Effects of sub-chronic dietary 2-monochloro-1,3-propanediol on oxidized lipids in rat heart, kidney, serum, and skeletal muscle

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I. Abstract

Chlorinated propanols are processing induced food contaminants formed in the manufacturing of vegetable oils and hydrolyzed vegetable protein. The 2-monochloro-propanediol (MCPD) isomer has been insufficiently studied for hazard assessment, while the 3-MCPD isomer has received more attention and has been classified as a possible human carcinogen. Xenobiotics and polyunsaturated fatty acids (PUFA) are known to be metabolized by some of the same classes of enzymes, namely cytochrome P450 (CYP), cyclooxygenase (COX), and lipoxygenase (LOX); and previous studies have shown PUFA metabolites to be altered by exposure to toxins. This study investigated the effects of dietary 2-MCPD by profiling PUFA metabolites called oxylipins, which have diverse homeostatic functions, and non-enzymatically produced oxidized phosphatidylcholines (OxPC) and isoprostanes (isoP) as markers of oxidative stress, in the heart, kidney, serum, and skeletal muscle of rats treated with control or 40 mg/kg body weight 2-MCPD for 90-days. It was observed that 2-MCPD treatment decreased n-3 PUFA derived oxylipins in the heart and decreased LOX produced oxylipins in the serum. In the kidney, 2-MCPD treatment altered few oxylipins with no clear grouping by enzyme pathway or PUFA precursor. In the skeletal muscle, there was an absence of oxylipin alterations with 2-MCPD treatment. 2-MCPD treatment did not alter the OxPC or isoP in any of the tissues quantified, indicating oxidative stress did not play a role in the mechanism of action of 2-MCPD. These findings of altered oxylipin profiles add to the weight of the evidence of 2-MCPD toxicity, as well as support the potential use of serum oxylipins as biomarkers of 2-MCPD exposure.

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VI. Abbreviations

1,3-DCP, (1,3-dichloro-2-propanol); 2-MCPD, (2-monochloro-1,3-propanediol); 2,3-DCP, (2,3-dichloro-1-propanol); 3-MCPD, (3-monochloro-1,2-propanediol); 4-HNE, (4-hydroxy-2-nonenal); 8-OHDG, (8-Oxo-2'-deoxyguanosine); AA, (arachidonic acid); ALA, (α-linolenic acid); BW, (body weight); COX, (cyclooxygenase); CP, (chloropropanols); CYP, (cytochrome P450); CYPe, (cytochrome P450 epoxygenase); CYPh, (cytochrome P450 hydroxylase); DGLA, (dihomo-γ-linolenic acid); DHA, (docosahexaenoic acid); DiHETrE, (dihydroxy-eicosatrienoic acid); DiHOME, (dihydroxy-octadecaenoic acid); EPA, (eicosapentaenoic acid); EpDPE, (epoxy-docosapentaenoic acid); EpETrE, (epoxy-eicosatrienoic acid); GI, (gastrointestinal); HDoHE, (hydroxydocosahexaenoic acid); HEPE, (hydroxy-eicoapentaenoic acids); HETE, (hydroxy-eicosatetraenoic acids); HHTrE, (hydroxyheptadecatrienoic acid); HMOX1, (heme oxygenase-1); HODE, (hydroxyoctadecaenoic acid); HpETE, (hydroperoxy-eicosatrienoic acid); HVP, (hydrolyzed vegetable protein); IARC, (International Agency for Research on Cancer); IS, (internal standard mixture); isoP, (isoprostanes); JECFA, (Joint FAO/WHO Expert Committee on Food Additives); LA, (linoleic acid); LC-MS/MS, (high performance liquid chromatography tandem mass spectrometry); LD_{50}, (median lethal dose); LOAEL, (lowest observed adverse effect level); LOD, (limit of detection); LOQ, (limit of quantitation); LOX, (lipoxygenase); MoA, (mechanism of action); NICI, (necrosis with inflammatory cell infiltrate); NOAEL, (no observed adverse effect level); oxoETE, (oxo-eicosatrienoic acid); OxPC, (oxidized phosphotidylcholine); PCB, (3,3',4,4',5-pentachlorobiphenyl); PG, (prostaglandin); PLA_2, (phospholipase A_2); PUFA, (polyunsaturated fatty acids); OxPC, (oxidized phosphatidylcholines); ROS, (reactive oxygen species); sEH, (soluble epoxide hydrolase); SPE, (solid phase extraction); SPM, (specialized pro-resolving mediators); TA, (tibialis anterior); TDI, (tolerable daily intake); TG, (test guideline); TX, (thromboxane).

1. Introduction

Chloropropanols (CP) have been identified as chemical process-induced food contaminants. CP occur as by-products of the refining of vegetable oils [1], the production of hydrolyzed vegetable protein (HVP) [2], and in water-resistant paper products [3]. 3-monochloro-1,2-propanediol (3-MCPD) is the most well studied CP isomer. It has been identified as a possible human carcinogen, with the kidneys and male reproductive organs as primary targets [4]. The CP isomer 2-monochloro-1,3-propanediol (2-MCPD), however, has been less studied, and Health Canada identified there to be insufficient data for regulatory assessment. Subsequently, Health Canada conducted repeated dose dietary exposure studies in rodents to investigate the hazard of 2-MCPD. A 28-day toxicity study in male and female F344 rats by [5] identified the heart and kidney as primary target organs, with elevated organ weights, non-cancerous lesions, and/or toxicogenomic changes involved in oxidative stress in these target organs [5].

Profiling lipid metabolites in toxicological studies is a novel approach. Oxylipins are the oxygenated metabolites of polyunsaturated fatty acids (PUFA) that function as lipid mediators of cellular processes, such as apoptosis, inflammation, and cell proliferation [6]. These molecules are produced enzymatically by three classes of enzymes, namely cytochrome P450 (CYP), cyclooxygenase (COX), and lipoxygenase (LOX) [6], which can also function in xenobiotic metabolism [7], [8]. Studies have shown oxylipins in various tissues to be altered by disease and diet differentially by sex [6], [9]–[14]. More recently, a few studies have focussed on the modulation of oxylipins as results of exposure to toxins [15]–[18], emphasizing their role in toxicology. Oxylipins as prospective biomarkers of CP exposure is an emerging field. A class of oxylipins, the isoprostanes (isoP), can be formed non-enzymatically by lipid peroxidation. Thus, the isoP can be utilized as markers of oxidative stress [19]–[22]. Another class of lipid produced

by lipid peroxidation are the oxidized phosphatidylcholines (OxPC), which can similarly be used as markers of oxidative stress [23]–[25]. By profiling these oxidized lipids in the heart, kidney, serum, and skeletal muscle of rats fed control or 2-MCPD, tissue-specific and exposure-related mode of action can be established. Also, the role of oxylipins as potential biomarkers of chloropropanols exposure may be derived.

2. Literature Review

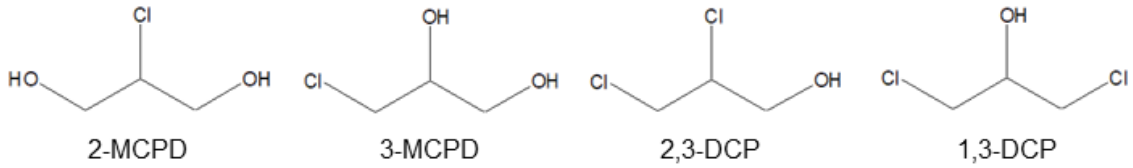
2.1. Chloropropanol Formation

CP are processing induced foodborne contaminants which include 2-MCPD, 3-MCPD, 2,3-dichloro-1-propanol (2,3-DCP), and 1,3-dichloro-2-propanol (1,3-DCP) (Figure 2.1.1). Several mechanisms of CP formation exist; CP were first identified as by-products of heat induced acid hydrolysis, a method used to produce the flavour enhancer HVP. In this method, hydrochloric acid is used to hydrolyze the protein containing by-products of vegetable oil or starch production [2]. One mechanism of CP formation is through the processing at a high temperature of glycerol under acidic conditions [26], resulting in a susceptibility to a nucleophilic substitution (S_N2) by chloride ions [27].

Another mode of CP formation is through the deodorization step in vegetable oil. The conditions of high temperature and pressure with the presence of chloride ions (from naturally occurring organic chloride compounds) allow for the chlorination of glycerol and acylglycerols. As a result, free and esterified (or bound) CP are formed [1]. Similarly, CP can be formed from cooking (e.g. grilling or frying) with the conditions of high temperature and presence of lipid and sodium chloride (naturally occurring or added) [27], [28].

Another source of CP is from water-resistant paper products (i.e. tea bags, coffee filters, paper cups, Styrofoam trays etc.). Synthetic polymeric resins are used in these products to improve their structural integrity when exposed to liquids. The resin polyamidoamine-epichlorohydrin, which can be used for this purpose, has been shown to undergo side reactions during processing that result in CP formation [3].

Figure 2.1.1 Molecular structures of chloropropanols.



2-MCPD, 2-monochloro-1,3-propanediol; 3-MCPD, 3-monochloro-1,2-propanediol; 2,3-DCP, 2,3-dichloro-1-propanol; 1,3-DCP, 1,3-dichloro-2-propanol.

2.2. 3-MCPD Toxicity

3-MCPD is the most well studied CP isomer, and its hazard is well characterized. For example, its median lethal dose (LD_{50}) was established in the 1970's as 152 mg/kg body weight (BW) [29]. Due to the regio-isomeric differences in 2-MCPD and 3-MCPD, the two isomers likely have differences in their mechanism of action (MoA) and detoxification. However, there is a paucity of data on 2-MCPD toxicity. Therefore, caution should be applied in extrapolating the 3-MCPD toxicity data to understand and inform the hazard of 2-MCPD.

Carcinogenicity studies in rat models have identified the kidney and testes as target organs of 3-MCPD toxicity. In a two-year study by [30], rats were given drinking water with 0, 25, 100 or 400 mg/kg BW 3-MCPD. At the highest doses, renal tubule carcinoma or adenomas were increased in males and females, as well as Leydig-cell tumors (in males). Renal tubule hyperplasia was significantly increased in all groups in a dose-dependent manner in males, while in females only the highest dose displayed increased hyperplasia. Similar results of nephrotoxicity and testicular toxicity were found in another two-year rat carcinogenicity study by Sunahara et al. (1993) (unpublished, reviewed in [4]). They reported a lowest observed effect level of 1.1 mg/kg BW, with renal tubule hyperplasia as the most sensitive end-point. They also reported increased Leydig-cell tumors which identify the testes as a target organ. These studies together with other studies of shorter exposure duration, have informed the International Agency for Research on Cancer (IARC) to deem 3-MCPD as a possible human carcinogen, Group 2B [31]. Based on the hazard information, the most recent evaluation by Joint FAO/WHO Expert Committee on Food Additives (JECFA) set the tolerable daily intake (TDI) for 3-MCPD at 4 μg/kg BW [4]. Due to the toxicity of 3-MCPD, Health Canada placed regulations on the 3-MCPD content of Asian-style sauces such as soy, oyster, mushroom sauces, etc. to 1 mg/kg [32].

2.2.1. **3-MCPD Detoxification**

There are two accepted mechanisms for 3-MCPD detoxification. The first is by oxidative metabolism of 3-MCPD by alcohol dehydrgoenase to β-chlorolactic acid, which is then converted to oxalic acid [33], [34]. This mechanism could contribute to renal toxicity because large amounts of oxalate can cause calcium oxalate deposition in renal tubules leading to acute oxalate nephropathy [35]. Oxalate and calcium oxalate have been shown *in vitro* to damage renal epithelial cells, particularly of the proximal convoluted tubule, through the release of free radicals [36].

The other mechanism of 3-MCPD detoxification is by a dehalogenation conversion to glycidol, followed by glutathione conjugation, to form N-acetyl-S-(2,3-dihydroxypropyl)-cysteine; a mercapturic acid [33], [34]. This mechanism has been shown to be dominant in rats, as shown in a study by [34], which measured β-chlorolactic acid and 3-MCPD mercapturates in the urine of rats fed 3-MCPD. They found a positive dose-dependent trend in urinary 3-MCPD mercapturates with each dose (the lowest being 1.84 mg/kg), that accounted for as much as 27.5% of the 3-MCPD intake; while urinary β-chlorolactic was below the limit of detection (LOD) at all doses [34]. The production of glycidol may impart toxic effects as glycidol is a known genotoxic carcinogen [37]. 3-MCPD; however, it has only been shown to have genotoxic effects *in vitro*; the effects *in vivo* have been classified as non-genotoxic [38], [39].

2.2.2. **Oxidative Stress**

Oxidative stress may play a role in the MoA of 3-MCPD. DJ-1 is an endogenous antioxidant protein, its normal function involves the reversible oxidation of the thiol group of Cys106. However, excessive oxidative stress can cause irreversible oxidation to sulfonic acid (SO_3H), which results in the loss of DJ-1 function [40]. DJ-1 is known to function in multiple redox

regulating mechanisms, and its irreversible oxidation is associated with disease states. In neuronal cells, the appearance of SO_3H on Cys106 has been found in Parkinson's, Huntington's, and Alzheimer's disease. Irreversible DJ-1 oxidation has also been associated with cancer, type II diabetes, male infertility, and stroke. Irreversible DJ-1 oxidation has also been associated with cancer, type II diabetes, and stroke [40]. 3-MCPD has been found to increase DJ-1 oxidation in rat kidney, testes, and liver [40]–[44]. 3-MCPD may not directly oxidize DJ-1; instead, the purported mechanism suggested is via the production of ROS. However, a previous 90-day 3-MCPD toxicity study in rats found testicular damage occurred despite a lack of increase to markers of lipid peroxidation [34]. Thus, the role of oxidative stress in the MoA of 3-MCPD remains to be elucidated.

2.3. 2-MCPD Toxicity

The toxicity of 2-MCPD has received less attention than 3-MCPD. A recent evaluation of CP by Health Canada identified a data gap for the regulatory risk assessment of 2-MCPD, because of insufficient toxicological information.

2.3.1. Health Canada Study (Raju et al., 2016): 28-day 2-MCPD Toxicity Study

To address the lack of data on 2-MCPD toxicity, a regulatory-study according to OECD TG-407 was ethically conducted (approved by Health Canada Ottawa Animal Care Committee and assigned as 2013-013) [5]. Briefly, male and female F344 rats were fed semi-synthetic purified diets (AIN-93G formulations) containing 0 (control), 25, 50, 100 or 200 mg/kg BW/day 2-MCPD, for 28 days. Male and female rats were staggered as different cohorts in a manner that each cohort consisted of all groups (including control) and were started a week apart. One week into the study, female rats receiving the two highest doses (100 or 200 mg/kg BW/day 2-MCPD) became moribund (weight loss and minimal food consumption or complete cessation of feeding)

and were euthanized. Given the moribund status of the high dose groups in female rats, the males at the same dose were spared from exposure to these high doses of 2-MCPD in the rest of the study. Male and female rats belonging to all other dose groups were included in the assessment of biochemical, hematological, immunophenotypical, and histological effects in the urine, blood, liver, heart, lungs, spleen, pancreas, kidney, adrenal glands, thymus, thyroids, tibialis anterior (TA) and sex organs. Gene expression profiling was also conducted in the heart, kidney, and liver [5].

No significant differences in body weight between groups occurred but multiple tissues weights were altered. Both the female and male 50 mg/kg groups had elevated tissue weights of the heart, kidney, spleen, and ovaries (female), while in the 25 mg/kg groups the kidneys and spleen (female only) weights were elevated. In the heart, compared to controls, non-neoplastic lesions were observed in the 50 mg/kg BW 2-MCPD exclusively in female rats. These lesions were characterized as multifocal acute myocardial necrosis and vacuolation accompanied by infiltrating mononuclear cells, a combination of lymphocytes (T cells, B cells, NK cells) and monocytes). In the kidney, the male 50 mg/kg group had increased mitosis of the proximal convoluted tubule. In the thyroid, increased follicular epithelial hypertrophy was found in the 25 and 50 mg/kg group for males and the 50 mg/kg group only for females [5].

There were no significant changes in leukocytes for males. In females, B cells (CD45RA+/CD3-) were elevated, and helper T cells and NK cells (CD161a+ (high)/CD3-) were decreased in the 25 mg/kg and 50 mg/kg groups compared to control [5].

Gene expression panels for cardiotoxicity, nephrotoxicity, hepatotoxicity, and oxidative stress showed sex and tissue specific alterations. In the liver, 4 genes were altered in females and none in males receiving 25 mg/kg, and 12 genes were altered in females and 2 in males receiving 50

mg/kg groups. The changes in gene expression that occurred were indicative of a response to oxidative stress [5].

In the heart, 4 genes were altered in females and 3 in males receiving 25 mg/kg BW, and 22 genes were altered in females and 6 in males receiving 50 mg/kg BW [5]. The genes *IL-6, Timp-1,* and *Serpine1,* were upregulated [5], and are related to wound healing as components of the hypoxia inducible factor 1 pathway which mediates the effects of ischemic hypoxia and oxidative stress [45]–[50]. Another gene, *Postn,* was the most altered by 2-MCPD exposure with a 15.49 and 3.64-fold increase in the 50 mg/kg females and males, respectively. The *Postn* gene codes for periostin; a matricellular protein involved in both homeostatic and pathological tissue remodelling of the heart [51]–[53].

In the kidney, 9 genes (*Abcc2*, *Bhmt*, *Calb1*, *Hmox1*, *Idh1*, *Nox4*, *Slc22a1*, *Ugt1a1*, and *Mpo*) were altered in females and 5 genes (*Abcb1b*, *Cdkn1a*, *Hmox1*, *Ptgs2*, *Sed1*) in males receiving 25 mg/kg BW, and 12 genes (*Abcc2*, *Atf3*, *Bhmt*, *Calb1*, *Havcr1*, *Hmox1*, *Idh1*, *Igfbp1*, *Slc22a1*, *Socs3*, *Ugt1a1*, *Mpo*, and *Ptgs2*) were altered in females and 6 genes (*Abcb1b*, *Atf3*, *Cdkn1a*, *Hmox1*, *Noxa1a*, and *Ptgs2*) in males receiving 50 mg/kg BW [5]. These kidney gene expression alterations were associated with functions, such as apoptosis, cell proliferation, and oxidative stress. Expression of the *Hmox1* gene was elevated in the kidneys of all groups and had the highest fold increase. *Hmox1* codes for heme oxygenase-1 (HMOX1), which functions to maintain redox balance and has been shown to diminish oxidative injury, be antiapoptotic, and antiproliferative [54]. HMOX1 has been shown to be upregulated in response to injury due to stressors, such as oxidative stress, pro-inflammatory cytokines, heavy metals, UV irradiation, and thiol-reactive substances [55].

There were specific pathological lesions in the skeletal muscle of the 2-MCPD exposure groups [5].

From these data the heart, kidney, skeletal muscle, spleen, and thyroid were targets of 2-MCPD exposure due to pathological changes. The results are being evaluated to derive a benchmark dose levels (BMDL) required for regulatory risk assessments. Primary evaluations of the pathology data suggest a No Observed Effect Level (NOEL) of 25 mg/kg BW 2-MCPD. The toxicogenomics effects of 2-MCPD observed in this sub-acute 28-days repeated dietary dose study were characteristic of increased wound healing, inflammation, and oxidative stress.

2.3.2. **Other 2-MCPD Toxicity Studies**

In another 28-day 2-MCPD toxicity study by Marchesini et al. (unpublished, reviewed in [56]), similar results were reported. This study included lower doses, feeding rats 0, 2, 16, and 30 mg/kg BW/day. They found severely toxic effects primarily in the heart and striated muscle, with lesser effects to the kidney and liver. The study had a NOAEL at the lowest dose of 2 mg/kg BW.

Unlike 3-MCPD toxicity, the heart and skeletal muscle were identified as targets of 2-MCPD toxicity, indicating a different toxicological mode of action. This is supported by two other studies which found little similarity in the transcriptomic and proteomic changes seen in the heart, kidney, liver, and testes of rats given non-toxic doses (10 mg/kg BW) of 2- or 3-MCPD [41], [57]. The differences in the organ-specific toxicity provides the impetus to assess the hazard of 2-MCPD toxicity.

2.3.3. **Potential 2-MCPD MoA**

2-MCPD was found to be non-genotoxic both *in vitro* and *in vivo* [38], [56]. Thus, 2-MCPD toxicity does not occur through direct alteration of DNA. The 2-MCPD MoA may differ from 3-

MCPD. One of the metabolites of 3-MCPD oxidation, β-chlorolactic acid (see Section 2.2.1.), cannot be produced from 2-MCPD due to their structural difference [41]. As a result, decreased nephrotoxicity may be expected because 2-MCPD metabolism will not lead to excess oxalate production. The metabolism of 2-MCPD by the other detoxification pathway that 3-MCPD can follow is, however, theoretically possible; thus, 2-MCPD can be dehalogenated to form glycidol and undergo subsequent glutathione conjugation [41]. It has been shown in rats that 50 mg/kg BW 2-MCPD results in increased urinary N-acetyl-S-(2,3-dihydroxypropyl)-cysteine [58]. This is the same metabolite found in the urine of 3-MCPD treated rats (see Section 2.2.1.), confirming 2-MCPD detoxification can occur through the glutathione conjugation pathway. Thus, the production of glycidol, as an intermediate in the pathway, is a potential source of toxicity because of its carcinogenicity [4].

2.3.4. Oxidative Stress

Similar to 3-MCPD, 2-MCPD exposure is thought to increase oxidative stress, which may contribute to its toxicity. For example, increased oxidative stress leads to increased lipid peroxidation, which, in excess, can cause cellular necrosis via the breakdown of the cellular membrane [7]. Proteomic analyses have shown increased DJ-1 oxidation with sub-toxic (10 mg/kg BW) 2-MCPD treatment in rat heart, kidney, liver, and testes [40], [41]. However, the oxidation occurred to a lesser extent than with 3-MCPD treatment at an equal dose, thus suggesting lesser ROS production by 2-MCPD. Both isomers caused irreversible DJ-1 oxidation, and therefore decreased DJ-1 antioxidant capacity, thus, impairing cellular ability to regulate redox balance [40].

2.4. Dichloropropanols

The other CP isomers, 1,3-DCP and 2,3-DCP, have also received little attention. From the few studies on 1,3-DCP that have been conducted, it has been determined to be hepatotoxic and nephrotoxic, and produced tumours in multiple organs in rats. It was shown to be genotoxic *in vitro*; however, two *in vivo* studies found it non-genotoxic. A TDI has not be established because the dose that caused tumours was 20,000 times that of the estimated high intake for humans [59]. Less is known of 2,3-DCP toxicity, as no oral toxicity data is available. However, 1,3-DCP and 2,3 DCP toxicity is likely different, as acute subcutaneous injection in rats showed more potent hepatotoxicity with 1,3-DCP [60].

2.5. CP Occurrence in Foods

Given the evidence of 2-MCPD toxicity in rodent models, it is justified to be concerned about human dietary 2-MCPD exposure, thus it is worthwhile to have an understanding of the extent to which 2-MCPD is occurring in foods. HVP is used as a flavour enhancer in Asian sauces. In a survey of soy sauces from Chinese markets, 2-MCPD content ranged from below the limit of quantitation (LOQ) of 5 μg/kg to 20,300 μg/kg, with a mean of 10 μg/kg. For 3-MCPD, the content ranged from <LOQ to 189,000 μg/kg, with values generally 10-fold that of 2-MCPD. The traditionally brewed soy sauces (no HVP) ranged from 4 to 17 μg/kg 2-MCPD [61]. Another study analyzed Asian sauces from UK markets in 2000 and 2002, as the European Commission enacted a regulatory limit of 20 μg/kg for 3-MCPD in 2002. In the 2000 survey, 2-MCPD content ranged from below the limit of detection (<10 μg/kg) to 19,800 μg/kg, and 3-MCPD ranged from 12 to 93,100 μg/kg. In 2002, 2-MCPD content ranged from <LOD-3330 μg/kg and 3-MCPD ranged from 11-21,200 μg/kg [62]. The study showed that industry was reducing the amount of CP; however, many products were still in excess of the 20 μg/kg 3-MCPD limit.

The identification of CP in refined vegetable oils is particularly concerning, because of their near ubiquity in most processed foods. This has spurred studies to gain a better knowledge of CP; however, the majority of studies, have only determined 3-MCPD content. Where 2-MCPD content determinations are unavailable, the 3-MCPD content in refined vegetable oils can still be used to estimate 2-MCPD content based on studies which have shown 2-MCPD has been found to occur at approximately half that of 3-MCPD [63], [64]. A study by [65] analyzed 10 refined and 15 virgin edible oils for free and bound 3-MCPD. The oils were sourced from various European countries and purchased in a Czech market. They found refined oils from soybean, rapeseed, sunflower, corn, olive, and olive pomace to contain bound 3-MCPD that ranged from <LOQ (<300 μg/kg) to 2462 μg/kg, while free 3-MCPD was below the LOQ (<9 μg/kg) in all refined oil samples. The virgin oils were all below the LOQ and 11 were below the LOD (<100 μg/kg) for bound 3-MCPD (except virgin roasted sesame seed oil, 337 μg/kg), and for free 3-MCPD all samples were below the LOQ (except virgin hazelnut oil 10 μg/kg, virgin soybean oil 12 μg/kg, and virgin wheat germ oil 24 μg/kg). The average ratio of bound:free 3-MCPD was 19 for virgin oils, while for refined oils it was 237. In the olive oil samples, there was an approximately two orders of magnitude increase from virgin to refined samples in 3-MCPD content [65]. The bound CP have been found to occur at much higher levels than the free forms, and, as expected, at much higher amounts in refined oils compared to virgin oils. It also been reported that 3-MCPD concentration varies by oil source, in the increasing order of: rapeseed oil < soya bean oil < sunflower oil < safflower oil < walnut oil < palm oil [4].

Another study examined 290 ready-to-eat food samples from markets in Hong Kong. They found bound 3-MCPD to occur in some or all products in the categories: breakfast cereals, noodles, biscuits, meat and its products, poultry and its products, fish and its products, fats and oils,

condiments and sauces, snacks, bakery, Chinese pastry, dairy products, and soup and non-alcoholic beverages. The highest mean content was 440 μg/kg in biscuits (range: 50-860 μg/kg), followed by 390 in fats and oils (range: <10-2500 μg/kg). None of the 15 products in the nuts and seeds category contained 3-MCPD [66]. This study showed that as expected there are a vast number of processed foods that contain 3-MCPD.

3-MCPD has been quantified in a variety of foods prepared with refined oils. A study by [67], determined the 3-MCPD content of Czech fried potato products. The French fries were prepared in blended frying oils which consisted primarily of palm oil. The 32 French fry samples had bound 3-MCPD content that ranged from 27 to 64 μg/kg and free 3-MCPD below the LOD; the 61 potato chip samples had bound 3-MCPD content that ranged from 98 to 2201 μg/kg and free 3-MCPD below the LOD to 46 μg/kg; while neither free nor bound 3-MCPD were found to occur in raw potatoes (below LOD <3 μg/kg) [67]. This study showed that when oil is used to fry foods, 3-MCPD can be imparted into that food.

Of the foodstuffs that contain refined oils, infant formula is of particular concern because it can be an infant's exclusive source of nutrition if not breastfed. In a study of 70 German retail infant formula samples, [64] found bound CP present in all samples. The concentration of 3-MCPD in formula was found to range from 65 to 148 μg/kg (free 3-MCPD equivalent) and 2-MCPD ranged from 23 to 71 μg/kg (free 2-MCPD equivalent). They also found a correlation of r^2=.81 for 3- & 2-MCPD, with a slope of 0.44. A U.S. sample of 55 infant formulas found 3-MCPD concentrations that ranged from 3 to 119 μg/kg [68].

There is a paucity of data on the occurrence of the DCP isomers in foods. However, a recent study by [63] described a novel method for the analysis of the four CP isomers. In this study 35 refined palm, rapeseed, and sunflower oil samples from were analyzed. The median values of

bound 3-MCPD, grouped by plant source, ranged from 230-1330 μg/kg, with palm oil being the highest, and the median values for bound 2-MCPD content ranged from 110-650 μg/kg. Despite the substantial 2- and 3-MCPD content, neither of the two DCP isomers were detected in any of the 35 refined oil samples analyzed; indicating DCP contamination of vegetable oils is not of concern.

The extent to which water-resistant paper-based food containers contribute to CP exposure is not clear. [3] tested the 2- and 3-MCPD leaching of some paper products. They found paper cups containing 200 ml hot water for 30 min leached <LOD (2 ng) to 16 ng of 3-MCPD and no 2-MCPD. Coffee filters imparted <LOD (10 ng) to 33.6 ng of 2-MCPD, and 18.2-7250 ng of 3-MCPD. Tea bags imparted 2.3-24.4 ng 3-MCPD and no 2-MCPD. These findings indicate that the CP in water-resistant paper products can be imparted into food and contribute to the dietary intake. Also, the relative amount of 2-MCPD was much less in these products, ranging from 0.5 to 10% of 3-MCPD.

2.5.1. **Dietary Intake**

Estimates of total 3-MCPD (free and bound) exposure using dietary intake data from 2012-2016 were calculated by [4], with values expressed as free 3-MCPD equivalents. For adults, the estimated mean 3-MCPD intake was 0.2 to 0.7 μg/kg BW and the 95th percentile intake was 0.5 to 2.6 μg/kg BW. For children aged 1 to 18 years, the estimated mean 3-MCPD intake was 0.4 to 1.3 μg/kg BW and the 95th percentile intake was 0.8 to 3.8 μg/kg BW. For infants aged less than 12 months, the estimated mean 3-MCPD intake was <1 to 10 μg/kg BW and the 95th percentile intake was 25 μg/kg BW [4]. Therefore, children are estimated to consume greater concentrations of CP than adults, with the high consumers from this population consuming just below the 3-MCPD TDI of 4 μg/kg BW. The 3-MCPD intake estimates for infants indicate

potentially hazardous 3-MCPD exposure, which are above the current adult TDI of 4 μg/kg BW and may warrant regulatory action.

While no dietary intake estimates for 2-MCPD have been conducted, it is possible to estimate the intakes based on 3-MCPD data. If we assume that the major dietary source of 2-MCPD is refined vegetable oil, the relative proportion of 2-MCPD to 3-MCPD of 0.44 can be used to convert the 3-MCPD intake values. The ratio of 0.44 was determined by [64] and is supported by the [63] data that showed 2-MCPD occurred approximately half that of 3-MCPD in refined edible oils from. Using 44% of the [4] 3-MCPD intake values, the estimated mean intake of 2-MCPD for adults would be 0.1 to 0.3 μg/kg BW and 0.2 to 1.1 μg/kg BW for the 95^{th} percentile intake. For children aged 1 to 18 years, the estimated mean 2-MCPD intake would be 0.2 to 0.6 μg/kg BW and the 95^{th} percentile intake would be 0.4 to 1.7 μg/kg BW, while for infants aged less than 12 months, the estimated mean 2-MCPD intake would be <1 to 4.4 μg/kg BW and the 95^{th} percentile intake would be 11 μg/kg BW. The infant estimates are likely the most accurate as the assumption made was that the only dietary 2-MCPD was from refined vegetable oils and that would be the case for infants which are exclusively formula fed. From these studies it is apparent that 2- and 3-MCPD are abundant in the food system. Soy sauces with added HVP can potentially contain very high free CP content. While lower CP content occurs in a variety of refined vegetable oil products, primarily in the bound form.

2.6. Chloropropanol Esters

Much of the CP in the food system exists esterified to fatty acids, referred to as bound CP. It has been shown that these esters are readily cleaved by lipases of the gastrointestinal (GI) tract. A study by [69] used a human intestinal model and found 3-MCPD sn-1 monoesters to be 95% hydrolyzed in 1 min, while diesters were 95% hydrolyzed at 90 minutes. The increased

hydrolyzation time was thought to be due to the lower affinity of GI lipases for sn-2 esters. The oral bioavailability of free 3-MCPD compared to its diester have been compared in rats. It was determined that the bioavailability of 3-MCPD dipalmitate was 86% that of the free form, and the maximum blood concentration of 3-MCPD was 4-fold higher in the free form. Also, 3-MCPD esters were not detected in the blood, with a LOD of 0.25 μg/g. This indicates that toxic effects may only occur through the free form if the bound form in not absorbed [70]. The determinations of the TDI for 3-MCPD assume complete hydrolysis of fatty acid ester by GI lipases due to the similarity to triacylglycerides [4].

Bound 2-MCPD has received less attention and its digestion and absorption have not yet been measured *in vivo*, but similar results to 3-MCPD have been shown *in vitro.* One study found free 2-MCPD, but not mono- and di-oleate and palmitate 2-MCPD esters, could cross a monolayer of Caco-2 cells (human model of colorectal epithelium). The bound 2-MCPD was instead hydrolyzed by the Caco-2 cells and then able to cross [71]. Another study compared the digestion of 2- and 3-MCPD dioleate esters by pancreatic lipase *in vitro*. It was found that after three hours free 2-MCPD appeared at approximately a 3-fold higher amount than the free 3-MCPD. This is due to the regio-isomeric differences in the two molecules. The sn-1 and sn-3 positions of the fatty acid esters on bound 2-MCPD make it more readily digested by pancreatic lipase than 3-MCPD diesters which include an sn-2 ester [72]. Thus, bound 2-MCPD can be assumed to have a greater bioavailability and maximal plasma concentration than bound 3-MCPD.

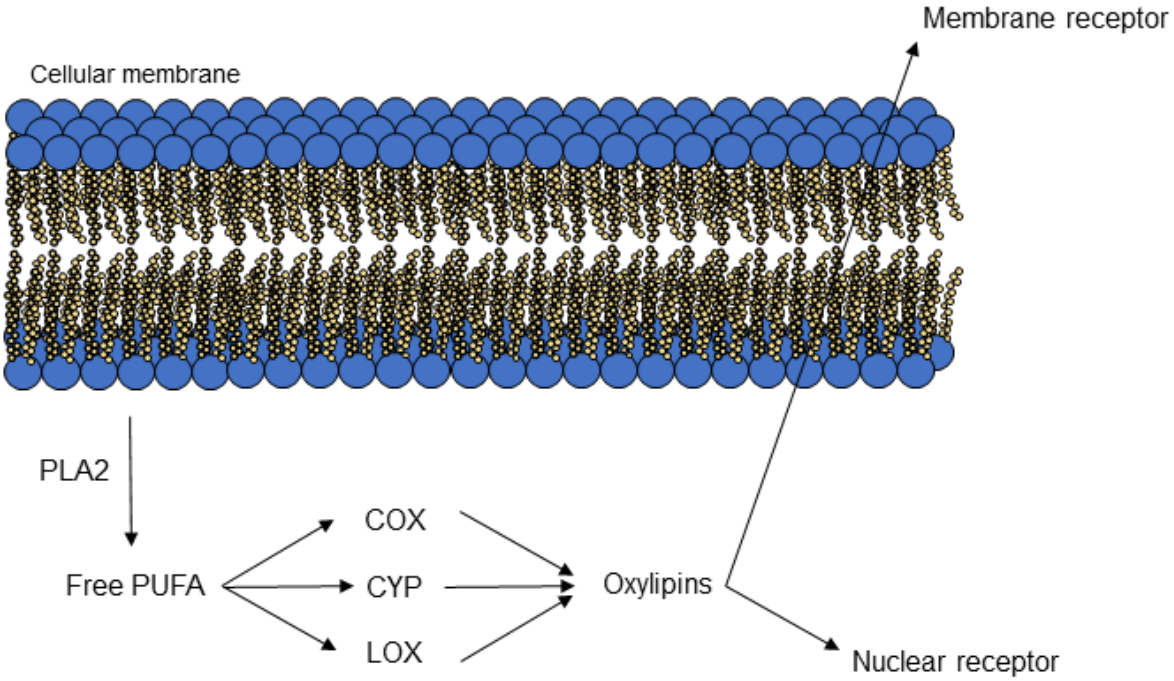
These findings indicate bound CP are highly bioavailable sources of free CP. Also, that the toxicity of bound CP is occurring via the liberated CP. The inverse relationship between toxicity and the number of esters seen with 3-MCPD may be assumed to be less pronounced for 2-

MCPD, because 2-MCPD diesters are in the sn-1 and sn-3 positions, which are sterically favoured by GI lipases over sn-2. Thus, there is likely less difference in the maximal plasma concentrations of 2-MCPD monoesters and diesters, and 2-MCPD diesters are more readily available, when compared to bound 3-MCPD. Of course, future *in vivo* studies are required to confirm this.

2.7. Oxylipins

Oxylipins are the oxygenated metabolites of cellular membrane PUFA. Oxylipin metabolism is first mediated by phospholipase A_2 (PLA_2). PLA_2 cleaves the fatty acid at the sn-2 position of the phospholipid, which is the preferred location for PUFA, while saturated FA are generally found in the sn-1 position. The liberated PUFA is available for metabolism by cyclooxygenase (COX), cytochrome p450 (CYP), and lipoxygenase (LOX) and enzymes. The CYP pathway can be further divided into products of hydroxylase (CYPh) or epoxygenase (CYPe) activity. The CYPe oxylipins can also undergo a further oxidation by soluble epoxide hydrolase (sEH), converting PUFA-epoxides to PUFA-diols. The generated oxylipins can transmit cellular signals by binding intercellularly to G protein-coupled receptors on the surface of cells, or intracellularly to nuclear receptors including the transcription factor peroxisome proliferator-activated receptor [6], [73] (Figure 2.7.1).

Figure 2.7.1 Diagram of oxylipin production and signalling.



COX, cyclooxygenase; CYP, cytochrome P450; LOX, lipoxygenase; PLA2, phospholipase A_2; PUFA, polyunsaturated fatty acid.

Oxylipins are known to have important biological functions by mediating cellular processes, such as, angiogenesis, apoptosis, cell proliferation, and inflammation [6]. However, the function of many oxylipins are not yet known or poorly understood. The known functions of oxylipins are often related to their parent fatty acid and whether it is an n-6 or n-3 PUFA. In the example of inflammation, the n-6 PUFA derived oxylipins are generally regarded as pro-inflammatory. The arachidonic acid (AA) COX derived prostanoids, have been well characterized as pro-inflammatory mediators. Certain non-steroidal anti-inflammatory drugs inhibit COX to reduce these molecules [6], [73]. The AA LOX derived HETE, oxo-ETE, and leukotrienes are also generally pro-inflammatory molecules [6], [73]. The n-3 PUFA oxylipins, in contrast, generally have anti-inflammatory functions. The most well studied of the eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) oxylipins are the maresins, resolvins, and protectins; which are termed specialized pro-resolving mediators (SPM) due to their potent anti-inflammatory and inflammation resolving function [6], [74]–[76].

The known functions of oxylipin isomers can be similar with varying potency or can have antagonistic activity [6]. For example, the AA COX derived prostaglandin (PG) I_2 is a highly active antiaggregatory factor for platelets. Other AA COX products, PGD_2, PGE_1, and $PGF_{1\alpha}$, have progressively less antiaggregatory function [77], while thromboxane $(TX)A_2$, another AA COX derived prostanoid, has pro-aggregatory activity [78]. Another example are the EpETrE, which are vasodilators, but when oxidized to DiHETrE by sEH, have opposing vasoconstricting function, although these data are not always consistent [6].

The variable potency and often opposing function of oxylipins highlight the importance of profiling a broad range of oxylipins in order to inform the larger biological picture; as opposed to measuring individual or limited oxylipins. With advancements in high performance liquid

chromatography tandem mass spectrometry (LC-MS/MS) technology, quantifying large numbers of oxylipins in a single sample to generate an oxylipin profile has become increasingly feasible. As the oxylipin profiles of tissues are being established it is of interest as to how they can differ. Multiple studies have found the amounts of oxylipins in a given tissue have been shown to be variable by sex [9]–[14], [79]. As shown in [9], the oxylipin profiles of rat kidney, liver, and serum, had amounts of oxylipins that were generally higher in males, except for the DHA derived oxylipins which were higher in females. The oxylipin profiles of tissues, such as adipose, heart, kidney, liver, and serum, have also been found to be altered in disease states and in response to diet; with variable responses dependent on tissue and sex [9]–[14], [79], [80]. For instance in [13], rats were fed diets high in either α-linolenic acid (ALA), EPA, or DHA. They found diet altered oxylipin profiles by most potently increasing oxylipins derived from that PUFA which was high in the diet. There were also tissue specific alterations, as with the kidney and serum, the ALA diet elevated DHA derived oxylipins; while in the liver, ALA did not alter DHA oxylipins. Diet and sex specific alterations also occurred to specific oxylipins, for example, in the liver PGE_3 in males only was increased by the ALA and DHA diets.

2.7.1. **Oxylipins and Toxins**

Recently, studies have begun to investigate alterations to PUFA metabolism with exposure to toxins [15]–[18]. The numerous roles of oxylipins as mediators of cellular processes, such as apoptosis, cell proliferation, angiogenesis, inflammation, and oxidative stress [6], make oxylipins potential biomarkers of toxicity. As well, oxylipin alterations may aid in elucidation of toxic MoA. Another rationale for analyzing oxylipins in toxicology is that many of the same enzymes involved in PUFA metabolism are also involved in xenobiotic detoxification.

The CYP super-family of enzymes are known for their role in detoxifying xenobiotics, but also

have endogenous metabolic functions. There are considered to be 57 CYP enzymes in humans, which are divided into families, generally based on greater than 40% genetic similarity [7]. The CYP families 1-3 are primarily responsible for xenobiotic metabolism; with over 2000 known substrates, many of which are pharmaceuticals [81]. The other CYP families are more selective and can be responsible for catalyzing particular metabolic steps [7]. There are specific CYP2 isozymes, however, that are known to have CYPe activity in both xenobiotic and lipid metabolism. Both CYP2C8 and CYP2C9, have lipid metabolism activity, with CYP2C8 known to convert AA to 11,12- and 14,15-epoxy-eicosatrienoic acid (EpETrE), while CYP2C9 can convert AA to 8,9-, 11,12-, and 14,15-EpETrE [82]. These isozymes also have well characterized xenobiotic metabolizing ability; with substrates including, warfarin, rosiglitazone, and ibuprofen [7], [83]. Similarly, CYP2S1, has been shown *in vitro* to convert PGG_2, 5-hydroperoxy-eicosatrienoic acid (HpETE), 12-HpETE, and 15-HpETE to PGH_2, 5-oxo-eicosatrienoic acid (oxoETE), 12-oxoETE, and 15-oxoETE, respectively [84]. CYP2S1 is also thought to be involved in carcinogen metabolism [85].

COX activity can simultaneously oxidize lipids and xenobiotics. In the first step of PG formation AA is oxidized by COX to PGG_2, an endoperoxide. Next, COX reduces PGG_2 to PGH_2 via peroxidase activity. It is during this second step that co-oxidation of xenobiotics, such as polycyclic aromatic hydrocarbons, has been shown to occur [7], [86]. Similarly, when LOX oxidizes PUFA to form a monohydroxy-PUFA, it first oxidizes a PUFA to form a PUFA-hydroperoxide, then by peroxidase activity forms a keto-PUFA, and it is during the second step that co-oxidation of xenobiotics occurs [8]. Through this mechanism LOX can oxidize xenobiotic toxins, such as 4-aminobiphenyl, 2-aminofluorene, and 1,2-dimethylhydrazine [8].

2.7.2. **Isoprostanes**

As described previously (Sections 2.2.2. and 2.3.4.), oxidative stress may play a role in CP toxicity. ROS generated by oxidative stress can cause lipid peroxidation of PUFA. This process is initiated by the ROS abstraction of a hydrogen atom adjacent to an unsaturated bond in a fatty acid chain. The PUFA then has an unpaired electron, forming a lipid radical, which will react with O_2, to form a lipid peroxyl radical. The lipid peroxyl radical can undergo a 5-exo cyclization to form a PG-like molecule. The class of oxylipins formed as a consequence of this process are the AA derived isoP [22]. The F_2-series isoP, which are isomeric to $PGF_{2\alpha}$ with their F-type prostanoid ring, are the most well studied of the isoP due to their stability and validation as gold-standard markers of oxidative stress *in vivo* [22]. In a comparison of multiple oxidative stress biomarkers, rats were exposed to acute CCl_4—a compound known to induce free radical damage of lipids, protein, and DNA. In the plasma, the isoP 8-iso$PGF_{2\alpha}$, as well as malondialdehyde, was found to be the most reproducible, sensitive, and stable biomarker for oxidative stress [87]. The F_2-series isoP are seen to be elevated in various oxidative stress related disease states and in smokers [22], and are considered a gold-standard marker for oxidative stress [22], [87], [88].

2.7.3. **Oxylipins in Chemical Exposure Toxicity Studies**

There have not yet been any studies which profiled oxylipins in response to CP intake. However, a few studies have reported the profile of oxylipins in response to other chemicals of toxicological relevance. Arsenic is a well-documented carcinogen known to affect multiple cell types via the induction of oxidative stress and apoptosis [89]. In a series of studies by [15]–[17], male mice were injected intraperitoneally with a single dose of 12.5 mg/kg arsenite (As(III)) or a saline control, and differential alterations to AA metabolism in the heart, lungs, liver, kidneys,

and brain were found. As(III) exposure differentially altered the expression of CYP genes; with more induced than inhibited in each tissue. In the heart, ω-hydroxylase expression and activity were measured, but no significant changes were found. As expected, levels of CYP ω-hydroxylase pathway metabolites 19- and 20-hydroxy-eicosatetraenoic acids (HETE) were unaltered. The *EPHX2* gene codes for sEH, which converts CYP pathway epoxides to diols. *EPHX2* expression was induced in the heart, lungs, liver, and kidney, but not in the brain. This translated to increased sEH activity in the heart and lungs, but not in the liver, and decreased activity in the kidney. The resulting effects on EpETrE and DiHETrE production were again differential by tissue. In the brain 11,12- and 14,15-EpETrE were decreased, while 8,9-, 11,12-, and 14,15-DiHETrE were increased. In the lungs, there were no significant changes to any EpETrE, while all (5,6-, 8,9-, 11,12-, and 14,15-) DiHETrE were elevated. In the liver, there were no changes to the EpETrE nor DiHETrE. In the kidneys, 8,9-, 11,12-, and 14,15-EpETrE were elevated, and 5,6-EpETrE was lowered, while 8,9-, 11,12-, and 14,15-DiHETrE were lowered. Finally, in the heart, 11,12-, and 14,15-EpETrE were decreased, while 8,9-, 11,12-, 14,15-DiHETrE were increased. In the brain only, additional lipid metabolism enzymes were analyzed. Both PLA_2 expression and activity were inhibited with As(III) exposure. Increased COX-2, 5- and 15-LOX, as well as a non-significant increase in COX-1 and decreased 12-LOX expression were found. The COX-1 metabolites PGD_2, $PGF_{2\alpha}$, and PGJ_2 were significantly decreased. The LOX metabolites 8/12-HETE (unseparated) were decreased [17].

A study by [18], found altered lipid metabolism of the liver and plasma in male rats injected with a dose-response of the environmental toxicant 3,3',4,4',5-pentachlorobiphenyl (PCB) intraperitoneally biweekly for three months. PCB exposure was also found to primarily alter the CYPe pathway and not the ω-hydroxylase metabolites, similarly to the previous study, but

including oxylipins derived from other PUFA. Few LOX and COX pathway oxylipins were altered in the liver, while none were significantly altered in the plasma. In the liver, most CYP epoxides increased; however, there were only clear dose-dependent increases for 14,15-EpETrE and 19,20-epoxydocosapentaenoic acid (EpDPE). While 5,6-EpETrE, 11,12-, and 14,15-epoxyeicosatetraenoic acid showed an inverse dose-dependent relationship in which the lowest PCB dose was significantly elevated from control, while the higher doses were not. Conversely, plasma CYP epoxides from each PUFA precursor generally decreased with PCB exposure, with 13,14- and 16,17-EpDPE decreasing significantly in the medium and high doses, but not low. The liver and plasma CYP sEH diols showed a clear pattern of results; all AA, 4/7 EPA, and all DHA (except 1 liver) derived CYP sEH diols increased in a dose-dependent manner [18]. From these studies, we see that toxic exposures can differentially alter oxylipins, particularly the CYP epoxides and diols.

There are known roles of the CYP epoxides and diols. The AA CYP derived EpETrE are anti-inflammatory and have cardioprotective effects in ischemia/reperfusion injury [6], [90]. The AA CYP epoxides and diols are known to have vasodilatory properties, as are the EPA and DHA epoxides. The EpETrE and DiHETrE, however, have opposing function, as the EpETrE are vasodilating and DiHETrE are vasoconstricting [6]. Coronary hyperemic response has been shown to be decreased when the ratio of EpETrE:DiHETrE decreases [90]. Also, the AA epoxides and diol 5,6-, 8,9-, 11,12-, 14,15-EpETrE, and 14,15-DiHETrE have been shown to promote angiogenesis and/or tumor progression in rodent models, while the DHA epoxides 16,17- and 19,20-EpDPE inhibit Met-1 tumour growth and angiogenesis [6].

The study by [5] found multiple genes associated with cell proliferation, apoptosis, and oxidative stress to be altered in the kidney with 2-MCPD exposure. *Hmox1* gene expression was

upregulated in male and female kidneys at all doses. HMOX1 catalyzes the rate-limiting step in the conversion of heme to bilirubin which produces carbon monoxide (CO). Endogenous CO acts as an intracellular signalling molecule mediating processes including inflammation, apoptosis, and oxidative stress. HMOX1 has been shown to be induced *in vitro* by PGE_2, Δ^{12}-PGJ_2, and 15-deoxy-$\Delta^{12,14}$-PGJ_2 [54]. Therefore, these oxylipins will be of particular interest in 2-MCPD toxicity.

2.8. Oxidized Phosphatidylcholines

The most plentiful phospholipids in mammalian tissues are phosphatidylcholines. OxPC are lipid peroxidation products of phosphatidylcholine bound PUFA. Their formation is similar to that of the isoP (see Section 2.6.2.), but instead of undergoing cyclization the lipid peroxyl radical abstracts a hydrogen atom from another PUFA (propagating the lipid peroxidation) and forms a hydroperoxy-PUFA which undergoes subsequent reactions to form the OxPC [88]. OxPC can be divided into two categories: fragmented and non-fragmented. Fragmented OxPC have an oxidation of the sn-2 PUFA in which a portion of the original fatty acid chain has been cleaved at an unsaturation, due to the formation of a carboxylic acid or aldehyde, creating a new terminal carbon. Non-fragmented OxPC are formed by the addition of peroxyl radicals at an unsaturated bond when but fragmentation does not occur; these molecules are prone to cyclization [23]. The numerous different OxPC that can be produced have altered biological function from their unoxidized precursors. They are found in atherosclerotic lesions have been shown to mediate monocyte binding to endothelium [91], apoptosis [24], and angiogenesis [25]. As a result, OxPC are predictors of acute coronary syndromes [92]. One study has shown OxPC to be increased in cardiomyocytes by exposure to chemotherapy the drug doxorubicin, known for its induction of oxidative stress and cardiotoxicity (Koleini, 2018). Similar to the isoP, due to lipid peroxidation

being the source of their formation, increases in OxPC can be used as markers of oxidative stress.

2.9. Literature Review Summary

In summary, CP are foodborne chemical contaminants formed in the manufacturing of HVP, refined vegetable oils, and water-resistant paper products. Of the CP isomers, 3-MCPD has received the most attention; it was categorized by the IARC as a possible human carcinogen (Group 2B) [31], and determined by JECFA to have a TDI of 4 μg/kg BW [4]. The hazard of 2-MCPD, however, is poorly understood. 28-day rodent oral toxicity studies have indicated cardiotoxicity, nephrotoxicity, and myotoxicity [5], [56], but further study is needed for hazard assessment and elucidation of the MoA. Oxylipins are enzymatically produced or auto-oxidation products of PUFA which have diverse cellular signalling function. OxPC are lipid peroxidation products of PUFA bound to phosphatidylcholine that also have altered function. The oxidized lipids, oxylipins and OxPC, can be altered with toxic exposures and can indicate changes in many cellular processes, including inflammation, oxidative stress, and tissue repair healing. Thus, these molecules have become of interest in a toxicological context.

2.10. Introduction Bibliography

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3. Health Canada Study (Raju et al., 2018): 90-day 2-MCPD Toxicity Study

To further investigate the effects of 2-MCPD, a 90-day sub-chronic dietary 2-MCPD exposure study in rats has been conducted [93]. This study is a follow-up to the 28-day 2-MCPD study by [5]. The contents of this chapter were conducted by Dr. Raju's laboratory from the Regulatory Toxicology Research Division, Food Directorate, Health Canada. The contribution of this thesis' author to the study will be described in the subsequent chapters.

3.1. Study Design

The animal protocol followed the Organization for Economic Co-operation and Development (OECD) test guideline (TG)-408, a repeated dose sub-chronic 90-day oral toxicity study in rodents. This method is a continuation of the OECD TG-407, a repeated dose 28-day oral toxicity study in rodents (see Section 2.3.1.). TG-408 provides a standardized framework for experimental procedures that can be used to determine the hazard of a test substance. The framework includes the minimum number of animals per group, minimum number of dosage groups, how to administer the test substance, maximum dosage, and measurements and analyses to perform [94].

In this study, 120 (n = 60/sex) 7-week old F344 rats were acclimatized for one week and then fed test diets *ad libitum* for 90 days. The F344 line is an in-bred healthy rat model. The diet provided was AIN-93G with corn oil (Envigo, Mississauga, ON) (Table 3.1.1), which contained 0, 0.5, 2, 10, or 40 mg/kg BW/d 2-MCPD (≥99.9%, Toronto Research Chemicals Inc., Toronto, ON). This AIN-93G rat diet contained corn oil (instead of soy oil) conforming to Health Canada's protocols for comparative and standardization purposes. Table 3.1.2 lists the fatty acid composition of the

corn oil. The concentrations of 2-MCPD in the diet (Table 3.1.3) were estimated based 7% dietary oil and on previous rat dietary consumption data of 15 g/d with an average weight of 250g. Rat weights and food consumption were measured weekly. After 90 days, the animals were terminated, and a detailed necropsy was performed. The tissues harvested were flash frozen in liquid nitrogen and stored at -80°C.

Table 3.1.1 AIN-93G semi-synthetic control diet composition.

Ingredient	g/kg	Kcal%
Casein	200	800
L-Cysteine	3	12
Corn Starch	397	1590
Maltodextrin-10	132	528
Sucrose	100	400
Cellulose BW-200	50	0
Corn oil	70	630
t-Butylhydroquinone	0.014	0
Mineral Mix S10022G	35	0
Vitamin Mix V10037	10	40
Choline bitartrate	2.5	0
Total	**1000**	**4000**

Table 3.1.2 Fatty acid composition of corn oil in the diet.

Fatty Acid		%
16:0	Palmitic	12
18:0	Stearic	2
18:1	Oleic	28
18:2	LA	57
18:3	ALA	1
20:4	AA	-
20:5	EPA	-
22:5	DPA	-
22:6	DHA	-
	SFA	14
	MUFA	28
	PUFA	58
	n-3	1
	n-6	57

AA, arachidonic acid; ALA, α-linolenic acid; DHA, docosahexanoic acid; DPA, docosapentaenoic acid; EPA, eicosapentaenoic acid; LA, linoleic acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid.

Table 3.1.3 Concentration of 2-MCPD in the diet.

Dietary concentration (ppm diet)	Estimated doses (mg/kg BW)
8.3	0.5
33.3	2
167.7	10
666.7	40

3.1.1. Ethics Approval

Ethics approval for animal experimentation was obtained from Health Canada Ottawa Animal Care Committee and was assigned as 2017-008. A pathologist (Dr. Don Caldwell) was assigned to the project with a Pathology Number of 187P (Appendix 10.1.).

3.2. Results

The analyses completed thus far are body and tissue weights, histopathology, serum biochemistry, hematology, immunophenotyping, and an oxidative stress panel. These data are currently unpublished and will be briefly described in this chapter.

3.2.1. Body and Tissue Weights

Body and tissue weights were compared between dose groups, but only within a sex. The rat body weights trended towards a decrease as 2-MCPD exposure increased, with a significant decrease in the 40 mg/kg female group only (Table 3.2.1). Tissue weights are listed in Table 3.2.2; alterations occurred in the heart, kidney, and spleen (female only), in which weights of the highest dose groups were elevated. Elevated heart weights were found in both the male and female 40 mg/kg groups. Kidney weights were elevated in both the male and female 40 mg/kg groups as well as the 10 mg/kg female group. The spleen of the 40 mg/kg female group only was also elevated. The significant alterations to tissue weights were the same when compared as absolute values and as values relative to body weight (g/kg BW); one exception was the 40 mg/kg male liver was significantly lower only as an absolute value, but not relative to body weight.

Table 3.2.1 Estimated 2-MCPD intakes and body weight.

	Control	0.5mg/kg 2-MCPD	2mg/kg 2-MCPD	10mg/kg 2-MCPD	40mg/kg 2-MCPD
Males					
Final Body Weight (g)	328.0 ± 5.1	323.1 ± 4.3	319.1 ± 3.4	320.9 ± 5.3	312.9 ± 4.2
Estimated 2-MCPD Consumption (mg/kg BW)	0.00 ± 0.00	0.44 ± 0.01	1.76 ± 0.01	8.96 ± 0.07	34.15 ± 0.35
Females					
Final Body Weight	176.2 ± 1.3	174.4 ± 2.7	170.5 ± 1.9	174.9 ± 1.3	168.8 ± 1.6*
Estimated 2-MCPD Consumption (mg/kg BW)	0.00 ± 0.00	0.47± 0.01	1.88 ± 0.01	9.39 ± 0.10	35.87 ± 0.88

Values given as mean ± standard error. Estimated 2-MCPD consumption is the mean taken over the entire duration of the study. *, denotes significant difference ($p<0.05$) from control.

Table 3.2.2 Tissue weights in g/100g BW of rats exposed to dietary 2-MCPD.

	Control	0.5mg/kg 2-MCPD	2mg/kg 2-MCPD	10mg/kg 2-MCPD	40mg/kg 2-MCPD
Males					
Liver	3.0363 ± 0.0529	3.0614 ± 0.0461	3.1780 ± 0.0758	3.1295 ± 0.0351	2.9635 ± 0.0315
Spleen	0.1999 ± 0.0025	0.1938 ± 0.0016	0.1956 ± 0.0020	0.1980 ± 0.0015	0.2042 ± 0.0022
Kidneys	0.5642 ± 0.0157	0.5548 ± 0.0064	0.5679 ± 0.0054	0.5744 ± 0.0077	0.6486 ± 0.0063*
Heart	0.2709 ± 0.0041	0.2687 ± 0.0034	0.2733 ± 0.0030	0.2730 ± 0.0031	0.3018 ± 0.0036*
Thymus	0.0717 ± 0.0023	0.0673 ± 0.0015	0.0675 ± 0.0027	0.0707 ± 0.0018	0.0681 ± 0.0015
Adrenals	0.0128 ± 0.0006	0.0133 ± 0.0003	0.0129 ± 0.0003	0.0123 ± 0.0003	0.0123 ± 0.0003
Prostate	0.1582 ± 0.0083	0.1425 ± 0.0058	0.1644 ± 0.0063	0.1558 ± 0.0067	0.1471 ± 0.0057
Testes	0.9493 ± 0.0115	0.9343 ± 0.0122	0.9711 ± 0.0091	0.9550 ± 0.0288	0.9440 ± 0.0093
Seminal Vesicles	0.3671 ± 0.0136	0.3813 ± 0.0142	0.3722 ± 0.0159	0.3641 ± 0.0132	0.3586 ± 0.0142
Epididymis	0.2991 ± 0.0046	0.2971 ± 0.0067	0.3017 ± 0.0031	0.2899 ± 0.0080	0.2926 ± 0.0049
Females					
Liver	2.9542 ± 0.0711	3.0466 ± 0.0621	2.9058 ± 0.0773	3.1457 ± 0.0584	2.7548 ± 0.0959
Spleen	0.2304 ± 0.0037	0.2283 ± 0.0053	0.2261 ± 0.0040	0.2341 ± 0.0037	0.2493 ± 0.0064*
Kidneys	0.5467 ± 0.0064	0.5429 ± 0.0060	0.5377 ± 0.0054	0.5835 ± 0.0077*	0.6709 ± 0.0060*
Heart	0.3117 ± 0.0053	0.3165 ± 0.0047	0.3053 ± 0.0036	0.3126 ± 0.0044	0.3335 ± 0.0032*
Thymus	0.1065 ± 0.0045	0.1048 ± 0.0044	0.1082 ± 0.0050	0.0997 ± 0.0033	0.1024 ± 0.0044
Adrenals	0.0240 ± 0.0006	0.0238 ± 0.0005	0.0228 ± 0.0007	0.0235 ± 0.0005	0.0232 ± 0.0008
Ovaries	0.0320 ± 0.0009	0.0329 ± 0.0009	0.0297 ± 0.0007	0.0331 ±0.0011	0.0297 ± 0.0012
Uterus	0.3090 ± 0.0335	0.2789 ± 0.0219	0.3027 ± 0.0268	0.2808 ± 0.0268	0.2286 ± 0.0150

Values given as mean ± standard error. *, denotes significant difference ($p<0.05$) from control.

3.2.2. **Histopathology**

The histopathology of the heart was conducted using the International Harmonization of Nomenclature and Diagnostic Criteria for Lesions in Rats and Mice, described in [95]. The results showed extensive lesions occurred in the left and right ventricles and septa, but not atria, of the 40 mg/kg 2-MCPD exposed hearts; for detailed lesion severity data see Table 3.2.3 & 3.2.4. Briefly, the 0, 0.5, 2, 10 mg/kg groups had instances of small foci of necrosis and inflammation in the right and left ventricle and septum. The 40 mg/kg groups had myofiber and interstitial vacuolation, increased interstitial cells, and multiple foci of necrosis with inflammatory cell infiltrate (NICI), visualized in Figures. 3.2.1 & 3.2.2. The leukocytes associated with the NICI were macrophages. These effects were comparable in males and females both in region, severity, and classification.

Other, less prominent, lesions were observed in the spleen with mild periarteriolar lymphoid sheath and follicle lesions reported in the female 40 mg/kg group only. There was also a slight increase in vacuolation of the testis in the 0.1, 10, and 40 mg/kg groups. Lesions were not observed in other tissues, including the kidneys and tibialis anterior, of either sex.

Table 3.2.3 Male rat lesion scores of the ventricles and septum.

	Control	0.5mg/kg 2-MCPD	2mg/kg 2-MCPD	10mg/kg 2-MCPD	40mg/kg 2-MCPD
Left Ventricle					
Interstitial Vacuolation	6/0/0/0/0	5/0/1/0/0	5/0/1/0/0	6/0/0/0/0	0/0/1/5/0*
Necrosis	5/1/0/0/0	6/0/0/0/0	5/1/0/0/0	6/0/0/0/0	1/4/1/0/0*
Interstitial Cells	6/0/0/0/0	6/0/0/0/0	6/0/0/0/0	6/0/0/0/0	0/0/3/2/1*
Cardiomyocyte Vacuolation	6/0/0/0/0	6/0/0/0/0	6/0/0/0/0	6/0/0/0/0	0/0/5/1/0*
Inflammation	6/0/0/0/0	5/1/0/0/0	4/2/0/0/0	5/1/0/0/0	4/1/1/0/0
NICI	4/2/0/0/0	1/5/0/0/0	0/6/0/0/0	2/4/0/0/0	0/0/1/3/2*
Fibrosis	6/0/0/0/0	6/0/0/0/0	5/1/0/0/0	4/2/0/0/0	0/0/6/0/0*
Right Ventricle					
Interstitial Vacuolation	6/0/0/0/0	6/0/0/0/0	6/0/0/0/0/0	6/0/0/0/0	2/1/3/0/0*
Necrosis	6/0/0/0/0	6/0/0/0/0	6/0/0/0/0/0	6/0/0/0/0	5/1/0/0/0
Interstitial Cells	6/0/0/0/0	6/0/0/0/0	6/0/0/0/0/0	6/0/0/0/0	0/2/2/2/0*
Cardiomyocyte Vacuolation	6/0/0/0/0	6/0/0/0/0	6/0/0/0/0/0	6/0/0/0/0	1/0/5/0/0*
Inflammation	4/2/0/0/0	5/1/0/0/0	5/1/0/0/0/0	5/1/0/0/0	3/3/0/0/0
NICI	5/1/0/0/0	5/1/0/0/0	5/1/0/0/0	2/4/0/0/0	0/1/2/1/1*
Fibrosis	6/0/0/0/0	5/1/0/0/0	4/1/1/0/0	5/0/1/0/0	0/2/4/0/0*
Septum					
Interstitial Vacuolation	6/0/0/0/0	6/0/0/0/0	6/0/0/0/0	6/0/0/0/0	0/0/2/3/0*
Necrosis	5/1/0/0/0	6/0/0/0/0	6/0/0/0/0	6/0/0/0/0	0/2/3/0/0
Interstitial Cells	6/0/0/0/0	6/0/0/0/0	6/0/0/0/0	6/0/0/0/0	0/0/3/2/0*
Cardiomyocyte Vacuolation	6/0/0/0/0	6/0/0/0/0	6/0/0/0/0	6/0/0/0/0	0/0/4/1/0*
Inflammation	6/0/0/0/0	6/0/0/0/0	3/3/0/0/0	3/3/0/0/0	1/4/0/0/0
NICI	3/3/0/0/0	1/5/0/0/0	2/4/0/0/0	2/4/0/0/0	0/0/3/2/0*
Fibrosis	5/1/0/0/0	5/1/0/0/0	5/1/0/0/0	6/0/0/0/0	0/2/3/0/0*

Values denote the number of individuals with lesions scored:
absent/mild/moderate/marked/severe. *, denotes significant difference ($p<0.05$) from control.
NICI, Necrosis with inflammatory cell infiltrate.

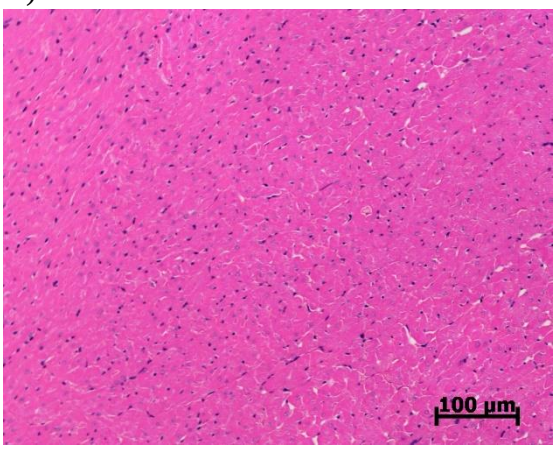
Table 3.2.4 Female rat lesion scores of the ventricles and septum.

	Control	0.5mg/kg 2-MCPD	2mg/kg 2-MCPD	10mg/kg 2-MCPD	40mg/kg 2-MCPD
Left Ventricle					
Interstitial Vacuolation	5/1/0/0/0	6/0/0/0/0	6/0/0/0/0	5/1/0/0/0	1/0/4/1/0*
Necrosis	6/0/0/0/0	6/0/0/0/0	6/0/0/0/0	6/0/0/0/0	1/0/2/3/0*
Interstitial Cells	6/0/0/0/0	6/0/0/0/0	6/0/0/0/0	5/1/0/0/0	0/0/2/4/0*
Cardiomyocyte Vacuolation	6/0/0/0/0	6/0/0/0/0	6/0/0/0/0	5/1/0/0/0	0/0/2/4/0*
Inflammation	4/2/0/0/0	4/2/0/0/0	6/0/0/0/0	5/1/0/0/0	1/0/3/2/0*
NICI	2/4/0/0/0	3/3/0/0/0	1/5/0/0/0	3/3/0/0/0	0/0/0/6/0*
Fibrosis	6/0/0/0/0	6/0/0/0/0	6/0/0/0/0	6/0/0/0/0	0/0/6/0/0*
Right Ventricle					
Interstitial Vacuolation	6/0/0/0/0	6/0/0/0/0	6/0/0/0/0	6/0/0/0/0	3/2/0/0/0
Necrosis	6/0/0/0/0	6/0/0/0/0	6/0/0/0/0	6/0/0/0/0	2/0/2/1/0
Interstitial Cells	6/0/0/0/0	6/0/0/0/0	6/0/0/0/0	5/1/0/0/0	0/0/0/5/0*
Cardiomyocyte Vacuolation	6/0/0/0/0	6/0/0/0/0	6/0/0/0/0	6/0/0/0/0	4/0/1/0/0
Inflammation	6/0/0/0/0	4/2/0/0/0	4/2/0/0/0	4/2/0/0/0	2/0/0/3/0
NICI	6/0/0/0/0	2/4/0/0/0	3/3/0/0/0	5/1/0/0/0	0/0/0/5/0*
Fibrosis	6/0/0/0/0	6/0/0/0/0	6/0/0/0/0	6/0/0/0/0	0/0/5/0/0*
Septum					
Interstitial Vacuolation	6/0/0/0/0	6/0/0/0/0	6/0/0/0/0	5/0/0/0/0	0/0/2/2/0*
Necrosis	6/0/0/0/0	6/0/0/0/0	6/0/0/0/0	5/0/0/0/0	0/0/3/1/0*
Interstitial Cells	6/0/0/0/0	6/0/0/0/0	6/0/0/0/0	5/0/0/0/0	0/0/0/4/0*
Cardiomyocyte Vacuolation	6/0/0/0/0	6/0/0/0/0	6/0/0/0/0	5/0/0/0/0	0/0/1/3/0*
Inflammation	4/2/0/0/0	4/2/0/0/0	5/1/0/0/0	2/3/0/0/0	0/0/2/2/0*
NICI	5/1/0/0/0	2/4/0/0/0	0/6/0/0/0	1/4/0/0/0	0/0/1/3/0*
Fibrosis	6/0/0/0/0	6/0/0/0/0	6/0/0/0/0	5/0/0/0/0	0/0/4/0/0*

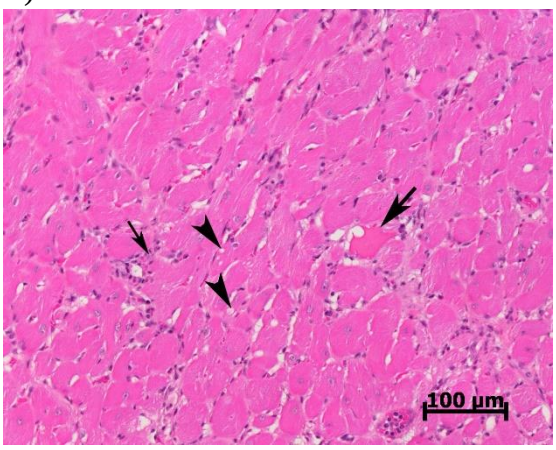
Values denote the number of individuals with lesions scored:
absent/mild/moderate/marked/severe. *, denotes significant difference ($p<0.05$) from control.
NICI, Necrosis with inflammatory cell infiltrate.

Figure 3.2.1 Histopathology image of female rat left ventricle fed A) control and B) 40 mg/kg 2-MCPD.

A)



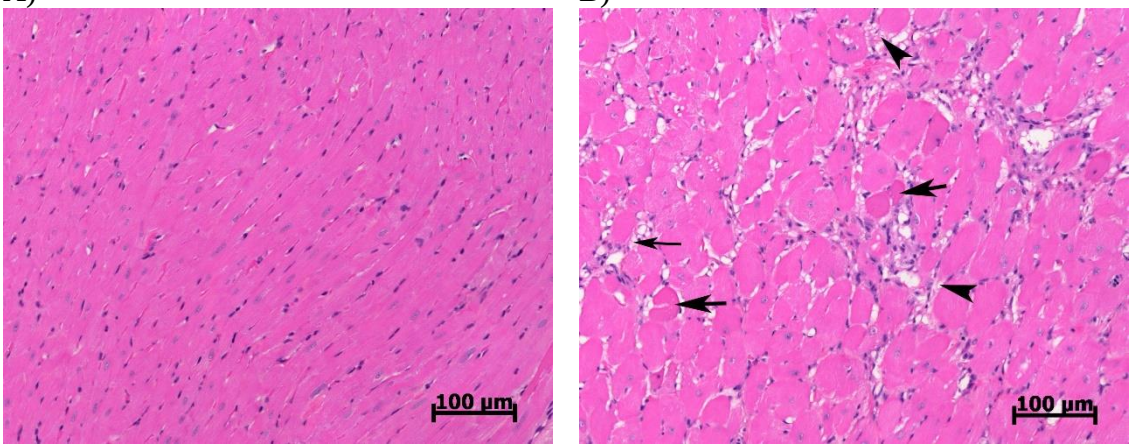
B)



Small arrow indicates increased interstitial cells. Large arrow indicates focal cardiomyocyte necrosis. Arrowheads indicates cardiomyocyte vacuolation.

Figure 3.2.2 Histopathology image of male rat left ventricle fed A) control and B) 40 mg/kg 2-MCPD.

A) B)



Small arrow indicates interstitial vacuolation with increased cellularity. Large arrows indicate focal cardiomyocyte necrosis. Arrowheads indicate cardiomyocyte vacuolation.

3.2.3. **Serum Biochemistry, Hematology, and Immunophenotyping**

While there were numerous differences in the serum biochemistry between groups, the only marker found outside of reference range was elevated inorganic phosphorous in 40 mg/kg males; the 40 mg/kg females were elevated but not outside of reference range. Elevated serum phosphorous can be a sign of impaired kidney function and is associated with increased mortality for humans with chronic kidney disease [96], [97].

The immunophenotyping analysis showed the leukocyte species in the female 40 mg/kg 2-MCPD group had increased B cells and NK/monocyte low expression cells, and decreased T cells, T cell:B cell ratio, and NKT cells. In the males, the only significant alteration was to the T cell:B cell ratio; however, B cells approached a significant increase ($p=0.054$) . Similar results were seen in the 28-day study, in which female B cells were increased and T cells decreased, and no significant changes occurred in males. The current results of increased B cells indicate an enhanced antibody mediated immune response.

3.2.4. **Oxidative Stress Panel**

A panel of serum markers for oxidative stress, which included 4-hydroxy-2-nonenal (4-HNE) a marker of lipid peroxidation, 8-Oxo-2'-deoxyguanosine (8-OHDG) a marker of DNA oxidation, and nitrotyrosine a marker of protein oxidation, showed no significant changes from control with 2-MCPD exposure (Table 3.2.5).

Table 3.2.5 Serum oxidative stress markers in rats exposed to 2-MCPD.

	Control	0.5 mg/kg 2-MCPD	2 mg/kg 2-MCPD	10 mg/kg 2-MCPD	40 mg/kg 2-MCPD
Males					
4-HNE (μg/μL)	2.35 ± 0.372	2.07 ± 0.566	2.45 ± 0.553	2.69 ± 0.692	2.48 ± 0.561
8-OHDG (ng/ μL)	0.223 ± 0.0537	0.255 ± 0.0423	0.172 ± 0.0326	0.140 ± 0.0142	0.184 ± 0.0244
Nitrotyrosine (μM)	361 ± 80.9	272 ± 36.0	294 ± 33.4	305 ± 49.7	256 ± 24.8
Females					
4-HNE (μg/μL)	4.56 ± 0.876	2.94 ± 0.775	4.61 ± 0.806	4.45 ± 0.816	3.91 ± 0.861
8-OHDG (ng/μL)	0.239 ± 0.00938	0.287 ± 0.0161	0.233 ± 0.0456	0.259 ± 0.0321	0.243 ± 0.0333
Nitrotyrosine (μM)	316 ± 63.5	255 ± 41.0	366 ± 53.3	317 ± 52.8	209 ± 41.4

No significant differences between groups occurred. 4-HNE, 4-hydroxy-2-nonenal; 8-OHDG, 8-Oxo-2'-deoxyguanosine.

3.3. Summary

This 90-day repeated dose sub-chronic oral feeding of 2-MCPD in rats, was conducted to fill a data gap for the assessment of the hazard of 2-MCPD. It was found that heart, kidney, and spleen (female only) weights were elevated in rats receiving the highest dose. Non-neoplastic lesions were found in the hearts of male and female rats receiving the highest dose; while, lesions were not significantly elevated in any other tissue. Despite the higher kidney weights, there were no 2-MCPD related changes in the pathological lesions found in the kidneys in males or females (controls and all dose groups had kidney lesions in both sexes). Thus, cardiotoxicity was the major outcome of 2-MCPD exposure, with a NOEL of 10 mg/kg BW. These data add to the weight of the evidence for 2-MCPD toxicity and identify heart as a main target organ.

4. Study Rationale, Hypotheses, Objectives

The 90-day repeated dose 2-MCPD oral toxicity study presented in Chapter 3, found 2-MCPD exposure of 40 mg/kg BW to have pathological effects, particularly to the heart [93]. Other studies have identified the kidney and skeletal muscle as sites of 2-MCPD toxicity, in addition to the heart (unpublished, reviewed in [56]). To further investigate the effects of 2-MCPD, this study conducted a targeted oxo-lipid analysis of the heart, kidney, serum, and TA of the control and 40 mg/kg groups.

4.1. Study Rationale

Review of literature suggests a toxicological knowledge gap in addressing the hazard of 2-MCPD. While the hazard of 3-MCPD has been assessed, there is less data on the effects of the isomeric 2-MCPD, which is known to occur concurrently. Few studies have analyzed oxylipins in conjunction with toxic exposures, and no studies have investigated the effects of any CP on oxylipin metabolism. Thus, an investigation of the effect of 2-MCPD on lipid metabolism is warranted. This study compared oxidized lipids, in the tissues previously reported as targets of 2-MCPD toxicity, from rats fed control or 40 mg/kg BW (the highest dose and LOAEL).

The effects of 3-MCPD are known to differ by sex, and similarly, the few 2-MCPD studies that have been performed have found evidence of differential effects (including toxicogenomics) between the sexes. Also, the few studies that have analyzed oxylipins with other toxicologically-relevant chemicals have only included male rodents. To address the address potential differences between sexes, this study included both males and females. Also, it is thought that oxidative stress may be a component of 2-MCPD toxicity; however, this is based on gene expression and

proteomic data. This study addressed oxidative stress by assaying the 'gold-standard' biomarkers the isoP and the OxPC.

4.2. Hypotheses

In this study, it was hypothesized that the oxo-lipid profiles of the heart, kidney, serum, and TA would be altered by 2-MCPD exposure. More specifically that:

i. The AA derived prostanoids would be elevated and the EPA and DHA derived SPM would be decreased with 2-MCPD treatment, due to increased inflammation.
ii. 2-MCPD treatment would increase the isoP and OxPC due to elevated oxidative stress.
iii. The oxo-lipid alterations would differ within tissues by sex.
iv. 2-MCPD treatment would specifically alter the CYP epoxides and diols as seen in other xenobiotic exposure studies.

4.3. Objectives

To address the hypotheses, the main objective of this study was to conduct a targeted oxo-lipidomic analysis using LC-MS/MS of the heart, kidney, serum, and TA from rats exposed to a 90-day sub-chronic repeated oral dose of 2-MCPD (Raju et al., 2018, described in Chapter 3), and to identify alterations in these molecules by comparing the control and high dose (40 mg/kg BW) treatment groups. This study also aimed to:

i. Address inflammation due to 2-MCPD treatment by comparing alterations from control of the AA prostanoids and the EPA and DHA SPM.
ii. To identify sex specific effects of 2-MCPD by including both male and female groups and conducting a two-way ANOVA that included sex.
iii. Include the CYP epoxides and diols in the analysis.

iv. Determine the role of oxidative stress in 2-MCPD toxicity by analyzing the isoP and OxPC.

5. Manuscript

The following is a manuscript of the portion of the study conducted by this thesis' author. This manuscript presents the methods, results, and discussion for this thesis. The manuscript's discussion will be expanded upon in Chapter 7. For additional information on method development see Appendix 10.2. & 10.3.

This manuscript has been submitted to Health Canada and is currently in the process of internal peer review.

Title: Bioactive lipids are altered in the heart, kidney, and serum of rats exposed to 2-MCPD.

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5.1. Abstract

CP have been identified as process-induced food contaminants which occur as by-products of the manufacturing of refined food oils and hydrolyzed vegetable protein. While the hazard of 3-MCPD has been well characterized, there has been a paucity of research on the concomitantly formed 2-MCPD isomer, thus forming a data gap for regulatory risk assessment. Studies that have investigated sub-acute 2-MCPD toxicity have found cardio-, nephro-, and myotoxic effects. Our study has profiled the toxic effects of 2-MCPD by conducting a targeted oxolipidomic analysis, using LC-MS/MS, of heart, kidney, serum, and skeletal muscle of F344 rats exposed to control and 2-MCPD (40 mg/kg BW) for 90 days. We found in the heart that n-3 PUFA derived oxylipins, particularly DHA oxylipins, were lower with 2-MCPD exposure, coincident with cardiac lesions. LOX derived oxylipins were decreased in the serum with a greater effect in the male 2-MCPD treatment group. Few oxylipin alterations were seen in the kidney and there was an absence of oxylipin alterations in the TA, which was consistent with the few physiological effects seen in these tissues. OxPC and isoP were not altered in this study, indicating that oxidative stress was not elevated by 2-MCPD. These findings will add to the weight of the evidence for 2-MCPD toxicity as well as support the use of serum oxylipins as potential biomarkers of 2-MCPD exposure.

Highlights:

- Rat heart, kidney, and serum oxylipin profiles were altered with 2-MCPD exposure of 40 mg/kg BW.
- DHA derived oxylipins were reduced in the heart by 2-MCPD exposure coincident with non-neoplastic lesions.
- LOX derived oxylipins were reduced in serum with 2-MCPD exposure.
- 2-MCPD did not alter oxidative stress based on OxPC and isoP data.

Keywords:
2-monochloro-1,3-propanediol (2-MCPD); Oxylipin; Oxidized phosphatidylcholine; Cardiotoxicity; Food contaminant; Serum.

5.2. Introduction

CP have been identified as process induced food-borne contaminants which occur in the refining of vegetable oils [1], the production of HVP [2], and in water-resistant paper products [3]. In vegetable oils, CP primarily occur as fatty acid esters which are readily hydrolyzed by gastrointestinal lipases [63], [65], [69], [70], [72]. 3-MCPD is the most well studied CP isomer; it has been identified as a possible human carcinogen, with the kidneys and male reproductive organs as primary targets [4]. Physiologically relevant levels of dietary 3-MCPD have been estimated to occur when babies are exclusively formula-fed [4].

The 2-MCPD isomer, which occurs concomitantly with 3-MCPD [63], [64], has been less studied and has received insufficient investigation for regulatory hazard assessment. The 28-day 2-MCPD rat toxicity studies which have been conducted have reported cardiotoxicity, nephrotoxicity, and myotoxicity [5], [56]. Transcriptomic and proteomic analyses have identified multiple sex and tissue specific alterations with 2-MCPD exposure. In the heart, alterations were related to tissue repair, necrosis, and mitochondrial dysfunction [5], [41], while in the kidney, alterations were associated with apoptosis, cell proliferation, and oxidative stress [5], [57].

Oxylipins are oxygenated metabolites of cellular membrane PUFA. The major precursors for oxylipins are the n-6 PUFA AA, dihomo-γ-linolenic acid (DGLA), and LA, and the n-3 PUFA ALA, EPA, and DHA. Their metabolism is first mediated by PLA_2, which cleaves PUFA from membrane phospholipids. The liberated PUFA is then available for metabolism by COX, CYP, LOX, or non-enzymatic oxidation. The CYP pathway can be further divided into hydroxylase (CYPh) or epoxygenase (CYPe) activities. Oxylipins transmit cellular signals by binding to G protein-coupled receptors on the surface of cells or to nuclear receptors including the peroxisome proliferator-activated receptor family [6], [73]. The induced processes include important cell

type-specific homeostatic functions such as vasodilation/vasoconstriction, angiogenesis, apoptosis, cell proliferation, and inflammation [6]. Oxylipins are potential biomarkers of toxic exposures because of the functions they mediate and the major enzymes in the production of oxylipins (COX, CYP, and LOX) can also have oxidative roles in xenobiotic metabolism [7], [8], [82], [83], [85], [86], [98]. Previous studies also have shown that oxylipins, and the activity of the enzymes that produce them, to be altered by toxic exposures to arsenic and PCB [15]–[18], suggesting that chronic exposure to 2-MCPD may alter oxylipins.

Oxidative stress can cause the non-enzymatic oxidation of lipids; forming molecules such as, OxPC and isoP. OxPC have altered biological function from their unoxidized precursors and are present in atherosclerotic lesions and induce apoptosis and angiogenesis [23]–[25]. The isoP are a class of oxylipin that have similar structure and function to their isomeric PG, which are potent lipid mediators of functions such as inflammation and the regulation of blood pressure, derived from COX metabolism [6], [20]–[22]. Due to the mechanism of their synthesis, both the OxPC and isoP are biomarkers of oxidative stress. The contribution of oxidative stress to CP toxicity is unclear. Proteomic and transcriptomic analyses of rats receiving sub-toxic doses of either 2- or 3-MCPD have found deregulation of PARK7/DJ-1, an endogenous redox regulating protein, in rat kidney, liver, and testes [40], [42]–[44]. However, a previous 90-day 3-MCPD toxicity study in rats found testicular damage occurred despite a lack of increase to markers of lipid peroxidation [34].

In this study, we profiled bioactive lipids in the heart, kidney, serum, and TA of rats exposed to dietary 2-MCPD. Our results demonstrate that the oxylipin profiles of the tissues analyzed were differentially affected by 2-MCPD exposure. These data will add to the weight of the evidence for 2-MCPD toxicity and aid in the determination of its MoA.

5.3. Methods

5.3.1. Animals and Experimental Design

The experimental procedures involving animals were approved by the Health Canada Health Protection Branch Animal Care Committee. The animal protocol followed the OECD TG-408; a sub-chronic repeated dose assay. In this study, 24 (*n* =12/sex) 8-week old F344 rats were fed an AIN-93G diet with corn oil containing 0 or 40 mg/kg BW/d 2-MCPD (Toronto Research Chemicals Inc., Toronto, Canada) for 90 days. The animals were then terminated, and a detailed necropsy was performed. The tissues harvested were flash frozen in liquid nitrogen and stored at -80°C.

5.3.2. Oxolipidomic Analysis

Flash frozen heart, kidney, and TA were dry pulverized (cryoPREP™ CP02). Free oxylipin extraction was performed as described in [9], [10], with some modifications. Briefly, a portion of frozen heart (250:3, m/v), kidney (75:1, m/v), or TA (100:1, m/v) was homogenized using the Omni Bead Ruptor 24 (Omni International, Inc., GA, USA) in ice-cold Tyrode's salt solution (9.6 g/L). Antioxidant cocktail [0.2 g/L BHT, 0.2 g/L EDTA, 2 g/L triphenylphosphine, and 2 g/L indomethacin in methanol/ethanol/water (2:1:1, v/v/v)] was added (1:20, v/v) to the homogenate or serum. Methanol/formic acid (100:1, v/v) was added to homogenates (4:3, v/v). A mixture of deuterated oxylipin internal standards (Cayman Chemical, MI) and di- 9:0-PC were also added. To prepare for solid phase extraction, samples were diluted in acidified water (1:5, v/v) to adjust to pH 3. Samples were applied to 33 μm polymeric reversed phase extraction columns (Phenomenex, CA, USA) pre-conditioned with methanol and pH 3 water. The columns were then rinsed with pH 3 water followed by hexane. Oxylipins and OxPC were then eluted from the column with methanol, collected, evaporated, and resuspended in MS grade

(70:30:0.02; v/v/v) water: acetonitrile: acetic acid for LC-MS/MS (Shimadzu Nexera XR coupled to a QTRAP 6500; Sciex, ON), modified from [99] and described in [9]. Oxylipins were quantified using the stable isotope dilution method described in [100].

The OxPC were analyzed from the same sample using a second injection into the LC-MS/MS. Their retention times were determined using matched standards. The resulting chromatograms were analyzed using MultiQuant software to identify unique oxylipin and OxPC peaks according to their retention time and mass/charge ratio of parent and daughter transitions. The LOD and LOQ were 3x and 5x the baseline peak intensity, respectively. For a listing of the oxylipins and OxPC scanned for but below the LOQ see Supplemental Table 1 (Appendix 10.4.). Dose response curves have not been established for the OxPC nor the oxylipins for which primary standards are not available. Therefore, these molecules were reported as relative amounts determined by their area ratio of peak intensity to internal standard intensity and were not included in enzyme pathway and precursor totals.

5.3.3. Statistical Analysis

Data were analyzed by two-way ANOVA with factors treatment (0 or 40 mg/kg BW 2-MCPD) and sex, with the null hypothesis rejected at the 0.05 level of probability. Data was transformed using Tukey's power ladder to optimize the normality of the distribution. Tukey's post-test was used to determine differences between groups. All statistics were performed using R (version 3.4.1).

5.4. Results

5.4.1. Histopathology and Oxidative Stress Panel

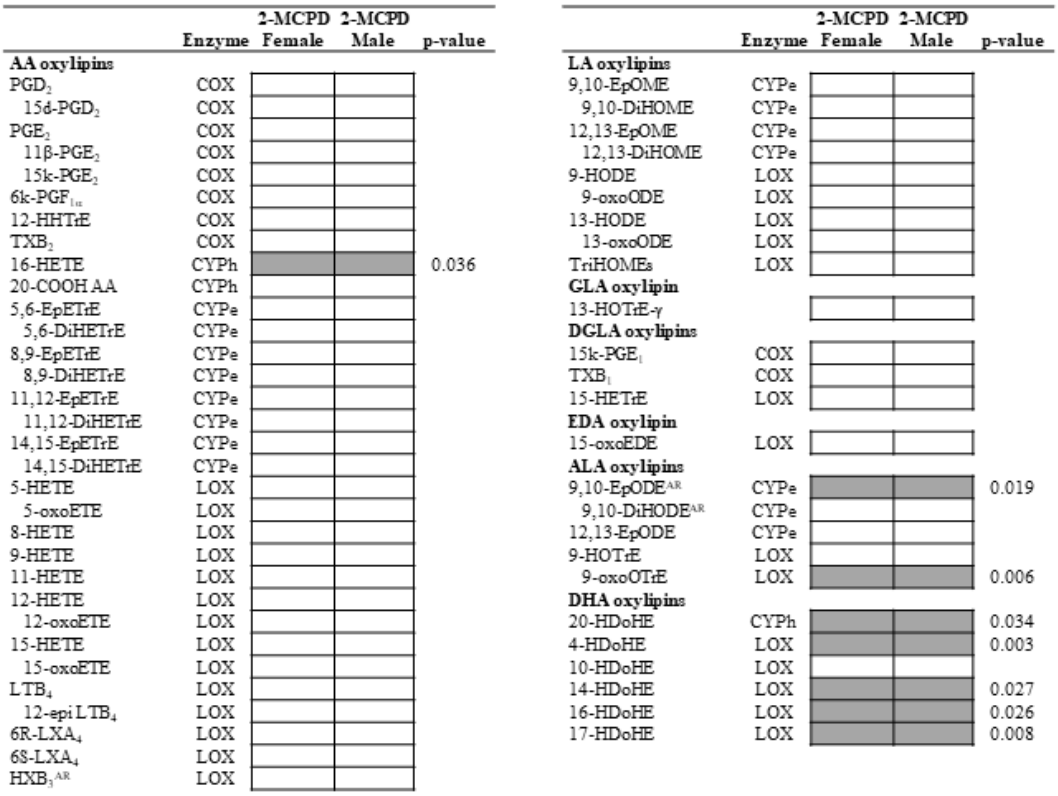
(Summary of results from Chapter 4)

The unpublished findings from this study include elevated heart and kidney wet weights in both males and females 2-MCPD treatment groups. The histopathology data from this study identified extensive lesions in the left and right ventricles and septa of the 2-MCPD-treated hearts of males and females. The lesions were described as multiple foci of NICI and myofibril and interstitial vacuolation. Lesions were not present in kidney or TA. Serum markers of oxidative stress, 4-HNE, 8-OHDG, and nitrotyrosine, were not different from control with 2-MCPD treatment in either sex.

5.4.2. Effects of 2-MCPD on Heart Oxylipins

In the heart, 57 unique oxylipins were quantified and the means, standard errors, and statistics can be found in Supplemental Table 2 (Appendix 10.4.). When compared to control, 8 of the 57 oxylipins were lower in 2-MCPD-treated rats. Almost all (7 of 8) were n-3 PUFA oxylipins, being derived from either DHA or ALA (Figure 5.4.1). This was reflected in the mass of DHA oxylipins which were lower than control with 2-MCPD exposure (Figure 5.4.2A). Total ALA oxylipins was not significantly different with 2-MCPD exposure (Figure 5.4.2B); however, 9,10-epoxyoctadecadienoic acid and 9,10-dihydroxyoctadecadienoic acid were not included in the ALA total because they were reported as area ratios due to the lack of available primary standards, so from this measure the effect on total ALA oxylipins was likely understated. Further indicating the specificity of the effect of 2-MCPD on n-3 derived oxylipins, the total of n-3 derived oxylipins was lower than control (Figure 5.4.3A). None of the n-6 PUFA oxylipin totals were altered by 2-MCPD exposure (Figure 5.4.2C-E), nor was the total n-6 oxylipins (Figure 5.4.3B). Treatment with 2-MCPD affected both CYP and LOX products derived from ALA and DHA. This effect, however, does not appear to be due an overall decrease in the activity of either enzyme as there were no alterations to their n-6 PUFA derived LOX analogues.

Figure 5.4.1 Heart oxylipin alterations from control by 40 mg/kg BW 2-MCPD.

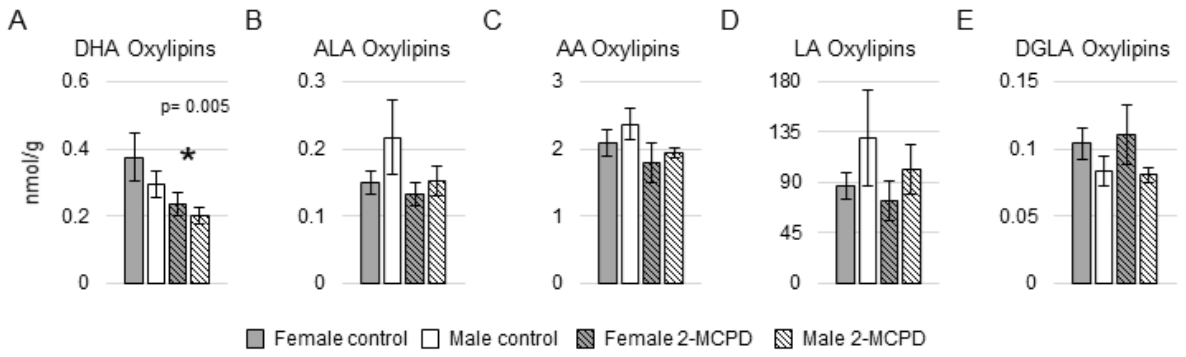


	Enzyme	2-MCPD Female	2-MCPD Male	p-value
AA oxylipins				
PGD_2	COX			
15d-PGD_2	COX			
PGE_2	COX			
11β-PGE_2	COX			
15k-PGE_2	COX			
6k-$PGF_{1\alpha}$	COX			
12-HHTrE	COX			
TXB_2	COX			
16-HETE	CYPh			0.036
20-COOH AA	CYPh			
5,6-EpETrE	CYPe			
5,6-DiHETrE	CYPe			
8,9-EpETrE	CYPe			
8,9-DiHETrE	CYPe			
11,12-EpETrE	CYPe			
11,12-DiHETrE	CYPe			
14,15-EpETrE	CYPe			
14,15-DiHETrE	CYPe			
5-HETE	LOX			
5-oxoETE	LOX			
8-HETE	LOX			
9-HETE	LOX			
11-HETE	LOX			
12-HETE	LOX			
12-oxoETE	LOX			
15-HETE	LOX			
15-oxoETE	LOX			
LTB_4	LOX			
12-epi LTB_4	LOX			
6R-LXA_4	LOX			
6S-LXA_4	LOX			
HXB_3^{AR}	LOX			

	Enzyme	2-MCPD Female	2-MCPD Male	p-value
LA oxylipins				
9,10-EpOME	CYPe			
9,10-DiHOME	CYPe			
12,13-EpOME	CYPe			
12,13-DiHOME	CYPe			
9-HODE	LOX			
9-oxoODE	LOX			
13-HODE	LOX			
13-oxoODE	LOX			
TriHOMEs	LOX			
GLA oxylipin				
13-HOTrE-γ				
DGLA oxylipins				
15k-PGE_1	COX			
TXB_1	COX			
15-HETrE	LOX			
EDA oxylipin				
15-oxoEDE	LOX			
ALA oxylipins				
$9,10\text{-EpODE}^{AR}$	CYPe			0.019
$9,10\text{-DiHODE}^{AR}$	CYPe			
12,13-EpODE	CYPe			
9-HOTrE	LOX			
9-oxoOTrE	LOX			0.006
DHA oxylipins				
20-HDoHE	CYPh			0.034
4-HDoHE	LOX			0.003
10-HDoHE	LOX			
14-HDoHE	LOX			0.027
16-HDoHE	LOX			0.026
17-HDoHE	LOX			0.008

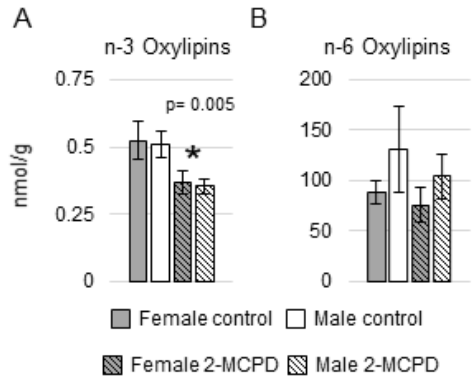
Significantly lower oxylipins are denoted by grey colouring. Means, SEs, and p-values are shown in Supplemental Table 2 (Appendix 10.4.). AA, arachidonic acid; ALA, α-linolenic acid; AR, area ratio; COX, cyclooxygenase; CYPe, cytochrome P450-epoxygenase; CYPh, cytochrome P450-hydroxylase; d, deoxy; DGLA, dihomo-γ-linolenic acid; DiHODE, dihydroxy-octadecadienoic; DiHOME, dihydroxy-octadecaenoic acid; DHA, docosahexaenoic acid; EDA, eicosadienoic acid; EpODE, epoxy-octadecadienoic acid; EpOME, epoxy-octadecaenoic acid; GLA, γ-linolenic acid; HDoHE, hydroxy-docosahexaenoic acid; HETE, hydroxy-eicosatetraenoic acid; HETrE, hydroxy-eicosatrienoic acid; HHTrE, hydroxyheptadecatrienoic acid; HODE, hydroxy-octadecadienoic acid; HOTrE, hydroxy-octadecatrienoic acid; HX, hepoxilin; k, keto; LA, linoleic acid; LOX, lipoxygenase; LT, leukotriene; oxoEDE, oxo-eicosadienoic acid; oxoETE, oxo-eicosatetraenoic acid; oxoODE, oxo-octadecadienoic acid; oxoOTrE, oxo-octadecatrienoic acid; PG, prostaglandin; TriHOMEs, 9,10,13- & 9,12,13-trihydroxy-octadecenoic acid (could not be separated); TX, thromboxane.

Figure 5.4.2 Effect of 40 mg/kg BW 2-MCPD on heart oxylipins derived from A) AA, B) LA, C) DGLA, D) ALA, and E) DHA.



AA, arachidonic acid; ALA, α-linolenic acid; DGLA, dihomo-γ-linolenic acid; DHA, docosahexaenoic acid; LA, linoleic acid.

Figure 5.4.3 Effect of 40 mg/kg BW 2-MCPD on heart oxylipins derived from A) n-6 PUFA, and B) n-3 PUFA.

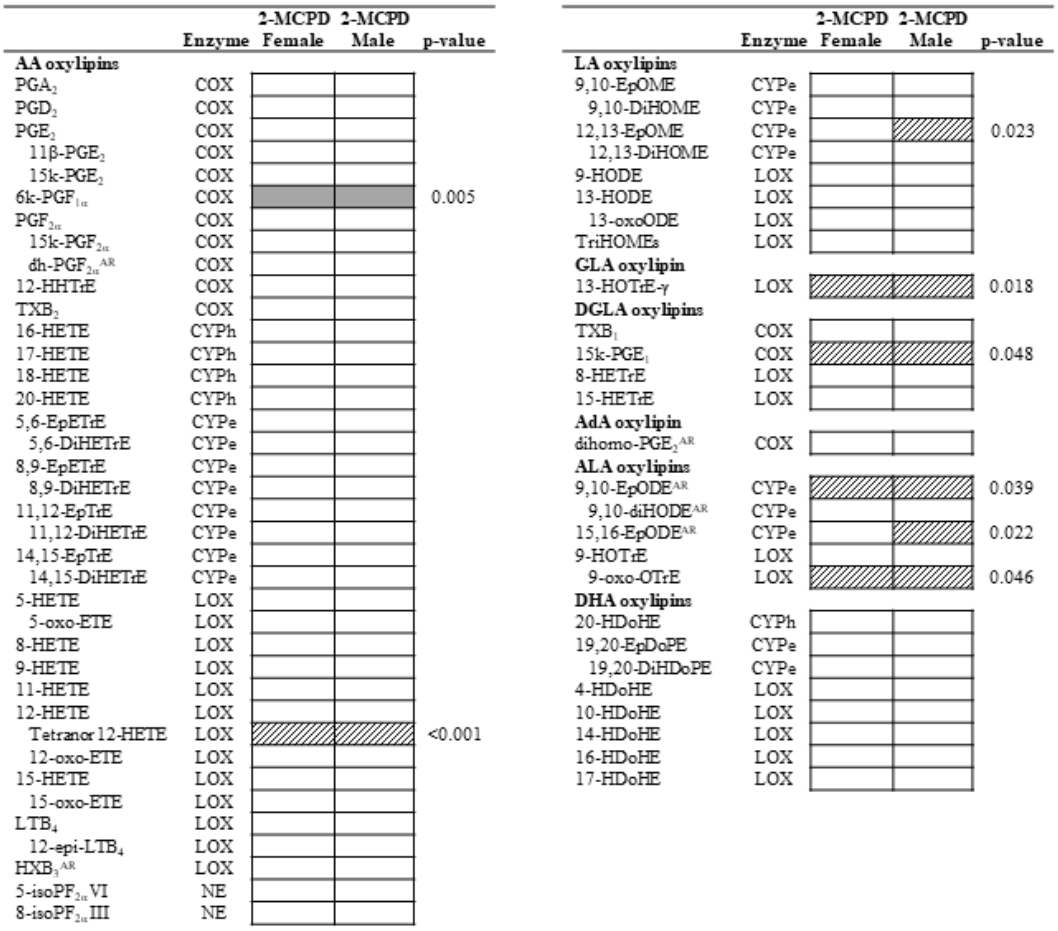


PUFA, polyunsaturated fatty acids.

5.4.3. **Effects of 2-MCPD on Kidney Oxylipins**

In the kidney, 65 unique oxylipins were quantified and the means, standard errors, and statistics can be found in Supplemental Table 3 (Appendix 10.4.). Of these, 2-MCPD exposure altered 8 oxylipins significantly from control, with 7 higher and 1 lower (Figure 5.4.4). Besides a potential effect on the ALA oxylipins (3 of 5 lower), there were no apparent patterns to the alterations, as no PUFA precursor totals, nor enzyme pathway totals were significantly altered (Supplemental Table 3 (Appendix 10.4.)).

Figure 5.4.4 Kidney oxylipin alterations from control by 40 mg/kg BW 2-MCPD.

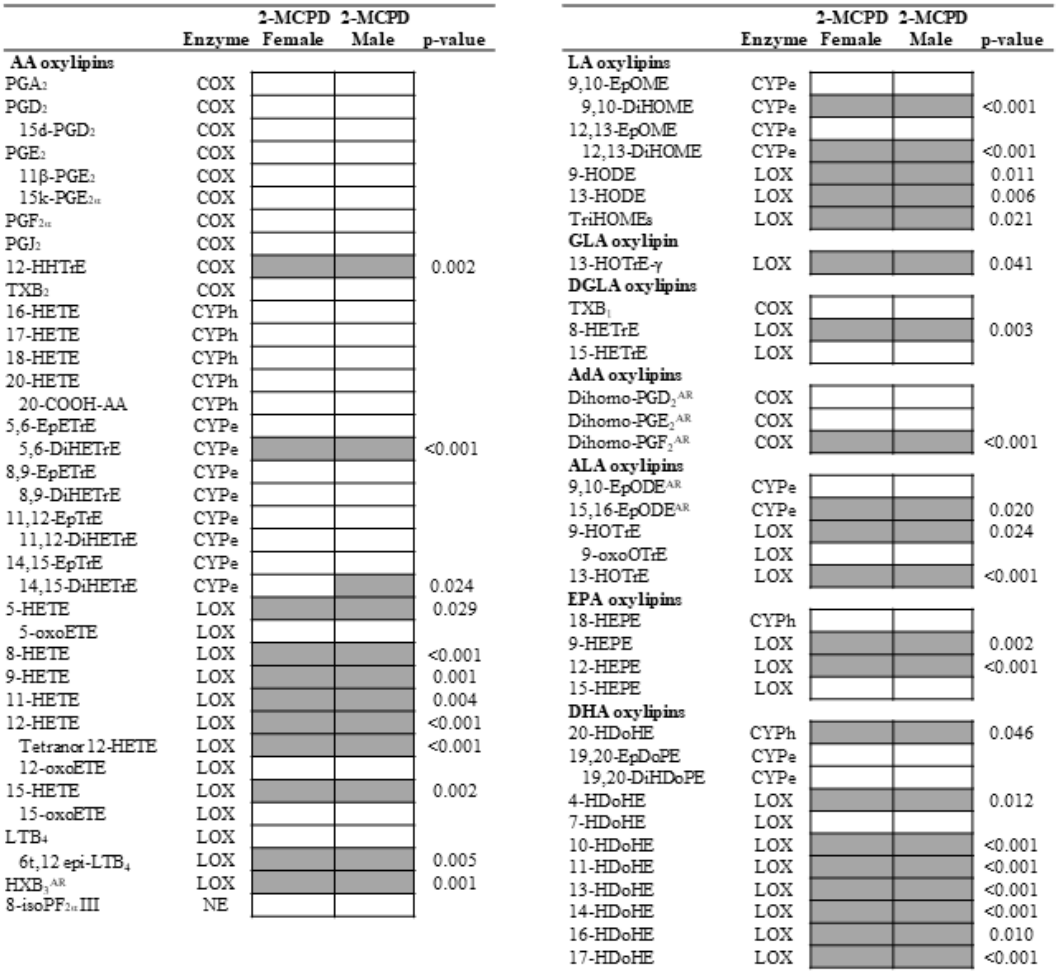


Significantly lower oxylipins are denoted by grey colouring and significantly higher oxylipins are denoted by diagonal lines. Means, SEs, and p-values are shown in Supplemental Table 3 (Appendix 10.4.). AA, arachidonic acid; AdA, adrenic acid; ALA, α-linolenic acid; AR, area ratio; COX, cyclooxygenase; CYPe, cytochrome P450-epoxygenase; CYPh, cytochrome P450-hydroxylase; DGLA, dihomo-γ-linolenic acid; DiHDoPE, dihydroxy-docosapentaenoic acid; DiHODE, dihydroxy-octadecadienoic; DiHOME, dihydroxy-octadecaenoic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; EpDoPE, epoxy-eicosadocosapentaenoic acid; EpODE, epoxy-octadecadienoic acid; EpOME, epoxy-octadecaenoic acid; GLA, γ-linolenic acid; HDoHE, hydroxy-docosahexaenoic acid; HETE, hydroxy-eicosatetraenoic acid; HEPE, hydroxy-eicosapentaenoic acid; HETrE, hydroxy-eicosatrienoic acid; HHTrE, hydroxyheptadecatrienoic acid; HODE, hydroxy-octadecadienoic acid; HOTrE, hydroxy-octadecatrienoic acid; HX, hepoxilin; isoP, isoprostane; LA, linoleic acid; LOX, lipoxygenase; LT, leukotriene; NE, non-enzymatic; oxoEDE, oxo-eicosadienoic acid; oxoETE, oxo-eicosatetraenoic acid; oxoOTrE, oxo-octadecatrienoic acid; PG, prostaglandin; TriHOMEs, 9,10,13- & 9,12,13-trihydroxy-octadecenoic acid (could not be separated); TX, thromboxane.

5.4.4. **Effects of 2-MCPD on Serum Oxylipins**

In the serum, 70 unique oxylipins were quantified and the means, standard errors, and statistics can be found in Supplemental Table 4 (Appendix 10.4.). The 2-MCPD exposure altered 31 oxylipins from control, all of which were lower (Figure 5.4.5). Oxylipins from all PUFA precursors were affected which was reflected in the oxylipin totals by each PUFA precursor (Supplemental Table 4 (Appendix 10.4.)). When examined by enzyme pathway, however, only 2 COX oxylipins were affected (Figure 5.4.5). The amount of one of these, 12-hydroxyheptadecatrienoic acid (HHTrE), represents more than half (59-70%) of the total COX derived oxylipins, such that when 12-HHTrE is not included, the remaining COX oxylipins were not altered by 2-MCPD (Figure 5.4.6A). Similarly, only 1 CYPh product was reduced by 2-MCPD exposure (Figure 5.4.5), which was reflected in the lack of 2-MCPD effect on total CYPh (Figure 5.4.6B). With respect to CYPe, there was an apparent treatment effect, but this was due to the 2 dihydroxy-octadecaenoic acids (DiHOME) (Figure 5.4.6C). When these two DiHOME were removed there was also no overall effect in total CYPe oxylipins (Figure 5.4.6D). The LOX oxylipins, however, were highly affected by 2-MCPD, with 25 of 33 individual LOX oxylipins (Figure 5.4.5) lower in the treatment groups. Interestingly, while most of the LOX pathway hydroxy-oxylipins were decreased, none of the four oxo-oxylipin metabolites quantified were altered (Figure 5.4.5). The total LOX oxylipins had a treatment by sex interaction in which 2-MCPD exposed groups were reduced from control with a greater difference in males than females (Figure 5.4.6E).

Figure 5.4.5 Serum oxylipin alterations from control by 40 mg/kg BW.

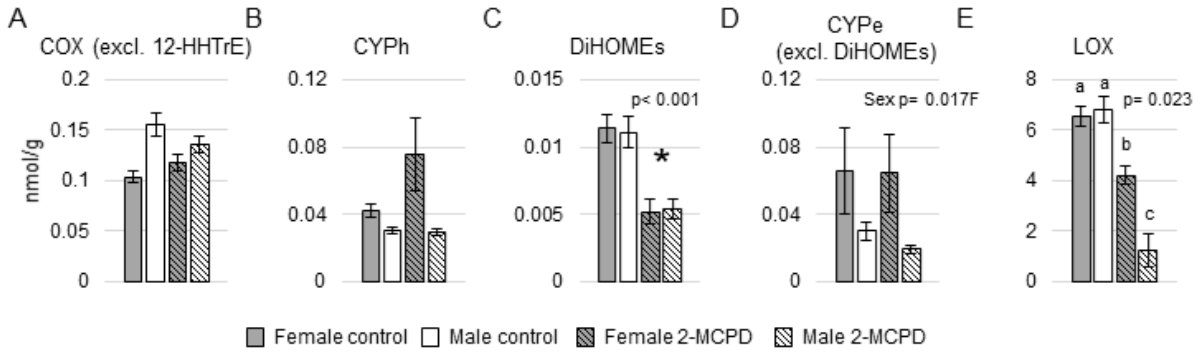


	Enzyme	2-MCPD Female	2-MCPD Male	p-value
AA oxylipins				
PGA_2	COX			
PGD_2	COX			
15d-PGD_2	COX			
PGE_2	COX			
11β-PGE_2	COX			
15k-$PGE_{2\alpha}$	COX			
$PGF_{2\alpha}$	COX			
PGJ_2	COX			
12-HHTrE	COX			0.002
TXB_2	COX			
16-HETE	CYPh			
17-HETE	CYPh			
18-HETE	CYPh			
20-HETE	CYPh			
20-COOH-AA	CYPh			
5,6-EpETrE	CYPe			
5,6-DiHETrE	CYPe			<0.001
8,9-EpETrE	CYPe			
8,9-DiHETrE	CYPe			
11,12-EpTrE	CYPe			
11,12-DiHETrE	CYPe			
14,15-EpTrE	CYPe			
14,15-DiHETrE	CYPe			0.024
5-HETE	LOX			0.029
5-oxoETE	LOX			
8-HETE	LOX			<0.001
9-HETE	LOX			0.001
11-HETE	LOX			0.004
12-HETE	LOX			<0.001
Tetranor 12-HETE	LOX			<0.001
12-oxoETE	LOX			
15-HETE	LOX			0.002
15-oxoETE	LOX			
LTB_4	LOX			
6t,12 epi-LTB_4	LOX			0.005
HXB_3^{AR}	LOX			0.001
8-iso$PF_{2\alpha}$ III	NE			

	Enzyme	2-MCPD Female	2-MCPD Male	p-value
LA oxylipins				
9,10-EpOME	CYPe			
9,10-DiHOME	CYPe			<0.001
12,13-EpOME	CYPe			
12,13-DiHOME	CYPe			<0.001
9-HODE	LOX			0.011
13-HODE	LOX			0.006
TriHOMEs	LOX			0.021
GLA oxylipin				
13-HOTrE-γ	LOX			0.041
DGLA oxylipins				
TXB_1	COX			
8-HETrE	LOX			0.003
15-HETrE	LOX			
AdA oxylipins				
Dihomo-PGD_2^{AR}	COX			
Dihomo-PGE_2^{AR}	COX			
Dihomo-PGF_2^{AR}	COX			<0.001
ALA oxylipins				
9,10-EpODE^{AR}	CYPe			
15,16-EpODE^{AR}	CYPe			0.020
9-HOTrE	LOX			0.024
9-oxoOTrE	LOX			
13-HOTrE	LOX			<0.001
EPA oxylipins				
18-HEPE	CYPh			
9-HEPE	LOX			0.002
12-HEPE	LOX			<0.001
15-HEPE	LOX			
DHA oxylipins				
20-HDoHE	CYPh			0.046
19,20-EpDoPE	CYPe			
19,20-DiHDoPE	CYPe			
4-HDoHE	LOX			0.012
7-HDoHE	LOX			
10-HDoHE	LOX			<0.001
11-HDoHE	LOX			<0.001
13-HDoHE	LOX			<0.001
14-HDoHE	LOX			<0.001
16-HDoHE	LOX			0.010
17-HDoHE	LOX			<0.001

Significantly lower oxylipins are denoted by grey colouring. Means, SEs, and p-values are shown in Supplemental Table 4 (Appendix 10.4.). AA, arachidonic acid; AdA, adrenic acid; ALA, α-linolenic acid; AR, area ratio; COX, cyclooxygenase; CYPe, cytochrome P450-epoxygenase; CYPh, cytochrome P450-hydroxylase; DGLA, dihomo-γ-linolenic acid; DiHDoPE, dihydroxy-docosapentaenoic acid; DiHODE, dihydroxy-octadecadienoic; DiHOME, dihydroxy-octadecaenoic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; EpDoPE, epoxy-eicosadocosapentaenoic acid; EpODE, epoxy-octadecadienoic acid; EpOME, epoxy-octadecaenoic acid; GLA, γ-linolenic acid; HDoHE, hydroxy-docosahexaenoic acid; HETE, hydroxy-eicosatetraenoic acid; HEPE, hydroxy-eicosapentaenoic acid; HETrE, hydroxy-eicosatrienoic acid; HHTrE, hydroxyheptadecatrienoic acid; HODE, hydroxy-octadecadienoic acid; HOTrE, hydroxy-octadecatrienoic acid; HX, hepoxilin; isoP, isoprostane; LA, linoleic acid; LOX, lipoxygenase; LT, leukotriene; NE, non-enzymatic; oxoEDE, oxo-eicosadienoic acid; oxoETE, oxo-eicosatetraenoic acid; oxoOTrE, oxo-octadecatrienoic acid; PG, prostaglandin; TriHOMEs, 9,10,13- & 9,12,13-trihydroxy-octadecenoic acid (could not be separated); TX, thromboxane.

Figure 5.4.6 Effect of 40 mg/kg BW 2-MCPD on serum oxylipins totalled by enzyme pathway A) COX oxylipins excluding 12-HHETrE, B) CYP hydroxylase oxylipins, C) CYP expoxygenase oxylipins excluding 9,10- and 12,13-DiHOME, D) 9,10- and 12,13-DiHOME, E) Lipoxygenase oxylipins.



F/M indicates females or males significantly higher, respectively. 12-HHTrE, 12-hydroxy-5,8,10-hydroxyheptadecatrienoic acid; COX, cyclooxygenase; CYPh, cytochrome P450 hydroxylase; DiHOME, dihydroxy-octadecaenoic acid; LOX, lipoxygenase.

5.4.5. Effects of 2-MCPD on Tibialis Anterior

In the TA, 47 unique oxylipins were quantified and the means, standard errors, and statistics can be found in Supplemental Table 5 (Appendix 10.4.). There was only one oxylipin significantly altered due to 2-MCPD exposure in the TA, 5-oxoETE and this was only in the female group (Supplementary Table 5 (Appendix 10.4.)).

5.4.6. Effects of Sex on Oxylipins

In the heart, 2-MCPD exposure affected oxylipins similarly in males and females, as evidenced by the absence of interaction effects. There were also few main effects of sex, with 4 of 57 oxylipins displaying a sex difference, 3 higher in males and 1 in females (Supplemental Table 2 (Appendix 10.4.)). In the kidney, 2-MCPD exposure generally had similar effects between males and females, with only 3 interaction effects. There were also few main sex effects, with 5 of 65 individual oxylipins being higher in males, and 2 oxylipins (both DHA derived) being higher in females. Despite the few sex effects present, the totals for LA, n-6, total, and LOX oxylipins were higher in males. These totals were highly influenced by the relative abundance of two LA LOX derived oxylipins (9-hydroxyoctadecaenoic acid (HODE) and 13-oxo-octadecadienoic acid) which were higher in males (Supplemental Table 3 (Appendix 10.4.)). In the serum, there were only 3 treatment by sex interaction effects only for individual oxylipins. The most main sex effects were observed in the serum. Of the 70 oxylipins, 7 were higher in males and 32 higher in females. Of the 7 oxylipins higher in males, 5 were from the COX pathway, and this was reflected in total COX oxylipins being higher in males. The total CYP, ALA, DHA, and total n-3 oxylipins were higher in females (Supplemental Table 4 (Appendix 10.4.)). In the TA, there was only 1 treatment by sex interaction to 5-oxoETE, and 7 of 47 individual oxylipins displayed a

main effect of sex, with all being higher in males and being derived from n-6 PUFA (Supplementary Table 5 (Appendix 10.4.)).

5.4.7. **Non-enzymatically Produced Oxo-lipids**

In the heart, kidney, and TA, 5 OxPC were quantified in each tissue (Table 5.4.1) and were not altered by treatment or sex. In the serum and kidney, 1 and 2 isoP were quantified, respectively, and similarly were unaltered by 2-MCPD exposure in each tissue (Figure 5.4.4 & Figure 5.4.5).

Table 5.4.1 Area ratios of oxidized phosphatidylcholines in the heart, kidney, and TA of rats fed control and 40 mg/kg BW 2-MCPD.

Oxidized Phospholipids (AR/g tissue)				
	Control Female	**Control Male**	**40 mg/kg 2-MCPD Female**	**40 mg/kg 2-MCPD Male**
Heart				
4-oxo-butyryl-PC	189±25.1	232±30.6	171±17.7	148±24.5
PGPC	338±88.6	420±46.2	486±83.3	382±68.6
PONPC	62.0±1.04	77.5±9.41	64.0±8.47	64.1±9.43
POVPC	587±67.8	849±117	645±78.9	608±76.0
Succinoyl-PC	85.5±18.5	101±5.14	94.4±14.5	74.6±11.2
Kidney				
4-oxo-butyryl-PC	108±23.8	100±14.0	70.6±19.9	103±17.2
PGPC	2.45±0.712	5.05±1.47	5.99±0.818	3.24±0.763
PONPC	53.4±17.1	75.8±16.4	82.7±8.10	51.6±12.9
POVPC	358±66.4	654±138	464±37.9	508±97.4
Succinoyl-PC	6.50±1.62	9.27±2.02	11.8±1.13	7.34±1.51
Tibialis Anterior				
4-oxo-butyryl-PC	850±223	555±84.6	706±93.0	698±116
PGPC	2625±739	2588±597	3265±530	3564±711
PONPC	423±102	254±48.1	416±57.4	409±66.2
POVPC	3643±941	3036±582	3587±543	4173±696
Succinoyl-PC	631±169	486±114	662±99.0	582±110

No significant differences occurred due to treatment or sex. PC, phosphatidylcholine; PGPC, 1-palmitoyl-2-glutaryl-sn-glycero-3-phosphatidylcholine; PONPC, 1-palmitoyl-2-oxononanoyl-sn-glycero-3-phosphatidylcholine; POVPC, 1-palmitoyl-2-(5-oxovaleryl)-sn-glycero-3-phosphatidylcholine.

5.5. Discussion

This study demonstrates that 2-MCPD alters oxylipins in the serum and in tissues that display physiological changes in response to exposure to this food contaminant. The heart was the tissue most affected by 2-MCPD exposure in this study, with extensive necrosis and vacuolation of the ventricles and septa. There was an inflammatory component to the cardiac lesions with increased NICI and a mild increase in inflammation. Our findings of a pattern of lower n-3 oxylipins in the hearts of the 2-MCPD treatment groups may help to explain the pathological effects observed. The process of inflammation occurs in two waves, an initial wave mediated by AA derived proinflammatory oxylipins, later followed by inflammation resolution mediated by n-3 derived SPM [101]. While there is little known of the ALA oxylipins which were decreased in the heart, more is known of the DHA oxylipins which were affected, particularly 14- and 17-hydroxy-DHA (HDoHE), which have anti-inflammatory functions through the inhibition of platelet aggregation and tumor necrosis factor α (TNFα) [102], [103]. Additionally, the SPM maresin-1 and -2 share the common precursor 14-hydroperoxy-DHA with 14-HDoHE, and protectin-1 and -X and resolvins D1-6 share the common precursor 17-hydroperoxy-DHA with 17-HDoHE. Thus, 14- and 17-HDoHE are activation markers of the SPM (which were below the LOD) and their decrease in the 2-MCPD exposed hearts indicates a possible decrease in SPM metabolites. While there were no increases in AA COX oxylipins classically associated with inflammation, the observed decrease in the inflammation resolving DHA oxylipins could allow for conditions of prolonged inflammation described in the histopathology. In addition to impaired resolution of inflammation, a decrease in SPM would also inhibit apoptotic cell clearance and tissue repair and regeneration [101], consistent with previous genomic and proteomic data [5], [41], and allow for

the progression of cardiac lesions. If this is the case, it would be of interest to test the efficacy of n-3 supplementation on mitigating 2-MCPD toxicity.

Nephrotoxicity from 2-MCPD exposure has been reported previously in rats [5], [56]. In this study, however, the only reported effect of 2-MCPD exposure to the kidney was elevated organ weight; the histopathology analysis found no pathological lesions in the kidneys. Thus, the dose provided in our study was enough to increase kidney weight, but not enough to develop lesions, suggesting that there was a successful homeostatic response. If we compare the effects of 2-MCPD exposure on the heart and the kidney, we see that the heart, which had pathological changes, only had reduced oxylipins with an effect primarily on the n-3 oxylipins; while in the kidney, there were no pathological changes and few oxylipin alterations that lacked an apparent pattern. The alterations in individual oxylipins in spite of a lack of nephrotoxic progression (elevated tissue weight but no lesions) may suggest that these oxylipins could serve as biomarkers of early 2-MCPD toxicity, but this remains to be investigated.

Our analysis of the serum oxylipins found 2-MCPD exposure decreased oxylipins from all PUFA, but primarily those derived from LOX. The LOX oxylipins are diverse in function; for instance, the AA LOX derived leukotrienes and HETE generally have pro-inflammatory function, while the DHA LOX derived HDoHE and SPM are anti-inflammatory [6], [101]. The decrease with 2-MCPD exposure occurred primarily to the hydroxy-oxylipins, but not the oxo-oxylipins, derived from LOX. A broad decrease in systemic LOX oxylipins may represent an impairment of LOX function due to 2-MCPD exposure. However, the effect to primarily the hydroxy-oxylipins may also be due to increased clearance of these oxylipins in response to 2-MCPD. One COX oxylipin which was reduced by 2-MCPD exposure was 12-HHTrE. This effect was not likely a decrease in 12-HHTrE production because TXB_2 levels were not altered.

TXB_2 is derived from TXA_2, which is produced simultaneously with 12-HHTrE, via TXA synthase metabolism of PGH_2 [104]. Thus, the decrease in 12-HHTrE could be due to increased degradation by 15-hydroxyprostaglandin dehydrogenase, an enzyme that oxidizes 12-HHTrE and the LOX derived 11-HETE, 15-HETE and 13-HODE, which were also decreased with 2-MCPD exposure [98], [105]. Similarly, an increase in the activity of other dehydrogenases, such as 5- and 12-hydroxyeicosanoid dehydrogenase, which oxidize 5- and 12-HETE, respectively [98], could account for the decrease in these LOX products and the concomitant lack of reduction in their metabolites, the oxo-oxylipins. Another enzyme that could contribute to this lack of change in the oxo-ETE is CYP2S1, which is thought to be involved in toxin metabolism and is induced by dioxin and polycyclic aromatic hydrocarbons (PAH). *In vitro* it converts the hydroperoxy-ETE, the precursors of HETE, directly to oxoETE [84].

The extensive oxylipin alterations in the serum indicates their potential utility as readily accessible biomarkers of 2-MCPD toxicity. As seen in the kidneys, oxylipin alterations can occur at levels below that which result in non-neoplastic lesions, which makes them ideal biomarkers. It will be pertinent to determine whether changes to oxylipins in the blood also occur at lower exposure doses, which would further support their use as potential biomarkers.

In our analysis of the TA, oxylipins were essentially unaffected by 2-MCPD exposure. Previous studies have reported myotoxicity due to 2-MCPD exposure [5], [56], but the lack of oxylipin alterations combined with an absence of histopathological findings suggests a NOAEL of up to 40 mg/kg BW 2-MCPD in the skeletal muscle.

Oxidative stress has been postulated as a factor in the MoA of 2-MCPD toxicity. Proteomic studies have found that in rat kidney, liver, and testes, non-toxic exposures of 3-MCPD (10 mg/kg BW, 28-days) resulted in the oxidative inactivation of DJ-1/PARK7, an endogenous redox

regulating protein [40]–[43]. The effect of 2-MCPD on DJ-1 deregulation, however, was less potent than 3-MCPD [40], [41]. The oxidation of DJ-1 is thought to occur via reactive oxygen species produced due to the presence of MCPD [40]–[44]. Our findings of a lack of alteration in the OxPC of the heart, kidney, and TA, and the isoP quantified in the kidney and serum, indicate that 2-MCPD exposure did not increase oxidative stress in these tissues in the current study. These findings are consistent with the lack of changes in serum 4-HNE, 8-OHDG, and nitrotyrosine with 2-MCPD exposure also observed in this study.

In conclusion, the altered oxylipins in the heart, kidney, and serum with 2-MCPD exposure add to the weight of the evidence for 2-MCPD toxicity. Also, this study illustrates the potential for the use of oxylipins as biomarkers for 2-MCPD exposure. Currently, there is an established TDI for 3-MCPD; however, this study indicates that 2-MCPD should also be included when considering the overall hazard of dietary CP.

6. Discussion

For the discussion of the targeted oxo-lipidomic analysis conducted, see Section 5.5.. This chapter will directly address the hypotheses and objectives of this thesis and expand on the limitations and future directions not discussed in the manuscript.

6.1. Discussion of Hypotheses

The main hypothesis of this study was that 2-MCPD treatment would alter the oxo-lipid profiles of the heart, kidney, serum, and TA. To address this, a targeted oxo-lipidomic profiling of heart, kidney, serum, and TA was performed on rats fed control or 40 mg/kg BW 2-MCPD for 90 days. When compared, it was found that the oxylipin profiles of the heart, kidney, and serum were altered by 2-MCPD treatment, while those of the TA were not. Also, none of the OxPC were significantly altered by 2-MCPD treatment. These findings indicate that 2-MCPD exposure can alter oxylipin profiles. From the histopathology data, the heart experienced cardiotoxic effects at the 40 mg/kg dose; and at the same dose, oxylipins were differentially altered. The serum had the most oxylipin alterations with 2-MCPD treatment, with decreases primarily to the LOX hydroxy-oxylipins. While in the kidneys, wet weights were increased in the 2-MCPD (40 mg/kg) group, but no differences in pathology were noted between the control and 40 mg/kg 2-MCPD. Kidney oxylipins were, however, altered with treatment of 40 mg/kg 2-MCPD. This illustrates the potential use for oxylipins as sensitive biomarkers for 2-MCPD exposure. The TA (skeletal muscle), which did not show signs of myotoxicity, appears to be a less sensitive tissue to the effects of 2-MCPD. The lack of oxylipin alteration at 40 mg/kg 2-MCPD dose suggests that the TA is more resistant to alteration of lipid metabolism.

It was also hypothesized that: (i) the AA derived prostanoids would be elevated and the EPA and DHA derived SPM would be decreased with 2-MCPD treatment, due to increased inflammation.

In the heart inflammation was found to be increased with 2-MCPD treatment, inflammation of both ventricles and septum of females was significantly increased with 40 mg/kg 2-MCPD, while there was a trend towards increased inflammation in males. Also, NICI was increased in both ventricles and septa of females and males receiving 40 mg/kg 2-MCPD. While the AA prostanoids, typically associated with inflammation, were not elevated with 2-MCPD in any tissue, there was a decrease in the n-3, and specifically DHA, oxylipins. This decrease in DHA oxylipins could prevent the resolution of inflammation, as discussed in Section 5.5.

Another hypothesis of this study was: (ii) 2-MCPD treatment would increase the isoP and OxPC due to elevated oxidative stress. Oxidative stress is considered to contribute to the toxic effects of 2-MCPD. However, the lack of alteration to the biomarkers oxidative stress, the isoP and OxPC, in the 2-MCPD treated rat hearts, in this study, indicates that any potential oxidative stress mechanisms in the cardiac tissues is independent of isoP and OxPC levels.

It was also hypothesized that: (iii) the oxo-lipid alterations would differ within tissues by sex. From Section 5.4.6., while many sex differences between oxylipins occurred, there were few treatment by sex interactions. The heart, which had no oxylipin interaction effects, also had comparable effects between 40 mg/kg 2-MCPD treatment groups in terms of elevated tissue weight and histopathological results. In the kidney, of the 8 oxylipins that were altered, 3 interaction effects occurred. In the serum, 3 interaction effects occurred to individual oxylipins, and the LOX total oxylipins was decreased in females and further decreased in males. Thus, interaction effects did occur; however, they were few, and indicate the effects of 2-MCPD treatment did not greatly differ between sexes.

Finally, it was hypothesized that: (iv) 2-MCPD treatment would specifically alter the CYP epoxides and diols as seen in other xenobiotic exposure studies. In this study, the CYP epoxides

and diols were largely unaffected in the heart, kidney, and TA. In the serum, 5/16 CYP epoxides or diols were altered. The lack of effect to the heart, which had pathological effects from 2-MCPD treatment, indicates that alteration to CYP metabolism seen in other studies did not occur. A major difference between this study and the previous studies that analyzed oxylipins with As(III) or PCB exposures was that the mode of delivery to animals was through direct injections and not exposed through the diet, suggesting toxicokinetic and toxicodynamic differences. The affect to CYP metabolism in the serum, however, may be of interest and would require further investigation to elucidate.

6.2. Strengths and Limitations

There were both strengths and limitations to the study design. First, the use of OECD TG-408 in this study provided a standardized procedure that is widely accepted in toxicology and this data will contribute to the literature. By following the OECD guidelines, there is less impetus for other groups to do similar studies and ultimately fewer animals will need to be sacrificed. This study was the first 90-day oral toxicity study that used 2-MCPD. Thus, the duration was a strength in that it advanced the literature by providing data on a sub-chronic oral exposure of 2-MCPD. However, effects of exposure over the lifetime of rats or in specific vulnerable windows of lifetime such as pregnancy, prenatal/neonatal stages of development, juvenile stages could not be derived from the OECD TG-408, and hence may be considered as a limitation. The use of free 2-MCPD was a strength of this study because it allowed for a direct assessment of the 2-MCPD toxicity. Much of the CP in foods exist esterified to fatty acids; thus, the use of free 2-MCPD could also be viewed as a limitation. The use of free 2-MCPD, however, was justified because it has been shown that CP are readily hydrolyzed by gastric lipases. The use of only 2-MCPD, instead of a mixture of 2-MCPD and 3-MCPD, could also be viewed as a limitation

because the mixture would better represent how dietary CP are consumed. However, the effects of 2-MCPD alone need to be elucidated before addressing mixtures.

This study used state of the art LC-MS/MS techniques to scan for over 150 oxylipins and 9 OxPC. This combination of oxylipin and OxPC analysis is an advancement in the methodology and is a strength of this study. This is also the first study to investigate oxylipin and OxPC with CP exposure.

For the purpose of testing this novel idea of addressing the effects of CP in modulating oxylipins, only control (0 mg/kg) and 40 mg/kg BW 2-MCPD groups were selected and 4 different organs (including serum) were subjected to analyses. The choice of utilizing the highest dose of 40 mg/kg BW 2-MCPD tested in the Health Canada 90-day exposure study was a strength because it provided both a dose in which pathological changes were seen in the heart and potentially a sub-toxic dose for the kidney and TA (lack of changes in lesions). This provided an opportunity to profile oxylipins in the heart which had clearly been affected by 2-MCPD and this analysis will add to the weight of the evidence for 2-MCPD toxicity as well as provide data that will contribute to the elucidation of the MoA. The use of only one treatment dose in the oxylipin analysis, is a limitation of this study as dose-response could not be derived. This will be an area for future study, as tissues from both male and female rats subjected to the other three lower doses are available for investigation.

The dose used in this study, however, was much higher than what a human would consume. The highest estimated intake of 11 ug/kg BW 2-MCPD (see Section 2.5.1.) is 3-orders of magnitude less than the 40 mg/kg BW treatment used. Typically, JECFA calculates a TDI they will typically apply an uncertainty factor of at least 100-fold to the benchmark dose level ($BMDL_{10}$) derived from these regulatory 28- or 90-day exposure studies, all bringing the dose into a more

relevant range. Additionally, one consideration of future studies is to address concurrent exposure of the two isomers – 2- and 3-MCPD in the ratio they naturally exist in foods.

A limitation of this study was that enzyme activity was not measured. Many of the oxylipin alterations indicate changes in enzyme activity; however, it is not clear whether enzymatic changes occur upstream or downstream of a metabolite. In other words, if an oxylipin is altered it is not clear whether its production has been altered or if its conversion to a new molecule has been changed, or both.

A limitation in the interpretation of the oxylipin data is that some oxylipins are produced by multiple enzyme pathways. For instance, 11- and 15-HETE and 5-, 12-, and 15-oxoETE are primarily produced by LOX oxidation of AA to form HpETE acids and then converted to HETE, followed by dehydrogenase oxidation to oxoETE [6], [98]; however, the HETE can also be produced by lipid peroxidation [106]. COX has also been shown to be able to convert minor amounts of 11- and 15-HpETE to 11- and 15-HETE, respectively [98], and the production of 5-, 12-, and 15-oxoETE has also been shown to be possible by direct conversion from HpETE by CYP [84]. So, for the purposes of interpretation of the data it was assumed that oxylipins were only derived from their primary enzyme pathway in order to create oxylipin enzyme pathway totals.

A limitation of this study, in reference to deriving conclusions on oxylipins profiling, was the use of corn oil instead of soy oil in the preparation of the AIN-93G diet, for standardization with previous toxicological studies. The use of corn oil in the rat diet is considered to be deficient in the essential fatty acid ALA, and its use in rat diets was changed to soy oil when the AIN-76 diets were updated to AIN-93 diets [107]. As a consequence of the use of dietary corn oil in this study, the oxylipin profiles were low in n-3 derived oxylipins when compared to previous studies

that profiled oxylipins in rats with a control diet fed adequate ALA [9], [14]. The rat heart oxylipin profile measured by [14], quantified 6 ALA, 8 EPA, and 16 DHA derived oxylipins. Compared to this study, in the heart, only 5 ALA, 0 EPA, and 6 DHA derived oxylipins were quantified. Also, by comparison of the more abundant n-3 oxylipins, the amounts of the n-3 oxylipins quantified were lower in this study. There was approximately 50% of the 9-hydroxy-octadecadienoic acid (HOTrE), 15% of the 20-HDoHE, and 5% of the 17-HDoHE, in the heart of this study compared to [14]. In the kidney, [9] quantified 4 ALA, 7 EPA, and 11 DHA oxylipins. While in this study, there were 5 ALA, 0 EPA, and 8 DHA oxylipins, with approximately 5% of the 9-HOTrE, 1% of the 20-HDoHE, and 10% of the 17-HDoHE. In the serum, [9] quantified 4 ALA, 7 EPA and 13 DHA oxylipins. While in this study, there were 5 ALA, 4 EPA, and 11 DHA oxylipins, with approximately 40% of the 9-HOTrE, similar 12-hydroxy-eicoapentaenoic acid (HEPE), 15% of the 15-HEPE, similar 20-HDoHE, and 25% of the 17-HDoHE. Thus, the number and amount of n-3 oxylipins was lower in our study compared to rats fed adequate ALA, variably by tissue. It should be noted that some of the variation in oxylipins may be due to differences in rat strains (F344 vs. Sprague-Dawley). The differences n-3 oxylipins are particularly relevant to our findings of decreased n-3 oxylipins in the heart with 2-MCPD exposure. Because of the lack of EPA oxylipins, whether this effect actually applies to EPA oxylipins, and not just the ALA and DHA oxylipins quantified in this study, could not be determined.

6.3. Future Directions

Now that this study has established oxylipins are altered by 2-MCPD exposure, it will be prescient to determine the oxylipin alterations at lower doses. This will elucidate whether there is a dose-response relationship for the oxylipins altered. It will also be of value to determine if

there are oxylipin alterations at doses below that of which pathological effects were observed. If oxylipins are altered in the heart at sub-toxic doses this could be informative of the early and specific changes directly related to the MoA, and not of unspecific signalling due to cell death or systemic toxicity.

To better understand the why the oxylipin alterations occurred, it will also be useful to investigate the enzymes that produce oxylipins. There are multiple approaches that could be used, such as gene expression, proteomics, or enzyme activity. The enzymes that will be of particular interest are PLA_2, LOX, CYP, and the various hydroxy-lipid dehydrogenases. Alterations to any of these enzymes' expression or activity could elucidate oxylipin differences, particularly whether oxylipins are altered due to changes in their production or turnover, providing additional explanatory insight to the 2-MCPD MoA.

Another future direction will be to use a CP exposure more like that which humans would be exposed to in the diet. This could entail exposing rats to a mixture of 2- and 3-MCPD. Some chemical mixtures have been shown to have synergistic toxic effects [108], [109]. Thus, whether this is the case for CP will be pertinent for regulatory hazard assessment, as these compounds are known to occur simultaneously in many foods [61]–[64].

To expand on the finding of decreased n-3 oxylipins with 2-MCPD exposure, a future study could test whether augmenting various dietary n-3 PUFA blunt the cardiotoxic effects of 2-MCPD. If there was a protective effect from an n-3 PUFA this could provide impetus for its inclusion in foods that are high in 2-MCPD or for its supplementation by high 2-MCPD consumers. This would also be directly relevant to infant formula, which, as described in section 2.5.1., when exclusively fed can provide potentially hazardous exposure to 2-MCPD. However,

infant formula also contains DHA and whether or not that may have a protective effect against 2-MCPD toxicity remains to be elucidated.

7. Conclusions and Implications

This study found the oxylipin profiles of heart, kidney, and serum to be altered by 90-day sub-chronic dietary 2-MCPD exposure, while the oxylipin profiles of the TA, and the OxPC in any tissue were not altered. The role of oxidative stress in the MoA of 2-MCPD is unclear, due to a lack of alterations to the OxPC and isoP quantified. The primary target organ based on histopathological data of 2-MCPD toxicity was the heart. This study found 2-MCPD exposure lowered n-3 oxylipins in the heart, which could decrease the capacity for inflammation resolution and wound healing, allowing for lesion progression. In the kidney, 2-MCPD exposure altered few oxylipins and with no apparent pattern. These alterations occurred at a dose at which pathological effects did not occur and are potentially indicative of early toxicity. The serum oxylipin profile was substantially altered by 2-MCPD exposure, and the accessibility of blood makes serum oxylipins appealing potential biomarkers of 2-MCPD toxicity. This study adds to the weight of the evidence for 2-MCPD toxicity and justifies the consideration of 2-MCPD in addition to 3-MCPD when refined vegetable oils and their products are manufactured and consumed. This study also provides impetus for the establishment of a TDI for 2-MCPD and subsequently for regulations to be placed on the allowable concentrations of 2-MCPD in foods.

8. References

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9. Appendices

9.1. Health Canada Study Ethics Approval

PROTOCOL FOR PROJECTS INVOLVING THE USE OF LABORATORY ANIMALS
ÉVALUATION DE PROTOCOLES DE RECHERCHES COMPORTANT L'UTILISATION D'ANIMAUX DE LABORATOIRE

HCD-ACC PROTOCOL NO
N° DE PROTOCOLE DE C.P.A.
2017-008

HC-Protected

Please refer to the HCD-ACC Policies and Procedures for additional information regarding the protocol approval process. This document can be found under "References" in the LAP database. / Veuillez vous référez au document des politiques et procédures pour de l'information additionelle relative au processus des approbations des protocoles. Vous trouverez ce document sous la rubrique "référence" dans la base de donnée du PAL

Title of project - Titre du projet	Planned Starting Date Date approximative du projet
90-day subchronic study of 2-MCPD in F344 rats	2017-03-03

SECTION 1 - INVESTIGATOR DATA - DONNÉES SUR LE CHERCHEUR PRINCIPAL		
Responsible Investigator - Chercheur principal Jayadev Raju	Telephone number Office - Bureau (613) 957-1233 Email jayadev.raju@hc-sc.gc.ca	Laboratory Animal User Training completion date Date d'achèvement de formation destiné aux utilisateurs d'animaux 2010-11-05
Organization HC-HPFB-FD-BCS	Unit - Division REGULATORY TOXICOLOGY RESEARCH DIVISION	
Research Staff - Personnel de Recherche Jennifer Roberts	Telephone number Office - Bureau (613) 957-0997	Laboratory Animal User Training completion date Date d'achèvement de formation destiné aux utilisateurs d'animaux 2009-12-18
Collaborator - Collaborateur(trice)	Telephone number Office - Bureau	Laboratory Animal User Training completion date Date d'achèvement de formation destiné aux utilisateurs d'animaux
Statistician - Statisticien(ne) Cunye Chao	Telephone number Office - Bureau (613) 954-6512	
Research ACC Member - Nom du membre du C.P.A. Ivan Curran	Telephone number - N° de téléphone Office - Bureau (613) 957-3052	
Responsible Manager - Gestionnaire Responsable Rekha Mehta	Telephone number - N° de téléphone Office - Bureau (613) 957-0988	

SECTION 2 - RELATED PROTOCOL - PROTOCOLE APPARENTÉ	
a) Has this or similar protocol been approved in the past? __ Yes ☐ No	Est-ce que ce projet ou un projet similaire a déjà été approuvé dan le passé? Oui ☐ Non
b) If yes, provide previous protocol number	Si oui, fournir le numéro de protocole précédent

SECTION 3 - ALTERNATE METHODOLOGIES - MÉTHODOLOGIES DE REMPLACEMENT	
Have alternate methodologies which address the 3-R philosophy for the project been explored? In the case of regulatory protocol the 3-R	A-t-on étudié la possibilité d'autres méthodologies respectant la philosophie des 3-R pour un tel projet? Dans le cas d'un protocole faisant l'objet d'une réglementation, la philosophie des 3-R doit

9.2. Oxo-Lipidomic Analysis Method Optimization

Method optimization was performed on rat serum, kidney, heart and TA in order to ensure that the maximum number of analytes were within the LOQ, without any exceeding the upper limit of the LC-MS/MS, nor outside of the linear range. The linear range refers to the theoretical linear relationship between the amount of an analyte per sample and the intensity of its response. The linear relationship can break down, however, as the amount of tissue used gets too low or high. When attempting to maximize the number of quantifiable analytes, as the amount of tissue used is increased there will eventually be a plateauing effect potentially due to factors, such as increased noise or analyte loss. To determine ideal volumes of tissue homogenates or serum to be used, a range of sample volumes with equal amounts of internal standard mixture (IS) were run through the solid phase extraction (SPE) procedure and then analyzed by LC-MS/MS. The IS was previously optimized so the concentration of each standard it contained would be in an ideal range given the LC-MS/MS injection volume. Analyte responses were assessed to ensure the parameters were met and homogenate volumes were selected for each tissue.

For heart and TA, an extra step was performed to decrease undissolved particulates in the samples after SPE, because muscle samples had previously clogged the LC-MS/MS column. The samples eluted in methanol were put through a column of $NaSO_4$ to filter out fibrous material and then evaporated and resuspended in (70:30:0.02; v/v/v) water: acetonitrile: acetic acid.

9.3. Oxidized Phospholipid Method peak determination

The OxPC method is a new method in which oxylipins and OxPC can be analyzed from the same sample using a second injection into the LCMS. The retention times for the OxPC had not yet been determined for the method and had to be determined. To do, this standards of 1-palmitoyl-2-(5-oxovaleryl)-sn-glycero-3-phosphatidylcholine (POVPC), 1-palmitoyl-2-glutaryl-sn-glycero-3- phosphatidylcholine (PGPC), 1-palmitoyl-2-oxononanoyl-sn-glycero-3-phosphatidylcholine (PONPC), 1-palmitoyl-2-azelaoylsn-glycero-3-phosphocholine (PAzPC), 1-palmitoyl-2-(4-keto-dodec-3-ene-dioyl)phosphatidylcholine (KDdiA-PC) were run. Oxidized 1-palmitoyl-2-docosahexaenoyl-sn-glycero3-phosphocholine (PDHPC) was used to determine retention times for its oxidation products 4-oxo-butyryl-PC and succinoyl-PC; and oxidized 1-stearoyl-2-arachidonoyl-sn-glycero-3-phosphocholine (SAPC) was used to determine retention times for its oxidation products 1-stearoyl -2-(5-oxovaleryl)-sn-glycero-3-phosphatidylcholine (SOVPC) and 1-stearoyl-2-glutaroyl-sn-glycero-3-phosphocholine (SGPC). The amounts of these molecules could not yet be quantified as the slopes of the linear range for each OxPC analyte have not been determined for this method. Thus, the OxPC were reported as relative amounts.

9.4. Manuscript Supplemental Tables

Tissue	Oxolipids scanned for but not quantified
Heart	**Oxylipins < LOD:** 10-Nitrooleate; 11β-$PGF_{2\alpha}$; 11β-dhk$PGF_{2\alpha}$; 11d-TXB_2; 11-HDoHE; 11-HEPE; 12,13-DiHODE; 12-oxoLTB_4; 13-oxoOTrE; 14,15-DiHETE; 14,15-EpETE; 14,15-LTC4; 14,15-LTE4; 15d-PGA_2; 15d-PGJ_2; 15-HEPE; 15k-PGD_2; 15k-$PGF_{1\alpha}$; 15k-$PGF_{2\alpha}$; 15R-LXA_4; 15t-PD1; 16,17-DiHDoPE; 16,17-EpDoPE; 17,18-DiHETE; 17,18-EpETE; 17-HETE; 17d-6k-$PGF_{1\alpha}$; 17k-DHA; 17k-DPA; 18-HEPE; 19,20-DiHDoPE; 19,20-EpDoPE; 19-HETE; 2,3-dinor-11β-$PGF_{2\alpha}$; 2,3-dinor-8-iso$PF_{2\alpha}$; 2,3-dinor-TXB_2; 2,3-dinor-6k-$PGF_{1\alpha}$; 20-COOH-LTB4; 20-HETE; 20-OH-LTB_4; 4k-DHA; 5-HEPE; 5-HETrE; 6k-PGE_1; 6t-LTB_4; 6t,12-epiLTB_4; 7-HDoHE; 7R-Maresin-1; 8-HDoHE; 8-HEPE; 8-iso-15k-$PGF_{2\beta}$; 8-iso$PF_{3\alpha}$; 9-Nitrooleate; dh-$PGF_{2\alpha}$; dhk-PGD_2; dhk-PGE_2; dhk-$PGF_{2\alpha}$; dihomo-15d-PGD_2; dihomo-PGD_2; dihomo-PGE_2; dihomo-$PGF_{2\alpha}$; dihomo-PGJ_2; HXA_3; LTC_4; LTD_4; LTE_4; LXB_4; PD1; PDX; PGA_2; PGB_2; PGD_1; PGD_3; PGE_1; PGE_3; $PGF_{1\alpha}$; $PGF_{3\alpha}$; PGJ_2; PGK_1; PGK_2; RvD1; RvD2; RvD5; RvE1; TXB_3. **LOD < Oxylipins < LOQ:** 12-HEPE; 13-HDoHE; 13-HOTrE; 14,15-LTD_4; 15,16-DiHODE; 15,16-EpODE; 18-HETE; 5,15-DiHETE; 5,6-DiHETE; 5-iso$PF_{2\alpha}$VI; 6,15-dk,dh-$PGF_{1\alpha}$; 8,15-DiHETE; 8-HETrE; 8-iso$PF_{2\alpha}$III; 9-HEPE; bicyclo-PGE_2; LXA_5; $PGF_{2\alpha}$. **OxPC < LOD:** KDdiaPC; SOVPC; SGPC. **LOD < OxPC < LOQ:** PAzPC.
Kidney	**Oxylipins < LOD:** 10-Nitrooleate; 11β-$PGF_{2\alpha}$; 11β-dhk-$PGF_{2\alpha}$; 11-HEPE; 12,13-DiHODE; 12,13-EpODE; 12-oxoLTB_4; 13-oxoOTrE; 14,15-diHETE; 14,15-EpETE; 14,15-LTC_4; 14,15-LTE_4; 15d-PGA_2; 15d-PGJ_2; 15k-PGD_2; 15k-$PGF_{1\alpha}$; 15-oxoEDE; 15R-LXA_4; 15t-PD1; 16,17-DiHDoPE; 16,17-EpDoPE; 17,18-DiHETE; 17,18-EpETE; 17d-6k-$PGF_{1\alpha}$; 17k-DHA; 17k-DPA; 19-HETE; 2,3-dinor-11β-$PGF_{2\alpha}$; 2,3-dinor-8-iso$PF_{2\alpha}$; 2,3-dinor-TXB_2; 2,3-dinor-6k-$PGF_{1\alpha}$; 20-COOH-AA; 20-COOH-LTB_4; 20-OH-LTB_4; 4k-DHA; 5-HEPE; 5-HETrE; 6k-PGE_1; 6R-LXA_4; 6S-LXA_4; 6t-LTB_4; 7R-Maresin-1; 8-HDoHE; 8-HEPE; 8-iso-15k-$PGF_{2\beta}$; 8-iso$PF_{3\alpha}$; 9-Nitrooleate; 9-oxoODE; dhk-PGE_2; dhk-$PGF_{2\alpha}$; dihomo-15d-PGD_2; dihomo-PGJ_2; HXA_3; LTC_4; LTD_4; LTE_4; LXB_4; PD1; PDX; PGB_2; PGD_1; PGD_3; PGE_1; PGE_3; $PGF_{1\alpha}$; PGK_1; PGK_2; RvD1; RvD2; RvD5; RvE1; TXB_3. **LOD < Oxylipins < LOQ:** 11d-TXB_2; 11-HDoHE; 12-HEPE; 13-HDoHE; 13-HOTrE; 14,15-LTD_4; 15,16-DiHODE; 15d-PGD_2; 15k-PGD_2; 15-HEPE; 18-HEPE; 5,15-DiHETE; 5,6-DiHETE; 6,15-dk,dh-$PGF_{1\alpha}$; 6t,12-epiLTB_4; 7-HDoHE; 8,15-DiHETE; 9-HEPE; bicyclo-PGE_2; dhk-PGD_2; dihomo-PGD_2; dihomo-$PGF_{2\alpha}$; LXA_5; $PGF_{3\alpha}$; PGJ_2. **OxPC < LOD:** KDdiaPC; SOVPC; SGPC.

	LOD < OxPC < LOQ: PAzPC.
Serum	**Oxylipins < LOD:** 10-Nitrooleate; 11β-$PGF_{2\alpha}$; 11β-dhk-$PGF_{2\alpha}$; 11-HEPE; 12,13-DiHODE; 12-epi-LTB_4; 12-oxoLTB4; 13-oxoOTrE; 13-oxoODE; 14,15-diHETE; 14,15-EpETE; 14,15-LTC_4; 14,15-LTD_4; 14,15-LTE_4; 15,16-DiHODE; 15d-PGA_2; 15d-PGJ_2; 15k-$PGF_{1\alpha}$; 15k-$PGF_{2\alpha}$; 15-oxoEDE; 15R-LXA_4; 15t-PD1; 16,17-DiHDoPE; 17,18-DiHETE; 17,18-EpETE; 17d-6k-$PGF_{1\alpha}$; 17k-DHA; 17k-DPA; 2,3-dinor-11β-$PGF_{2\alpha}$; 2,3-dinor-8-iso$PF_{2\alpha}$; 2,3-dinor-TXB_2; 2,3-dinor-6k-$PGF_{1\alpha}$; 20-COOH-LTB_4; 20-OH-LTB_4; 5,15-DiHETE; 5,6-DiHETE; 5-HEPE; 5-HETrE; 5-iso$PF_{2\alpha}$VI; 6,15-dk,dh-$PGF_{1\alpha}$; 6k-PGE_1; 6k $PGF_{1\alpha}$; 6R-LXA_4; 6S-LXA_4; 6t-LTB_4; 7R-Maresin-1; 8,15-DiHETE; 8-HDoHE; 8-HEPE; 8-iso-15k-$PGF_{2\beta}$; 8-iso$PF_{3\alpha}$; 9,10-diHODE; 9-Nitrooleate; 9-oxoODE; bicyclo-PGE_2; dh-$PGF_{2\alpha}$; dhk-PGD_2; dhk-PGE_2; dhk-$PGF_{2\alpha}$; dihomo-15d-PGD_2; dihomo-PGJ_2; HXA_3; LTB_4; LTC_4; LTD_4; LTE_4; LXA_5; LXB_4; PD1; PDX; PGB_2; PGD_1; PGD_3; PGE_1; PGE_3; $PGF_{1\alpha}$; PGK_1; PGK_2; RvD1; RvD2; RvD5; RvE1; TXB_3. **LOD < Oxylipins < LOQ:** 11d-TXB_2; 12,13-EpODE; 15k-PGD_2; 15k PGE_1; 16,17-EpDoPE; 19-HETE; 4k-DHA; $PGF_{3\alpha}$. **OxPC < LOD:** KDdiaPC; SOVPC; SGPC. **LOD < OxPC < LOQ:** PAzPC.
Tibialis anterior	**Oxylipins < LOD:** 10-Nitrooleate; 11β-$PGF_{2\alpha}$; 11β-dhk-$PGF_{2\alpha}$; 11βPGE2; 11d-TXB_2; 11-HDoHE; 11-HEPE; 12,13-DiHODE; 12-HEPE; 12-oxoLTB4; 13-oxoOTrE; 14,15-diHETE; 14,15-EpETE; 14,15-LTC_4; 14,15-LTD_4; 14,15-LTE_4; 15,16-DiHODE; 15d-PGA_2; 15d-PGD_2; 15d-PGJ_2; 15-HEPE; 15k-PGD_2; 15k-$PGF_{1\alpha}$; 15k-$PGF_{2\alpha}$; 15-oxoEDE; 15t-PD1; 16,17-DiHDoPE; 16,17-EpDoPE; 17,18-DiHETE; 17,18-EpETE; 17-HETE; 17d-6k-$PGF_{1\alpha}$; 17k-DHA; 18-HEPE; 18-HETE; 19,20-DiHDoPE; 19-HETE; 2,3-dinor-11β-$PGF_{2\alpha}$; 2,3-dinor-8-iso$PF_{2\alpha}$; 2,3-dinor-TXB_2; 2,3-dinor-6k-$PGF_{1\alpha}$; 20-COOH-LTB_4; 20-HETE; 20-OH-LTB_4; 5,15-DiHETE; 5,6-DiHETE; 5-HEPE; 5-HETrE; 5-iso$PF_{2\alpha}$VI; 6,15-dk,dh-$PGF_{1\alpha}$; 6k-PGE_1; 6t-LTB_4; 6t,12-epiLTB4; 7R-Maresin-1; 8,15-DiHETE; 8-HDoHE; 8-HEPE; 8-iso-15k-$PGF_{2\beta}$; 8-iso$PF_{2\alpha}$III; 8-iso$PF_{3\alpha}$; 9,10-diHODE; 9,10-EpODE; 9-HEPE; 9-Nitrooleate; bicyclo-PGE_2; dhk-PGD_2; dhk-PGE_2; dhk-$PGF_{2\alpha}$; dihomo-15d-PGD_2; dihomo-PGD_2; dihomo-PGE_2; dihomo-$PGF_{2\alpha}$; dihomo-PGJ_2; HXA_3; LTC_4; LTD_4; LXA_5; LXB_4; PD1; PDX; PGA_2; PGB_2; PGD_1; PGD_3; PGE_1; PGE_3; $PGF_{1\alpha}$; $PGF_{3\alpha}$; PGJ_2; PGK_1; PGK_2; RvD1; RvD2; RvD5; RvE1; tetranor 12-HETE; TXB1; TXB_3. **LOD < Oxylipins < LOQ:** 12-epi-LTB_4; 13-HOTrE; 13-HOTrE-γ; 15,16-EpODE; 17k-DPA; 19,20-EpDoPE; 20-COOH-AA; 4k-DHA; 5,6 DiHETrE; 6R-LXA_4; 8,9-DiHETrE; 9-oxoOTrE; 9-HOTrE; dh-$PGF_{2\alpha}$; LTB_4; LTE_4. **OxPC < LOD:** KDdiaPC; SOVPC; SGPC.

LOD < OxPC < LOQ: PAzPC.

Supplemental Table 1. List of oxolipids scanned for but below the limit of quantitation. AA, arachidonic acid; DiHDoPE, dihydroxy-docosapentaenoic acid; DiHETE, dihydroxyeicosatetraenoic acid; DiHODE, dihydroxy-octadecadienoic; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid; EpDoPE, epoxy-eicosadocosapentaenoic acid; EpETE, epoxy-eicosatetraenoic acid; HDoHE, hydroxy-docosahexaenoic acid; HETE, hydroxy-eicosatetraenoic acid; HEPE, hydroxy-eicosapentaenoic acid; HETrE, hydroxy-eicosatrienoic acid; HOTrE, hydroxy-octadecatrienoic acid; HX, hepoxilin; isoP, isoprostane; LT, leukotriene; oxoEDE, oxo-eicosadienoic acid; oxoOTrE, oxo-octadecatrienoic acid; PD, protectin PG, prostaglandin; Rv, resolvin; TX, thromboxane.

Heart Oxylipins (pmol/g tissue)

Oxylipin	Enzyme	Control Female	Control Male	2-MCPD Female	2-MCPD Male	p-value Trt	p-value Sex
LA oxylipins							
9,10-EpOME	CYPe	287±33.6	357±81.9	232±51.9	331±66.8		
9,10-DiHOME	CYPe	93.6±15.3	111±26.5	78.9±12.4	109±19.9		
12,13-EpOME	CYPe	303±46.6	397±115	270±64.1	352±83		
12,13-DiHOME	CYPe	91.2±15.4	103±27.6	80.3±11.8	108±23.4		
9-HODE	LOX	31056±4753	46997±18676	27935±6873	36409±8947		
9-oxoODE	LOX	18349±1993	26058±6234	14064±2618	21251±3713		
13-HODE	LOX	22712±4100	36168±13319	19845±5536	28314±7011		
13-oxoODE	LOX	8672±1037	12398±3433	7625±1548	10140±2078		
TriHOMEs	LOX	4818±616	6691±1458	3914±1028	5064±1277		
LA total		86383±11725	129280±42717	74045±17632	102078±22406		
GLA oxylipins							
13-HOTrE-γ	LOX	80.9±11.3	59.9±9.22	88.7±19.3	58.4±4.60		
DGLA oxylipins							
15k-PGE_1	COX	3.84±0.533	3.45±0.702	4.47±0.59	4.42±0.269		
TXB_1	COX	10.5±1.90	11.9±2.96	9.54±2.49	10.2±2.30		
15-HETrE	LOX	8.85±1.53	7.98±1.65	8.2±1.66	7.65±1.37		
DGLA total		23.1±1.46	23.3±2.58	22.2±3.41	22.3±1.29		
EDA oxylipins							
15-oxoEDE	LOX	19.4±2.20	34.7±8.20	20.6±4.29	35.9±7.60		0.026 M
AA oxylipins							
PGD_2	COX	29.7±2.75	44.4±13.2	36.5±3.84	39.0±9.87		
15d-PGD_2	COX	30.7±10.5	51.7±24.5	25.8±6.79	40.9±13.3		
PGE_2	COX	18.5±2.22	20.1±4.16	18.1±2.55	20.0±3.40		
11β-PGE_2	COX	10.3±1.19	11.8±2.97	8.17±1.94	13.5±3.88		
15k-PGE_2	COX	26.7±3.62	30.4±7.77	28.4±4.77	29.6±5.39		
6k-$PGF_{1\alpha}$	COX	52.1±15.4	132±45.1	42.1±14.9	203±89.7		0.012 M
12-HHTrE	COX	122±12.0	121±5.12	110±11.9	107±9.60		
TXB_2	COX	12.6±0.774	15.6±1.31	13.4±2.56	16.8±2.69		
16-HETE	CYPh	27.8±1.86	26±2.77	24.6±3.29	19.7±1.57	0.036	
20-cooh AA	CYPh	39.9±7.45	40.7±12.9	44.7±18.6	69.8±28.0		
5,6-EpETrE	CYPe	16.4±5.95	18.2±7.40	10.5±1.50	11.2±2.74		

5,6-DiHETrE	CYPe	23.7±4.74	21.4±3.65	25.2±4.23	25.9±4.67		
8,9-EpETrE	CYPe	70.3±4.66	71.1±11.2	70.9±9.66	78.7±3.44		
8,9-DiHETrE	CYPe	10.1±1.26	11.0±1.23	11.4±1.18	13.2±1.34		
11,12-EpETrE	CYPe	42.6±1.85	46.1±5.05	42.7±6.31	44.8±2.00		
11,12-DiHETrE	CYPe	8.07±1.24	9.01±1.42	9.28±1.04	10.3±1.07		
14,15-EpETrE	CYPe	19.6±2.21	20.1±2.58	22.4±4.05	17.5±1.09		
14,15-DiHETrE	CYPe	14.8±2.29	16.4±1.86	16.4±2.10	18.6±2.43		
5-HETE	LOX	48.0±11.7	47.7±8.29	53.3±20.5	32.8±4.70		
5-oxoETE	LOX	51.9±11.7	62.6±16.9	38.9±11.9	31.3±4.15		
8-HETE	LOX	74.1±12.1	73.0±13.3	61.2±22.6	54.5±10.4		
9-HETE	LOX	63.2±8.40	57.4±7.62	54.3±16.1	44.7±6.04		
11-HETE	LOX	111±29.7	109±30.0	71.9±20.3	64.6±10.4		
12-HETE	LOX	84.7±18.6	90.5±18.2	67.6±22.1	63.9±13.9		
12-oxoETE	LOX	230±56.2	236±62.4	166±32.4	182±24.6		
15-HETE	LOX	340±64.9	387±96.4	293±95.6	238±52.9		
15-oxoETE	LOX	263±52.6	292±68.5	214±46.8	194±32.6		
LTB_4	LOX	27.3±4.89	30.3±6.56	27.0±5.75	32.3±7.95		
12-epi LTB_4	LOX	22.4±3.98	25.1±5.47	22.6±5.19	28.1±7.45		
6R-LXA_4	LOX	117±22.2	142±39.3	97.8±18.0	113±19.0		
6S-LXA_4	LOX	81.9±12.2	107±27.6	67.1±10.7	86.0±15.9		
HXB_3^{AR}	LOX	28.5±5.74	33.9±9.49	31.9±8.57	39.7±14.2		
AA total		2090±199	2367±239	1795±303	1945±75.3		
ALA oxylipins							
9,10-$EpODE^{AR}$	CYPe	320±71.3	558±230	287±85.1	432±129	0.019	0.045 M
9,10-$DiHODE^{AR}$	CYPe	298±25.5	370±46.1	205±38.1	285±34.5		
12,13-EpODE	CYPe	3.79±0.385	3.79±0.592	2.27±0.353	3.69±0.592		
9-HOTrE	LOX	124±14.8	186±50.5	115±15.4	132±19.2		
9-oxoOTrE	LOX	20.9±2.72	26.6±4.43	14.0±1.69	16.3±2.55	0.006	
ALA total		149±17.3	217±55.1	132±17.0	152±22.0		
DHA oxylipins							
20-HDoHE	CYPh	117±37.7	72.6±16.5	69.0±13.7	32.6±5.36	0.034	0.042 F
4-HDoHE	LOX	3.73±1.21	3.7±0.485	2.60±2.05	0.778±0.240	0.003	
10-HDoHE	LOX	73.3±12.0	57.0±7.59	47.2±9.72	60.8±6.62		
14-HDoHE	LOX	109±17.0	95.7±11.9	66.5±8.09	72.7±24.6	0.027	
16-HDoHE	LOX	9.74±2.65	7.49±1.94	6.86±3.56	2.47±0.692	0.026	
17-HDoHE	LOX	61.4±11.1	57±11.1	42.5±12.5	32.6±3.45	0.008	
DHA total		375±71.4	294±40.2	235±34.8	202±25.9	0.005	

Total Oxylipins	89100±11529	132240±42658	76317±17635	104458±22462	
n-6 total	88577±11549	131730±42623	75951±17618	104104±22467	
n-3 total	523±69.9	510±47.0	366±43.0	354±29.9	0.003
COX	317±25.7	443±79.1	296±44.8	485±135	
CYP	1052±107	1253±262	941±180	1213±225	
LOX	87451±11436	130354±42358	74918±17445	102630±22146	

Supplemental Table 2. Oxylipins in hearts of rats treated with 0 (control) and 40 mg/kg BW 2-MCPD for 90 days. Values are mean ± standard error. Blue highlighting indicates a significant decrease from control with 2-MCPD exposure. M indicates males significantly higher, F indicates females significantly higher. ^{AR} values are reported as area ratios because these oxylipins could not be absolutely quantified due to no available primary standard. AA, arachidonic acid; ALA, α-linolenic acid; COX, cyclooxygenase; CYPe, cytochrome P450-epoxygenase; CYPh, cytochrome P450-hydroxylase; DGLA, dihomo-γ-linolenic acid; DiHODE, dihydroxy-octadecadienoic; DiHOME, dihydroxy-octadecaenoic acid; DHA, docosahexaenoic acid; EDA, eicosadienoic acid; EpODE, epoxy-octadecadienoic acid; EpOME, epoxy-octadecaenoic acid; GLA, γ-linolenic acid; HDoHE, hydroxy-docosahexaenoic acid; HETE, hydroxy-eicosatetraenoic acid; HETrE, hydroxy-eicosatrienoic acid; HHTrE, hydroxyheptadecatrienoic acid; HODE, hydroxy-octadecadienoic acid; HOTrE, hydroxy-octadecatrienoic acid; HX, hepoxilin; isoP, LA, linoleic acid; LOX, lipoxygenase; LT, leukotriene; oxoEDE, oxo-eicosadienoic acid; oxoETE, oxo-eicosatetraenoic acid; oxoODE, oxo-octadecadienoic acid; oxoOTrE, oxo-octadecatrienoic acid; PG, prostaglandin; TriHOMEs, 9,10,13- & 9,12,13-trihydroxy-octadecenoic acid (could not be separated); Trt, treatment; TX, thromboxane.

Kidney Oxylipins (pmol/g tissue)

Oxylipin	Enzyme	Control Female	Control Male	2-MCPD Female	2-MCPD Male	p-value Trt	p-value Sex
LA oxylipins							
9,10-EpOME	CYPe	108±16.7	101.4±12.8	133±24.5	185±42.1		
9,10-DiHOME	CYPe	77.8±12.0	53.4±14.7	92.5±24.2	103±26.5		
12,13-EpOME	CYPe	148±27.8	135±20.3	176±37.9	236±41.2		
12,13-DiHOME	CYPe	50.2±7.75^{ab}	40.1±7.83^{b}	46.0±11.2^{ab}	94.5±22.6^{a}		0.023*
9-HODE	LOX	5928±1224	9090±1842	7109±1395	13376±2968		0.042 M
13-HODE	LOX	5229±1029	7897±1555	6940±1548	11768±2393		
13-oxoODE	LOX	16874±3274	32559±7837	23201±4448	48370±11238		0.030 M
TriHOMEs	LOX	885±90.5	1336±280	1332±205	1663±334		
LA total		29300±5411	51211±11338	39028±7329	75795±16184		0.031 M
GLA oxylipins							
13-HOTrE-γ	LOX	45.6±9.53	42.0±6.72	89.1±18.1	61.4±9.96	0.018	
DGLA oxylipins							
TXB_1	COX	5.44±0.494	6.96±1.07	8.86±1.84	8.23±1.62		
15k-PGE_1	COX	5.69±0.965	4.93±0.735	8.36±1.25	6.77±1.12	0.048	
8-HETrE	LOX	24.0±5.87	34.3±8.13	48.2±12.9	38.6±7.79		
15-HETrE	LOX	49.0±10.4	80.1±15.6	93.6±21.6	96.9±16.7		
DGLA Total		84.2±16.9	126±23.9	159±37.2	150±25.3		
AA oxylipins							
PGA_2	COX	50.1±6.76	47.9±4.83	54.3±11.9	93.6±22.7		
PGD_2	COX	113±29.1	150±36.4	79.3±23.6	137±39.1		
PGE_2	COX	339±101	371±158	161±61.5	346±158		
11β-PGE_2	COX	362±117	381±143	184±67.0	384±191		
15k-PGE_2	COX	83.7±15.3	89.7±11.9	109±22.5	103±21.6		
6k-$PGF_{1\alpha}$	COX	106±19.1	147±66.1	30.1±5.57	91.5±46.9	0.005	
$PGF_{2\alpha}$	COX	45.4±9.93	63.5±13.9	34.0±6.49	67.8±23.3		0.035 M
15k-$PGF_{2\alpha}$	COX	5.50±0.796^{b}	11.8±1.46^{a}	8.78±1.98^{ab}	6.45±1.09^{ab}		0.011*
dh-$PGF_{2\alpha}^{AR}$	COX	3.04±0.259	5.04±0.717	5.29±1.01	3.64±0.494		

12-HHTrE	COX	2392±534	4364±1008	3168±1048	3824±1049		
TXB_2	COX	27.6±2.74	42.9±7.77	30.5±5.67	41.6±9.30		
16-HETE	CYPh	240±50.9	392±69.3	394±93.7	448±84.8		
17-HETE	CYPh	13.1±2.16	12.8±2.07	15.6±3.09	14.3±2.42		
18-HETE	CYPh	8.49±1.60	9.50±2.02	14.1±4.95	12.8±1.53		
20-HETE	CYPh	31.3±7.64	69.8±22.3	53.6±18.8	81.0±10.6		0.021 M
5,6-EpETrE	CYPe	99.2±29.2	89.5±15.8	108±20.9	121±26.6		
5,6-DiHETrE	CYPe	23.7±6.33	28.8±6.31	25.9±5.80	37.3±7.36		
8,9-EpETrE	CYPe	115±29.0	132±27.8	125±20.7	153±24.6		
8,9-DiHETrE	CYPe	31.9±7.42	33.5±8.6	37.8±12.3	39.5±5.62		
11,12-EpTrE	CYPe	66.1±16.2	85.5±13.0	79.0±20.1	83.9±15.4		
11,12-DiHETrE	CYPe	41.8±9.03	39.8±14.1	58.6±22.4	40.5±4.67		
14,15-EpTrE	CYPe	51.5±8.83	62.1±7.36	73.3±15.1	57.4±12.9		
14,15-DiHETrE	CYPe	30.1±5.15	34.7±8.47	36.6±7.822	36.8±5.15		
5-HETE	LOX	687±174	1499±319	1292±381	1276±266		
5-oxo-ETE	LOX	489±119	1053±228	833±202	930±203		
8-HETE	LOX	498±107	917±216	651±150	816±137		
9-HETE	LOX	353±71.8	673±154	479±105	590±100		
11-HETE	LOX	1706±513	2095±437	1695±375	2197±492		
12-HETE	LOX	1284±404	1599±336	1350±326	1849±355		
Tetranor 12-HETE	LOX	10.0±2.48	9.75±1.57	44.4±11.3	33.0±9.55	<0.001	
12-oxo-ETE	LOX	4448±1109	7520±1393	7487±1680	9140±1795		
15-HETE	LOX	3398±733	6629±1526	4294±989	4669±801		
15-oxo-ETE	LOX	3152±757	6139±1228	4111±749	4680±1367		
LTB_4	LOX	41.4±7.36	115±31.4	88.1±28.9	70.5±13.1		
12-epi-LTB_4	LOX	39.6±7.34	90.1±21.7	75.7±23.7	61.1±14.9		
HXB_3^{AR}	LOX	121±25.5	289±79.0	251±63.4	208±43.1		
5-isoP$F_{2\alpha}$ VI	NE	3.96±0.303	4.42±0.542	5.11±0.785	4.63±0.540		
8-isoP$F_{2\alpha}$ III	NE	8.38±1.08	8.25±1.52	5.81±1.03	8.54±1.70		
AA Total		20395±4169	35010±6026	27291±6193	32545±5907		
ADA oxylipin							
dihomo-PGE_2^{AR}	COX	18.7±8.46	18.1±8.34	6.01±4.06	13.7±7.13		

ALA oxylipins							
9,10-EpODE[AR]	CYPe	64.1±7.21	96.0±19.2	99.7±16.5	121±16.0	0.039	
9,10-DiHODE[AR]	CYPe	90.9±18.3	133±34.4	131 ±28.4	182±43.6		
15,16-EpODE[AR]	CYPe	15.3±2.28[ab]	12.9±1.34[b]	15.9±4.06[b]	27.7±2.95[a]	0.022*	
9-HOTrE	LOX	58.9±13.2	88.4±22.4	74.1±14.4	188±66.7		
9-oxoOTrE	LOX	35.9±6.88	77.2±22.0	57.7±12.3	143±36.3	0.046	0.018 M
ALA total		94.8±19.8	166±42.8	132±26.0	331±101		0.024 M
DHA oxylipins							
20-HDoHE	CYPh	118±31.0	74.5±15.7	176±49.0	89.3±40.2		
19,20-EpDoPE	CYPe	5.02±1.13	3.21±0.27	7.28±1.49	3.41±0.783		0.024 F
19,20-DiHDoPE	CYPe	10.1±1.71	7.33±1.55	14.2±2.85	7.30±0.425		0.008 F
4-HDoHE	LOX	41.4±31.3	47.2±9.29	83.0±19.8	52.3±16.5		
10-HDoHE	LOX	44.4±13.8	32.7±7.54	47.9±11.3	32.4±4.95		
14-HDoHE	LOX	28.6±5.76	35.7±7.49	37.0±7.22	28.2±5.15		
16-HDoHE	LOX	71.7±16.6	60.0±11.8	85.1±16.2	53.8±7.31		
17-HDoHE	LOX	424±95.2	364±72.1	529±124	345±55.4		
DHA total		743±173	625±115	980±226	611±94.6		
Total oxylipins		50664±9691	87180±16438	67679±13441	109494±21417		0.040 M
n-6 total		49826±9508	86390±16324	66567±13230	108552±21265		0.038 M
n-3 total		838±188	790±134	1112±243	942±184		
COX total		3417±585	5519±950	3798±1197	4967±1443		
CYP total		1270±231	1405±184	1666±350	1844±298		
LOX total		45846±8979	80081±15552	62127±12024	102527±20470		0.038 M

Supplemental Table 3. Oxylipins in kidneys of rats treated with 0 (control) and 40 mg/kg BW 2-MCPD for 90 days. Values are mean ± standard error. Blue highlighting indicates a significant decrease from control with 2-MCPD exposure, Red highlighting indicates a significant increase from control with 2-MCPD exposure. M indicates males significantly higher, F indicates females significantly higher. [AR] values are reported as area ratios because these oxylipins could not be absolutely quantified due to no available primary standard. * indicates treatment x sex interaction effect; [K] indicates Kruskal Wallis test was used due to non-parametric data. AA, arachidonic acid; AdA, adrenic acid; ALA, α-linolenic acid; COX, cyclooxygenase; CYPe, cytochrome P450-epoxygenase;

CYPh, cytochrome P450-hydroxylase; DGLA, dihomo-γ-linolenic acid; DiHDoPE, dihydroxy-docosapentaenoic acid; DiHODE, dihydroxy-octadecadienoic; DiHOME, dihydroxy-octadecaenoic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; EpDoPE, epoxy-eicosadocosapentaenoic acid; EpODE, epoxy-octadecadienoic acid; EpOME, epoxy-octadecaenoic acid; GLA, γ-linolenic acid; HDoHE, hydroxy-docosahexaenoic acid; HETE, hydroxy-eicosatetraenoic acid; HEPE, hydroxy-eicosapentaenoic acid; HETrE, hydroxy-eicosatrienoic acid; HHTrE, hydroxyheptadecatrienoic acid; HODE, hydroxy-octadecadienoic acid; HOTrE, hydroxy-octadecatrienoic acid; HX, hepoxilin; isoP, isoprostane; LA, linoleic acid; LOX, lipoxygenase; LT, leukotriene; NE, non-enzymatic; oxoEDE, oxo-eicosadienoic acid; oxoETE, oxo-eicosatetraenoic acid; oxoOTrE, oxo-octadecatrienoic acid; PG, prostaglandin; TriHOMEs, 9,10,13- & 9,12,13-trihydroxy-octadecenoic acid (could not be separated); Trt, treatment; TX, thromboxane.

Serum Oxylipins (nmol/L)

Oxylipins	Enzyme	Control Female	Control Male	2-MCPD Female	2-MCPD Male	p-value Trt	p-value Sex
LA oxylipins							
9,10-EpOME	CYPe	10.6±4.62	3.58±0.736	7.30±3.03	2.23±0.505		0.031 F
9,10-DiHOME	CYPe	6.53±0.518	5.93±0.758	2.87±0.435	2.98±0.345	<0.001	
12,13-EpOME	CYPe	32.5±13.9	13.7±2.49	33.4±13.3	8.13±1.55		0.017 F
12,13-DiHOME	CYPe	4.88±0.540	5.15±0.493	2.33±0.595	2.44±0.394	<0.001	
9-HODE	LOX	128±39.6	78.9±14.9	101±35.9	38.7±2.80	0.011	
13-HODE	LOX	164±43.6	116±17.2	123±40.9	49.8±3.40	0.006	
TriHOMEs	LOX	67.8±25.4	26.4±12.3	48.9±16.4	11.8±0.775	0.021	<0.001 F
LA total		346±101	224±34.3	271±93.3	104±7.51	0.008	
GLA oxylipin							
13-HOTrE-γ	LOX	5.58±0.793	3.17±0.445	6.38±2.04	1.49±0.181	0.041	0.002 F
DGLA oxylipins							
TXB_1	COX	0.984±0.0659	1.46±0.142	1.13±0.064	1.23±0.0747		0.006 M
8-HETrE	LOX	2.41±0.303	2.15±0.286	2.16±0.638	0.818±0.0613	0.003	0.020 F
15-HETrE	LOX	1.35±0.178	1.32±0.224	1.53±0.371	0.839±0.0526		
DGLA total		4.74±0.440	4.93±0.589	4.82±0.997	2.89±0.176	0.024	
AA oxylipins							
PGA_2	COX	1.44±0.499	1.18±0.559	1.59±0.589	0.755±0.247		
PGD_2	COX	7.94±0.921	8.30±0.576	9.69±1.06	9.44±0.647		
15d-PGD_2	COX	0.431±0.0971	0.321±0.0581	0.352±0.0944	0.199±0.0268		
PGE_2	COX	6.98±0.330	8.08±1.10	7.42±0.739	7.56±0.773		
11β-PGE_2	COX	8.71±0.251	10.4±1.46	9.58±0.786	9.43±0.694		
15k-PGE_2	COX	3.04±1.68	1.12±0.744	3.04±1.23	0.314±0.0562		0.025 F
$PGF_{2\alpha}$	COX	3.29±0.260	4.37±0.399	3.05±0.445	3.55±0.139		0.025 M
PGJ_2	COX	0.581±0.0796	0.824±0.180	0.574±0.106	0.652±0.0617		
12-HHTrE	COX	230±28.6	357±26.9	166±16.9	242±18.3	0.002	<0.001 M
TXB_2	COX	68.8±8.14	119±6.99	78.3±7.40	103±5.64		<0.001 M
16-HETE	CYPh	1.61±0.553	0.909±0.129	1.68±0.388	0.624±0.0597		0.008 F
17-HETE	CYPh	0.197±0.0362[a]	0.137±0.00858[ab]	0.205±0.0269[a]	0.0927±0.0100[b]		0.048*
18-HETE	CYPh	0.487±0.0608	0.558±0.0469	0.613±0.116	0.443±0.0409		
20-HETE	CYPh	1.78±0.181	1.74±0.0832	2.44±0.421	1.94±0.195		

20-COOH-AA	CYPh	34.9±3.92	25.3±1.64	68.2±20.9	25.5±1.79		0.008 F
5,6-EpETrE	CYPe	6.26±2.10	2.75±0.730	6.96±2.38	2.00±0.286		0.006 F
5,6-DiHETrE	CYPe	0.584±0.0759	1.08±0.107	0.308±0.0365	0.475±0.0506	<0.001	<0.001 M
8,9-EpETrE	CYPe	4.01±1.55	1.98±0.455	4.35±1.45	1.12±0.135		0.005 F
8,9-DiHETrE	CYPe	0.255±0.0257	0.349±0.0204	0.274±0.0471	0.296±0.0164		
11,12-EpTrE	CYPe	4.71±2.02	2.07±0.692	4.77±1.61	1.29±0.114		0.001 F
11,12-DiHETrE	CYPe	0.314±0.445[b]	0.630±0.534[a]	0.528±0.157[ab]	0.540±0.0768[a]		0.031*
14,15-EpTrE	CYPe	4.71±1.47	2.13±0.409	5.06±1.46	1.73±0.132		0.006 F
14,15-DiHETrE	CYPe	0.727±0.0690[b]	1.22±0.0387[a]	0.730±0.0989[b]	0.849±0.0138[b]		0.024*
5-HETE	LOX	51.4±16.3	32.0±7.80	50.8±14.4	17.8±1.09	0.029	0.004 F
5-oxoETE	LOX	15.1±6.93	5.66±2.96	13.9±4.95	2.19±0.225		<0.001 F
8-HETE	LOX	54.2±5.75	54.6±4.39	41.2±5.50	26.4±1.73	<0.001	
9-HETE	LOX	13.0±2.83	8.82±0.974	10.8±2.88	4.28±0.198	0.001	<0.001 F
11-HETE	LOX	53.6±5.62	54.1±3.80	41.8±2.83	39.6±2.99	0.004	
12-HETE	LOX	5225±477	5867±513	3138±204	2823±176	<0.001	
Tetranor 12-HETE	LOX	15.5±2.29	16.1±1.44	6.40±0.785	4.04±0.502	<0.001	
12-oxoETE	LOX	540±157	365±65.2	500±71.7	291±21.8		
15-HETE	LOX	52.6±8.94	41.2±5.67	44.6±9.30	24.9±0.785	0.002	0.008 F
15-oxo-ETE	LOX	13.3±5.83	5.49±2.63	9.28±2.66	2.18±0.176		<0.001 F
6t,12 epi-LTB4	LOX	2.99±0.913	1.11±0.324	1.84±0.617	0.271±0.0597	0.005	<0.001 F
HXB_3[AR]	LOX	16817±1987	16200±1224	13292±1355	10410±752	0.001	
8-iso$PF_{2\alpha}$ III	NE	0.286±0.0397	0.297±0.0189	0.342±0.0534	0.275±0.0218		
AA total		6431±400	7000±522	4234±268	3648±204	<0.001	
ADA oxylipins							
dihomo-PGD_2[AR]	COX	135±21.6	141±21.6	220±72.1	108±7.93		
dihomo-PGE_2[AR]	COX	144±9.32	209±24.3	142±26.4	144±12.3		
dihomo-$PGF_{2\alpha}$[AR]	COX	447±39.1	623±69.5	264±55.3	369±31.6	<0.001	0.012 M
ALA oxylipins							
9,10-EpODE[AR]	CYPe	2603±1201	952±487	2386±954	258±74.2		0.029 F
15,16 EpODE[AR]	CYPe	0.784±0.203	0.512±0.0894	0.526±0.129	0.260±0.0332	0.020	0.036 F
9-HOTrE	LOX	2.32±0.696	1.40±0.234	1.82±0.571	0.758±0.0973	0.024	0.035 F
9-oxo-OTrE	LOX	0.794±0.379	0.268±0.0726	0.438±0.137	0.143±0.0143		0.037 F
13-HOTrE	LOX	1.19±0.201	1.20±0.101	0.805±0.209	0.447±0.0444	<0.001	
ALA total		4.30±1.23	2.86±0.359	3.06±0.91	1.35±0.136	0.005	0.045 F

EPA oxylipins							
18-HEPE	CYPh	0.102±0.0274	0.0692±0.0219	0.225±0.0660	0.106±0.0436		
9-HEPE	LOX	5.66±0.542	6.60±0.786	4.69±0.655	3.62±0.271	0.002	
12-HEPE	LOX	34.3±3.12	40.5±6.44	23.6±3.46	20.1±2.16	<0.001	
15-HEPE	LOX	0.128±0.0243	0.246±0.0145	0.126±0.0430	0.138±0.0254		0.024 M
EPA total		40.2±3.54	47.4±7.21	28.6±4.17	24.0±2.37	<0.001	
DHA oxylipins							
20-HDoHE	CYPh	3.18±0.555	1.46±0.355	2.56±0.468	0.860±0.0526	0.046	<0.001 F
19,20-EpDoPE	CYPe	0.485±0.131	0.213±0.0312	0.476±0.110	0.217±0.0260		0.006 F
19,20-DiHDoPE	CYPe	0.351±0.0395	0.511±0.0225	0.454±0.0762	0.353±0.0343		
4-HDoHE	LOX	15.8±5.06	7.34±2.78	14.2±4.22	2.88±0.194	0.012	<0.001 F
7-HDoHE	LOX	0.898±0.235	0.352±0.130	0.999±0.187	0.304±0.0445		<0.001 F
10-HDoHE	LOX	4.39±0.445	3.27±0.534	1.74±0.157	0.928±0.0768	<0.001	<0.001 F
11-HDoHE	LOX	4.68±0.470	3.74±0.671	1.52±0.107	0.947±0.120	<0.001	0.005 F
13-HDoHE	LOX	4.13±0.526	3.26±0.380	1.98±0.216	1.15±0.128	<0.001	0.003 F
14-HDoHE	LOX	60.5±5.77	47.8±6.54	23.3±2.55	11.9±0.744	<0.001	<0.001 F
16-HDoHE	LOX	0.903±0.246	0.415±0.0809	0.789±0.185	0.200±0.0142	0.010	<0.001 F
17-HDoHE	LOX	6.90±1.49	5.00±0.554	5.04±1.07	1.81±0.128	<0.001	0.001 F
DHA total		102±8.51	73.3±9.01	53.1±8.60	21.5±1.21	<0.001	<0.001 F
Total oxylipins		7002±424	7382±549	4649±354	3816±207	<0.001	
n-6 total		6856±415	7258±535	4565±344	3769±204	<0.001	
n-3 total		147±11.1	124±14.4	84.8±13.2	46.9±3.54	<0.001	0.009 F
COX total		334±34.0	512±36.7	284±20.8	378±25.2	0.011	<0.001 M
CYP total		119±26.3	71.5±6.39	146±36.9	54.2±3.32		0.001 F
LOX total		6551±406[a]	6799±531[a]	4223±331[b]	1253±655[c]	0.023*	

Supplemental Table 4. Oxylipins in serum of rats treated with 0 (control) and 40 mg/kg BW 2-MCPD for 90 days. Values are mean ± standard error. Blue highlighting indicates a significant decrease from control with 2-MCPD exposure, Dark blue highlighting indicates a decrease from control lower than another group. M indicates males significantly higher, F indicates females significantly higher. ^{AR} values are reported as area ratios because these oxylipins could not be absolutely quantified due to no available primary standard. * indicates treatment x sex interaction effect. AA, arachidonic acid; AdA, adrenic acid; ALA, α-linolenic acid; COX, cyclooxygenase; CYPe, cytochrome P450-epoxygenase; CYPh, cytochrome P450-hydroxylase; DGLA, dihomo-γ-linolenic acid;

DiHDoPE, dihydroxy-docosapentaenoic acid; DiHODE, dihydroxy-octadecadienoic; DiHOME, dihydroxy-octadecaenoic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; EpDoPE, epoxy-eicosadocosapentaenoic acid; EpODE, epoxy-octadecadienoic acid; EpOME, epoxy-octadecaenoic acid; GLA, γ-linolenic acid; HDoHE, hydroxy-docosahexaenoic acid; HETE, hydroxy-eicosatetraenoic acid; HEPE, hydroxy-eicosapentaenoic acid; HETrE, hydroxy-eicosatrienoic acid; HHTrE, hydroxyheptadecatrienoic acid; HODE, hydroxy-octadecadienoic acid; HOTrE, hydroxy-octadecatrienoic acid; HX, hepoxilin; isoP, isoprostane; LA, linoleic acid; LOX, lipoxygenase; LT, leukotriene; NE, non-enzymatic; oxoEDE, oxo-eicosadienoic acid; oxoETE, oxo-eicosatetraenoic acid; oxoOTrE, oxo-octadecatrienoic acid; PG, prostaglandin; TriHOMEs, 9,10,13- & 9,12,13-trihydroxy-octadecenoic acid (could not be separated); Trt, treatment; TX, thromboxane.

Tibialis Anterior Oxylipins (pmol/g)

Oxylipins	Enzyme	Control Female	Control Male	2-MCPD Female	2-MCPD Male	p-value Trt	p-value Sex
LA oxylipins							
9,10-EpOME	CYPe	18.2±3.09	49.9±14.2	34.9±7.24	38.6±10.6		
9,10-DiHOME	CYPe	4.01±1.70	5.82±1.60	3.43±0.600	5.46±1.06		
12,13-EpOME	CYPe	19.1±2.04	44.7±12.5	39.0±8.72	37.5±12.2		
12,13-DiHOME	CYPe	5.31±2.19	7.67±2.42	4.01±0.841	7.03±1.68		
9-HODE	LOX	454±87.6	779±242	446±53.0	740±119		
9-oxoODE	LOX	715±212	850±281	677±45.5	1205±261		
13-HODE	LOX	463±107	745±201	430±47.4	696±126		
13-oxoODE	LOX	827±289	1003±303	731±77.5	1554±447		
TriHOMEs	LOX	213±89.3	251±58.5	171±17.2	277±48.6		0.037 M
LA total		2719±770	3736±1084	2536±221	4560±938		
DGLA total							
15k-PGE_1	COX	0.858±0.269	0.763±0.127	0.803±0.109	0.764±0.147		
8-HETrE	LOX	3.71±1.06	5.72±0.987	4.77±0.340	7.87±1.50		0.034 M
15-HETrE	LOX	1.28±0.198	1.97±0.288	1.43±0.141	1.91±0.255		0.022 M
DGLA Total		5.85±1.26	8.45±1.33	7.00±0.553	10.5±1.58		
AA oxylipins							
PGD_2	COX	54.7±41.6	22.5±6.41	26.8±9.16	47.3±18.1		
PGE_2	COX	5.64±1.38	4.74±0.443	5.42±0.350	5.56±0.668		
15k-PGE_2	COX	6.39±1.42	5.36±0.561	5.82±1.39	7.61±1.25		
6k-$PGF_{1\alpha}$	COX	10.9±7.98	5.24±1.33	3.15±1.15	16.9±7.84		
$PGF_{2\alpha}$	COX	2.44±1.08	1.69±0.0841	1.24±0.330	3.25±1.07		
12-HHTrE	COX	18.5±5.68	23.4±5.29	13±2.48	32.5±11.9		
TXB_2	COX	1.73±0.847	1.26±0.194	0.910±0.182	1.75±0.499		
16-HETE	CYPh	4.10±0.312	5.60±0.667	4.78±0.596	4.82±0.463		
5,6-EpETrE	CYPe	3.44±0.575	7.27±1.71	6.81±1.22	4.57±0.821		
8,9-EpETrE	CYPe	7.21±1.15	14.1±3.50	13.3±2.56	10.5±2.15		
11,12-EpTrE	CYPe	5.01±0.831	10.5±2.55	9.92±2.35	7.83±1.83		

11,12-DiHETrE	CYPe	0.447±0.0441	0.705±0.0607	0.503±0.0274	0.751±0.0468	<0.001 M
14,15-EpTrE	CYPe	4.38±0.644	9.11±1.79	8.84±2.06	6.20±1.17	
14,15-DiHETrE	CYPe	0.802±0.140	1.23±0.139	0.735±0.0395	1.11±0.0893	0.001 M
5-HETE	LOX	99.6±16.7	159±27.4	142±21.0	141±26.4	
5-oxoETE	LOX	25.7±5.28^{b}	56.0±16.5^{ab}	75.0±16.2^{a}	36.6±5.81^{ab}	0.014*
8-HETE	LOX	109±9.48	126±40.3	81.4±20.9	143±17.5	
9-HETE	LOX	59.4±6.86	64.8±14.6	46.2±6.62	79.1±7.83	
11-HETE	LOX	32.5±3.49	40.4±6.90	26.8±4.22	44.0±5.13	0.020 M
12-HETE	LOX	29.7±2.63	29.4±4.44	22.5±2.45	34.4±6.14	
12-oxoETE	LOX	110±15.0	59.8±21.1	96.5±41.9	123±34.7	
15-HETE	LOX	75.4±6.18	120±19.0	89.8±11.0	102±10.8	0.042 M
15-oxoETE	LOX	146±27.6	165±36.1	180±23.6	216±38.6	
6S-LXA_4	LOX	12.8±1.55	23.2±3.44	24.9±6.68	14.0±19.0	
15R-LXA_4^{AR}	LOX	0.106±0.00499	0.131±0.0102	0.120±0.00994	0.112±0.0106	
HXB_3^{AR}	LOX	0.152±0.0288^{b}	0.337±0.0451^{a}	0.288±0.0467^{ab}	0.191±0.0328^{ab}	0.001*
AA Total		825±101	957±173	887±88.5	1084±115	
ALA oxylipins						
12,13-EpODE	CYPe	0.599±0.0851	0.814±0.209	0.686±0.0969	0.812±0.196	
DHA oxylipins						
20-HDoHE	CYPh	17.2±4.16	17.6±2.91	18.0±2.61	20.8±5.04	
4-HDoHE	LOX	34.8±7.46	52.6±8.43	58.3±6.56	47.1±9.84	
7-HDoHE	LOX	24.3±2.49	18.8±4.27	17.1±4.25	24.4±3.01	
10-HDoHE	LOX	4.19±0.397	5.90±0.600	5.16±0.851	5.49±0.781	
13-HDoHE	LOX	16.0±2.15	31.3±7.73	29.2±7.21	29.5±7.85	
14-HDoHE	LOX	4.71±0.454	5.31±0.404	5.65±0.719	5.62±0.649	
16-HDoHE	LOX	6.73±0.849	8.36±1.14	8.29±1.22	9.13±1.19	
17-HDoHE	LOX	54.8±7.74	77.4±14.8	80.5±14.9	73.6±12.9	
DHA total		163±23.3	217±34.8	222±34.1	216±37.6	
Total oxylipins		3713±854	4919±1277	3653±274	5871±1065	
n-6 oxylipins		3550±843	4701±1256	3430±254	5654±1042	
n-3 oxylipins		163±23.2	218±34.9	223±34.2	217±37.7	

COX total	101±56.4	64.9±11.9	57.2±11.9	116±36.0
CYP total	89.9±12.3	175±40.9	145±25.0	146±31.7
LOX total	3522±828	4679±1242	3451±249	5609±1047

Supplemental Table 5. Oxylipins in tibialis anterior of rats treated with 0 (control) and 40 mg/kg BW 2-MCPD for 90 days. Values are mean ± standard error. Red highlighting indicates a significant increase from control with 2-MCPD exposure. M indicates males significantly higher, F indicates females significantly higher. ^{AR} values are reported as area ratios because these oxylipins could not be absolutely quantified due to no available primary standard. * indicates treatment x sex interaction effect. AA, arachidonic acid; ALA, α-linolenic acid; COX, cyclooxygenase; CYPe, cytochrome P450-epoxygenase; CYPh, cytochrome P450-hydroxylase; DGLA, dihomo-γ-linolenic acid; DiHOME, dihydroxy-octadecaenoic acid; DiHETrE, dihydroxy-eicosatrienoic acid; DHA, docosahexaenoic acid; EpODE, epoxy-octadecadienoic acid; EpOME, epoxy-octadecaenoic acid; HDoHE, hydroxy-docosahexaenoic acid; HETE, hydroxy-eicosatetraenoic acid; HETrE, hydroxy-eicosatrienoic acid; HHTrE, hydroxyheptadecatrienoic acid; HODE, hydroxy-octadecadienoic acid; HX, hepoxilin; LA, linoleic acid; LOX, lipoxygenase; LX, lipoxin; oxoETE, oxo-eicosatetraenoic acid; PG, prostaglandin; TriHOMEs, 9,10,13- & 9,12,13-trihydroxy-octadecenoic acid (could not be separated); Trt, treatment; TX, thromboxane.