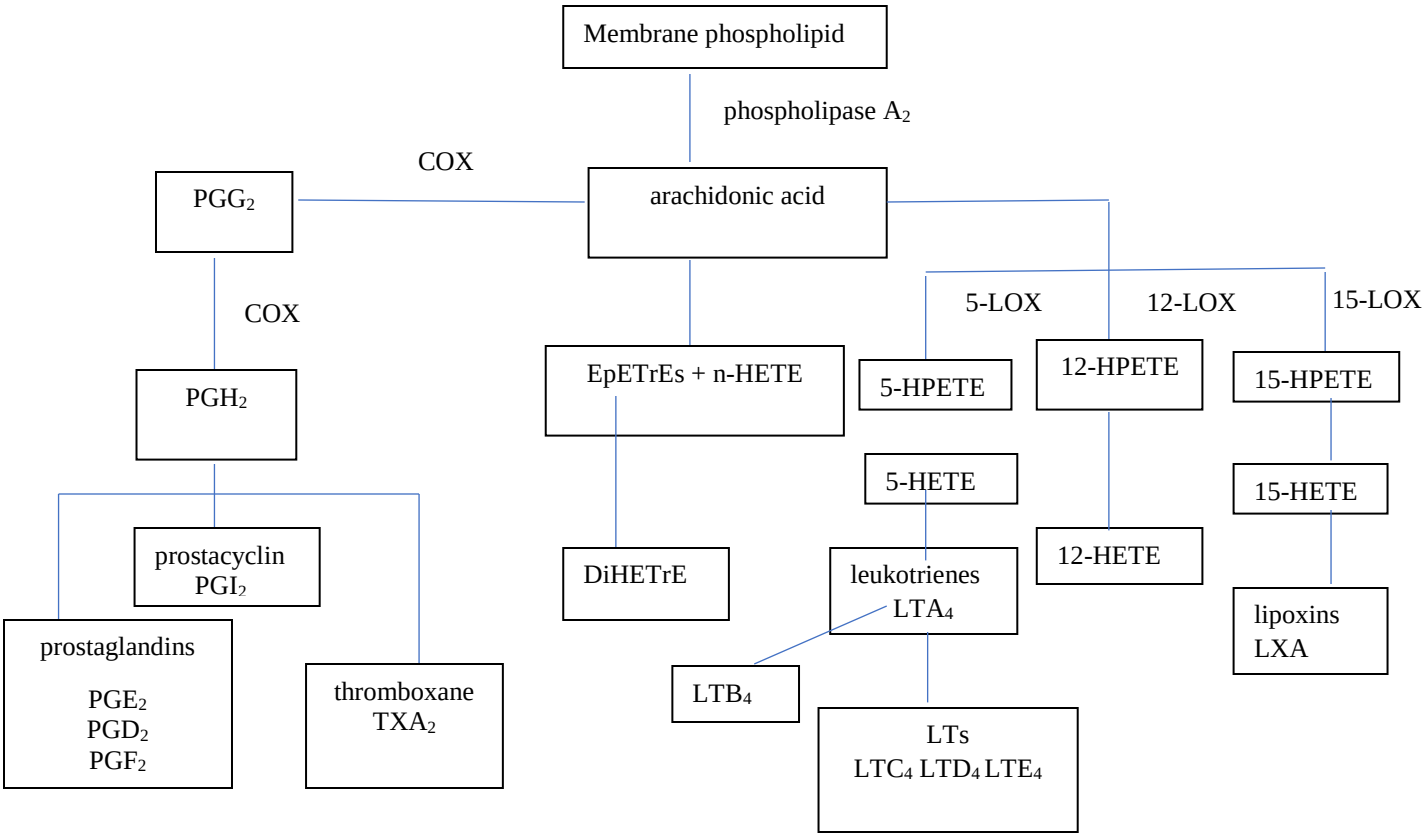
Eicosanoids are oxylipins derived from AA which is an important constituent of membrane phospholipids. Eicosanoids include the PG, TX, leukotrienes, hydroxyeicosatetraenoic acid, epoxyeicosatetraenoic acids and lipoxins [125]. The key oxylipin PUFA precursors DGLA, AA, EPA and DHA can be obtained directly from the diet or from the elongation and desaturation of LA and ALA. LA and ALA also ca be converted to oxylipins. Production of oxylipins from n-6 fatty acids is proportional to high n-6 fatty acid consumption and similarly high n-3 fatty acid consumption is related to high production of n-3 fatty acid derived oxylipins [17].

Oxylipins are formed by the action of three classes of enzymes: a) cyclooxygenase (COX), b) lipoxygenase (LOX), and c) cytochrome P450 (CYP). The first pathway converts 20-carbon PUFA into prostanoids – PG and TX by the action of COX enzymes. COX converts DGLA, AA and EPA into one, two and three series prostanoids, respectively, such as PGD_1, PGD_2 and PGD_3 [126].

The second pathway involves LOX enzymes that catalyze the hydroperoxidation of PUFA, including LA, ALA, DGLA, ARA, AdA, EPA and DHA, to form oxylipins such as leukotrienes, lipoxins, and hydroxy fatty acids [127].

Cytochrome P450 enzymes that produce oxylipins via epoxygenase or omega-hydroxylase activity. Oxidation via the CYP pathway produces epoxides such as AA derived epoxyeicosatrienoic acids and LA derived epoxyoctadecenoic acids. They have role in cardiovascular functions such as vasodilatation of coronary arteries. Biosynthesis of oxylipins are shown in figure 1.2.

Figure 1.2: Biosynthesis of oxylipins



Different oxylipins vary in their length and double bond configurations. The type of oxylipin formed depends mainly on the type of PUFA being oxidized and on the PUFA metabolizing oxygenases and enzyme preference for specific fatty acids [128]. The predominant source of oxylipins is LA and AA. EPA and DHA are two other important sources of oxylipins and can be obtained directly from the diet or can be synthesized from alpha-linolenic acid (ALA), however to a lesser extent [129]. Overall, oxylipins derived from AA are considered pro-inflammatory while oxylipins derived from EPA or DHA are considered anti-inflammatory or less potent. For example, EPA-derived LTB_5 is 10 to 100 fold less potent in stimulating neutrophils compared to LTB_4 [130].

Oxylipins play various functions, they may have similar or opposing effects including apoptosis, tissue repair, blood clotting, proliferation of cell, blood vessel permeability, pain, inflammation, immune actions, and regulation of blood pressure [17].

1.6.3 Oxylipins in kidney, liver and serum

The kidney is a key site of oxylipin synthesis where they are important in the maintenance of renal homodynamics, the renin-angiotensin system, water-electrolyte balance and release of neurotransmitters [131,132]. Not only do oxylipins play a vital role in maintaining GFR and salt-water homeostasis but they also are involved in proliferation and inflammation in response to renal insults. For instance, PGE_2 stimulates cell proliferation during injury such as glomerulonephritis, and over production of such oxylipins, common during disease conditions, leads to abnormal proliferation of renal cells and inflammation [133]. TXA_2 stimulates cell proliferation in culture, and is a potent vasoconstrictor. In addition, TXA_2 synthase inhibitors reduce progressive glomerular destruction in rat kidneys [134,135]. Another oxylipin, 20-HETE, produced from AA is an effective vasoconstrictor and mediates inflammation [136]. 12-HETE

inhibits renin release from renal cortical slices [137]. 12/15-HETE is a key player in pathogenesis of diabetic kidney disease [138]. Oxylipins can activate peroxisome proliferator-activated receptors (PPARs), which are normally expressed in the kidneys and mediate cellular proliferation, inflammation and fibrosis [139].

Oxylipins mediate their effects via coupling to stimulatory or inhibitory G proteins and are important and complex regulators of cAMP levels in the kidney [140–142]. Oxylipins play an important role in maintaining GFR in diseased kidneys by regulating hemodynamics, salt/water homeostasis and inflammatory and proliferative processes [143]. Their effects are varied depending on both the type and location of receptors. For example, depending on the presence of diverse E-prostanoid receptors, PGE_2 shows both vasodilitory and vasconstrictory effects, while PGI_2 has primarily vasodilatory effects. PGI_2 also influences tubular transport processes, regulates cell growth and apoptosis whereas inflammation processes can be influenced by PGE_2 [144]. In addition, 12-HETE modulates the activity of MAP kinases, and cAMP-dependent transcription factors in renal cells [145,146].

In previous literature, LA derived oxylipins such as EpOMEs and DiHOMEs have been shown to have a large range of harmful effects on health, and are termed as leukotoxins. In particular, DiHOMEs exert cytotoxicity in renal proximal tubular cells [147,148] and are involved in a variety of pathophysiological consequences including inducing chemotaxis, inflammation, cardiovascular disease and pulmonary diseases [149–152]. Other LA derived oxylipins such as HODE and oxoODEs play roles in oxidative stress, vasodilation, proliferation and inflammation and in the pathogenesis of atherosclerosis [27,126,153,154]. Some LA derived oxylipins in serum are associated with pain and Achilles tendinopathy [28,155], and lower levels of 13-HODE have been reported in colon and lung cancer [156,157]. Oxylipins in liver and

blood have been associated with a number of diseased liver conditions and other disorders [22–28], but these have not been examined in the context of HP diets.

1.6.4 Influence of dietary protein on oxylipins

Functional changes in kidney such as increased glomerular filtration rate and renal plasma flow are accompanied by augmentation of the urinary excretion of the vasoactive eicosanoid prostaglandin PGE_2, 6-keto-$PGF_{1\alpha}$ and thromboxane TXB_2 [158,159]. Renal plasma flow and glomerular filtration rate are regulated synergistically by vasodilatory eicosanoids PGE_2 and the vasoconstrictor TxA_2 [160,161].

In vitro glomerular production of vasodilatory eicosanoids is increased by high intake of dietary protein. This involves activation of the PLA_2-COX pathway because of higher levels of plasma and/or intrarenal angiotensin II, which results changes in renal hemodynamics [162]. A study by Yanagisawa et al. showed that the activity of membrane associated PLA_2, COX and the production of PGE_2, 6-keto $PGF_{1\alpha}$ and TxB_2 are remarkably increased in glomeruli from rats that consume a HP diet (40% casein) than those from rats consuming a low-protein diet (6% casein) for 8 weeks [163,164]. Increased production of thromboxane in normal rats provided HP diet also has been demonstrated by Ichikawa et al. [165] and Paller et al. [166] who showed higher urinary excretion of PGE_2 in normal rats that consumed HP diets.

Effects of dietary protein on tubular eicosanoid production were analyzed by Yanagisawa et al. who found that, medullary tubules from rats provided a HP diet produced considerably higher amounts of PGE_2 and 6-keto $PGF_{1\alpha}$. The quantitative order of synthetic rates of eicosanoids by the renal tubules was found to be different in various studies. For example in one study [162] the order was found to be $PGE_2 > TxB_2 >$ 6-keto $PGF_{1\alpha}$ whereas it was $PGE_2 >$ 6-

keto $PGF_{1\alpha}$ > TxB_2 in other studies [163,167]. These indicated that PGE_2 was the major eicosanoid in renal tubules and also, medullary tubules form more PGE_2, 6-keto $PGF_{1\alpha}$, and TxB_2 than cortical tubules compared to LP group. The production rates of these three were not same between tubules of rats fed a HP or a LP diet. The enhanced activity of COX could play role for higher eicosanoids production by using AA released more in the tubules [162].

By increasing the levels of TXB_2, decreasing 6-keto-$PGF_{1\alpha}$ HP diet and high-grade proteinuria may quicken kidney damage. By influencing the expression of kidney vasoactive substances, and decreasing anti-oxidation, HP diets can speed up the kidney damage through prompting high-grade proteinuria in five sixth nephrectomy rats [168].

The glomerular eicosanoid production rates depends on the quantity of protein in the diet. A study was conducted in normal animals and in rats with three distinct models of experimental renal disease: diabetes mellitus, Heymann nephritis, and partial renal ablation. PGE_2, $PGF_{2\alpha}$, and TxB_2 was greater in the rats that fed the HP diet (40% protein), an increment that ranged between 52 and 257%. Glomerular (isolated) production of PGE_2 and TxB_2 was also noticeably greater in rats that consumed the HP diet when studied in the presence of AA. However, no major differences were observed between the two dietary protein groups in the production of eicosanoids by preparations of renal papillae or in the release of TxB_2 from clotting blood for low (8.5%) vs. HP (40%) diet [18].

Similar findings were shown in another in vitro study on rats with remnant kidney, (after decrease of 70% renal mass) fed HP or LP diet for 2 weeks. Formation of PGE_2, 6-keto $PGF_{1\alpha}$, and TxB_2 in isolated rat glomeruli were increased in rats that consumed the HP diet. PGE_2 and 6-keto $PGF_{1\alpha}$, biosynthesis was increased by 2.5 and 12 times, respectively, in HP vs. LP

groups. On the other hand TxB_2 formation was increased by only 40% compared with LP fed rats. Papillary prostanoid production was apparently unaffected by the dietary protein [15].

To summarize, it can be said that increased synthesis of eicosanoids (PGE_2, $PGF_{2\alpha}$, 6-keto $PGF_{1\alpha}$, and TxB_2) was observed in vitro study in medullary tubules in HP compared to LP fed groups. To date there is no study that looked on effect of HP diet on liver and serum oxylipin profile.

Chapter 2: Study Rationale, Objective and Hypothesis

2.1 Rationale

It is well accepted that healthy lifestyle changes focused primarily on weight loss are the first-line approach to obesity but the optimal diet to attain this is not clear. A popular approach to weight loss is to use HP diets but there is insufficient information on the benefits and risks of HP diets. According to the IOM 2005 report, "Research is needed on HP intakes (>145 mg N/kg/day) in relationship to: positive nitrogen balance and requirement estimates; metabolic and possible toxic effects and pathways impacted by these high intakes" (p 737)

Previous studies have shown that a HP diet increases COX oxylipins in the normal and diseased kidney in vitro [18,162]. However, these and other classes of oxylipins (LOX and CYP 450 products) have not yet been examined in vivo in normal rats. Previous studies mainly put emphasis on the association between dietary protein level and kidney health by examining clinical parameters of kidney function. To date, no studies have examined the effect of dietary HP on the complete kidney oxylipin profile. Liver and blood oxylipins have been associated with a number of diseased liver conditions such as hepatitis, cirrhosis, acute liver failure and other disorders [22–28], but these also have not been examined in the context of HP diets. In this study, livers were taken since they are the main site for protein metabolism and blood was taken as a possible tissue to be used to reflect changes in the kidney and/or liver.

In our study we have used casein since it is in the standard protein in the AIN 93M diet and most commonly used by other researchers. Understanding how different dietary protein levels affect the profile of kidney, liver and serum oxylipins will provide basic information on

the effects of dietary protein. Examination of the tissue oxylipin profile will provide insight into the etiology and progression of alterations of their functions and therefore targets for future experiments.

2.2 Hypothesis

Oxylipins present in kidneys, livers and serum of normal adult rats will be increased with higher protein level.

2.3 Objective

To examine the kidney, liver and serum oxylipin profile in normal adult rats provided LP, NP and HP diets for two weeks.

Chapter 3 Manuscript: Linoleic Acid Derived Oxylipins are Elevated in Kidney and Liver and Reduced in Serum in Rats Given a High Protein Diet

3.1 Introduction

HP diets are increasingly being offered as an approach to reduce weight gain and obesity, but whether they should be recommended for long-term consumption is not clear. On the one hand, HP diets possess many reported beneficial effects such as increasing weight loss and satiety and improving body composition, lipid-related risk factors and fasting insulin levels [5–7]. They also appears to have anti-steatotic and anti-inflammatory effects in the liver in obesity and non-alcoholic fatty liver disease [40,50,117–119,169]. On the other hand, consumption of HP diets results in enlarged kidneys, increased glomerular filtration rates, renal and glomerular hypertrophy, fibrosis and renal glomerulosclerosis in both the short- and long-term in normal kidneys [8–12,15,18]. In diseased kidneys, it is well-established that HP diets worsen and LP diets mitigate the increased glomerular hyperfiltration, hyperemia and acceleration of chronic kidney disease [13,14,40,170,171]. Thus despite the many potential benefits, the safety of HP diets remains unclear, as evidenced by the latest dietary protein recommendations from the Institute of Medicine that concluded that there is insufficient data to support the long-term safety of HP diets [1].

One group of bioactive molecules that may shed light on dietary protein effects in relation to this question is the oxylipins. These bioactive lipids are oxidized metabolites of polyunsaturated fatty acids produced by COX, LOX and CYP pathways [17,172–174]. The most well-known oxylipins are the eicosanoids derived from AA, and these are important regulators of physiological events in health and disease in all tissues, including the kidney and liver, the two tissues that appear to be most affected by HP diets. In the kidney, COX derived oxylipins

maintain normal renal function [131,132], but several of these COX oxylipins [e.g. (PG)E_2, (TX)B_2] are elevated in vitro in glomeruli isolated from normal or diseased rats consuming high protein diets, and in diseased rats this is associated with worsening of renal function [15,18,175]. This suggests a potentially negative effect of these oxylipins, which is supported by studies showing that COX inhibitors can reduce renal disease progression [144,176–179]. The effects of HP diets on other renal oxylipins have not been examined in this regard, but it is known that some derived from AA via the LOX and CYP pathways [e.g. leukotriene (LT)B_4 and D_4, 12- and 20-hydroxyeicosatetraenoic acid (HETE)] can be inflammatory in the kidney [180–182], and thus could potentially be involved in renal HP effects. Additionally, LA derived oxylipins such as EpOME and DiHOME appear to be cytotoxic in renal and other cells, but whether they are altered in tissues with HP feeding is unknown [147,149–152]. Similarly, liver and blood oxylipins have been associated with a number of diseased liver conditions and other disorders [22–28], but these also have not been examined in the context of HP diets.

Therefore to understand the effects of HP diets on these bioactive lipids, the current study examined the in vivo renal and hepatic oxylipin profile in response to low (LP), normal (NP) and HP diets for two weeks. Changes in the size of the kidney in response to dietary protein level occur in less than two weeks [10,15,18], so changes in this time period would be expected they play a role in mediating physiological effects of the HP diets. Serum oxylipins also were analyzed to determine how well they reflect tissue oxylipin profiles. Herein we report that the LA oxylipins are the oxylipins most affected by the high protein diet. These fundamental data may have implications for dietary protein recommendations and, paired with functional studies, will ultimately help to interpret how dietary HP effects on oxylipins relate to health and physiological function.

3.2 Material and methods

3.2.1 Animals and diets

A total of thirty normal male Sprague-Dawley rats at 10 wk of age were randomly divided into three groups and were provided isocaloric diets with LP (8% protein by weight), NP (14% protein by weight) or HP (50% protein by weight) diets for two weeks. The NP diet was the AIN 93M standard diet for adult rodents [183], and the other diets were achieved by interchanging casein and carbohydrate sources, as detailed in Table 3.1. A level of 50% protein was chosen for the HP diet to determine effects of an extreme protein diet, but one that has been promoted for weight loss [113,184–186]. Rats were given free access to water and diet for the duration of the study.

At the end of feeding period, rats were anesthetized with isofluorane and terminated via decapitation to collect trunk blood to obtain serum, which was stored at -80°C until analysis. The left kidneys were removed, weighed and bisected longitudinally across the hilium, with one half fixed in 10% formalin for 24 hours followed by transfer to phosphate buffered saline at 4°C until further processing for histologic analysis. The right kidney and liver were immediately snap-frozen in liquid N_2 and stored at -80°C until analysis for oxylipins and fatty acids. All research procedures were approved by the University of Manitoba Committee on Animal Care and were in accordance with the Canadian Council on Animal Care Guidelines (see Appendix 2.1 and Appendix 2.15 for details).

3.2.2 Oxylipin analysis

Kidney cortex, medulla and liver were homogenized in Tyrode's salt solution (pH 7.6) and samples for oxylipin analysis were prepared and analyzed by HPLC-MS/MS multiple-reaction monitoring as described [187–189], which is based on the methods of Deems et al.

[190]. Briefly, deuterated internal standards (Cayman Chemical, MI, USA) and 10 µL of antioxidant solution [(0.2mg/mL BHT, 0.2mg/mL EDTA, 2mg/mL triphenylphosphine, 2mg/mL indomethacininmethanol/ethanol/water (2:1:1, v/v/v)] were added to 200 µL tissue homogenates or serum. Sample pH was adjusted to <3 and solid phase extraction was with Strata-X SPE columns (Phenomenex, CA, USA) preconditioned with methanol and pH 3 water. Samples were loaded onto the columns, rinsed with 10% methanol, and eluted with methanol. After drying under nitrogen, samples were resuspended in 100 µL solvent A [(water/acetonitrile/acetic acid, 70/30/0.02, v/v/v)] for analysis by HPLC-MS/MS as described [187–189]. Details of all oxylipins screened for but below the limit of detection (<3 times above baseline) or below the limit of quantitation (<5 times above baseline), internal standards and retention times are provided in Supplemental Tables S1 and S2. Quantification of oxylipins was determined using the stable isotope dilution method [191] and expressed as ng/g of wet tissue or ng/mL of serum (see Appendix 2.2 to 2.10 for details).

3.2.3 Fatty acid analysis

For fatty acid analysis, total lipids were extracted via solvent-solvent extraction from 250 µL aliquots of the tissue homogenates or serum as described [192,193]. Phospholipids were then separated by thin layer chromatography (heptane/isopropyl/acetic acid, 60/40/3, v/v/v) [194] since PUFA in phospholipid are the source of oxylipins in tissue [195,196]. Oxylipins in blood come from multiple tissues, so serum total fatty acids were determined. Fatty acids were methylated using methanolic H_2SO_4 and quantified by gas chromatography as described [197,198] (see Appendix 2.11, 2.12, 2.13 for details).

3.2.4 Mean glomerular volume analysis

Formalin fixed kidneys were embedded in paraffin, sectioned at 5 µm and processed using our published methods [144,199]. Transverse tissue sections were stained with haemotoxylin and eosin (H&E) for glomerular volume measurement. Images were captured with an AMG-EVOS-XL microscope (Life Technologies, MA, USA) and were analyzed with ImageJ software (NIH, USA). An average of thirty randomly chosen glomeruli images (x20 magnification) per kidney were collected for histological analysis and all measurements were carried out by a blinded investigator. Standard stereological techniques developed by Weibel [200] as described previously [199] was used to calculate mean glomerular volumes (see Appendix 2.14 for details).

3.2.5 Statistical analysis

Data were analyzed by 1- or 2-way ANOVA using the GLM procedure of SAS 9.4 (SAS Institute Inc, NC, USA). The Shapiro-Wilk test was used to test normality and non-normal data was normalized by log transformation where possible. If normality was not achieved, a nonparametric test was used (Kruskal-Wallis). When the overall main or interaction effect was significant, post hoc analysis (Tukey's test) was used for simple effect comparisons. Outliers were removed if they were outside of the mean±3SD. All data were presented as mean±SE. Significance was set at $p<0.05$ for main and interaction effects.

Table 3.1 Experimental diet composition

Ingredients	LP	NP	HP
		g/kg diet	
Cornstarch	**510**	**465**	**192**
Casein (87% protein)	**80**	**140**	**500**
Dextrinized cornstarch	**169**	**155**	**63.9**
(90-94% tetrasaccharides)			
Sucrose	100	100	100
Soybean oil (no additives)	40	40	40
Fiber (90-95% cellulose)	50	50	50
Mineral mix (AIN-93M-MX)	35	35	35
Vitamin mix (AIN-93-VX)	10	10	10
L-cysteine	**1.03**	**1.80**	**6.43**
Choline bitartrate (41.1% choline)	2.50	2.50	2.50
Tert-butylhydroquinone	0.008	0.008	0.008
% calories from protein	9	15	53

3.3 Results

3.3.1 General results

After two weeks of feeding, body weights of rats given HP compared to NP were not different, but LP rats had lower body weights. Kidneys and livers were both enlarged in HP compared to NP and LP fed rats, and kidneys but not livers were smaller in LP compared to NP rats (Table 3.2). The enlarged kidneys were associated with higher mean glomerular volumes, which were 28% and 22% higher in HP compared to LP and NP fed rats, respectively (Fig 3.1).

3.3.2 HP increases LA oxylipins and decreases other n-6 oxylipins in the kidney

Medulla LA oxylipins derived via the CYP epoxygenase pathway, namely 9,10-DiHOME, 12,13-EpOME and 12,13-DiHOME, were 2-3 times higher in HP compared to NP or LP rats. The levels of 9,10-EpOME followed a similar trend but differences by post-hoc analyses did not reach statistical significance. In contrast, 4/5 renal oxylipins derived from GLA and DGLA (formed via desaturation and elongation of LA) were lower in HP compared to LP rats, with NP rat values being in between. Additionally, only 4/31 AA derived oxylipins (PGE_2, TxB_2, 5-HETE, 15-oxoETE) were lower in HP compared to LP rats only and one AA oxylipin (15-keto PGE_2) and one AdA oxylipin (dihomo $PGF_{2\alpha}$) in the medulla was lower in HP compared to both LP and NP rats. No n-3 oxylipins were altered in the kidney by dietary protein level. The only oxylipin that was different in LP compared to NP was 13-HODE, which was higher in the LP compared to NP rats (Oxylipins that are different presented in Fig. 3.2 and all oxylipins are shown in Appendix 1.1).

Oxylipin changes were not necessarily reflective of fatty acid changes in the kidney. For example, GLA and DGLA were higher despite lower levels of most oxylipins derived from these

fatty acids (Appendix 1.2). Also, some AA oxylipins are higher in cortex, while others are higher in medulla even though AA levels are not different in cortex compared to medulla.

3.3.3 Oxylipin distributions in the cortex and medulla

Half of the oxylipins had differing levels in cortex compared to medulla, with 11/32 being higher in cortex and 21/32 being higher in medulla. These differences tended to be associated with specific pathways: oxylipins produced via 5- and 8-LOX and CYP hydroxylase pathways were generally higher in the cortex, while oxylipins produced via COX and 12- and 15-LOX pathways were generally higher in the medulla.

3.3.4 HP increases LA and other n-6 oxylipins in liver

The effects of dietary protein level on liver oxylipins were observed primarily when HP rats were compared to LP rats, with NP values being in between these. Similar to kidney, LA oxylipins formed in the liver via the epoxygenase pathway (2 of 4 oxylipins) were 1.5-4 times higher in HP rats, but only when compared to LP rats. These changes were observed in the EpOMEs, while the DiHOMEs were largely unaffected, resulting in higher DiHOME/EpOME ratios in LP fed rat livers. A similar pattern was observed for the 14,15-DiHETrE/14,15-EpETrE ratio, the only other oxylipins present in liver representing the product/substrate pairing for the soluble epoxide hydrolase (sEH) enzyme (Fig. 3.3). In addition, LA oxylipins formed via the LOX pathway (3 of 5 oxylipins) also were higher in HP compared to LP rats, so the total mass of LA oxylipins was higher in HP compared to LP livers. In contrast to the lower levels of AA oxylipins in kidneys of HP rats, 4 of 22 AA oxylipins were higher in HP compared to LP fed rats and 1 AA derived oxylipin ($PGF_{2\alpha}$) was increased in the HP compared to the NP diet only. Only one n-3 fatty acid derived oxylipin (7-HDoHE) was higher in HP compared to LP rats (Oxylipins that are different presented in Fig. 3.4 and all oxylipins are shown in Appendix 1.3).

Similar to what was observed in the kidney, fatty acid changes were not necessarily reflective of oxylipin changes in the liver. None of the oxylipin fatty acid precursors were different from each other in rats given the three diets (Select fatty acids are presented in Fig. 3.4 and all fatty acids are shown in Appendix 1.4).

3.3.5 HP decreases only LA oxylipins in serum

Similar to the kidney and liver, LA oxylipins were altered by dietary protein levels in the serum. However, these oxylipins were lower in rats given the HP diets: 3 out of the 4 LA CYP epoxygenase and 2 of the 5 LA LOX oxylipins were reduced in HP compared to LP and/or NP rats. As a result, total serum LA oxylipins were lower in HP compared to NP rats. Unlike the kidney and liver, no AA derived oxylipins in serum were altered by dietary protein level, but similar to the tissues, few n-3 oxylipins in serum were affected by dietary protein level (Oxylipins that are different presented in Fig. 3.5 and all oxylipins are shown in Appendix 1.5). Similar to kidney and liver, serum fatty acids results were not consistent with serum oxylipins. AA and DHA were higher in HP compared to LP rats even though no AA oxylipins were altered and one DHA were lower with HP feeding compared to LP diets (Select fatty acids are presented in Fig. 3.5 and all fatty acids are shown in Appendix 1.6).

Table 3.2 Body, kidney and liver weights in 12 wk old male rats provided LP, NP and HP diets for 2 weeks

	LP	NP	HP	P Value
Body, 1st day (g)	421±2.57	421±4.60	422±4.24	
Body, 7th day (g)	446±4.11^{B}	458±5.70^{AB}	465±2.73^{A}	0.0192
Body, 14th day (g)	489±6.13^{B}	509±3.51^{A}	522±5.54^{A}	0.0004
Kidney (g)	2.88±0.07^{C}	3.29±0.07^{B}	4.09±0.08^{A}	<.0001
Kidney/body (g/100g)	0.59±0.01^{C}	0.65±0.02^{B}	0.78±0.01^{A}	<.0001
Liver (g)	18.3±1.30^{B}	19.6±0.53^{B}	23.1±0.63^{A}	0.0021
Liver/body (g/100g)	3.72±0.23^{B}	3.85±0.10^{B}	4.42±0.09^{A}	0.0082

Values represent mean±SE. Values with different superscript letters are significantly different from each other. P values ≥ 0.05 are not shown.

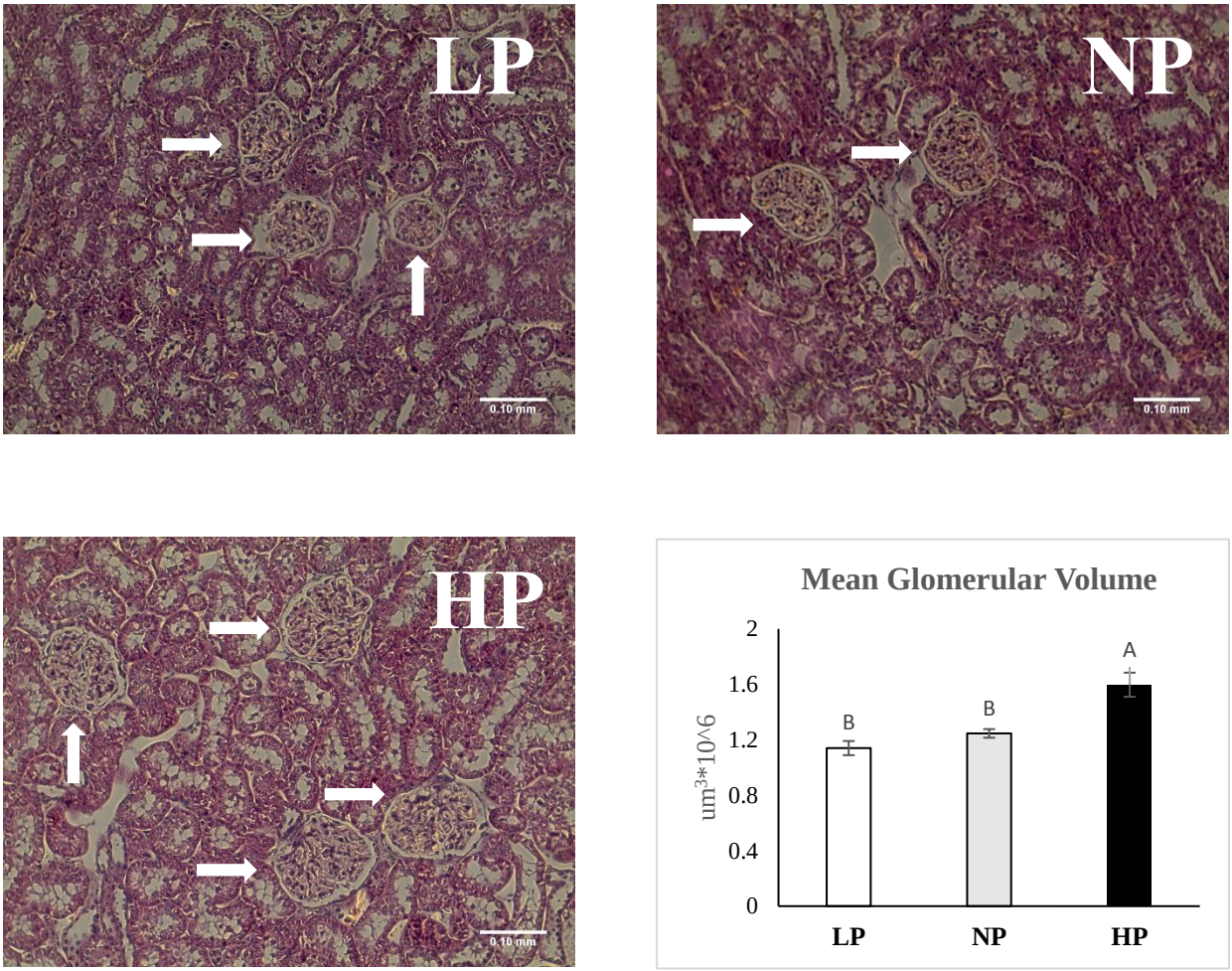
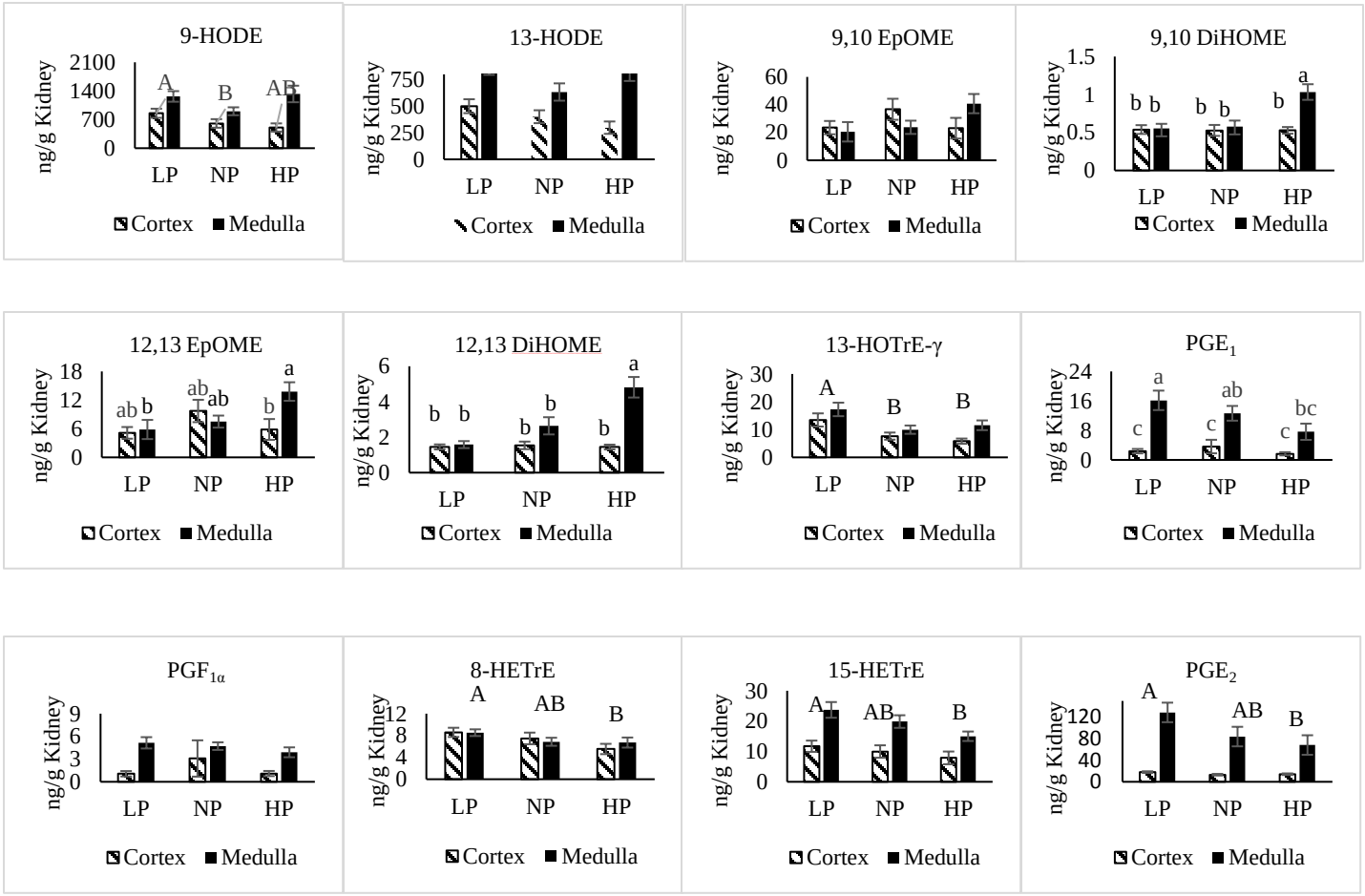


Fig 3.1. Representative images of glomeruli and mean glomerular volumes in rats given the LP, NP and HP diet for two weeks. Sections were stained with H&E. Arrows indicate glomeruli. Differing letters indicate differences among diets.


9-HODE
ng/g Kidney
2100
1400
700
0
A
B
AB
LP
NP
HP
Cortex
Medulla
13-HODE
ng/g Kidney
750
500
250
0
LP
NP
HP
Cortex
Medulla
9,10 EpOME
ng/g Kidney
60
40
20
0
LP
NP
HP
Cortex
Medulla
9,10 DiHOME
ng/g Kidney
1.5
1
0.5
0
b b
b b
b
a
LP
NP
HP
Cortex
Medulla
12,13 EpOME
ng/g Kidney
18
12
6
0
ab b
ab
ab
b
a
LP
NP
HP
Cortex
Medulla
12,13 DiHOME
ng/g Kidney
6
4
2
0
b b
b
b
a
LP
NP
HP
Cortex
Medulla
13-HOTrE-γ
ng/g Kidney
30
20
10
0
A
B
B
LP
NP
HP
Cortex
Medulla
PGE1
ng/g Kidney
24
16
8
0
c
a
c
ab
c
bc
LP
NP
HP
Cortex
Medulla
PGF1α
ng/g Kidney
9
6
3
0
LP
NP
HP
Cortex
Medulla
8-HETrE
ng/g Kidney
12
8
4
0
A
AB
B
LP
NP
HP
Cortex
Medulla
15-HETrE
ng/g Kidney
30
20
10
0
A
AB
B
LP
NP
HP
Cortex
Medulla
PGE2
ng/g Kidney
120
80
40
0
A
AB
B
LP
NP
HP
Cortex
Medulla

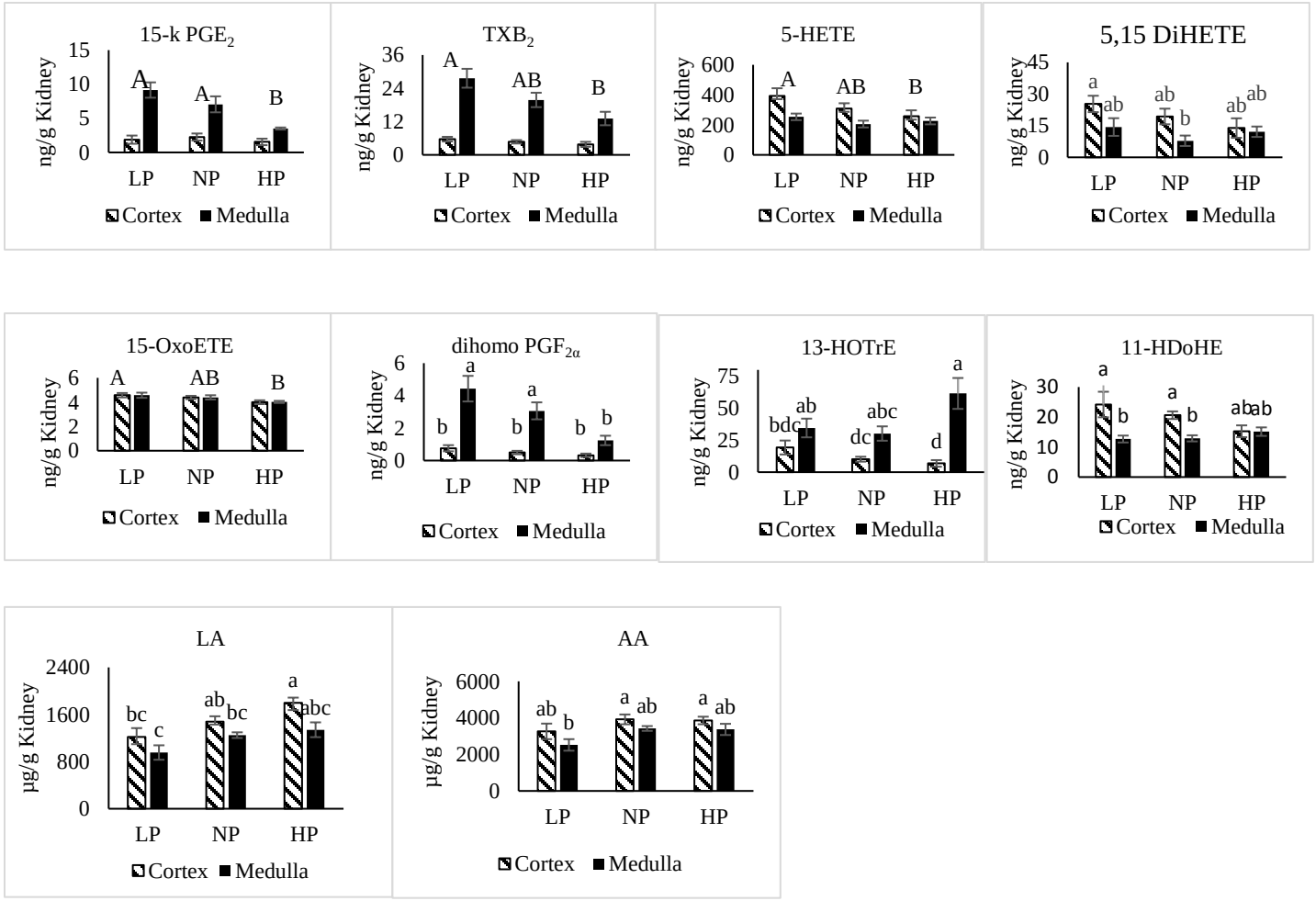


Fig. 3.2. Effect of dietary protein level on rat cortex and medulla oxylipins. Oxylipins with a p value <0.05 and the precursor fatty acids of oxylipins most affected (LA, AA) are shown. Differing UPPER case superscript letters indicate differences among diets. Differing lower case superscript letters indicates simple effects when interactions were present. Values are mean±SE (n=8-10), and are based on wet tissue weight.

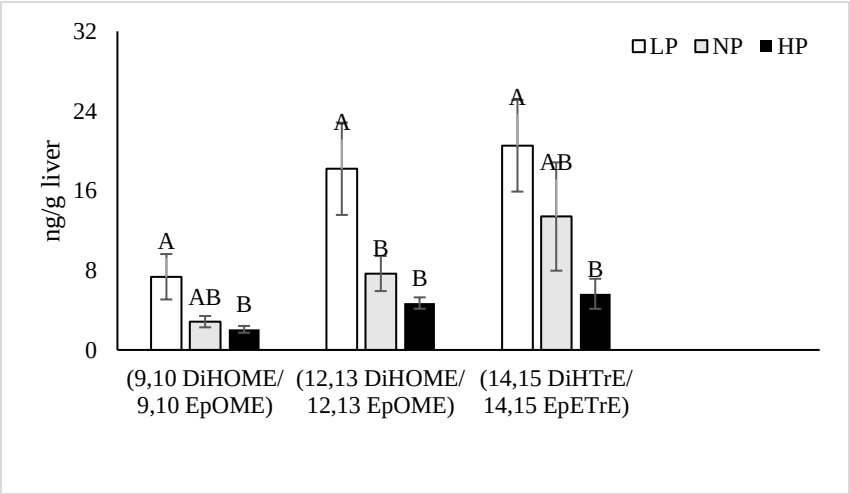


Fig. 3.3. Oxylipin product to substrate ratios for soluble epoxide hydrolase (sEH) in the liver. Differing letters indicate differences among diets within each ratio.

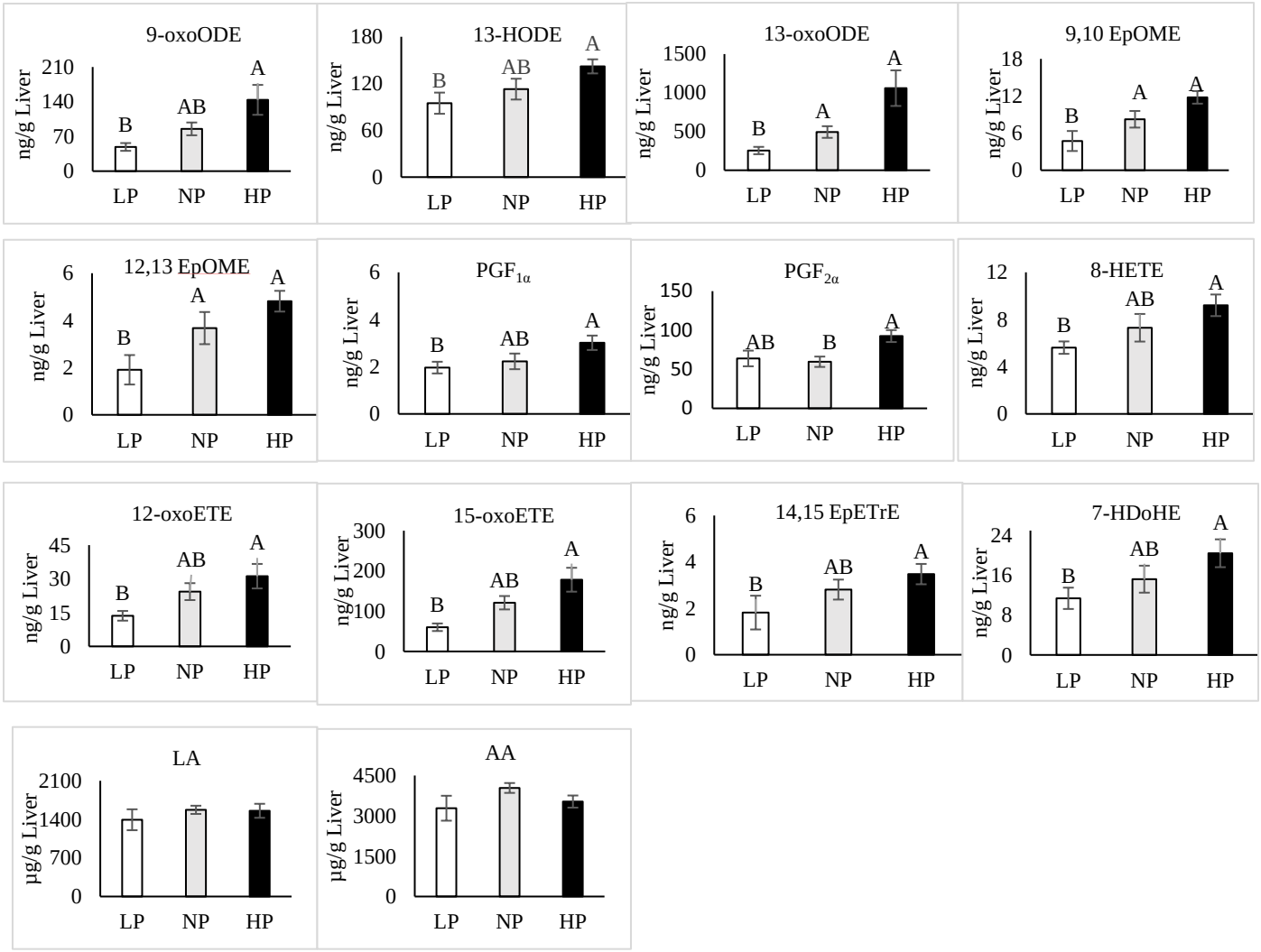


Fig. 3.4. Effect of dietary protein level on rat liver oxylipins. Oxylipins with a p value <0.05 and the precursor fatty acids of oxylipins most affected (LA, AA) are shown. Differing UPPER case superscript letters indicate differences among diets. Values are mean±SE (n=8-10), and are based on wet tissue weight.

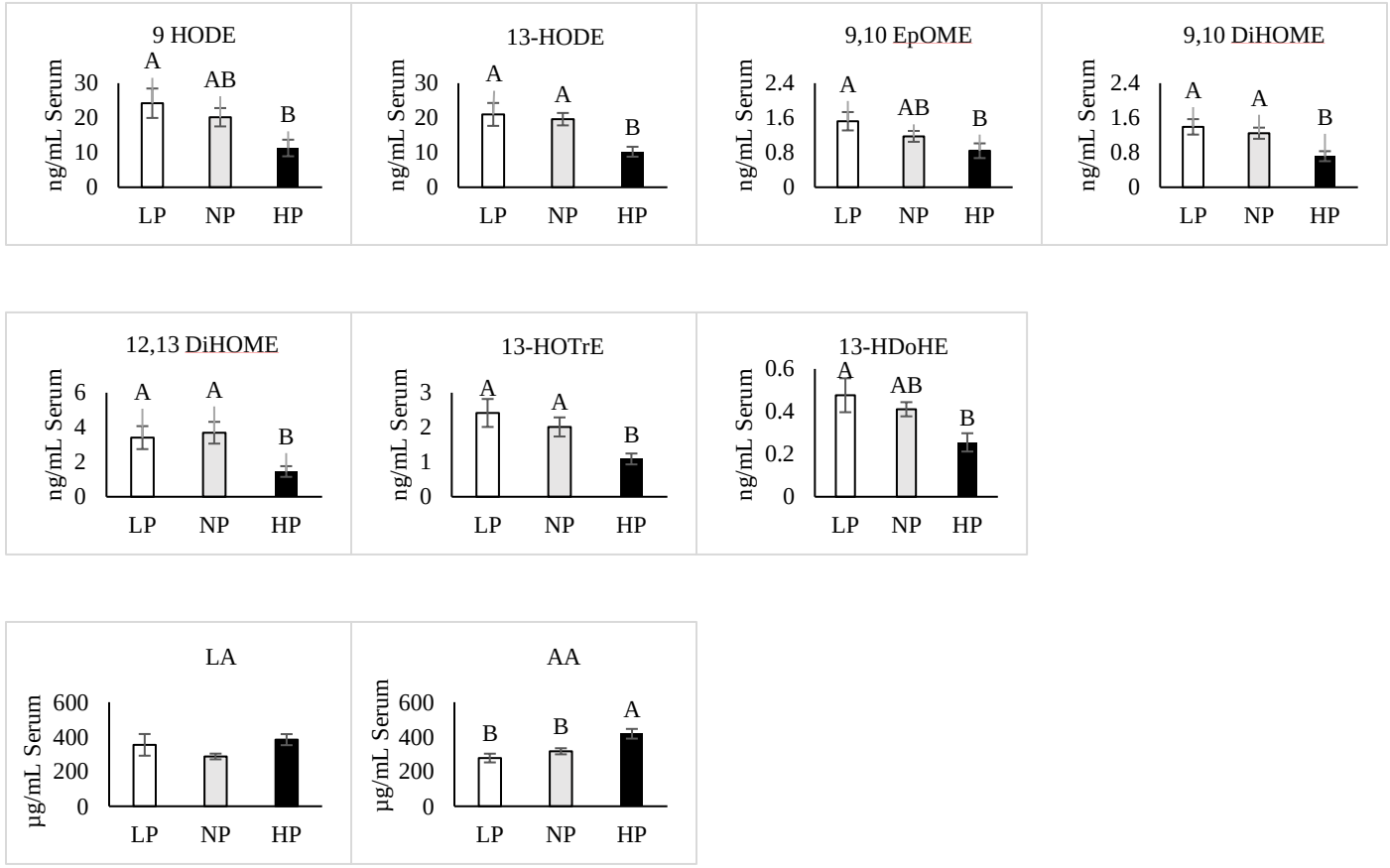


Fig. 3.5. Effect of dietary protein level on rat serum oxylipins. Oxylipins with a p value <0.05 and the precursor fatty acids of oxylipins most affected (LA, AA) are shown. Differing UPPER case superscript letters indicate differences among diets. Values are mean±SE (n=8-10), and are based on wet tissue weight.

3.4 Discussion

The present findings reveal that dietary protein level has the greatest effect on LA derived oxylipins in vivo, with the HP diets resulting in higher LA oxylipins in kidney and liver and lower LA oxylipins in serum. Although the functions of the LA oxylipins are not well-known, the mass of these oxylipins is similar to that of the levels of AA derived oxylipins in all three sites, indicating that they may have physiological effects comparable in importance to the more well-known AA derived oxylipins known as eicosanoids. The CYP epoxygenase products of LA (EpOMEs) are sometimes referred to as leukotoxins and iso-leukotoxins, as they were first identified in leukoyctes and are elevated in inflammation [201,202]. However, their diol metabolites (DiHOMEs) also are toxic in renal proximal tubular cells and may be responsible for the toxic effects of the monoepoxide precursors [203,204], although they appear to have differing mechanisms of action [56].

These CYP LA oxylipins are involved in a variety of pathophysiological situations, including inflammation, cardiovascular disease and pulmonary diseases [149–152]. Other LA derived oxylipins such as HODE and oxoODEs play a role in oxidative stress, vasodilation, proliferation, inflammation and in the pathogenesis of atherosclerosis [27,126,153,154]. They are also factors in the progression of liver injury, and may be useful in the diagnosis and prognosis of non-alcoholic hepatic steatosis [23,25,205]. On the other hand, other studies of LA oxylipins suggest that they may be associated with beneficial effects. Higher HODE levels are associated with reduced inflammation and skin proliferation, and HODEs have been shown to inhibit platelet aggregation [206–208]. Thus whether the altered levels of these LA oxylipins in the kidney and liver of HP rats indicate detrimental or beneficial effects remains to be determined. Interestingly,

LA oxylipins also are the oxylipins most affected by the type of protein in mice provided NP diets [209], suggesting that LA oxylipins may be uniquely affected by dietary proteins.

Similarly, the implications of the lower levels of LA oxylipins in serum remain to be elucidated. The most prevalent LA oxylipins in plasma/serum were 9- and 13-HODE, consistent with previous studies [27,28], and these were lower in HP rat serum. Higher levels of LA oxylipins in serum are associated with pain and Achilles tendinopathy [28,155], and lower levels of 13-HODE have been reported in colon and lung cancer [156,157].

The observed changes in the levels of other n-6 oxylipins also may have physiological effects, although fewer of these were altered by dietary protein level. AA oxylipins in the kidney are important in the maintenance of renal homodynamics, the renin-angiotensin system, water-electrolyte balance and release of neurotransmitters [131,132]. Previous studies have shown that in vitro production of vasoactive COX derived AA oxylipins (PGE_2, 6-keto $PGF_{1\alpha}$ and TxB_2) is increased in isolated glomeruli obtained from normal rats given diets containing 40% casein compared to 6% casein for 8 weeks [163,164]. Similar findings were observed in glomeruli isolated from rats with diabetes mellitus, Heymann nephritis, and partial renal ablation [15,18]. In the current study, however, these oxylipins were not increased in vivo in kidney, and several were even reduced with HP compared to LP feeding even though glomerular volume was higher and hypertrophy observed. This discrepancy between in vitro vs in vivo effects is not clear, but may reflect differences in effects of in vitro stimulation of isolated cells vs the steady-state levels observed in vivo. The lower in vivo levels may more closely reflect normal kidney responses, while the in vitro studies may reflect the disease condition in which elevated levels appear to be detrimental.

In contrast to the lower levels of AA oxylipins in HP rat kidneys, some AA oxylipins were higher in HP rat livers, but like the kidney, most were unaffected. In the liver the PGs appear to be associated with protective effects in a variety of liver diseases, but whether $PGF_{2\alpha}$, the one PG that was elevated by dietary HP in the current study also exhibits these effects is unclear [22,210–212]. Similarly, both 12- and 15-oxoETE were elevated in liver with HP feeding, and while their roles in the liver have not been examined, they do appear to have anti-inflammatory and -proliferative activity or have less pro-inflammatory and -proliferative activity than their parent HETE compounds [213–216]. Whether the altered levels of these select AA oxylipins in HP livers contributes to the beneficial effects of HP diets on the liver remains to be determined.

Similarly, the oxylipin product to substrate ratios for sEH in the liver were lower in HP compared to LP rats. The liver has higher levels of CYP oxylipins relative to other tissues [217], and although the current study was with normal weight rats without hepatic steatosis, this lower ratio may relate to studies that indicate that lower sEH activity can reduce hepatic steatosis and endoplasmic reticulum stress[218–220]. This may indicate a potential mechanism by which the HP diets could reduce liver pathology associated with obesity.

Oxylipins derived from the desaturase and elongase products of LA (DGLA and AA) in the kidney tended to be lower, possibly indicating that metabolism of LA may be impaired with HP feeding. This is reminiscent of the inhibition of LA metabolism by dietary soy protein compared to casein [221,222], but in the current study only the oxylipins and not their fatty acid precursors were lower. Indeed oxylipin changes were not necessarily reflective of fatty acid changes in any of these sites, as has previously been observed [189,193]. This may be because phospholipid may not be the only source of tissue oxylipins [223], or because the oxylipin levels

are 2-3 orders of magnitude lower than the fatty acid pool and fatty acids can be metabolized by multiple pathways, such as β-oxidation or conversion to other fatty acids and lipid mediators [124,224,225].

Oxylipins produced via 5- and 8-LOX and CYP hydroxylase pathways were generally higher in the renal cortex, while oxylipins produced via COX and 12- and 15-LOX pathways were generally higher in medulla. These findings were generally consistent with limited data on gene expression of these enzymes, as mRNA localization for 5-LOX is mainly in the glomerulus, for 15-LOX and COX-1 predominantly in the collecting duct, and COX-2 mostly in the cortical thick ascending limb of Henle [226,227]. The only oxylipins for which mRNA localization of the enzyme may have been different were those derived via 12-LOX, which is localized mainly to the glomerulus, although it also was found a low levels in the cortex [226]. It has previously been shown that oxylipin levels and mRNA or protein levels of the enzyme do not necessarily coincide, possibly explaining this discrepancy [143].

In conclusion, this comprehensive examination of the effects of dietary protein level on the oxylipin profile in the rat kidney, liver and serum demonstrates that the oxylipins most changed by protein level are those derived from LA. Studies delineating the physiological effects of these novel oxylipins in these tissues and blood are necessary to understand whether these changes are adaptive or detrimental responses to the dietary changes. In addition, determining at what level these effects occur will have implications for dietary recommendations for high protein diets.

Chapter 4 Thesis Discussion

4.1 Thesis discussion

There are controversies surrounding the consumption of high protein diets. Regarding these debates, our study examined the effects of dietary LP, NP and HP diets on oxylipin production. The current study showed that dietary protein level has the greatest effect on LA derived oxylipins, with LA oxylipins in the kidney and liver being increased and serum oxylipins being decreased with HP diets.

After two weeks of feeding, kidney and liver weights were both enlarged in HP compared to NP and LP fed rats, consistent with previous studies [10,15,18]. The enlarged kidneys were associated with higher mean glomerular volumes, which were 28% and 22% higher in HP compared to LP and NP fed rats, respectively, so changes in this short time period would be expected to play a role in mediating physiological effects of the HP diets.

The present findings demonstrate that LA derived oxylipins are mostly affected in the tissues and serum which also has been observed in mice with differing sources of dietary protein [209]. The function of these LA derived oxylipins, especially EpOMEs and DiHOMEs, are not well studied. In previous literature, EpOMEs and DiHOMEs have been shown to have a large array of harmful effects on health, and are termed as leukotoxins. In particular, DiHOMEs exert cytotoxicity in renal proximal tubular cells [147,148]. Other LA derived oxylipins such as HODE and oxoODEs play roles in oxidative stress, induction of vasodilation, suppression of proliferation, inflammation and in the pathogenesis of atherosclerosis [27,126,153,154]. They are also major factors in the progression of liver injury and, they could be useful in the diagnosis and prognosis of non-alcoholic steatohepatitis [23,25,205].

In contrast to increased production of renal AA derived oxylipins in previous in vitro studies, reduced levels of AA derived COX oxylipins was observed in our in vivo study [15,18,162,163]. The observed changes in the levels of AA and other n-6 oxylipins also may have physiological effects, although fewer of these were altered by dietary protein level in tissues and none of them were altered in serum. This discrepancy between in vitro vs in vivo effects is not clear, but may reflect differences in effects of in vitro stimulation of isolated cells vs the steady-state levels observed in vivo. The lower in vivo levels may more closely reflect normal kidney responses, while the in vitro studies may reflect the disease condition in which elevated levels appear to be detrimental. In the liver the PGs appear to be associated with protective effects in a variety of liver diseases [22,210–212].

With regards to kidney cortex and medulla effects, it appears that oxylipin distributions are pathway specific: oxylipins produced via 5- and 8-LOX and CYP hydroxylase pathways were generally higher in cortex, while oxylipins produced via COX and 15-LOX pathways were generally higher in medulla which is also consistent with gene expression of these enzymes [226,227].

Oxylipin fatty acid precursor results were not consistent with oxylipin changes in the kidney, liver and serum. LA oxylipins were mostly affected by dietary protein but LA fatty acids did not change in the tissues and serum. AA levels remained unchanged in the kidney and liver although some AA oxylipins were altered with dietary protein level. Interestingly, serum AA levels were higher with HP diets even though serum AA oxylipins were unchanged. It indicates that oxylipins changes are not necessarily reflective of fatty acid changes in the tissues and blood as has previously been observed [189,193].

4.2 Strengths

- The advanced methods using HLPC-MS/MS of the current study allowed for analysis of the kidney, liver and serum oxylipin profile to an extent never done before with greater than 160 oxylipins scanned. Previously with ELISA few oxylipins could be analyzed and it was time consuming. With LCMS the whole profile can be examined in a short run and it is capable of detecting many novel oxylipins for which functions are unknown.
- It is the first study to look at the effect of different levels of protein on the complete oxylipin profile in any species or tissue.
- The varying levels of protein (LP, NP and HP) provided excellent comparisons to understand the effect of dietary protein on oxylipin profiles. The NP diet was formulated to reflect the average Canadian intake of daily protein and represents an excellent control.
- During histological analysis, the researcher was blinded to avoid any bias, thereby increasing credibility.

4.3 Significance of the study

- Our study is the first study to comprehensively examine the effect of protein level on the oxylipin profile in the rat kidney, liver and serum in vivo, going beyond studies that have so far only examined COX metabolites in vitro.
- This basic data will be applicable to health and may also have implications for dietary protein recommendations, with the ultimate aim of understanding how these dietary effects on oxylipins relate to health and physiological function.

4.4 Limitations

- Using a male rat model is a limitation as female and other species may have different results.
- The feeding period also is limited and will not indicate long term feeding effects.
- Only one type of protein (casein, most commonly used) was tested; other protein types may have different effects.
- The HP diet is a very high level of dietary protein and conclusions cannot be extrapolated to more moderate level of dietary protein.
- The diet induced changes in healthy tissues may not necessarily be reflected in diseased kidneys, so any findings of safety or damage cannot be extrapolated to renal disease, but it will provide background information for such further studies.
- The HP effects could be due to reductions in carbohydrate level, since the increased dietary protein was at the expense of carbohydrate, although this is less likely because the change relative to baseline levels of each of these macronutrients is much greater for protein than carbohydrate. Nevertheless, this will need to be tested before more definitive conclusions in this regard can be made.

4.5 Future directions

- Future studies could include long term effects of differing levels of protein (eg. 15%, 25%, 35%) and observe at what level positive/negative effects occurs) on kidney, liver, serum.
- In our study we used casein protein. Future research investigating effects of plant vs animal protein on different tissues are required.
- Sex is in important factor in renal health and disease. Renal disease progression is directly affected by various gender related factors such as the size of the kidney, renal blood flow, hormonal differences and signaling molecules. Female sex hormones (such as 17ß-estradiol) may reduce inflammatory processes and cell apoptosis and have protective effects on renal tissue, whereas male sex hormones demonstrate opposite effects [228]. Fatty acid metabolism can also be influenced by gender. Therefore, effects of dietary protein levels on oxylipins and fatty acid profile in both male and female rat tissues should be analyzed.
- Research in models of renal disease examining the role of oxylipins in HP diet effects on disease would be of interest, since HP diets adversely affect diseased kidney.
- Further investigation of functions of oxylipins in the kidney, liver and serum are needed to understand whether the changes herein reflect beneficial or harmful effects.
- Effects of addition or inhibition of specific oxylipins such as EpOMEs, DiHOMEs in the kidney, liver cell line to understand their functions.

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Chapter 5 Appendices

Appendix 1

Appendix 1.1 Kidney oxylipins in normal 12 wk old rats given LP, NP and HP diets for 2 weeks

	LP		NP		HP		P value	
	Cortex	Medulla	Cortex	Medulla	Cortex	Medulla		
Oxylipin				ng/g kidney			Diet	Tissue
				LA oxylipins				
9-HODE	853±111^{A}	1265±129	605±104^{B}	902±96.9	505±100^{AB}	1325±202	0.0357	<0.0001
9oxoODE	4.23±0.12	4.61±0.17	4.10±0.17	4.41±0.08	3.82±0.13	4.59±0.11		<0.0001
13-HODE	500±65.5	881±86.1	401±60.7	633±81.5	297±58.1	887±150	0.0456	<0.0001
13oxoODE	4.63±0.17	5.09±0.18	4.49±0.14	4.89±0.15	4.18±0.11	4.91±0.11		<0.0001
9,12,13 triHOME	713±196	468±80.3	397±71.2	350±50.0	490±119	453±53.2		
9,10 EpOME	23.5±4.79	20.5±7.00	36.7±7.57	23.7±4.77	23.1±7.44	40.8±6.92		0.0248*
9,10 diHOME	0.54±0.06^{b}	0.55±0.06^{b}	0.53±0.07^{b}	0.58±0.08^{b}	0.53±0.04^{b}	1.03±0.10^{a}		0.0030
12,13 EpOME	5.13±1.22^{ab}	5.84±2.01^{b}	9.72±2.34^{ab}	7.49±1.26^{ab}	5.85±2.18^{b}	13.8±1.94^{a}		0.0245
12,13 diHOME	1.45±0.14^{b}	1.58±0.20^{b}	1.54±0.21^{b}	2.64±0.49^{b}	1.44±0.14^{b}	4.82±0.58^{a}		0.0012
Sum	2106±376	2652±306	1460±257	1930±220	1333±275	2736±417		0.003
				GLA oxylipin				
13-HOTrE-y	13.5±2.37^{A}	17.3±2.43	7.66±1.28^{B}	9.97±1.50	5.83±0.95^{B}	11.5±1.74	0.001	0.0095
				DGLA oxylipins				
PGE_1	2.38±0.60^{c}	16.2±2.64^{a}	3.63±1.84^{c}	12.6±2.00^{ab}	1.65±0.43^{c}	7.64±2.20^{bc}		<0.0001
$PGF_{1\alpha}$	1.06±0.36	5.15±0.75	3.08±2.40	4.71±0.50	1.09±0.34	3.90±0.65		<0.000
8-HETrE	8.58±0.84^{A}	8.52±0.61	7.47±1.06^{AB}	6.86±0.74	5.53±0.94^{B}	6.71±0.92	0.0243	
15-HETrE	11.7±1.86^{A}	23.7±2.59	9.98±2.04^{AB}	19.9±2.07	7.88±2.10^{B}	15.0±1.56	0.0172	<0.0001
Sum	23.7±3.29^{A}	53.5±4.36	24.2±2.92^{AB}	44.0±3.95	16.1±3.49^{B}	33.3±4.60	0.0029	<0.0001
				AA oxylipins				
PGD_2	23.0±5.17	75.3±9.94	16.5±3.88	64.5±8.92	18.9±5.72	55.9±9.02		<0.0001
15d PGD_2	2.31±0.47	2.11±0.43	2.12±0.70	2.49±0.73	2.29±0.75	1.42±0.18		
PGE_2	18.0±3.64^{A}	128±17.56	13.2±3.37^{AB}	83.9±10.5	14.1±3.84^{B}	68.2±14.8	0.0231	<0.0001
15k PGE_2	1.87±0.60^{A}	9.15±1.12	2.26±0.52^{A}	7.05±1.17	1.54±0.48^{B}	3.51±0.15	0.0103	<0.0001
dhk PGE_2	13.2±5.41	29.6±6.28	8.99±3.54	14.1±5.03	12.3±5.11	16.8±10.4		
$PGF_{2\alpha}$	21.8±3.51	90.9±9.5	19.2±4.58	82.0±10.3	22.2±7.13	73.1±11.2		<0.0001
6k PGF1α	4.30±0.54	100±15.3	3.97±0.73	91.3±15.3	4.25±1.50	62.5±12.5		<0.0001
TXB_2	5.68±0.84^{A}	27.6±3.41	4.72±0.61^{AB}	19.8±2.60	3.80±0.89^{B}	13.1±2.43	0.0005	<0.0001
12-HHTrE	105±25.9	976±339	59.2±9.53	1023±337	95.1±49.9	1054±344		<0.0001
5-HETE	392±50.0^{A}	253±21.9	309±34.5^{AB}	205±22.6	257±39.3^{B}	226±22.7	0.0404	**0.0014**
5,15 diHETE	25.3±3.89^{a}	14.4±4.20^{ab}	19.4±3.70^{ab}	7.90±2.47^{b}	13.9±4.62^{ab}	12.1±2.46^{ab}		0.032
5oxoETE	3.53±0.09	3.52±0.15	3.43±0.19	3.39±0.16	3.24±0.13	3.41±0.09		
8-HETE	36.1±4.94	27.4±3.34	32.1±3.18	23.6±2.39	29.7±1.69	27.6±2.24		**0.0078**
8,15 diHETE	240±51.0	131±27.8	173.7±18.4	123±35.6	143±40.8	101±13.5		**0.0384**
9-HETE	123±16.6	70.3±3.82	86.9±7.89	64.9±6.12	82.9±10.9	71.4±7.38		**0.0006**
11-HETE	100±8.79	204±16.2	86.5±6.65	169±13.0	85.81±10.7	164±16.3		<0.0001
12-HETE	170±22.2	173±16.8	142±15.4	188±17.5	109±17.2	174±28.8		0.0185
12oxoETE	1.96±0.22	1.95±0.29	1.93±0.15	2.01±0.18	1.72±0.16	2.01±0.15		
15-HETE	546±75.5	452±31.9	460±57.4	395±30.9	384±73.8	401±35.6		
15oxoETE	4.57±0.18^{A}	4.57±0.21	4.38±0.14^{AB}	4.40±0.17	4.00±0.16^{B}	4.02±0.09	0.0042	
LTB4	7.95±3.40	4.79±1.86	6.44±2.25	3.70±1.42	3.85±1.19	3.47±1.01		
16-HETE	43.9±7.87	26.5±3.53	30.7±5.87	21.0±2.96	26.0±5.85	23.6±2.17		**0.0183**
5,6 DiHETrE	3.91±0.87	2.75±0.66	3.75±0.95	2.40±0.71	3.62±0.78	3.71±0.95		
8,9 DiHETrE	1.70±0.14	1.57±0.23	1.53±0.35	1.19±0.22	1.98±0.29	1.50±0.18		
11,12 EpETrE	0.87±0.14	0.91±0.23	1.18±0.20	0.92±0.11	1.03±0.17	1.12±0.22		
11,12 DiHETrE	1.92±0.26	1.53±0.17	1.91±0.39	1.30±0.18	2.13±0.41	1.77±0.18		
14,15 EpETrE	2.71±0.49	2.92±0.91	3.76±0.55	2.53±0.21	3.05±0.55	3.11±0.56		
14,15 DiHETrE	1.73±0.23	1.72±0.25	1.43±0.20	1.57±0.15	1.62±0.27	1.92±0.22		
5-iso $PGF_{2\alpha}$VI	52.6±12.4	24.7±2.72	40.7±7.39	20.4±3.84	41.3±12.8	24.6±5.22		**0.0018**
8-iso PGF2αIII	18.7±3.48	24.5±3.53	15.7±2.45	13.2±4.03	14.9±5.10	21.9±3.74		
8-iso 15k $PGF_{2\beta}$	2.46±1.09	4.25±3.61	1.97±0.49	10.2±4.20	3.67±1.92	2.04±1.37		
Sum	1979±304	2874±403	1559±199	2654±364	1394±262	2626±452		0.0002
				ADA oxylipin				
dihomo $PGF_{2\alpha}$	0.76±0.19^{b}	4.43±0.79^{a}	0.49±0.11^{b}	3.06±0.52^{a}	0.32±0.11^{b}	1.24±0.29^{b}		<0.0001
				ALA oxylipins				
9-HOTrE	24.5±7.33	36.1±9.70	9.00±2.35	17.7±2.84	7.64±2.35	45.0±9.14		<0.0001

	LP		NP		HP		P value	
	Cortex	Medulla	Cortex	Medulla	Cortex	Medulla		
Oxylipin				ng/g kidney			Diet	Tissue
13-HOTrE	19.3±5.45^{bdc}	34.6±7.32^{ab}	10.2±1.93^{dc}	30.2±5.62^{abc}	6.84±2.59^{d}	61.6±12.1^{a}	0.0041	
				EPA oxylipins				
5-HEPE	40.8±5.08	31.7±4.07	32.6±3.98	26.6±2.78	38.4±4.62	35.3±4.03		
12-HEPE	8.37±1.11	10.8±1.98	6.72±1.13	9.61±1.58	6.99±1.28	11.6±1.57		0.0098
15-HEPE	7.51±1.28	8.71±1.23	5.13±0.90	10.7±1.74	5.90±0.90	12.0±1.38		0.0002
18-HEPE	14.6±4.28	12.8±1.78	13.6±3.15	10.4±1.41	19.0±4.02	16.5±3.53		
Sum	71.2±10.0	63.9±6.83	58.0±6.54	57.2±6.20	70.2±9.30	75.5±8.40		
				DHA oxylipins				
4-HDoHE	113±17.34	63.4±5.40	96.9±7.73	49.8±3.89	80.8±10.9	63.6±5.50		**<0.0001**
7-HDoHE	29.2±6.08	14.0±1.46	25.3±3.69	16.2±2.13	23.9±2.06	17.6±2.94		**0.001**
8-HDoHE	35.2±7.12	17.8±1.69	28.3±2.18	15.4±1.56	27.8±4.27	19.8±1.66		**<0.0001**
10-HDoHE	16.5±2.15	8.44±1.02	11.8±0.94	9.07±0.68	12.7±1.19	11.2±1.55		**0.0007**
11-HDoHE	24.2±4.25^{a}	12.7±1.15^{b}	20.6±1.27^{a}	12.9±1.03^{b}	15.3±1.97^{ab}	15.1±1.42^{ab}	0.0238	
13-HDoHE	15.7±2.36	20.7±2.00	14.6±1.81	18.1±3.11	13.5±1.98	18.5±1.49		0.0153
14-HDoHE	28.5±6.38	31.9±6.38	28.4±1.66	44.5±4.92	19.6±3.43	46.2±8.09		0.001
16-HDoHE	25.0±3.97	15.7±1.29	22.6±3.48	14±1.68	20.2±3.19	16.8±1.42		
17-HDoHE	93.0±13.3	86.4±11.9	77.1±3.60	113.9±9.33	67.1±7.44	115±18.7		0.0054
20-HDoHE	71.5±16.5	53.4±9.54	127±56.2	47.9±8.33	87.5±19.7	47.7±10.1		**0.0123**
Sum	451±74.1	324±29.2	452±60.1	341±21.2	368±46.1	371±38.1		

Differing UPPER case superscript letters within a row indicate differences among diets. Differing lower case superscript letters within a row indicates simple effects when interactions were present. *P values listed across diet and tissue are the P value for diet×tissue interaction or for Wilcoxin's test P value if the data were not normally distributed. **Bold** P values are higher in cortex than medulla. P values ≥ 0.05 are not shown. Values are mean±SE (n=8-10), and are based on wet tissue weight.

Appendix 1.2 Kidney fatty acids in normal 12 wk old rats given LP, NP and HP diets for 2 weeks

	LP		NP		HP			
Fatty Acids	Cortex	Medulla	Cortex	Medulla	Cortex	Medulla	P Value	
			µg/g kidney				Diet	Tissue
14:0	19.2±1.01^{B}	16.3±0.60	19.1±0.67^{B}	17.7±1.18	24.8±1.42^{A}	20.8±1.06	<0.0001	0.0014
16:0	2803±90.2	2336±104	2817±159	2451±67	2849±88.6	2449±92.9		<0.0001
16:1t	14.4±2.18^{bc}	12.6±1.50^{c}	18.0±1.11^{bc}	18.1±0.91^{bc}	29.1±2.50^{a}	21.2±1.64^{b}	0.0002*	
16:1	98.5±18.1	74.5±14.2	115±8.70	100±6.07	110±15.8	81.3±7.94		0.0372
17:0	21.0±1.05^{B}	17.2±0.99	22.3±1.90^{B}	18.7±1.46	45.9±1.75^{A}	35.9±2.13	<0.0001	<0.0001
18:0	2539±69.9^{ab}	1888±69.1^{c}	2621±152^{a}	1997±79.1^{c}	2634±78.1^{a}	2176±99.1^{bc}	<0.0001	
18:1	748±43.3^{b}	786±50.4^{ab}	793±49.3^{ab}	877±27.2^{ab}	894±53.3^{ab}	956±50.2^{a}	0.0315	
18:1n7c	299±32.7^{ab}	222±23.9^{b}	355±25.4^{a}	291±11.0^{ab}	342±22.9^{a}	273±18.9^{ab}	0.0065	
18:2n6	1219±149.8^{bc}	954±122^{c}	1477±92.3^{ab}	1249±48.3^{bc}	1800±86.1^{a}	1341±125^{abc}	0.0003	
18:3n6	3.31±0.67^{bc}	1.56±0.44^{c}	4.36±0.58^{b}	3.80±0.40^{bc}	9.54±0.71^{a}	5.29±0.70^{b}	0.0125	
18:3n3	5.81±1.57^{B}	3.50±1.04	6.01±0.40^{AB}	4.77±0.47	8.48±0.73^{A}	6.25±1.08	0.0305	0.0243
20:0	23.4±1.31^{c}	32.2±1.52^{bc}	25.3±2.12^{c}	35.1±1.62^{b}	24.6±3.66^{c}	48.0±3.06^{a}	<0.0001	
20:1	8.06±1.46^{b}	9.02±1.25^{ab}	9.86±1.36^{ab}	11.5±0.56^{ab}	12.1±1.13^{ab}	13.2±1.14^{a}	0.0222	
20:2n6	18.7±2.39^{bc}	13.6±2.16^{c}	20.6±1.02^{abc}	18.6±0.67^{bc}	27.3±1.54^{a}	22.7±2.21^{ab}	0.0003	
20:3n6	90.0±14.6^{c}	111±16.7^{bc}	129±10.73^{abc}	160±6.34^{ab}	153.1±7.77^{ab}	177±14.3^{a}	0.0002	
20:4n6	3260±425^{ab}	2520±315^{b}	3916±269^{a}	3420±130^{ab}	3857±206^{a}	3368±306^{ab}	0.0127	
20:3n3	3.18±1.11	4.30±1.37	1.93±1.03	3.77±1.26	4.63±1.41	5.45±1.66		
20:5n3	17.3±3.12^{c}	19.2±3.43^{c}	22.5±2.15^{bc}	29.3±2.09^{bc}	51.1±5.00^{a}	35.1±3.81^{b}	0.0044	
22:0	72.3±4.71	67.7±5.23	77.3±8.38	70.8±4.76	73.8±5.90	74.5±4.00		
22:2n6	6.30±1.92	5.50±1.63	11.3±2.29	18.8±11.6	12.6±2.79	12.9±3.53		
22:4n6	57.2±9.06^{b}	61.8±8.67^{ab}	62.5±7.78^{ab}	90.4±2.90^{a}	61.3±7.03^{ab}	77.0±7.39^{ab}	0.0114	
22:5n6	11.1±2.76^{B}	9.65±1.27	17.7±2.17^{A}	15.6±1.71	19.2±2.17^{A}	14.5±3.05	0.0068	
22:5n3	32.1±6.14	26.9±3.98	31.0±3.87	29.7±2.76	26.5±2.98	22.2±2.21		
22:6n3	269±41.1^{a}	155±22.1^{b}	321±25.8^{a}	224±13.0^{ab}	328±21.6^{a}	228±22.7^{ab}	0.0002	
24:0	615±18.3^{a}	394±18.5^{b}	620±37.3^{a}	406±16.9^{b}	580±19.8^{a}	396±19.2^{b}	<0.0001	
24:1	119±16.5	107±13.1	137±13.6	122±15.9	141±13.2	112±13.5		

Differing UPPER case superscript letters within a row indicate differences among diets. Differing lower case superscript letters within a row indicates simple effects when interaction were present. *P values listed across diet and tissue are the P value for diet×tissue interaction or for Wilcoxin's test P value if the data were not normally distributed. P values ≥ 0.05 are not shown. Values are mean±SE (n=8-10), and are based on wet tissue weight.

Appendix 1.3 Liver oxylipins in normal 12 wk old rats given LP, NP and HP diets for 2 weeks

Oxylipin	LP	NP	HP	P Value
		ng/g liver		
		LA oxylipins		
9-HODE	237±37.8	279±47.9	321±41.5	
9-oxoODE	48.8±7.82^{B}	85.2±12.8^{AB}	143±30.2^{A}	0.0019
13-HODE	94.8±13.6^{B}	112±13.3^{AB}	142±9.04^{A}	0.0193
13-oxoODE	254±47.4^{B}	493±75.2^{A}	1059±230^{A}	0.0001
9,12,13 triHOME	231±34.7	322±134	211±26.7	
9,10 EpOME	4.73±1.61^{B}	8.24±1.34^{A}	11.7±1.02^{A}	0.0003
9,10 diHOME	17.0±3.47	19.3±2.85	21.9±2.48	
12,13 EpOME	1.91±0.62^{B}	3.68±0.68^{A}	4.82±0.44^{A}	0.0006
12,13 diHOME	19.3±2.57	20.3±2.38	21.1±2.22	
Sum	910±110^{B}	1344±213^{AB}	1938±305^{A}	0.0056
		DGLA oxylipins		
PGD_1	4.39±0.53	4.47±0.66	4.86±0.72	
$PGF_{1\alpha}$	1.96±0.25^{B}	2.23±0.33^{AB}	3.02±0.31^{A}	0.0485
15-HETrE	7.94±0.69	10.3±2.76	6.27±0.72	
Sum	14.3±0.99	17.0±3.13	14.1±1.11	
		AA oxylipins		
PGB_2	777±229	358±65.4	339±90.1	
PGD_2	129±33.5	89.2±10.5	110±12.8	
PGE_2	78.8±10.0	75.5±11.0	96.6±12.4	
11bPGE_2	127±16.3	128±17.1	159±21.9	
$PGF_{2\alpha}$	63.8±10.0^{AB}	59.7±6.62^{B}	92.5±7.67^{A}	0.0173
6k $PGF_{1\alpha}$	2.17±0.44	1.43±0.33	1.38±0.13	
TXB_2	6.13±1.42	4.07±0.74	3.99±0.74	
12-HHTrE	133±22.8	130±29.4	102±9.19	
5-HETE	43.4±4.30	52.4±6.00	59.2±5.21	
5-oxoETE	39.9±6.12	62.6±10.1	69.8±13.5	
8-HETE	5.62±0.53^{B}	7.30±1.17^{AB}	9.21±0.92^{A}	0.0147
11-HETE	34.8±4.93	37.4±4.83	44.6±5.22	
12-HETE	84.0±15.4	86.7±14.5	81.8±17.8	
12-oxoETE	13.6±2.15^{B}	24.3±3.76^{AB}	31.2±5.44^{A}	0.0155
15-HETE	76.1±8.12	99.8±17.8	89.8±9.95	
15-oxoETE	60.1±9.38^{B}	121±16.6^{AB}	178±29.6^{A}	0.0015
16-HETE	70.1±8.29	95.7±28.9	44.6±8.20	
5,6 DiHETrE	2.94±0.40	2.17±0.29	1.91±0.19	
8,9 DiHETrE	6.50±1.40	8.94±1.59	6.61±1.09	
11,12 DiHETrE	14.5±2.76	20.3±3.31	15.0±2.19	
14,15 EpETrE	1.82±0.73^{B}	2.81±0.43^{AB}	3.48±0.44^{A}	0.0059
14,15 DiHETrE	19.1±2.72	23.1±3.74	17.9±4.01	
Sum	1792±320	1470±135	1559±167	
		ALA oxylipin		
9-HOTrE	15.7±2.57	22.9±9.75	20.1±3.71	
		EPA oxylipins		
PGE_3	9.95±1.43	13.7±2.95	14.0±2.57	
12-HEPE	10.5±1.73	9.63±2.01	7.51±1.37	
Sum	20.0±2.57	23.3±3.77	21.5±3.05	
		DHA oxylipins		
4-HDoHE	53.7±9.71	63.3±9.39	68.6±7.63	
7-HDoHE	11.3±2.14^{B}	15.2±2.71^{AB}	20.4±2.80^{A}	0.0281
8-HDoHE	12.4±1.87	12.6±2.22	15.6±2.75	
10-HDoHE	7.50±0.95	9.35±1.58	11.7±1.42	
11-HDoHE	8.41±1.12	11.1±1.87	12.7±1.67	
13-HDoHE	12.4±1.85	15.2±2.92	14.6±2.68	
14-HDoHE	29.7±5.53	27.7±4.17	33.0±7.44	
16-HDoHE	12.9±1.63	14.2±2.62	13.8±1.53	
17-HDoHE	43.5±4.49	50.9±6.96	61.9±8.47	
20-HDoHE	26.2±3.08	31.2±4.43	21.4±3.17	
19,20 DiHDoPE	8.52±1.35	10.3±1.34	10.5±1.18	
Sum	224±28.5	260±33.2	283±35.3	

Differing UPPER case superscript letters within a row indicate differences among diets. P values ≥ 0.05 are not shown. Values are mean±SE (n=8-10), and are based on wet tissue weight.

Appendix 1.4 Liver fatty acids in normal 12 wk old rats given LP, NP and HP diets for 2 weeks

Fatty Acids	LP	NP	HP	P Value Diet
		µg/g kidney		
14:0	20.7±2.10	22.4±1.90	23.0±2.31	
16:0	2682±145^{AB}	2780±108^{A}	2251±113^{B}	0.0123
16:1t	12.9±1.85	15.6±2.38	31.3±17.9	
16:1	177±23.0	186±24.5	122±26.0	
17:0	19.9±2.97^{B}	21.7±1.67^{B}	41.0±3.73^{A}	<.0001
18:0	3356±181^{B}	3642±137^{AB}	4181±164^{A}	0.0043
18:1	582±41.0^{AB}	693±48.0^{A}	465±77.5^{B}	0.0337
18:1n7c	333±61.7	487±40.7	370±42.3	
18:2n6	1392±189	1572±74.8	1555±127	
18:3n6	13.4±3.37	9.60±0.79	7.66±1.19	
18:3n3	5.06±1.35	4.86±0.27	6.19±0.79	
20:0	9.64±0.43^{AB}	10.0±0.41^{A}	8.58±0.26^{B}	0.0248
20:1	12.4±2.25^{B}	19.7±1.33^{A}	10.9±1.30^{B}	0.0021
20:2n6	23.7±4.06^{B}	35.3±2.66^{AB}	39.2±4.11^{A}	0.016
20:3n6	145±20.8	209±13.9	190±18.4	
20:4n6	3283±463	4037±183	3531±225	
20:5n3	36.5±7.66	38.6±3.82	45.7±4.71	
22:0	39.1±1.99	36.9±1.61	35.7±1.06	
22:2n6	8.77±2.32	11.7±2.23	4.88±2.10	
22:4n6	42.7±5.60	44.6±1.64	36.6±2.42	
22:5n6	52.6±6.16^{B}	96.8±11.2^{A}	89.5±10.8^{A}	0.007
22:5n3	122±17.2^{A}	128±10.8^{A}	60.1±7.42^{B}	0.0009
22:6n3	1005±154	1318±64.6	1153±79.3	
24:0	136±7.88^{A}	133±3.48^{A}	110±2.64^{B}	0.0011
24:1	52.5±8.02^{AB}	57.2±4.03^{A}	38.0±2.53^{B}	0.047

Differing UPPER case superscript letters within a row indicate differences among diets. P values ≥ 0.05 are not shown. Values are mean±SE (n=8-10), and are based on wet tissue weight.

Appendix 1.5 Serum oxylipins in normal 12 wk old rats given LP, NP and HP diets for 2 weeks

Oxylipin	LP	NP	HP	P Value
		ng/mL serum		
		LA oxylipins		
9-HODE	24.2±4.24^{A}	20.1±2.64^{AB}	11.3±2.39^{B}	0.0247
9-oxoODE	2.15±0.50	1.80±0.33	1.29±0.31	
13-HODE	21.0±3.30^{A}	19.6±1.79^{A}	10.2±1.43^{B}	0.0018
13 oxoODE	6.95±1.03	6.54±0.92	3.74±1.02	
9,12,13 triHOME	8.87±2.31	6.24±0.71	5.43±1.83	
9,10 EpOME	1.52±0.21^{A}	1.17±0.13^{AB}	0.84±0.17^{B}	0.0329
9,10 diHOME	1.39±0.18^{A}	1.25±0.13^{A}	0.72±0.12^{B}	0.0063
12,13 EpOME	4.37±1.17	3.59±0.59	2.14±0.74	
12,13 diHOME	3.41±0.66^{A}	3.69±0.63^{A}	1.45±0.31^{B}	0.0165
Sum	73.8±10.4^{A}	64.0±6.22^{AB}	37.1±7.30^{B}	0.0108
		DGLA oxylipins		
PGD_1	0.83±0.13	0.55±0.09	0.45±0.09	
PGE_1	0.47±0.08	0.50±0.09	0.57±0.09	
15-HETrE	0.91±0.14	0.79±0.11	0.60±0.08	
Sum	1.73±0.03	1.34±0.19	1.05±0.01	
		AA oxylipins		
PGD_2	6.86±1.33	5.22±0.82	5.40±0.93	
PGJ_2	0.21±0.04	0.12±0.02	0.15±0.03	
PGE_2	5.77±1.00	5.04±0.78	5.35±0.99	
11bPGE$_2$	9.56±1.67	8.28±1.28	9.01±1.68	
$PGF_{2\alpha}$	2.88±0.54	2.23±0.43	2.75±0.51	
TXB_2	80.3±25.7	80.5±17.3	73.0±22.8	
12-HHTrE	77.8±14.9	67.1±9.90	76.8±13.7	
5-HETE	2.84±0.22	3.62±0.80	3.69±0.98	
5-oxoETE	1.36±0.39	1.04±0.12	1.16±0.14	
8-HETE	1.87±0.31	1.73±0.22	1.37±0.23	
9-HETE	4.12±0.86	3.63±0.75	2.82±0.47	
11-HETE	15.1±3.17	14.3±2.25	11.8±2.86	
12-HETE	310±64.7	248±32.8	208±31.3	
12-oxoETE	3.67±0.73	4.12±0.63	3.95±0.72	
15-HETE	10.0±1.41	8.99±1.26	8.18±1.11	
15-oxoETE	0.84±0.17	0.76±0.08	0.73±0.09	
5,6 DiHETrE	0.15±0.02	0.14±0.02	0.13±0.02	
11,12 EpETrE	0.43±0.07	0.36±0.04	0.33±0.08	
11,12 DiHETrE	0.14±0.03	0.13±0.02	0.10±0.02	
14,15 EpETrE	0.72±0.11	0.86±0.06	0.65±0.09	
14,15 DiHETrE	0.34±0.04	0.32±0.07	0.31±0.06	
8-iso 15k $PGF_{2\beta}$	0.03±0.01	0.02±0.00	0.04±0.01	
20cooh AA	7.14±6.39	7.89±4.53	7.14±6.22	
Sum	542±107	465±55.2	423±66.5	
		ALA oxylipins		
9-HOTrE	1.00±0.17	0.76±0.09	0.60±0.16	
13-HOTrE	2.42±0.40^{A}	2.01±0.28^{A}	1.09±0.16^{B}	0.0117
13 oxoOTrE	90.0±22.0	78.0±4.85	94.7±7.75	
Sum	93.4±22.1	80.7±4.90	96.4±7.76	
		EPA oxylipin		
12-HEPE	8.28±2.39	5.73±0.75	4.39±0.98	
		DHA oxylipins		
4-HDoHE	0.38±0.05	0.42±0.04	0.42±0.07	
7-HDoHE	0.29±0.07	0.34±0.09	0.14±0.03	
10-HDoHE	0.21±0.07	0.24±0.06	0.18±0.06	
13-HDoHE	0.48±0.08^{A}	0.41±0.03^{AB}	0.26±0.04^{B}	0.0264
14-HDoHE	3.45±1.01	3.33±0.45	2.29±0.29	
16-HDoHE	0.39±0.05	0.40±0.03	0.32±0.05	
17-HDoHE	2.86±0.25	3.28±0.37	2.38±0.39	
20-HDoHE	0.52±0.07	0.58±0.07	0.53±0.12	
7R Maresin-1	0.07±0.02	0.07±0.01	0.06±0.01	
Sum	8.64±1.41	9.05±0.64	6.58±0.08	

Differing UPPER case superscript letters within a row indicate differences among diets. P values ≥ 0.05 are not shown. Values are mean±SE (n=8-10).

Appendix 1.6 Serum fatty acids in normal 12 wk old rats given LP, NP and HP diets for 2 weeks

Fatty Acids	LP	NP	HP	P Value
		µg/mL		Diet
10:0	0.08±0.01	0.07±0.01	0.15±0.03	
12:0	0.39±0.05^{AB}	0.31±0.04^{B}	0.60±0.10^{A}	0.0178
14:0	12.5±2.92	8.43±0.95	10.3±1.54	
14:1	1.22±0.45	0.48±0.08	0.49±0.12	
16:0	467±78.3	370±17.1	392±40.7	
16:1t	8.38±2.67	5.66±0.44	6.82±0.59	
16:1	82.7±18.5	58.4±5.10	48.8±7.54	
17:0	2.33±0.23^{B}	2.14±0.33^{B}	3.88±0.57^{A}	0.0108
18:0	146±25.5^{B}	175±9.52^{B}	274±24.9^{A}	0.0009
18:1	370±76.6	282±12.8	277±36.2	
18:1n7c	96.0±51.5	44.0±3.11	54.0±5.93	
18:2n6	354±62.4	287±16.5	384±31.9	
18:3n6	5.45±0.95	3.90±0.31	3.10±0.50	0.0515
18:3n3	19.5±4.61	12.2±1.68	17.0±3.20	
20:0	1.91±0.27	1.38±0.20	1.80±0.18	
20:1	4.48±1.23	3.52±0.34	3.70±0.46	
20:2n6	3.01±0.68	3.32±0.17	4.40±0.47	
20:3n6	11.1±2.89	11.0±0.81	10.1±2.39	
20:4n6	278±25.1^{B}	317±17.5^{B}	418±27.9^{A}	0.0014
20:3n3	0.26±0.07	0.22±0.03	0.74±0.33	
20:5n3	7.89±1.60	7.25±0.60	8.14±0.66	
22:0	4.43±0.22	4.20±0.31	4.40±0.46	
22:1	0.23±0.05	0.21±0.05	0.38±0.09	
22:2n6	2.02±0.37	1.77±0.38	1.40±0.42	
22:4n6	4.56±1.20	5.43±0.70	4.87±0.41	
22:5n6	3.46±0.58	3.51±0.28	5.57±0.96	
22:5n3	8.54±2.01	8.34±1.07	6.29±0.80	
22:6n3	36.5±5.17^{B}	44.9±2.66^{B}	63.0±3.49^{A}	0.0003
24:0	9.61±0.51	9.03±0.50	9.53±0.33	
24:1	9.70±0.56	9.65±0.76	9.93±0.55	

Differing UPPER case superscript letters within a row indicate differences among diets. P values ≥ 0.05 are not shown. Values are mean±SE (n=8-10).

Appendix 1.7 Oxylipins scanned but below the level of detection (<3 times baseline)

Kidney

COX

PGD_1; PGK_1; TXB_1; 15-k-$PGF_{1\alpha}$; dihomo-15deoxy-PGD_2; dihomo-PGD_2; dihomo-PGE_2; dihomo-PGJ_2; bicyclo-PGE_2; dihydro-$PGF_{2\alpha}$; dihydro-k-PGD_2; PGA_2; PGB_2; PGJ_2; PGK_2; tetranor-PGDM; tetranor-PGEM; tetranor-PGFM; 2,3-dinor-11β-$PGF_{2\alpha}$; 2,3-dinor-TXB_2; 2,3-dinor-6-k-$PGF_{1\alpha}$; 6-k-PGE_1; 6,15-diketo-dihydro-$PGF_{1\alpha}$; 11β-$PGF_{2\alpha}$; 11β-dihydro-keto$PGF_{2\alpha}$; 11β-PGE_2; 11-dehydro-TXB_2; 15-deoxy-PGA_2; 15-deoxy-PGJ_2; 15-k-PGD_2; 15-k-$PGF_{2\alpha}$; 19-hydroxy-PGE_2; 19-hydroxy-$PGF_{2\alpha}$; 20-carboxy-AA; 20-hydroxy-PGE_2; 20- hydroxy-$PGF_{2\alpha}$; Δ17-6 k-$PGF_{1\alpha}$; PGD_3; PGE_3; $PGF_{3\alpha}$; TXB_3

LOX

9,10,13-TriHOME; HXA_3; HXB_3; LTC_4; LTD_4; LTE_4; LXB_4; 6R-LXA_4; 6S-LXA_4; 6-trans-LTB_4; 6-trans,12epi-LTB_4; tetranor-12-HETE; 12epi-LTB_4; 12-oxo-LTB_4; 14,15-LTC_4 (EXC_4); 14,15-LTD_4 (EXD_4); 14,15-LTE_4 (EXE_4); 15R-LXA_4; 20-hydroxy-LTB_4; 20-carboxy- LTB_4; 5,6-DiHETE; 13-oxoOTrE; RvE_1; 8-HEPE; 11-HEPE; 15-oxoEDE; 17 keto- PA/17oxo-DPA; 7R-Maresin-1; PD_1; RvD_1; RvD_2; 10S,17S-DiHDoHE (PDX); 15-trans-PD_1; 17-k-DHA/ 17-oxo-DHA; 5-HETrE

CYP

5,6-EpETrE; 12,13-DiHODE; 15,16-EpODE; 15,16-DiHODE; 14,15-EpETE; 17,18-EpETE; 16,17-EpDPE; 19,20-EpDPE; 19,20-DiHDPA; 17-HETE; 18-HETE; 19-HETE; 20-HETE

Non-enzymatic oxylipins

8-iso-$PGF_{3\alpha}$; 2,3-dinor-8-iso-$PGF_{2\alpha}$; 10-Nitrooleate; 9-Nitrooleate

Liver

COX

PGK_1; TXB_1; 15-k-$PGF_{1\alpha}$; dihomo-15deoxy-PGD_2; dihomo-PGD_2; dihomo-PGE_2; dihomo-$PGF_{2\alpha}$; dihomo-PGJ_2; bicyclo-PGE_2; dihydro-$PGF_{2\alpha}$; dihydro-k-PGE_2; PGA_2; PGK_2; tetranor-PGDM; tetranor-PGEM; tetranor-PGFM; 2,3-dinor-11β-$PGF_{2\alpha}$; 2,3-dinor-TXB_2; 2,3-dinor-6-k-$PGF_{1\alpha}$; 6-k-PGE_1; 6,15-diketo-dihydro-$PGF_{1\alpha}$; 11β-$PGF_{2\alpha}$; 11β-dihydro-keto$PGF_{2\alpha}$; 11-dehydro-TXB_2; 15-deoxy-PGA_2; 15-deoxy-PGD_2; 15-deoxy-PGJ_2; 15-k-PGD_2; 15-k- PGE_2; 15-k-$PGF_{2\alpha}$; 19-hydroxy-PGE_2; 19-hydroxy-$PGF_{2\alpha}$; 20-carboxy-AA; 20-hydroxy-PGE_2; 20- hydroxy-$PGF_{2\alpha}$; Δ17-6 k-$PGF_{1\alpha}$; PGD_3; $PGF_{3\alpha}$; TXB_3

LOX

9,10,13-TriHOME; 13-HOTrE-γ; 8-HETrE; HXA_3; HXB_3; LTB_4; LTC_4; LTD_4; LTE_4; LXB_4; 6R-LXA_4; 6S-LXA_4; 6-trans-LTB_4; 6-trans,12epi-LTB_4; 9-HETE; tetranor-12-HETE; 12epi-

LTB_4; 12-oxo-LTB_4; 14,15-LTC_4 (EXC_4); 14,15-LTD_4 (EXD_4); 14,15-LTE_4 (EXE_4); 15R-LXA_4 20-hydroxy-LTB4; 20-carboxy- LTB_4; 5,6-DiHETE; 5,15-DiHETE; 8,15-DiHETE; 9-oxoOTrE; 13-HOTrE; 13-oxoOTrE; LXA_5; RvE_1; 5-HEPE; 8-HEPE; 9-HEPE; 11-HEPE; 15-HEPE; 15-oxoEDE; 17 keto- PA/17oxo-DPA; 7R-Maresin-1; PD_1; RvD_1; RvD_2; 10S,17S-DiHDoHE (PDX); 15-trans-PD_1; 17-k-DHA/ 17-oxo-DHA; 5-HETrE

CYP

5,6-EpETrE; 8,9-EpETrE; 11,12-EpETrE; 14,15-EpETrE; 9,10-EpODE; 9,10-diHODE; 12,13-EpODE; 15,16-EpODE; 17,18-EpETE; 16,17-EpDPE; 19,20-EpDPE; 17-HETE; 18-HETE; 19-HETE; 20-HETE; 18-HEPE

Non-enzymatic oxylipins

5-iso-$PGF_{2\alpha}$VI; 8-iso-$PGF_{2\alpha}$III; 8-iso-$PGF_{3\alpha}$; 8-iso-15k-$PGF_{2\beta}$; 2,3-dinor-8-iso-$PGF_{2\alpha}$; 10-Nitrooleate; 9-Nitrooleate

Serum

COX

$PGF_{1\alpha}$; TXB_1; 15-k-$PGF_{1\alpha}$; dihomo-15deoxy-PGD_2; dihomo-PGD_2; dihomo-PGE_2; dihomo-$PGF_{2\alpha}$; dihomo-PGJ_2; bicyclo-PGE_2; dihydro-$PGF_{2\alpha}$; dihydro-k-PGD_2; PGA_2; PGB_2; PGK_2; tetranor-PGDM; tetranor-PGEM; tetranor-PGFM; 2,3-dinor-11β-$PGF_{2\alpha}$; 2,3-dinor-TXB_2; 2,3-dinor-6-k-$PGF_{1\alpha}$; 6-k-PGE_1; 6-k-$PGF_{1\alpha}$; 6,15-diketo-dihydro-$PGF_{1\alpha}$; 11β-$PGF_{2\alpha}$; 11β-dihydro-ketoPGF$_{2\alpha}$; 11-dehydro-TXB_2; 15-deoxy-PGA_2; 15-deoxy-PGD_2; 15-deoxy-PGJ_2; 15-k-PGD_2; 15-k- PGE_2; 15-k-$PGF_{2\alpha}$; 19-hydroxy-PGE_2; 19-hydroxy-$PGF_{2\alpha}$; 20-carboxy-AA; 20-hydroxy-PGE_2; 20- hydroxy-$PGF_{2\alpha}$; Δ17-6 k-$PGF_{1\alpha}$; PGD_3; PGE_3; $PGF_{3\alpha}$; TXB_3

LOX

9,10,13-TriHOME; 8-HETrE; HXA_3; HXB_3; LTB_4; LTC_4; LTD_4; LTE_4; LXB_4; 6R-LXA_4; 6S-LXA_4; 6-trans-LTB_4; 6-trans,12epi-LTB_4; tetranor-12-HETE; 12epi-LTB4; 12-oxo-LTB_4; 14,15-LTC_4 (EXC_4); 14,15-LTD_4 (EXD_4); 14,15-LTE_4 (EXE_4); 15R-LXA_4; 20-hydroxy-LTB_4; 20-carboxy- LTB_4; 5,6-DiHETE; 5,15-DiHETE; 8,15-DiHETE; 9-oxoOTrE; LXA_5; RvE_1; 5-HEPE; 8-HEPE; 9-HEPE; 11-HEPE; 15-HEPE; 15-oxoEDE; 17 keto- PA/17oxo-DPA; 8-HDoHE; PD_1; RvD_1; RvD_2; 10S,17S-DiHDoHE (PDX); 11-HDoHE; 15-trans-PD_1; 17-k-DHA/ 17-oxo-DHA; 5-HETrE

CYP

5,6-EpETrE; 8,9-EpETrE; 8,9-DiHETrE; 9,10-EpODE; 9,10-diHODE; 12,13-EpODE; 12,13-DiHODE; 15,16-EpODE; 15,16-DiHODE; 14,15-EpETE; 17,18-EpETE; 16,17-EpDPE; 19,20-EpDPE; 19,20-DiHDPA; 16-HETE; 17-HETE; 18-HETE; 19-HETE; 20-HETE; 18-HEPE

Non-enzymatic oxylipins

5-iso-$PGF_{2\alpha}$VI; 8-iso-$PGF_{2\alpha}$III; 8-iso-$PGF_{3\alpha}$; 2,3-dinor-8-iso-$PGF_{2\alpha}$; 10-Nitrooleate; 9-Nitrooleate

Appendix 1.8 Oxylipins detected (>3times baseline) but below the level of quantitation (<5 times baseline)

Kidney

9-oxoOTrE; LXA_5; 9-HEPE; 8,9-EpETrE; 9,10-EpODE; 9,10-diHODE; 12,13-EpODE

Liver

PGE_1; dhk-PGD_2; PGJ_2; 12,13-DiHODE; 15,16-DiHODE

Serum

PGK_1; dhk-PGE_2; Dhk-$PGF_{2\alpha}$; 13-HOTrE-γ

Appendix 1.9 Oxylipin mass transitions, retention times, deuterated internal standards and standard curve slopes[1]

Oxylipin	Precursor PUFA	Mass Transition	Retention Time	Internal Standard	Slope
COX derived oxylipins					
PGD_1	DGLA	353.0 / 235.0	7.74	PGD2-d4	1.0006
PGE_1	DGLA	353.0 / 235.0	7.67	PGE2-d4	1.4269
$PGF_{1\alpha}$	DGLA	355.0 / 293.0	7.24	PGF2α-d4	3.0675
PGK_1	DGLA	351.0 / 251.0	7.76	PGE2-d4	4.2079
TXB_1	DGLA	371.0 / 171.0	6.33	TXB2-d4	3.6782
15-k-$PGF_{1\alpha}$	DGLA	353.0 / 221.0	7.68	PGF2α-d4	No Primary[2]
dihomo-15deoxy-PGD_2	ADA	361.0 / 299.0	14.08	15deoxy-PGJ2-d4	No Primary
dihomo-PGD_2	ADA	379.0 / 299.0	9.70	PGD2-d4	No Primary
dihomo-PGE_2	ADA	379.0 / 299.0	9.40	PGE2-d4	No Primary
dihomo-$PGF_{2\alpha}$	ADA	381.0 / 337.0	9.20	PGF2α-d4	2.812
dihomo-PGJ_2	ADA	361.0 / 299.0	12.52	15-deoxy-PGJ2-d4	No Primary
bicyclo-PGE_2	AA	333.0 / 175.0	11.00	PGE2-d4	0.79425
dihydro-$PGF_{2\alpha}$	AA	355.0 / 283.0	7.97	PGF2α-d4	No Primary
dihydro-k-PGD_2	AA	351.0 / 207.0	9.15	PGD2-d4	2.34
dihydro-k-PGE_2	AA	351.0 / 207.0	8.44	PGE2-d4	0.529
PGA_2	AA	333.0 / 271.0	9.68	15-deoxy-PGJ2-d4	11.454
PGB_2	AA	333.0 / 271.0	12.29	15-deoxy-PGJ2-d4	1.0891
PGD_2	AA	351.0 / 271.0	7.78	PGD2-d4	1.6144
PGE_2	AA	351.0 / 271.0	7.43	PGE2-d4	7.8596
$PGF_{2\alpha}$	AA	353.0 / 193.0	7.25	PGF2α-d4	3.4181
PGJ_2	AA	333.0 / 189.0	9.87	15-deoxy-PGJ2-d4	3.6267
PGK_2	AA	349.0 / 249.0	7.71	PGE2-d4	1.8211
tetranor-PGDM	AA	327.0 / 247.0	2.39	PGD2-d4	0.0983
tetranor-PGEM	AA	327.0 / 291.0	2.30	PGE2-d4	0.4032
tetranor-PGFM	AA	329.0 / 247.0	2.30	PGF2α-d4	No Primary
TXB_2	AA	369.0 / 169.0	6.61	TXB2-d4	6.3658
2,3-dinor-11β-$PGF_{2\alpha}$	AA	325.0 / 227.0	5.40	PGF2α-d4	1.2716
2,3-dinor-TXB_2	AA	341.0 / 137.0	5.22	TXB2-d4	0.3628
2,3-dinor-6-k-$PGF_{1\alpha}$	AA	363.0 / 281.0	6.60	6-k-PGF1α-d4	No Primary
6-k-PGE_1	AA	367.0 / 331.0	5.65	PGE2-d4	0.9207
6-k-$PGF_{1\alpha}$	AA	369.0 / 245.0	5.34	6-k-PGF1α-d4	3.6495
6,15-diketo-dihydro-$PGF_{1\alpha}$	AA	369.0 / 267.0	6.52	PGF2α-d4	0.4899
11β-$PGF_{2\alpha}$	AA	353.0 / 335.0	6.74	PGF2α-d4	0.1703
11β-dihydro-ketoPGF$_{2\alpha}$	AA	353.0 / 113.0	8.20	PGF2α-d4	0.8673
11β-PGE_2	AA	353.0 / 335.0	6.74	PGE2-d4	0.1703
11-dehydro-TXB_2	AA	367.0 / 305.0	7.39	TXB2-d4	2.3749
12-HHTrE	AA	279.0 / 217.0	13.53	15-HETE-d8	0.3646
15-deoxy-PGA_2	AA	315.0 / 271.0	15.19	15-deoxy-PGJ2-d4	2.6609
15-deoxy-PGD_2	AA	333.0 / 271.0	11.81	15-deoxy-PGJ2-d4	29.722
15-deoxy-PGJ_2	AA	315.0 / 203.0	14.57	15-deoxy-PGJ2-d4	1.4436
15-k-PGD_2	AA	349.0 / 235.0	8.64	PGD2-d4	No Primary
15-k-PGE_2	AA	349.0 / 235.0	7.78	PGE2-d4	1.3377
15-k-$PGF_{2\alpha}$	AA	351.0 / 219.0	7.68	PGF2α-d4	0.6609
19-hydroxy-PGE_2	AA	367.0 / 243.0	3.25	PGE2-d4	0.3835
19-hydroxy-$PGF_{2\alpha}$	AA	369.0 / 192.0	2.95	PGF2α-d4	No Primary
20-cαrboxy-AA	AA	333.0 / 271.0	14.38	ARA-d8	1.1049
20-hydroxy-PGE_2	AA	367.0 / 175.0	3.15	PGE2-d4	No Primary
20-hydroxy-$PGF_{2\alpha}$	AA	369.0 / 165.0	2.85	PGF2α-d4	No Primary
Δ17-6 k-$PGF_{1\alpha}$	EPA	367.0 / 163.0	4.68	PGF2α-d4	2.0835
PGD_3	EPA	349.0 / 269.0	6.79	PGD2-d4	0.5356
PGE_3	EPA	349.0 / 269.0	6.52	PGE2-d4	1.3408
$PGF3_{\alpha}$	EPA	351.0 / 193.0	6.27	PGF2α-d4	1.2284
TXB_3	EPA	367.0 / 169.0	5.73	TXB2-d4	5.4993
LOX derived oxylipins					
9-HODE	LA	295.0 / 171.0	15.95	9-HODE-d4	1.4704
9-oxoODE	LA	293.0 / 185.0	16.28	5-oxoETE-d7	1.4353
9,10,13-TriHOME	LA	329.0 / 171.0	7.32	9,10-DiHOME-d4	3.1287
9,12,13-TriHOME	LA	329.0 / 211.0	7.19	12,13-DiHOME-d4	6.1871
13-HODE	LA	295.0 / 195.0	15.8	13-HODE-d4	2.9546
13-oxo-ODE	LA	293.0 / 167.0	15.96	5-oxoETE-d7	0.2716

Oxylipin	Precursor PUFA	Mass Transition	Retention Time	Internal Standard	Slope
13-HOTrE-γ	GLA	293.0 / 193.0	14.89	13-HODE-d4	1.6998
8-HETrE	DGLA	321.0 / 157.0	17.12	5-HETE-d8	1.8141
15-HETrE	DGLA	321.0 / 221.0	16.74	15-HETE-d8	2.9424
HXA3	AA	335.0 / 195.0	14.6	LTB4-d4	No Primary
HXB3	AA	335.0 / 183.0	14.8	LTB4-d4	No Primary
LTB4	AA	335.0 / 195.0	11.93	LTB4-d4	1.2507
LTC4	AA	624.0 / 272.0	10.75	LTB4-d4	0.3364
LTD4	AA	495.0 / 177.0	9.19	LTB4-d4	1.04755
LTE4	AA	438.0 / 333.0	10.57	LTB4-d4	0.93675
LXB4	AA	351.0 / 221.0	7.58	LTB4-d4	0.3733
5-HETE	AA	319.0 / 115.0	17.19	5-HETE-d8	1.1690
5-oxoETE	AA	317.0 / 203.0	17.56	5-oxoETE-d7	0.8498
6R-LXA_4	AA	351.0 / 217.0	8.50	LTB4-d4	0.1535
6S-LXA_4	AA	351.0 / 115.0	8.78	LTB4-d4	0.292
6-trans-LTB_4	AA	335.0 / 195.0	11.44	LTB4-d4	0.9015
6-trans,12epi-LTB_4	AA	335.0 / 195.0	11.58	LTB4-d4	0.6674
8-HETE	AA	319.0 / 155.0	16.71	5-HETE-d8	2.311
9-HETE	AA	319.0 / 123.0	16.84	5-HETE-d8	0.2492
11-HETE	AA	319.0 / 167.0	16.41	5-HETE-d8	5.3158
12-HETE	AA	319.0 / 135.0	16.6	15-HETE-d8	0.189
tetranor-12-HETE	AA	265.0 / 109.0	13.28	15-HETE-d8	2.0436
12-oxoETE	AA	317.0 / 153.0	16.6	5-oxoETE-d7	1.5173
12epi-LTB_4	AA	335.0 / 195.0	11.89	LTB4-d4	1.4374
12-oxo-LTB_4	AA	333.0 / 179.0	12.6	LTB4-d4	1.4903
14,15-LTC_4 (EXC_4)	AA	624.0 / 272.0	7.50	LTB4-d4	No Primary
14,15-LTD_4 (EXD_4)	AA	495.0 / 177.0	10.98	LTB4-d4	No Primary
14,15-LTE_4 (EXE_4)	AA	438.0 / 333.0	9.00	LTB4-d4	No Primary
15-HETE	AA	319.0 / 175.0	16.07	15-HETE-d8	0.9018
15-oxoETE	AA	317.0 / 113.0	16.14	5-oxoETE-d7	2.2341
15R-LXA_4	AA	351.0 / 165.0	8.50	LTB4-d4	No Primary
20-hydroxy-LTB_4	AA	351.0 / 195.0	5.60	LTB4-d4	0.6127
20-carboxy-LTB_4	AA	365.0 / 195.0	5.35	LTB4-d4	0.1690
5,6-DiHETE	AA	335.0 / 115.0	15.29	LTB4-d4	0.4905
5,15-DiHETE	AA	335.0 / 201.0	11.46	LTB4-d4	0.5267
8,15-DiHETE	AA	335.0 / 235.0	10.98	LTB4-d4	0.1268
9-HOTrE	ALA	293.0 / 171.0	14.46	9-HODE-d4	2.006
9-oxoOTrE	ALA	291.0 / 185.0	14.98	5-oxoETE-d7	2.2547
13-HOTrE	ALA	293.4 / 195.0	14.59	13-HODE-d4	0.62155
13-oxoOTrE	ALA	291.0 / 247.0	14.8	5-oxoETE-d7	0.0856
LXA_5	EPA	349.0 / 215.0	7.32	LTB4-d4	0.3762
RvE_1	EPA	349.0 / 195.0	5.51	LTB4-d4	0.2271
5-HEPE	EPA	317.0 / 115.0	15.81	5-HETE-d8	1.1101
8-HEPE	EPA	317.0 / 155.0	15.34	5-HETE-d8	1.1697
9-HEPE	EPA	317.0 / 149.0	15.48	5-HETE-d8	0.6765
11-HEPE	EPA	317.0 / 215.0	15.23	5-HETE-d8	No Primary
12-HEPE	EPA	317.0 / 179.0	15.33	15-HETE-d8	1.7238
15-HEPE	EPA	317.0 / 219.0	15.0	15-HETE-d8	1.8731
15-oxoEDE	EPA	321.0 / 223.0	17.92	5-oxoETE-d7	1.1611
17 keto-DPA/17oxo-DPA	DPA	343.0 / 247.0	16.89	LTB4-d4	0.619
4-HDoHE	DHA	343.0 / 101.0	17.37	5-HETE-d8	0.4409
7-HDoHE	DHA	343.0 / 141.0	16.7	5-HETE-d8	0.5523
7R-Maresin-1	DHA	359.0 / 177.0	11.33	LTB4-d4	0.2837
8-HDoHE	DHA	343.0 / 109.0	16.77	5-HETE-d8	0.4476
PD_1	DHA	359.0 / 153.0	10.8	LTB4-d4	No Primary
RvD_1	DHA	375.0 / 141.0	8.36	LTB4-d4	0.5530
RvD_2	DHA	375.0 / 175.0	7.64	LTB4-d4	0.3639
10-HDoHE	DHA	343.0 / 153.0	16.36	5-HETE-d8	2.1831
10S,17S-DiHDoHE (PDX)	DHA	359.0 / 153.0	11.19	LTB4-d4	1.0292
11-HDoHE	DHA	343.0 / 149.0	16.5	5-HETE-d8	0.8501
13-HDoHE	DHA	343.0 / 221.0	16.18	15-HETE-d8	0.60295
14-HDoHE	DHA	343.0 / 205.0	16.3	15-HETE-d8	0.7806
15-trans-PD_1	DHA	359.0 / 153.0	11.0	LTB4-d4	No Primary
16-HDoHE	DHA	343.0 / 233.0	16.0	15-HETE-d8	3.3544
17-HDoHE	DHA	343.0 / 245.0	16.06	15-HETE-d8	0.2779
17-k-DHA/ 17-oxo-DHA	DHA	341.0 / 297.0	16.31	LTB4-d4	1.1412
5-HETrE	MA	321.0 / 205.0	18.65	5-HETE-d8	0.2746
CYP-epoxygenase derived oxylipins					
9,10-EpOME	LA	295.0 / 171.0	17.41	9,10 EpOME-d4	3.7629

Oxylipin	Precursor PUFA	Mass Transition	Retention Time	Internal Standard	Slope
9,10-DiHOME	LA	313.0 / 201.0	13.22	9,10 diHOME-d4	16.426
12,13-EpOME	LA	295.0 / 195.0	17.21	12,13 diHOME-d4	10.516
12,13-DiHOME	LA	313.0 / 183.0	12.66	12,13 diHOME-d4	11.798
5,6-EpETrE	AA	319.0 / 191.0	18.13	11,12 DiHETrE-d11	3.7275
5,6-DiHETrE	AA	337.0 / 145.0	15.56	11,12 DiHETrE-d11	3.6065
8,9-EpETrE	AA	319.0 / 155.0	17.9	8,9 DiHETrE-d11	1.2994
8,9-DiHETrE	AA	337.0 / 127.0	14.85	8,9 DiHETrE-d11	5.5366
11,12-EpETrE	AA	319.0 / 167.0	17.7	11,12 DiHETrE-d11	9.1336
11,12-DiHETrE	AA	337.0 / 167.0	14.27	11,12 DiHETrE-d11	16.4237
14,15-EpETrE	AA	319.0 / 175.0	17.24	14,15 DiHETrE-d11	1.0437
14,15-DiHETrE	AA	337.0 / 207.0	13.54	14,15 DiHETrE-d11	10.939
9,10-EpODE	ALA	293.0 / 171.0	16.39	9,10 diHOME-d4	No Primary
9,10-diHODE	ALA	311.0 / 201.0	11.62	9,10 diHOME-d4	No Primary
12,13-EpODE	ALA	293.0 / 183.0	16.19	12,13 diHOME-d4	2.4048
12,13-DiHODE	ALA	311.0 / 183.0	11.12	12,13 diHOME-d4	4.4379
15,16-EpODE	ALA	293.0 / 235.0	15.49	12,13 diHOME-d4	No Primary
15,16-DiHODE	ALA	311.0 / 223.0	10.62	12,13 diHOME-d4	No Primary
14,15-EpETE	EPA	317.0 / 207.0	16.27	14,15 DiHETrE-d11	2.6835
17,18-EpETE	EPA	317.0 / 259.0	15.88	14,15 DiHETrE-d11	1.8647
16,17-EpDPE	DHA	343.0 / 193.0	17.36	14,15 DiHETrE-d11	0.2435
19,20-EpDPE	DHA	343.0 / 241.0	16.91	14,15 DiHETrE-d11	2.2213
19,20-DiHDPA	DHA	361.0 / 229.0	13.31	14,15 DiHETrE-d11	1.1322
CYP-hydroxylase derived oxylipins					
16-HETE	AA	319.0 / 189.0	15.51	15-HETE-d8	0.5383
17-HETE	AA	319.0 / 247.0	15.4	15-HETE-d8	3.0768
18-HETE	AA	319.0 / 261.0	15.28	15-HETE-d8	3.2074
19-HETE	AA	319.0 / 231.0	14.89	20-HETE-d6	0.2715
20-HETE	AA	319.0 / 245.0	15.02	20-HETE-d6	0.3998
18-HEPE	EPA	317.0 / 215.0	14.5	20-HETE-d6	1.3858
20-HDoHE	DHA	343.0 / 241.0	15.72	20-HETE-d6	1.3316
Non-enzymatic oxylipins					
5-iso-$PGF_{2\alpha}$VI	AA	353.0 / 115.0	7.02	PGF2α-d4	1.5803
8-iso-$PGF_{2\alpha}$III	AA	353.0 / 193.0	6.50	PGF2α-d4	1.9210
8-iso-$PGF_{3\alpha}$	EPA	351.0 / 307.0	5.50	PGF2α-d4	No Primary
8-iso-15k-$PGF_{2\beta}$	AA	351.0 / 219.0	6.96	PGF2α-d4	3.6218
2,3-dinor-8-iso-$PGF_{2\alpha}$	AA	325.0 / 237.0	5.09	PGF2α-d4	5.3857
10-Nitrooleate	OA	326.0 / 169.0	19.65	EPA-d5	No Primary
9-Nitrooleate	OA	326.0 / 168.0	19.65	EPA-d5	No Primary

[1]Abbreviations: ADA, Adrenic acid; DGLA, Dihomo-γ-linolenic acid; DiHEPE, Dihydroxy-eicosapentaenoic acid; DiHETE, Dihydroxy-eicosatetraenoic acid; DiHODE, Dihydroxy-octadecadienoic acid; DiHOTrE,Dihydroxy-octadecatrienoic acid; DPA, Docosapentaenoic acid; EpDPE, Epoxy-docosapentaenoic acid; EpEDE, Epoxy-eicosadienoic acid; EpETE, Epoxy-eicosatetraenoic acid; EpETrE, Epoxy-eicosatrienoic acid; EpODE, Epoxy-octadecadienoic acid; EpOME, Epoxy-octadecenoic acid; Ex,Eoxin; GLA, γ-linolenic acid; HpODE, Hydroperoxy-octadecadienoic acid; Hx,Hepoxilin; Lt,Leukotriene; Lx,Lipoxin; MaR, Maresin; OA, Oleic acid; oxo-EPE, oxo-Eicosapentaenoic acid; PD, Protectin; PGEM, Prostaglandin E metabolite.

[2]For those with no primary, the retention time was estimated based on comparisons of known and unknown retention time of oxylipins in our samples and the retention times of oxylipins reported by the following papers.

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

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

Appendix 1.10 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

Appendix 1.11 Volume and concentration of deuterated internal standards

Internal Standard	Volume to Add (μl)	Stock Conc (ng/μl)	Final Conc (ng/μl)
6-k-$PGF_{1\alpha}$-d4	30.0	25	0.75
TXB_2-d4	20.0	25	0.50
$PGF_{2\alpha}$-d4	20.0	50	1.00
PGE_2-d4	10.0	50	0.50
PGD_2-d4	50.0	25	1.25
13,14-dihydro-15-keto-$PGF_{2\alpha}$	20.0	50	1.00
LTB4-d4	80.0	25	2.00
20-HETE-d6	80.0	25	2.00
15-HETE-d8	40.0	25	1.00
5-HETE-d8	80.0	25	2.00
13-HODE-d4	40.0	25	1.00
9-HODE-d4	40.0	25	1.00
12,13-DiHOME-d4	20.0	25	0.50
9,10-DiHOME-d4	20.0	25	0.50
14,15-DHET-d11	10.0	25	0.25
11,12-DHET-d11	10.0	25	0.25
8,9-DHET-d11	40.0	25	1.00
15d-PGJ_2-d4	80.0	25	2.00
EPA-d5	40.0	50	2.00
5-OxoETE-d7	270.0	25	6.75
Total	1000.0		

20μL was added to each sample (per 200μL kidney and liver homogenate and per 200μL serum) for oxylipin analysis.

Appendix 2 Protocol

Appendix 2.1 Diet preparation

All ingredients except cornstarch, dextrose, fibre (cellulose), and sucrose are found in the walk-in refrigerator. Labelled containers for weighing ingredients can be found in the refrigerator (these containers do not need to be washed after every use but must be kept in the refrigerator)

1. Measure out all ingredients according to recipe.
2. In a large bowl add sucrose and all ingredients weighing less than 200g (eg. Min mix, L-cysteine, methionine, etc). Mix by hand using a small utensil so all ingredients are thoroughly combined. This is an important step to ensure that smaller ingredients are well distributed in the diet.
3. In the mixing bowl (Hobart Mixer bowl) add half of all remaining ingredients and half of the mixture of smaller ingredients. Mix thoroughly with utensil and break up any large clumps. Add the remaining half of all ingredients and mix again with utensil.
4. Attach Hobart Mixer bowl to the machine. Lock into place and raise the bowl up using the lever. Attach plastic shield.
5. Set mixing speed to 1 and timer to 3 minutes. Only turn the power switch on after timer has been set. (Keep hands free of mixer)
6. When mixing stops turn the power switch off, lower the bowl, and scrape the sides of bowl and the beater. Also, make sure to blend ingredients at the bottom of the bowl as the beater can not reach them. Once finished raise bowl back into place and attach the shield.
7. Set mixing speed to 2 and the timer to 5 minutes. Do not start the mixer yet. At this step you will need to add soybean oil.

8. Tare the scale with the container of soybean oil without the lid. Turn on the mixer and slowly begin to add the oil.
9. When mixing stops turn the power switch off, lower the bowl, and scrape the sides of bowl, the beater, and the bottom of the bowl again. Once finished raise bowl back into place and attach the shield.
10. Set mixing speed to 2 and the timer to 5 minutes again.
11. When mixing stops turn the power switch off, lower the bowl, and scrape the sides of bowl, the beater, and the bottom of the bowl again. Once finished raise bowl back into place and attach shield.
12. Set mixer speed to 2 and the timer to 5 minutes one last time.
13. Once mixing is finished remove the bowl from the machine and mix contents by hand and break up any remaining clumps.
14. The finished diet should be grainy in consistency.
15. Diet can now be placed in a large container and stored in the walk-in freezer.

Clean all equipment used. Wash down counters and machine. Sweep floor. Wash weigh containers at the end of once every four diets are made.

Appendix 2.2 Homogenization protocol

Use Tyrode's (pH 7.6) salt solution (see Appendix 2.4 and 2.5) to homogenize kidney tissue.

Only kidney and liver need to be homogenized. Serum does not need to be homogenized.

Therefore, 350mg of wet tissue = 2000uL of Tyrode's (pH7.6) required.

Ensure there is enough prepared of:

- Tyrode's salt solution (pH 7.6) (Check for deterioration. (See Appendix 2.4 and 2.5).
- 1% Triton Solution as per instructions (see Appendix 2.6).
- 12 mL test tubes with lids that have been soaked overnight in Contrad solution, rinsed and dried.
- 100:1 Methanol : Formic Acid
- pH 3 water – (water that has had pH adjusted to 3.0 using 1M HCL)
- Antioxidant Cocktail

1. Turn on water bath to 37°C.
2. Turn on the Accuspin 3R centrifuge to 4°C and ensure microtube rotor is in place.
3. Label 16 x 125mm disposable glass test tubes with sample ID's.
4. Obtain a large container of ice.
5. Prepare and label three disposable glass tubes (16 x 125mm) with 100% ethanol and three disposable glass tubes (16 x 125mm) with ultrapure water for cleaning the homogenizer.
6. Remove the required kidney/liver samples from the -80°C freezer and keep on ice.
7. Weigh and record kidney/liver sample into labeled tubes, cover with parafilm and immediately place on ice.
8. Return the remaining kidney/liver samples to the -80°C freezer.

9. Calculate, record, and add required amount of Tyrode's (pH7.6) to each weighed kidney sample.

350 mg of tissue = 2000 μL of Tyrode's required

10. Clean homogenizer before use, after use, and in between each sample by:
    - 3 tubes ethanol x 30 seconds each at speed 15
    - dab with kimwipe to dry
    - 3 tubes ultrapure water x 30 seconds each at speed 15
    - dab with kimwipe to dry
11. Place test tube containing kidney/liver tissue in a small plastic container (yogurt container) containing an ice slurry.
12. Insert rotor into test tube and homogenize at speed 20 for 30 seconds. Avoid generating bubbles. Stop and check that all kidney tissue is at the bottom of the tube. If not, use rotor tip to push everything to the bottom of the test tube.
13. Homogenize again for another 30 seconds (speed 20).
14. Repeat steps 13 – 15 for each sample. Remember to keep tubes covered with parafilm and on ice as much as possible.
15. Any tools contaminated with biological hazards can be wiped off with 10% bleach then washed normally with contrad/ other detergent.

Appendix 2.3 Homogenate aliquoting

1. **Fatty acids:** Aliquot 250 µL into 12 mL tubes with screw top for fatty acid analysis (make sure these tubes have been soaked overnight in Contrad solution, rinsed and dried).

    To clean tubes, prepare a 2-5% solution of Contrad and distilled water in a large beaker, submerge tubes and lids and let soak overnight. Once they are finished soaking thoroughly rinse with tap water, 3 to 5 times, followed by distilled water (3 to 5 times) and let dry upside-down on a rack.

   Immediately add 8.34 µL of the antioxidant cocktail to the fatty acid analysis tubes (ratio is 6.67 µL antioxidant for every 200 µL of homogenate) and put back on ice if proceeding directly to fatty acid analysis. Otherwise, flush with nitrogen, cap and store at -80°C.

2. **Oxylipins:** Remove an aliquot (200 µL for each tube) for the oxylipin analysis and place in a microcentrifuge tube for liver and kidney; 200 µL serum is used directly.

1. Add 1 µL of a 1% Triton solution for every 100 µL aliquot for oxylipin analysis. (Final concentration of Triton in homogenate should be 0.01%: 0.01x 100=1 µL)
2. Vortex for 10 seconds.
3. Incubate, covered with parafilm, on ice for 10 minutes.
4. Vortex again for 10 seconds.
5. Incubate, covered, on ice for 10 minutes.
6. Vortex again for 10 seconds.
7. Incubate, covered, on ice for 10 minutes.
8. Working quickly, add in the same order below
    a. 500 µL of 100:1 methanol : formic acid
    b. 800 µL of pH3 water

c. 90 µL of ethanol

d. 10 µL of antioxidant cocktail

e. And vortex for 5 seconds

9. Store samples in -80°C freezer for future oxylipin extraction – see Appendix 2.9

Appendix 2.4 Reconstitution of Tyrode's salts without sodium bicarbonate

Product# T2145 (Sigma)

Tyrode's salts powder comes prepackaged from Sigma-Aldrich and is kept in the fridge. Please refer to the product insert for full product information. Powdered salts are hygroscopic and should be protected from moisture. The entire contents of each package should be used immediately after opening. Preparing a concentrated salt solution is not recommended as precipitates may form. Tyrode's Salts are meant to maintain cellular pH and osmotic balance.

1. Measure out 1L of ultrapure water in a graduated cylinder. Water temperature should be 15-20°C.
2. Transfer about 800 mL of measured water into a 2000 mL beaker. Add a large stir bar (careful not to splash) and place on stir plate. Begin gentle stirring.
3. Add powdered Tyrode's salts and continue stirring until dissolved. Do NOT heat.
4. Rinse original Tyrode's salts packaging with some (NOT all) of the remaining 200 mL measured water to remove all traces of powder. Add to solution in step 3. Rinse the package 3 times.
5. Transfer solution to a 1L volumetric flask and bring to volume using some of the remaining 200 mL measured water in graduated cylinder from step 1. Rinse the flask 3 times. Can use a pipette to bring the flask up to volume w/ water.
6. Insert volumetric stopper and invert 10x to mix.
7. Transfer to a 1L glass bottle covered with tin foil to protect from light and clearly label as Tyrode's salts WITHOUT NaHCO3. Store in the refrigerator (2-8°C).

Appendix 2.5 Tyrode's preparation

1. Measure 100 mL of reconstituted Tyrode's salt solution into a graduated cylinder.
2. Weigh 100mg of powdered sodium bicarbonate (Sigma, S5761) into a 125 mL Erlenmeyer flask.
3. Cover flask with tin foil to protect from light. Add a stir bar.
4. Transfer about 80 mL of measured Tyrode's in graduated cylinder from step 1 to the Erlenmeyer flask.
5. Completely dissolve powder into solution by placing on stir plate and stirring (apprx 15 min). Powder must be completely dissolved before adjusting pH.
6. While continuing to stir, adjust the pH of the solution to pH 7.6 using 1N HCl or 1N NaOH. Normally to achieve pH 7.6, a couple of drops of 1N HCl are required.
7. Transfer solution to a 100mL volumetric flask and bring to volume using some of the remaining 20 mL measured Tyrode's in graduated cylinder from step 1.
8. Insert volumetric stopper and invert 10x to mix.
9. Transfer to a 100mL glass bottle covered with tin foil to protect from light and clearly label as Tyrode's salts (pH 7.6). Store in the refrigerator (2-8°C).

NOTE: Tyrode's that has sodium bicarbonate added and has been pHed can deteriorate.

Deterioration can be recognized by:

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

Check all these signs before using.

Appendix 2.6 1% Triton preparation

This solution mixes best if Tyrode's (pH 7.6) is at room temperature.

1. Weigh out 0.02 g of Triton solution in a 20 mL scintillation vial.
2. Add 2.0 mL of room temperature Tyrode's (pH 7.6) using a 1.0 mL eppendorf pipette.
3. Cover with cap and vortex well.
4. Cover with tin foil to protect from light and chill on ice or in fridge. Store remains in refrigerator.

A final concentration of 0.01% Triton is required in the homogenate to disrupt lipids and release proteins. This will ONLY be added to the LC-MS/MS fraction.

Therefore add 10 µL of 1% Triton (pH 7.6) to 1000uL of kidney homogenate for the LC-MS/MS fraction only to make a 0.01% Triton final solution

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

Appendix 2.7 Antioxidant cocktail preparation

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

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

Appendix 2.8 Solvent A and Solvent B preparation

Solvent A

Water – Acetonitrile – Acetic Acid [70:30:0.02; v/v/v]

MS Grade

Prevent evaporation of prepared solutions using paraffin around cap seals

To make 1000 mL:

- 700 mL water
- 300 mL acetonitrile
- 200 µL acetic acid

Vacuum filter through Whatman #4 filter paper

Solvent B

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

MS Grade

To make 1000 mL:

- 500 mL Acetonitrile
- 500 mL Isopropyl alcohol
- Vacuum filter through Whatman #4 filter paper

Appendix 2.9 Solid phase extraction of oxylipins

Ensure that there is enough of the following:

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

Samples should be kept on ice whenever possible.

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

Sample Preparation for Oxylipin Analysis

1. Remove homogenized kidney and liver samples or serum directly from -80^{o}C freezer and defrost on ice.
2. Cool-down centrifuge to 4°C.
3. Turn on nitrogen evaporator water bath to 37°C.
4. For serum and homogenate, add 1mL of pH3 water to a small test tube (13*100mm or 5mL). One thawed vortex and transfer 1ml of the sample into the test tube.
5. Add 20 µL of internal standard to each glass tube. Vortex.
6. Before putting your internal standard back into the freezer run some nitrogen gas over it before closing the cap.
7. Acidify samples to pH 3 with 1N HCl if necessary (usually 4.5 µL of 1N HCl suffices for rat kidney). ATTENTION: Vortex to mix before reading pH. Use pH-indicator strips to test pH.

8. Centrifuge for 5 min at 3000 rpm at 4°C to remove debris.
9. In the fume hood, set-up and label a Strata-X SPE (Phenomenex) (60 mg/3mL) column for each sample using the wooden rack designed for columns.
10. Place a waste vial under each column.
11. Pre-condition the column with 2ml methanol. Allow the methanol to drip through for 1 minute, then gently apply pressure with a BD 10ml syringe to increase flow. Do not allow column to go dry.
12. Pre-condition with 2ml pH3 water. Push through after 1min in the same way as the previous step.
13. Apply sample to the column, avoiding the pellet at the bottom. The entire sample will be too much volume for the column. Let the sample drip through then add the remainder when there is enough room.
14. Add 1ml of 10% methanol in pH3 water to the sample vial. Vortex, then centrifuge at 4°C, 3000rpm for 5 minutes. Apply this to the column avoiding the pellet.
15. Rinse column with 2 ml pH3 water, and then with 1 ml Hexane. The Hexane will not flow through without pressure applied. Push through until dry.
16. Remove waste vials and place 1.5ml microtubes underneath columns. Elute with 1ml methanol. Push the methanol through and give a few additional pushes to get the last drops.
17. If the samples are not being run that day on the LCMS, displace air with nitrogen gas and store at -80°C until the day they will be run. Then dry down. When you remove the samples from the -80°C, vortex then spin down with minicentrifuge to get all solvent/analyte out of the cap.

18. Drying down in the nitrogen evaporator:

    a. Make sure that the water bath in the nitrogen evaporator is at 37°C.
    b. Open one or two of the needles (so that when you turn on the pressure it has somewhere to go).
    c. Open the valve on top of the nitrogen tank, the pressure on the right gauge should increase.
    d. Open the regulator on the far left (the one that says "Parker"), the pressure on the left gauge should increase.
    e. You can adjust the amount of nitrogen gas coming out of the needles with the knob that is attached to the side of the water bath (LPM AIR).
    f. Open a needle for each tube that you will be drying.
    g. Clean the needle with 100% chloroform, which should be in a 20 mL vial, labeled, next to the evaporator or in Flammables cabinet. Dip the needles into the chloroform. You can watch the bubbles to see how strong your flow of nitrogen gas is.
    h. Put your tubes into the evaporator, and lower the needles so that they are gently blowing nitrogen gas on the surface of the solution in the tube. Do not let the needles touch the solution.
    i. Leave your samples to dry for about an hour. Check on them every 15 minutes to ensure the temperature is kept at 37°C. As the samples evaporate you can lower the needle.

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

a. Once your samples have dried (there should be no methanol left in the tubes, just dried residue from the sample), take them out of the water bath.
b. Take the solvent A out of the fridge (4°C) and add 100 µL to each tube.
c. Vortex each tube so that all the dried sample is mixed in with the solvent A.
d. Centrifuge the samples at 14000g (rcf) for 10 minute at 4°C.
e. Transfer (using 100µL pipette) into labeled GC/LC vials containing a 200µl polypropylene conical insert.
f. Run on LCMS that day.

20. Clean up:

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

Make sure all solvents and tools are put away.

Appendix 2.10 Analysis of HPLC-MS/MS Data using Multi-Quant

General Rules and Tips for Selecting Peaks

1. Click on small icon with the graphs to view the data in graph form. Click on the magnifying glass icon to zoom in.
2. Now the data is set up to go back and forth between the table and the graphs.
3. The Internal standards (IS) are listed first. These usually have the correct peak selected but it is a good idea to go through all of them to make sure.
4. After the IS list all analytes are listed and shows all samples for that one analyte in the graphs.
5. If the specific analyte has a deuterated internal standard select the peak according to the peak in the internal standard.
6. If the analyte does not have a deuterated internal standard look at the printed primary list. If there is a primary for that analyte look at the primary's graph and the peak will tell you the retention time for the samples.
7. If there is no primary for the analyte, the next table to look at is the one that lists all analytes with their expected retention times. These retention times are just approximate to your samples and your samples will generally come slightly after these.
8. The samples are in order that they run in (index number). If not, sort them in order of index number. It is expected that there will be some drift of the retention time as you go through the samples.
9. The peaks should be >5 times the baseline. If not, click "select peak to not found icon" at the top.
10. The peaks should also include an area ≥ 3.000e3. If not, click the "select peak to not found icon."

11. Some peaks will have a “shoulder” or two peaks in one. To view them as 2 separate peaks in the space for “Gaussian Smooth Width” change the 2 to 1 and “Apply.” You should then be able to select the correct peak.
12. Sometimes it is hard to determine the baseline. To zoom in on a graph, click and drag along the axis of the graph to where you want to zoom. If you want to zoom in on all graphs right click on the graphs and select “Options”, go into the tab called “Zooming” and under “zooming access intensity” select 1000cps. This will zoom in all of the samples but make sure to put it back to original setting (100 percent of largest peak) after looking at the samples.
13. Don’t be misled by very small peaks. If the baseline is very low the peak could still be 5x above the baseline. Zoom in for a closer look to determine this.
14. Finally select all analytes and from edit tool copy entire table and export to excel for further calculations.

Appendix 2.11 Fatty acid extraction

Always use volatile solvents in fume hood (most especially chloroform and any of the ethers)! Volatile (flammable) solvents are stored in the yellow flammable cabinets.

Glass Tubes must always be soaked in Contrad overnight and rinsed thoroughly with distilled water, so prepare clean tubes the day before you run samples. To speed up the drying time they may be placed in the oven at 100^0C until completely dry.

Keep samples on ice

1. Take your prepared glass 12ml tube with 250ul of tissue (liver and kidney) homogenate (sample + Tyrode's) or serum out of the -80^0C freezer and put on ice to let thaw.

2. Turn on the Accuspin 3R centrifuge to 4°C. Turn on the Nitrogen bath to 37°C. Make sure the bath is filled with distilled water until the water reaches the bottom of the sample holder.

3. Put the TLC plate in a 110°C oven for 1 hour. Doing so completely dries out the plate. After an hour, place the TLC plate into a dessicator. Turn oven down to 80°C.

4. Prepare a blank tube with 250µL of Tyrodes with every set of samples you do.

5. Add 2.5mL (2500µl) 2:1 chloroform:methanol with 0.01% BHT to your homogenate and blank. To prepare solution: 0.03g butylated hydroxytoluene, 200mL chloroform, 100mL methanol = 0.06g BHT, 400mL chloroform, 200mL methanol. Vortex.

6. Add 10µL of C15:0 standard and 10µL of C17:0 mix using a 100µL pipette.

Standards:

C15:0 Phospholipid (10mg/ml) – add 10µL for Phospholipid (PL) analysis

C17:0 Free Fatty Acid (2mg/ml) and C17:0 Triglyceride (5.5mg/ml)

1:1(mass:mass) mixture– add 10µL for other fatty acids which excludes phospholipid analysis.

For serum only add 10µL for Phospholipid (PL) analysis.

7. Add 2.25mL (2250µl) of 2:1 chloroform:methanol to the homogenate.

8. Cap and vortex for 15 seconds.

9. Add 950µl 0.73% NaCl. To prepare solution: 0.73g NaCl in 100mL de-ionized water = 3.65g NaCl in 500mL de-ionized water.

10. Cap and vortex for 30 seconds, put on ice. (Takes approx. 45min to get to this step.)

11. Centrifuge for 10 minutes at 800g or RCF. It is crucial to place and balance tubes properly or breakage may occur. If you have an odd number of tubes, add an extra tube with similar solvents and water volumes to balance the load.

12. Get 4mL glass vials with Teflon lids and label with sample ID's using tape.

13. Take the tubes out of the centrifuge when completed. You should see 2 layers. Using a glass Pasteur pipette with small yellow bulb, carefully extract the lower phase without taking the upper phase. Slide the tip of the Pasteur pipette down the side while pushing out bubbles, and slowly draw up the lower phase and release it into your newly labeled 4ml vials.
Be careful not to extract the upper phase. If the 2 layers were accidently disturbed and mixed, re-centrifuge the tube (make sure to balance in the centrifuge).

14. Before placing your samples in the nitrogen bath, turn on the nitrogen tank so that a gentle stream comes out the open the needles that you will be using. Wash the needles with chloroform (place the needle in a scintillation vial with 100% chloroform for 5 seconds) and then let any remaining chloroform volatilize.

15. Place your samples (in the 4mL vials) in the nitrogen bath with the lids off. Carefully lower the needles close to the sample and turn the nitrogen gas on until you see a gentle ripple in the sample. Keep in the nitrogen bath until the vial is completely dry.

16. Remove samples from nitrogen one at a time adding 100µL of 2:1 chloroform:methanol . Blow nitrogen over sample, cap and vortex. Samples are vulnerable to oxidation between being removed from nitrogen and adding solvent.

17. If temporarily stopping at this point, store 4mL vials in -20°C freezer to continue later. (Warning: If the liquid extract is not clear or cloudy and takes too long to dry check with Tanja to ensure samples are okay to proceed).

Appendix 2.12 Thin layer chromatography

This step is only for Kidney and liver. For serum we directly go to acid methylation by skipping TLC.

1. Turn on oven to around 88°C (or lower heat if still on from heating TLC plate). The temperature desired for methylation is 80°C, but when the oven is opened, the temperature rapidly drops. 8-10°C above desired temperature gives some leeway.

2. Make up the solvent tank and let it equilibrate for at least half an hour.

- Cut a piece of chromatography paper to fit in tank so that it covers the back and sides. You should only need to fold the paper in half lengthwise and tear.
- To make solvent for tank, get a glass graduated cylinder (100ml or 250ml), in fume hood fill graduated cylinder with 60ml of Heptane, 40ml of Isopropyl ether, and 3ml of acetic acid. You can pour Heptane and isopropyl ether into graduate cylinder so it reaches 100ml then pipette 3ml of acetic acid in. Pour solvent mixture into tank and close, fastening on lid. Do this in the fume hood and leave tank in the fume hood for solvent to volatilize.

3. Using the TLC template with 8 lanes, create equal and parallel vertical lanes on your silica plate (that was previously heated) using the dull side of a razor blade. Be careful not to make any marks on any other part of the plate or to touch the plate (you could accidentally transfer lipids onto the plate from your gloves). Leave at least 1cm on each side of plate. Only use pencil on the plate, no ink.

4. Turn on nitrogen tank with small drying hose attached. Using a 100µl pipette, draw up 50uL of your sample and slowly spot it along the horizontal line drop by drop in the center of the lane. After each drop, dry with nitrogen hose.

5. Place the plate in the tank until the solvent line reaches 1 cm from the top. Keep plate as vertical as possible. This will take approximately 20-30 minutes. Make sure you check the solvent front at 20min. While plate is running, label your 12ml tubes with sample ID and either PL or NL (for kidney and liver samples only) and get your weigh paper ready for scraping plate (fold creases)

6. Let solvent volatilize off TLC plate in the fume hood. You'll know it has when the plate is removed from the fume hood and you don't smell a strong odour of solvent (a slight odour of acetic acid, ie vinegar, is fine). Dry the plate with a gentle stream of nitrogen gas. Samples are again susceptible to oxidation, so don't dry for any longer than 10 minutes. It should only take a minute or two using nitrogen gas to dry.

7. Protect the back of the fume hood and other samples in the fume hood by placing a large piece of cardboard along the back of the fume hood. Stand up TLC plate in the center of the cardboard.

8. Attach hose to the nitrogen gas in fume hood and to bottle with 0.1 % ANS solution in water and spray a fine layer onto the TLC plate. 0.1% ANS can last about a week. Check the color of the solution, if it is a dark yellowish/brown, it is no longer good to use. To prepare 0.1% ANS, weigh out 100 mg ANS (8-Anilino-1-Naphthalene-Sulfonic Acid) and add 100 mL of milli-Q water and stir on stir plate for about 30 minutes. Protect from light using tinfoil.

9. In a dark room wearing UV safety glasses, use the hand held UV light to mark the PL, NL lines using a pencil, indicating where to scrape. R_f (retention factor) for PL = 0, R_f for TG =0.61 (PL is at the origin and TG is about 60% of the way to the solvent front).

10. Since static is a factor, wipe your gloves with a dryer sheet and keep it handy for further use if needed. Be careful not to contaminate your samples with the dryer sheet.

11. Wear a mask when scraping as silica dust is extremely hazardous. Also, work in an area of the lab away from others. Using a razor blade, scrape the indicated portions onto a creased weigh paper and carefully transfer to the corresponding, labeled 12mL screw top test tube.

12. Add 1.2mL (1200µl) of methanolic HCl (or methanolic sulfuric acid) kept in fume hood. A solution of 6% methanol sulfuric acid is made by slowly adding 6ml of sulfuric acid to 94ml of methanol and can only be used fresh (up to 1 week). Use Teflon tape, cap tightly and vortex. Ensure that caps are in good condition and that the top of the test tube has no cracks so that it can be sealed completely. If stopping here, store overnight in -20°C. Note methanolic sulphuric acid is only good for about 1 week. Then dispose of it in appropriate waste bottle.

Appendix 2.13 Fatty acid methylation

Turn on oven (about 80°C) and allow it to reach temperature, if it is not already on

1 With a marker, mark the liquid level of each tube. Place tubes in a metal rack in a preheated 80^0C oven for 1.5 hours. After 30 minutes if the volume decreases, remove the tubes from the oven, let cool, add more acid to achieve the original volume and place back in oven

2. After 1.5 hours**,** remove rack from oven and cool for 10-15 minutes.

3. In the fume hood, add 1.5mL (1500µl) toluene to the tubes, cap tightly (very important!) and vortex for 30 seconds.

4. Add 1mL of ultrapure water to the tubes, cap and vortex for 15 seconds. Centrifuge for 5 minutes at 800g or RCF.

5. Using glass Pasteur pipette tip with yellow rubber bulb, transfer the top layer to a clean 2mL GC vial (glass), being VERY careful not to transfer the bottom layer and cap. Place in the -20°C freezer.

Later you can thaw the sample, dry under nitrogen using a dry bath (you can use the water bath, just be careful). It is very important not to get any water in your sample at this point.

6. Once the solvent has evaporated, immediately add 100µL of hexane for kidney and liver samples and 50 µL of hexane for serum to the tubes (adjust volume according to concentration on GC after samples have been run) and cap. Work with one sample at a time. Again, samples are susceptible to oxidation between removal from nitrogen gas and addition of solvent. Store the samples in the -20^0C freezer in a labeled box.

When they are ready to be run on the GC, label GC vials with sample number, date and your initials and transfer 100uL for kidney and liver samples and 50 µL for serum into a glass insert

with spring. Cap and store in -20^{0}C freezer (top shelf, right in front) in a sample box clearing stating that they are ready to be run on GC and marked to place an analytical service request. (Warning: Your samples should be clear, not supposed to have any color on it. If you see anything suspicious do not place an analytical request or at least warn them.

During transferring the top layer, pay close attention. Sometimes it is hard to see whether you are transferring also part of bottom layer. You may transfer little less amount to be safe)

Appendix 2.14 Kidney histology protocol

Use of microscope

1. To begin, you must take photos of your slides using the AMG-EVOS-XL microscope (Life Technologies).
2. Turn on microscope (small black switch on right back side of microscope)
3. Place a slide under the microscope lens using the 20x objective lens for analyzing mean glomerular volume (MGV).
4. Make sure you have the same light settings every time you take photographs
5. 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.
6. Note: if the color of your photos appear different (ex: purple when they should be red) re-calculate the white balance values and follow directions accordingly.
7. 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.
8. If taking photographs for MGV take enough photos to capture 30 glomeruli.
9. Once focused, click the bottom button of the microscope to capture the image.
10. Then move your slide to take a new photo, focus if you need to, then click continually take photos.

Mean glomerular volume (MGV)

Using the same glomeruli that you analyzed for glomerulosclerosis, now analyze the MGV using Image-J Software (NIH).

1. Open the Image J program.
2. Choose: File→Open to open your images, including the micrometer slide image.
3. Working first with the micrometer slide image, choose the Straight Line tool in Image J and trace a known distance. Note: The total distance of the micrometer is 1 mm. The large divisions are 0.1 mm, and the small divisions are 0.01 mm.
4. Choose: Analyze→Set Scale. Enter the known distance you have traced and the units. Do not change the "Distance in pixels." Select: Global, which will apply the scale to all images you have opened. Select: OK. Note: You must ensure you have set the scale for every image you want to measure. If you open a new image after setting the scale, you will need to repeat this step.
5. Choose: Analyze→Set Measurements. Select the outcomes (area in mm unit)
6. Using the Free-Hand tool in Image J, very carefully and precisely, trace the area you would like to measure.
7. Choose: Analyze→Measure. The results tab will appear automatically.

Copy the results into an Excel file and save. Then calculate the volume by using the following formula.

Mean glomerular volume (MGV) was calculated as:

$MGV = 1.25 (MGA)^{3/2}$

where, 1.25 is derived from β/K, where β equals 1.38 (pertains to spheres) and K equals 1.10 (a distribution coefficient).

Appendix 2.15 Research ethics and compliance

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

Animal Care & Veterinary Services
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veterinaryservices@umanitoba.ca

15 April 2016

TO: Dr. H. Aukema
HNS/FAFS

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

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

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

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

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

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

Category of Invasiveness: **D**

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

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

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

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

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

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

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

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

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

MF/tvo

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