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Liang, Nuanyi
Hennebelle, Marie
Gaul, Susanne
et al.

Publication Date

2021-09-01

DOI

10.1016/j.plefa.2021.102316

Peer reviewed

HHS Public Access

Author manuscript

Prostaglandins Leukot Essent Fatty Acids. Author manuscript; available in PMC 2022 June 01.

Published in final edited form as:

Prostaglandins Leukot Essent Fatty Acids. 2021 September ; 172: 102316. doi:10.1016/j.plefa.2021.102316.

Feeding mice a diet high in oxidized linoleic acid metabolites does not alter liver oxylipin concentrations

Nuanyi Liang[a], **Marie Hennebelle**[a,1], **Susanne Gaul**[b,c], **Casey D Johnson**[b], **Zhichao Zhang**[a], **Irina A Kirpich**[d,e,f], **Craig J McClain**[d,e,f,g], **Ariel E Feldstein**[b], **Christopher E Ramsden**[h,i], **Ameer Y Taha**[a,*]

[a]Department of Food Science and Technology, College of Agriculture and Environmental Sciences, University of California Davis, Davis, CA, Unites States

[b]Department of Pediatrics, University of California San Diego, La Jolla, CA, Unites States

[c]Klinik und Poliklinik für Kardiologie, University Hospital Leipzig, Leipzig University, Germany

[d]Division of Gastroenterology, Hepatology, and Nutrition, University of Louisville, Louisville, KY

[e]Hepatobiology and Toxicology Program, University of Louisville, Louisville, KY

[f]Department of Pharmacology and Toxicology and University of Louisville Alcohol Center

[g]Veterans Affairs San Diego Healthcare System, San Diego, CA; and Robley Rex Veterans Medical Center, Louisville, KY

[h]Laboratory of Clinical Investigation, National Institute on Aging, National Institutes of Health, Baltimore, MD, Unites States

[i]National Institute on Alcohol Abuse and Alcoholism, National Institutes of Health, Bethesda, MD, United States

Abstract

The oxidation of dietary linoleic acid (LA) produces oxidized LA metabolites (OXLAMs) known to regulate multiple signaling pathways in vivo. Recently, we reported that feeding OXLAMs to

*Corresponding author: Department of Food Science and Technology, College of Agriculture and Environmental Sciences, University of California Davis, One Shields Avenue, Davis, CA, USA 95616. ataha@ucdavis.edu (A.Y. Taha).

[1]Current address: Wageningen University & Research, Laboratory of Food Chemistry, Bornse Weilanden 9, 6708 WG Wageningen, The Netherlands.

CRediT authorship contribution statement

Nuanyi Liang: Project administration, Formal analysis, Writing – original draft. **Marie Hennebelle:** Data curation, Formal analysis, Writing – review & editing. **Susanne Gaul:** Conceptualization, Funding acquisition, Investigation, Methodology, Writing – review & editing. **Casey D Johnson:** Conceptualization, Funding acquisition, Investigation, Methodology, Writing – review & editing. **Zhichao Zhang:** Data curation, Formal analysis, Writing – review & editing. **Irina A Kirpich:** Conceptualization, Funding acquisition, Investigation, Methodology, Writing – review & editing. **Craig J McClain:** Conceptualization, Funding acquisition, Investigation, Methodology. **Ariel E Feldstein:** Conceptualization, Funding acquisition, Investigation, Methodology, Writing – review & editing. **Christopher E Ramsden:** Conceptualization, Funding acquisition, Investigation, Methodology, Writing – review & editing. **Ameer Y Taha:** Conceptualization, Funding acquisition, Investigation, Methodology, Writing – review & editing, Resources, Software, Supervision, Writing – review & editing.

Declaration of Competing Interest

None to declare

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.plefa.2021.102316.

mice resulted in liver inflammation and apoptosis. However, it is not known whether this is due to a direct effect of OXLAMs accumulating in the liver, or to their degradation into bioactive shorter chain molecules (e.g. aldehydes) that can provoke inflammation and related cascades. To address this question, mice were fed a low or high LA diet low in OXLAMs, or a low LA diet supplemented with OXLAMs from heated corn oil (high OXLAM diet). Unesterified oxidized fatty acids (i.e. oxylipins), including OXLAMs, were measured in liver after 8 weeks of dietary intervention using ultra-high pressure liquid chromatography coupled to tandem mass-spectrometry. The high OXLAM diet did not alter liver oxylipin concentrations compared to the low LA diet low in OXLAMs. Significant increases in several omega-6 derived oxylipins and reductions in omega-3 derived oxylipins were observed in the high LA dietary group compared to the low LA group. Our findings suggest that dietary OXLAMs do not accumulate in liver, and likely exert pro-inflammatory and pro-apoptotic effects via downstream secondary metabolites.

Keywords

Liver; Lipid mediators; Linoleic acid; Oxidized fatty acids; Free oxylipins; UPLC-MS/MS

1. Introduction

Oxylipins are oxygenated derivatives of polyunsaturated fatty acids (PUFAs) formed through auto-oxidation [1] or cyclooxygenase (COX) [2], lipoxygenase (LOX) [3], cytochrome P450 (CYP) [4], 15-hydroxyprostaglandin dehydrogenase (PGDH) [5] and soluble epoxide hydrolase (sEH) enzymes [6, 7]. Oxylipins and their metabolites are important mediators of inflammation [8–10], the resolution of inflammation [11–13], cellular proliferation [14, 15], apoptosis [16, 17] and vascular function [18, 19].

Circulating and tissue oxylipin concentrations are known to be regulated by dietary PUFA levels [20–22]. For instance, feeding rats a diet high in linoleic acid (LA, 18:2n-6) was shown to increase LA-derived oxylipins, known as ‘oxidized linoleic acid metabolites’ (OXLAMs) in plasma, brain and peripheral tissues [20, 21]. Similarly, in humans, lowering dietary LA reduced circulating LA and OXLAM concentrations [23].

In vivo, OXLAMs are involved in mediating inflammation and apoptosis. Feeding mice a high LA diet, which increases plasma and tissue OXLAM concentrations, was shown to exacerbate chronic alcohol-induced liver injury [24, 25] by stimulating pro-inflammatory and pro-apoptotic pathways [26, 27]. Preclinical and clinical studies have reported an association between circulating OXLAMs and liver inflammatory diseases of alcoholic [28–30] and non-alcoholic origin [31–33]. For instance, the OXLAMs, 9- and 13-hydroxyoctadecadienoic acid (HODE) and 9- and 13-oxo-octadecadienoic acid (oxo-ODE) were observed to be higher in patients with nonalcoholic steatohepatitis, a severe form of nonalcoholic fatty liver disease, compared to patients with hepatic steatosis (a less severe form of nonalcoholic fatty liver disease) [31].

Diet is a source of OXLAMs, originating mainly from high LA oils (e.g. soybean oil) used in food processing [34–36]. OXLAMs constitute over 57% of oxylipins in high-LA oils

[36], and their concentration has been shown to increase during short or long-term thermal processing [37–40].

Dietary OXLAMs can be absorbed [41–43] and are therefore bioavailable to tissues and organs [44]. However, it is not known, whether they accumulate in tissues, such as the liver, where they can locally act to provoke inflammation and hepatocyte injury [45]. Recently, we reported that feeding mice a high LA diet low in OXLAMs, and a low LA diet enriched with OXLAMs (i.e. high OXLAM diet), provoked liver inflammatory and pro-apoptotic pathways compared to a low LA diet low in OXLAMs [26]. The high OXLAM diet in particular, was associated with increased oxidative stress, hepatocyte cell apoptosis, and the activation of the NLRP3 inflammasome pathway [26]. However, in the same study, we did not observe an increase in plasma OXLAM concentrations in the high OXLAM dietary group [26]. We hypothesized that this could be due to increased deposition of OXLAMs into peripheral tissues such as the liver.

Thus, in the present study we measured OXLAMs in liver samples archived from the same experiment, to determine whether feeding a high OXLAM diet increases hepatic OXLAM concentrations in mice. Additionally, oxylipins derived from omega-6 dihomo-γ-linolenic acid (DGLA, 20:3n-6) and arachidonic acid (ARA, 20:4n-6), and omega-3 alpha-linolenic acid (ALA, 18:3n3), eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) were measured to determine whether dietary OXLAMs impacted oxylipins derived from these PUFAs.

2. Materials and methods

2.1. Preparation of the study diets

The preparation of the three diets used in this study and their nutrient composition has been previously reported [26, 46]. Briefly, the three isocaloric diets (Dyets Inc., Bethlehem, PA) contained (% calories) 44% carbohydrates, 16% protein and 40% fat. Each diet contained casein (200 g/kg), DL-methionine (3 g/kg), sucrose (348.6 g/kg), cornstarch (150 g/kg), cellulose (50 g/kg), fat (201.4 g/k), mineral mix (#200000 from Dyets Inc, 35 g/kg), vitamin mix (#300050 from Dyets Inc, 10 g/kg) and choline bitartrate (2 g/kg). The three diets varied only by fat composition – one diet contained low LA (4.3% of energy), the other contained high LA (17% of energy) and the third contained low LA (4.3% energy) supplemented by 13% energy-equivalent of Crisco™ corn oil heated at 115 °C for ~4 weeks with daily stirring (i.e. high OXLAM diet) [26]. The low LA diet contained 40 g/kg corn oil, 141.8 g/kg coconut oil and 19.6 g/kg flaxseed oil as the source of fat (Dyets Inc. catalogue # 104097). The high LA diet contained 158.5 g/kg corn oil, 23.3 g/kg coconut oil and 19.6 g/kg flaxseed oil (catalogue # 104099). The high OXLAM diet contained 67.7 g/kg corn oil, 114.1 g/kg coconut oil and 19.6 g/kg flaxseed oil (catalogue # 104098). As previously reported, the low LA diet contained 20.2 ± 4.7 nmol/g of OXLAMs, the high LA diet contained 33.4 ± 2.0 nmol/g and the high OXLAM diet contained 259.6 ± 21.6 nmol/g OXLAMs [26]. The fatty acid composition was also reported previously [26]. The low LA diet contained (% of total fatty acids) 16% LA, 6% ALA, 69% saturated fatty acids (SFAs) and 9% monounsaturated fatty acids (MUFAs). The high LA diet contained 47% LA, 6%

ALA, 22% SFAs and 25% MUFAs. The high OXLAM diet contained 12% LA, 7% ALA, 67% SFAs and 14% MUFAs.

2.2. Animal experiments

Animal experiments and liver sample collection procedures were also reported previously [26, 46]. In brief, 7-week-old wild-type male C57BL/6 mice (n = 8 / group) were randomized to the three diets for 8 weeks after 1 week of normal chow diet adaptation upon arrival. The animal experiments were performed following the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals and approved by the University of California, San Diego Institutional Animal Care and Use Committee.

After 8 weeks of feeding, mice were food-restricted for 3–4 h to minimize potential postprandial effects on oxylipins [47, 48], and euthanized by carbon dioxide. Livers were harvested immediately, cut into several pieces, frozen in isobutane chilled in dry ice, and stored at −80 °C until the time of oxylipin analysis.

2.3. Sample preparation and analysis of free oxylipins in liver

Weighted liver samples (30–100 mg) were mixed with 200 μL of extraction solvent (methanol containing 0.1% acetic acid and 0.1% butylated hydroxytoluene), 10 μL antioxidant mix (0.2 g/L EDTA, butylated hydroxytoluene, and triphenylphosphine), 10 μL deuterated surrogate standard mix (0.5 μM) and 2 beads. The surrogate standard mix contained d11–11(12)-EpETrE, d11–14,15-DiHETrE, d4-6-keto-PGF1a, d4-9-HODE, d4-LTB4, d4-PGE2, d4-TXB2, d6-20-HETE and d8-5-HETE (all from Cayman Chemical, Ann Arbor, MI). The mixture was stored in a −80 °C freezer for 30 min and homogenized for 2 min with a bead homogenizer (Bullet Blender, Next Advance). This process was repeated to ensure that the liver was thoroughly homogenized. The homogenized sample was centrifuged for 10 min at 0 °C at 15,871 g. The supernatant (200 μL) was transferred to a new tube and 1800 μL of ultrapure water was added.

Unesterified oxylipins in the diluted supernatant were isolated using solid phase (SPE) extraction on Oasis HLB (Waters Cooporation) 60 mg extraction columns. The columns were conditioned with one column volume of ethyl acetate followed by two column volumes of methanol and two column volumes of SPE buffer (0.1% acetic acid and 5% methanol in ultra-pure water). Samples were loaded onto the column, and washed with 2 column volumes of SPE buffer. Oxylipins were eluted from the SPE column with 0.5 mL methanol followed by 1.5 mL of ethyl acetate. The collected fractions were dried under nitrogen, re-dissolved in 100 μL methanol, vortexed for 2 min, and centrifuged at 15,871 g for 2 min at 0 °C. The oxylipin extract was transferred to Ultrafree-MC VV Centrifugal Filters and centrifuged at 15,871 g for 20 min at 0 °C at. The filtered extract containing oxylipins was transferred to LCMS amber vials containing inserts and subjected to ultra-high pressure liquid chromatography coupled to tandem mass-spectrometry (UPLC-MS/MS) analysis.

UPLC-MS/MS analysis was performed on an Agilent 1290 Infinity UPLC system coupled with a 6460 triple-quadrupole tandem MS with electrospray ionization (Agilent Corporation, Palo Alto, CA, USA) as previously described [49].

2.4. Statistical analysis

All statistical analysis was performed on GraphPad Prism version 8.4.3 for Mac or 9.1.0 for Windows (GraphPad Software, San Diego, California USA, www.graphpad.com). The normality of distribution for each oxylipin per dietary group was assessed using Shapiro-Wilk's test. As shown in Supplementary Table S1, most oxylipins were not normally distributed, so a Kruskal-Wallis and a Dunn's multiple comparisons test was performed. For oxylipins that were normally distributed in all three diet groups, an ordinary one-way ANOVA and a Tukey's multiple comparisons test was performed. Non-detected data in up to 3 out of 8 samples per dietary group were imputed by dividing the limit of detection (LOD) by the square root of 2. The LOD was defined as the lowest observable point on the calibration standard curve. The number of imputed values per group (for a total of 7 compounds in which the imputation was applied) is shown in Supplementary Table S2. Data are expressed as median ± interquartile ranges (IQR) encompassing 25th and 75th percentiles. Significance was set at $P < 0.05$, and denoted in the figures (below) as *, **, and *** for $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively.

3. Results

3.1. Effect of OXLAM feeding on liver n-6 PUFA-derived oxylipins

Fig. 1 shows omega-6 LA, DGLA and ARA derived oxylipin concentrations in the three dietary groups. Compared to the low LA diet, the high OXLAM diet did not significantly alter liver OXLAM concentrations (Fig. 1-A). The high LA diet significantly increased LA-derived 9-oxo-ODE by 58% and 87% compared to the low LA and high OXLAM groups, respectively. 9-HODE, 13-HODE, 12(13)-epoxyoctadecenoic acid (EpOME), 9(10)-EpOME and 12,13-dihydroxyoctadecenoic acid (DiHOME), were significantly higher by 98–260% in mice on the high LA diet compared to mice on the high OXLAM diet. No significant differences were observed among the groups for the DGLA metabolite, 15 (S)-hydroxyeicosatrienoic acid (15(*S*)-HETrE) (Fig. 1-B).

Hydroxylated ARA species were altered by the high LA diet. ARA-derived 11-hydroxyeicosatetraenoic acid (HETE) and 15-HETE were significantly higher by 68–194% in the high LA dietary group compared to the low LA and high OXLAM dietary groups (Fig. 1C). 12-HETE was 10-fold higher in the high LA group compared to the high OXLAM group.

3.2. Effect of OXLAM feeding on liver n-3 PUFA-derived oxylipins

ALA-derived 9-hydroxyoctadecatrienoic acid (HOTrE) and 13-HOTrE were least detected in the high OXLAM group compared to the low or high LA groups. A scatter plot of detected values per group is shown in Supplementary Figure S1. As shown, 9-HOTrE was detected in 87.5%, 87.5% and 25% of samples (out of 8) in the low LA, high LA and high OXLAM dietary groups, respectively. 13-HOTrE was detected in 62.5%, 75% and 37.5% of samples (out of 8) in the low LA, high LA and high OXLAM dietary groups, respectively.

Fig. 2 shows the median concentration of oxylipins derived from EPA and DHA. EPA-derived 12-hydroxyeicosapentaenoic acid (HEPE) was 67% lower in the high LA group

compared to the low LA group (Fig. 2-A). 17(18)-EpETE was detected in 7 out of 8 mice in the high OXLAM group and at a frequency of 50% or less in the low and high LA groups (Supplementary Figure S2).

DHA-derived oxylipins did not differ significantly among the groups (Fig. 2-B).

4. Discussion

This study demonstrated that feeding mice a high OXLAM diet for 8 weeks did not alter liver OXLAM concentrations. In contrast, the high LA diet increased several LA- and ARA-derived oxylipins, and decreased EPA-derived 12-HEPE compared to the low LA diets with or without OXLAMs. These observations suggest that dietary LA but not dietary OXLAMs modify liver oxylipin concentrations.

Previously, we reported (in the same mice) that the high OXLAM diet increased liver markers of inflammation, apoptosis and oxidative stress compared to the low and high LA diets (both low in OXLAMs) [26]. Additionally, both the high LA and high OXLAM diets increased transcription markers of fatty acid oxidation (e.g. CPT1) and apoptosis (e.g. TXNIP) compared to the low LA diet [26]. While the effects of the high LA diet can be attributed to the increase in LA or ARA-derived oxylipins (relative to the low LA diet), the effects of the high OXLAM diet on liver inflammation, oxidative stress and apoptosis are difficult to explain in the absence of changes in liver oxylipin concentrations.

There are a few plausible explanations for the lack of changes in liver OXLAMs following chronic administration of the high OXLAM diet. One explanation is that OXLAMs were rapidly metabolized into shorter-chain aldehydes and ketones following incorporation into the liver due to rapid turnover [44]. This is consistent with our finding of increased liver malonaldehyde concentration in mice on the high OXLAM diet compared to those on the low and high LA diets [26]. Another possibility is that we did not have the analytical sensitivity needed to measure subtle changes in OXLAM concentrations following their incorporation into hepatocytes, where they may concentrate in certain organelles due to targeted transport via fatty acid binding proteins [50, 51]. Lastly, the duration of the study may have been too short to observe marked changes in OXLAM accumulation. A longer feeding period may lead to measurable changes in liver OXLAM concentrations.

The lack of changes in liver OXLAM concentrations parallels our findings in plasma and brain measured in the same mice [26, 46]. In both matrices, we did not observe an increase in OXLAM concentrations in response to dietary OXLAM feeding. It appears, therefore, that plasma and tissues are not sensitive to dietary OXLAMs, possibly due to their rapid turnover within tissues [44]. This is in agreement with studies showing that dietary OXLAMs degrade into aldehydes in the stomach, which can accumulate in liver and potentially other organs [26, 43].

The low detectability of ALA-derived 9-HOTrE and 13-HOTrE and high detectability of EPA-derived 17(18)-EpETE in the high OXLAM group relative to the low or high LA groups is interesting but difficult to interpret. A low or high percent detectability in one group over the other may be caused by group-specific matrix effects (e.g. ion suppression

or enhancement). Matrix effects are typically caused by non-specific compounds interfering with the ionization of the analyte during UPLC-MS/MS analysis. Our data suggest that livers from the high OXLAM group may have different matrix features compared to livers from the low and high LA groups. We cannot exclude the possibility that differences in oxylipin detectability are metabolic in nature. Future studies involving tracers could further inform on this hypothesis in OXLAM-fed mice.

The increase in OXLAMs and ARA-derived oxylipins, and the reduction in EPA-derived 12-HEPE in the high LA group compared to the low LA group is consistent with prior studies showing that a high LA diet increases plasma and tissue *n*-6 oxylipins and decreases *n*-3 oxylipins [21, 41, 42, 46, 50]. This is because elevated intake of dietary LA increases circulating and tissue n-6 PUFA concentrations, while reducing n-3 PUFA concentrations due to increased turnover (i.e. increased loss of EPA and DHA due to metabolism) [52]. Overall, these observations lend support to the notion that dietary LA is an important modulator of tissue n-3 and n-6 PUFA derived oxylipin concentrations.

A limitation of this study is that we did not measure unesterified PUFA precursors to oxylipins. Doing so would have allowed us to determine whether dietary-induced changes in oxylipins were linked to changes in the availability and turnover of their unesterified PUFA precursors. Recently, we reported that a high OXLAM diet altered the percent composition of PUFAs in the brain, suggesting potential pathways linking dietary OXLAMs to in vivo PUFA metabolism [46].

In summary, this study showed that dietary OXLAMs did not alter liver unesterified oxylipin concentrations. Our findings suggest that the previously reported effects of the high OXLAM diet on liver inflammation and apoptotic markers are likely caused by aldehyde/ketone degradation products of OXLAMs [26]. A direct effect of OXLAMs is also plausible, particularly if they concentrate in organelles. Labeled OXLAMs could be used in future studies to better understand OXLAM partitioning and metabolism within the liver.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Funding

This study was supported by USDA National Institute of Food and Agriculture, Hatch/Taha (project #1,008,787, A.Y.T.); National Institutes of Health Grants R01AA024102–01A1 (I.A.K.), U01AA022489 (A.E.F., C.J.M.), 1U01AA021901–01 (C.J.M.), 1U01AA021893–01 (C.J. M.), and R01AA023681 (C.J.M.); U.S. Department of Veterans Affairs Grant 1I01BX002996 (C.J.M.); the Intramural Programs of the National Institute on Aging and the National Institute on Alcohol Abuse and Alcoholism (C.E.R.); National Institute of General Medical Sciences Grant P20GM113226 (C.J.M.; Institutional Development Award); National Institute on Alcohol Abuse and Alcoholism Grant P50AA024337 (C.J.M.); and German Research Foundation Grant SCHU3146/1–2 (S.G.).

Abbreviations

ALA	Alpha-linoleic acid
ARA	Arachidonic acid

COX	Cyclooxygenase
CYP	Cytochromes P450
DHA	Docosahexaenoic acid
DiHETE	Dihydroxyeicosatetraenoic acid
DiHOME	Dihydroxyoctadecenoic acid
DGLA	Dihomo-γ-linolenic acid
EPA	Eicosapentaenoic acid
EPOME	Epoxyoctadecenoic acid
HODE	Hydroxyoctadecadienoic acid
HETE	Hydroxyeicosatetraenoic acid
HOTrE	Hydroxyoctadecatrienoic acid
HEPE	Hydroxyeicosapentaenoic acid
IQR	Interquartile range
LA	Linoleic acid
LOX	Lipoxygenase
MUFAs	Monounsaturated fatty acids
OxoODE	Oxo-octadecadienoic acid
OXLAMs	Oxidized linoleic acid metabolites
PGDH	15-Hydroxyprostaglandin dehydrogenase
PUFAs	Polyunsaturated fatty acids
sEH	Soluble epoxide hydrolase
SFAs	Saturated fatty acids
UPLC-MS/MS	Ultra-high pressure liquid chromatography coupled to tandem mass-spectrometry

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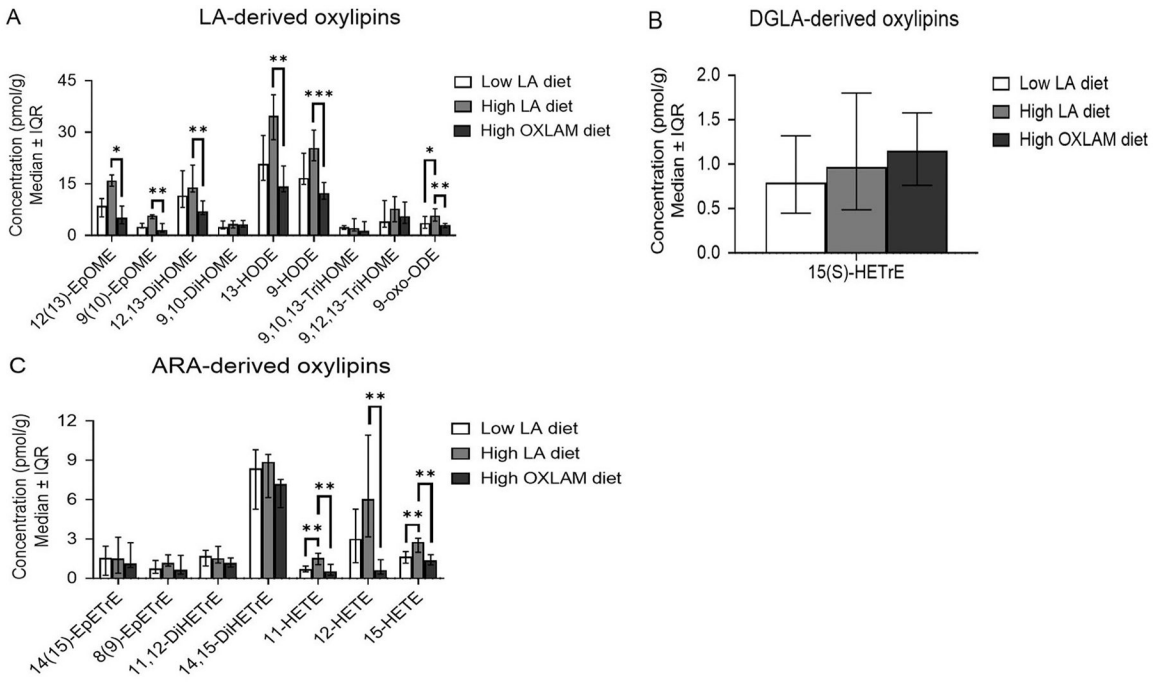

Fig. 1.

Concentration of n-6 fatty acid-derived oxylipins, including oxylipins derived from A) LA, B) DGLA, and C) ARA, in liver of mice fed a low LA diet, high LA diet, or a high OXLAM diet for 8 weeks. Data were analyzed by Kruskal-Wallis and Dunn's multiple comparisons test, or an ordinary one-way ANOVA and a Tukey's multiple comparisons test, and reported as median and interquartile ranges (IQR, 25% and 75%) of $n = 8$ mice per group.

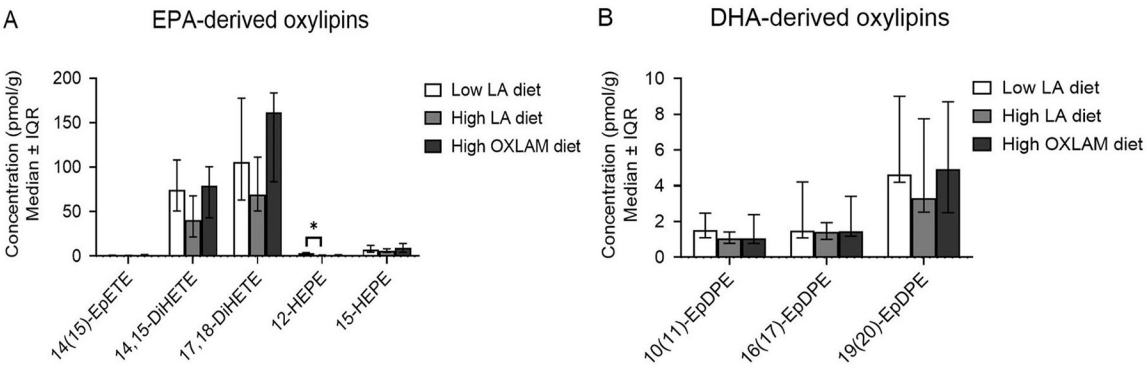

Fig. 2.

Concentration of n-3 fatty acid-derived oxylipins, including oxylipins derived from A) EPA and B) DHA in liver of mice fed a low LA diet, high LA diet or a high OXLAM diet for 8 weeks. Data were analyzed by Kruskal-Wallis and Dunn's multiple comparisons test, or an ordinary one-way ANOVA and a Tukey's multiple comparisons test, and reported as median and interquartile ranges (IQR, 25% and 75%) of $n = 8$ mice per group.