OXIDIZED LIPIDS AS POTENTIAL MEDIATORS AND MARKERS OF DISEASE IN PREGNANCY AND DEVELOPMENT

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A thesis submitted in fulfilment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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February 20, 2020

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Abstract

Oxidized lipids derived from omega-3 (n-3) and n-6 polyunsaturated fatty acids (PUFAs)—collectively known as oxylipins—are bioactive mediators that affect functions of multiple organs. There are plausible mechanisms whereby excessive or inadequate exposures to specific oxylipins could adversely affect placenta, predispose to preterm birth and/or impact brain development. Yet there are large gaps in our understanding of the effects of oxylipins in pregnancy.

The long-term objective of this research is to determine the impacts of oxylipins on human pregnancy and neurodevelopment. In this research I aimed to: (i) establish proof-of-concept that the largest alteration in dietary PUFAs in modern times (increased linoleic acid (LA) and preformed oxidized LA metabolite (OXLAM) consumption) alters brain biochemistry in a mammalian model; (ii) design and develop an assay for targeted profiling of oxylipins and precursor unesterified fatty acids; (iii) determine effects of delayed processing, typical of human studies, on oxylipin concentrations in plasma; (iv.) profile oxylipins and unesterified precursors in plasma from pregnant Australian participants in the Omega-3 fats to Reduce the Incidence of Prematurity (ORIP) study. ORIP samples were used to investigate: (a) concentrations of specific oxylipins throughout pregnancy, (b) effects of docosahexaenoic acid (DHA) supplementation on oxylipins; and (c) whether oxylipin concentrations measured in first and second trimesters predict risk of preterm birth.

In study 1, increasing dietary LA in a mouse model increased n-6 and decreased n-3 fatty acids, and increased OXLAMs in brain. Dietary OXLAMs had no effect on brain oxylipins but decreased brain PUFAs. Thus, brain fatty acids are modulated by both dietary LA and OXLAMs, while brain OXLAMs are regulated by endogenous synthesis from LA, rather than incorporation of preformed OXLAMs.

In study 2, I investigated effects of delayed blood processing typical of human studies, on oxylipin concentrations in plasma and established that most oxylipins were reasonably stable when blood is stored on wet ice for up to 2 hours prior to processing. The lone exception was substantial time-dependent increases in 12-lipoxygenase-derived and platelet-derived oxylipins. These findings gave me confidence to proceed with study 3.

In study 3, I observed that in the placebo group without DHA supplementation unesterified arachidonic acid (AA) and several AA-derived oxylipins decreased between weeks 14-24 of pregnancy. DHA supplementation increased plasma unesterified DHA and DHA-derived oxylipins in plasma. Participants with higher concentrations of 5-lipoxygenase-derived oxylipins at 14 weeks, or unesterified AA at 24 weeks, had higher risk of preterm birth in exploratory analysis. The hypothesis that 5-lipoxygenase-derived oxylipins and/or unesterified AA could serve as mechanism-based biomarkers for preterm birth should be evaluated in larger, adequately powered studies.

Collective findings stimulated and informed design and conduct of animal and human studies investigating the roles of oxylipins in pregnancy and development in humans. Findings suggest the need to move past the traditional measure of esterified fatty acid levels in blood and tissues and to measure the likely mediators of change – unesterified fatty acids their oxylipin derivatives – in humans. Such work could ultimately have important implications for human populations by informing strategies for manipulating oxylipins as in a manner to decrease the risk preterm birth and/or favorably impacting neurodevelopmental trajectory.

Declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint award of this degree.

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Christopher Edward Ramsden

February 2020

Work arising from candidature

1.1 Publications

1. **C.E. Ramsden**, M. Hennebelle, S. Schuster, G.S. Keyes, C. Johnson, I. Kirpich, J. E. Dahlen, M.S. Horowitz, D. Zamora, A.E. Feldstein, C. McClain, B. Muhlhausler, M. Makrides, R.A. Gibson, A.Y. Taha. Effects of diets enriched in linoleic acid and its peroxidation products on brain fatty acids, oxylipins, and aldehydes in mice. *Biochim Biphys Acta (BBA) - Molecular and Cell Biology of Lipids*. 2018 Oct; 1863(10):1206-1213. doi:10.1016/j.bbalip. 2018.07.007.

2. **C.E. Ramsden**, Z. Yuan, M.S. Horowitz, D. Zamora, S. Majchrzak-Hong, B. Muhlhausler, A.Y. Taha, M. Makrides, R.A. Gibson. Temperature and time-dependent effects of delayed blood processing on oxylipin concentrations in human plasma. *Prostaglandins, Leukotrienes and Essential Fatty Acids*. 2019; 150; 31-37; Sept 4 https://doi.org/10.1016/j.plefa.2019.09.001

3. **C.E. Ramsden**, M. Makrides, Z. Yuan, M.S. Horowitz, D. Zamora, L.N. Yelland, K. Best, J. Jensen, A.Y. Taha, R.A. Gibson. Plasma oxylipins and unesterified precursor fatty acids are altered by DHA supplementation in pregnancy: Can they help predict risk of preterm birth? *Prostaglandins, Leukotrienes and Essential Fatty Acids*. 2020; 153; 102041 https://doi: 10.1016/j.plefa.2019.102041 (published online 2019 Dec 13, 2019).

1.2 Presentations

Event	Type	Title	Authors
SAHMRI (South Australian Health and Medical Research Institute) Invited Presentation, 2016	Oral	Effects of dietary linoleic acid and OXLAMs on clinical endpoints and mortality in randomized controlled trials	**C. Ramsden**
Fatty Acid & Cell Signalling-12, 2017	Oral	Oxylipins in inflammation and pain and their modulation by diet	**C. Ramsden**
Oxidized Lipids Symposium, University of California-Davis	Oral	Effects of manipulating dietary linoleic acid in humans. Symposium entitled "Oxidized lipids in pregnancy and development"	**C. Ramsden**
National Institute on Aging Retreat, 2017	Poster	Biochemical, metabolic and behavioural effects dietary heated oils and hydroperoxy-octadecadienoates	J. Jensen, A. Domenichiello, **C. Ramsden** (presented by J. Jensen)
National Institute on Aging Retreat, 2018	Poster	Lipidomic profiling of targeted oxylipins with ultra-performance liquid chromatography tandem mass spectrometry	Z. Yuan, **C. Ramsden** (presented by Z. Yuan)

Acknowledgements

I would like to acknowledge and express profound gratitude to Professor Robert Gibson, my principal supervisor, for his wisdom, motivation, excellent mentorship, and generosity with his time throughout this Ph.D. program, and to Professor Maria Makrides for her mentorship, enthusiasm, leadership and perspective. Thank you, Bob and Maria, for your generosity with your time, keen insights and training on the potential roles of lipids in pregnancy and development, and for setting the outstanding example for rigorous design, conduct, and interpretation of human clinical trials. Your mentorship will impact me far beyond these Ph.D. projects to enhance my scientific career, and its potential to impact human health. I would also like to provide a special thanks to Professor Ameer Taha, for mentorship, collaboration, and generosity in sharing time and expertise, particularly in the analytical aspects of my Ph.D. training. I would like to acknowledge the contributions of collaborators on these thesis projects especially Beverly Muhlhausler, David Jeffries, Liu Ge, Maria Hennebelle, Daisy Zamora, Mark Horowitz, Sharon Majchrzak-Hong and Greg Keyes. I would like to provide a special acknowledgement to Zhi-Xin Yuan for leading mass spectrometry assay development and validation for oxylipins and free fatty acids, which were applied in thesis projects 2 and 3. I would like to acknowledge the University of Adelaide including Renae Jordan and Kay Anderson for administrative assistance, and Christopher Ford for his guidance and time. I would like to acknowledge the ORIP investigators and staff for transformational work in conducting the rigorous ORIP trial and for sharing the unique plasma samples and high-quality clinical data used in analyses herein, and the ORIP participants, as without them these projects would not be

possible. Finally, I would like to thank my wife and daughters for their support, patience and humor during this tremendously busy and scientifically enriching period of my life.

Acronyms

AA	Arachidonic acid
ALA	Alpha-linolenic acid
BHT	Butylated hydroxytoulene
BLQ	Below limit of quantitation
COX	Cyclooxygenase
CYP	Cytochrome P450
DGLA	Dihomo-gamma-linolenic acid
DHA	Docosahexaenoic acid
DIHETE	Dihydroxy-eicosatetraenoic acid
DiHETrE	Dihydroxy-eicosatrienoic acid
DIHOME	Dihydroxy-octadecenoic acid
DPA	Docosapentaenoic acid
DTA	Docosatetraenoic acid
EDTA	Ethylenediaminetetraacetic acid
EET	Epoxyeicosatrienoic acids
EPA	Eicosapentaenoic acid
EpDPA	Epoxy-docosapentaenoic acid
EpETE	Epoxy-eicosatetraenic acid
EpETrE	Epoxy-eicosatrienoc acid
EPOME	Epoxy-octadecenoic acid
ETA	Eicosatetraenoic acid
FLAP	5-lipoxygenase activating protein
GC/FID	Gas Chromatrography/Flame Ionization Detector
GLA	Gamma-linolenic acid
HDHA	Hydroxy-docosaheaenoic acid
H-E	Hydroxy-epoxide
HEPE	Hydroxy-eicosapentaenoic acid
HETrE	Hydroxy-eicosatrienoic acid

HNE	Hydroxynonenal
HODE	Hydroxy-octadecadienoic acid
HPLC	High-performance liquid chromatography
HPODE	Hydroperoxy-octadecadienoic acid
K-E	Keto-epoxide
KG	Kilogram
LA	Linoleic acid
LC-MS/MS	Liquid chromatography tandem mass spectrometry
LO	Lipoxygenase
LOQ	Limit of quantitation
LT	Leukotriene
MUFA	Monounsaturated fatty acid
n-3	Omega-3
n-6	Omega-6
NMR	Nuclear magnetic resonance
NSAID	Non-steroidal anti-inflammatory drug
ORIP	Omega-3 fats to Reduce the Incidence of Prematurity
OXLAM	Oxidized linoleic acid metabolite
OXO-ODE	Oxo-octadecadienoic acid
PG	Prostaglandin
PG	Prostaglandin
PUFA	Polyunsaturated fatty acid
RV	Resolvin
SDA	Stearidonic acid
SEH	Soluble Epoxide Hydrolase
SFA	Saturated fatty acid
SPE	Solid phase extraction
TRIHOME	Trihydroxy-octadecenoic acid
TX	Thromboxane

CHAPTER 1:

INTRODUCTION AND CONTEXT

1.1 Overview of oxylipins

Oxidized lipids derived from omega-3 (n-3) and omega-6 (n-6) polyunsaturated fatty acids (PUFAs), collectively known as oxylipins, comprise a large, heterogeneous family of labile, bioactive signaling molecules that play diverse roles in human health and disease (1). Classic oxylipins such as prostaglandins and leukotrienes have been exhaustively investigated and well documented to have numerous bioactivities (reviewed in 1.1.1). More recent evidence has suggested that a growing list of oxylipins play pivotal regulatory roles in the initiation and resolution of inflammation (2-5), endothelial cell activation (6), coagulation (7), vasoconstriction and vasodilation (8), nociception and anti-nociception (9-13), and parturition (2, 14-18). Accordingly, alterations in oxylipin concentrations have been implicated in numerous physiological responses, and in the pathogenesis of conditions ranging from chronic pain (19), to cardiovascular diseases (20), neurodegenerative diseases (21), and preterm birth (22).

The initial step in oxylipin generation is oxidation of one or more 1,4-*cis*-*cis*-pentadiene structures, which are unique structural features of PUFAs such as arachidonic acid (AA, 20:4n-6), docosahexaenoic acid (DHA, 22:6:n-3), and linoleic acid (LA, 18:2n-6)(**Fig 1A**). Major PUFA oxidation reactions include peroxidation, which involves the addition of a hydroperoxide moiety to the 1 or 4 carbon of the 1,4-*cis*-*cis*-pentadiene structure which shifts the double bonds into conjugation (**Fig 1B**), and epoxidation which adds oxygen to form an epoxide moiety, eliminating one carbon-carbon double bond (**Fig 1C**). Three major iron-containing enzyme classes—cyclooxygenases (COX), lipoxygenases (LO), and cytochrome P450 (CYP) epoxygenases—are known to catalyze oxidation of PUFAs to generate regio- and stereospecific oxylipin derivatives. PUFAs can also undergo free-radical mediated peroxidation under

conditions of oxidative stress, producing racemic mixtures of hydroperoxy- and epoxy-fatty acids.

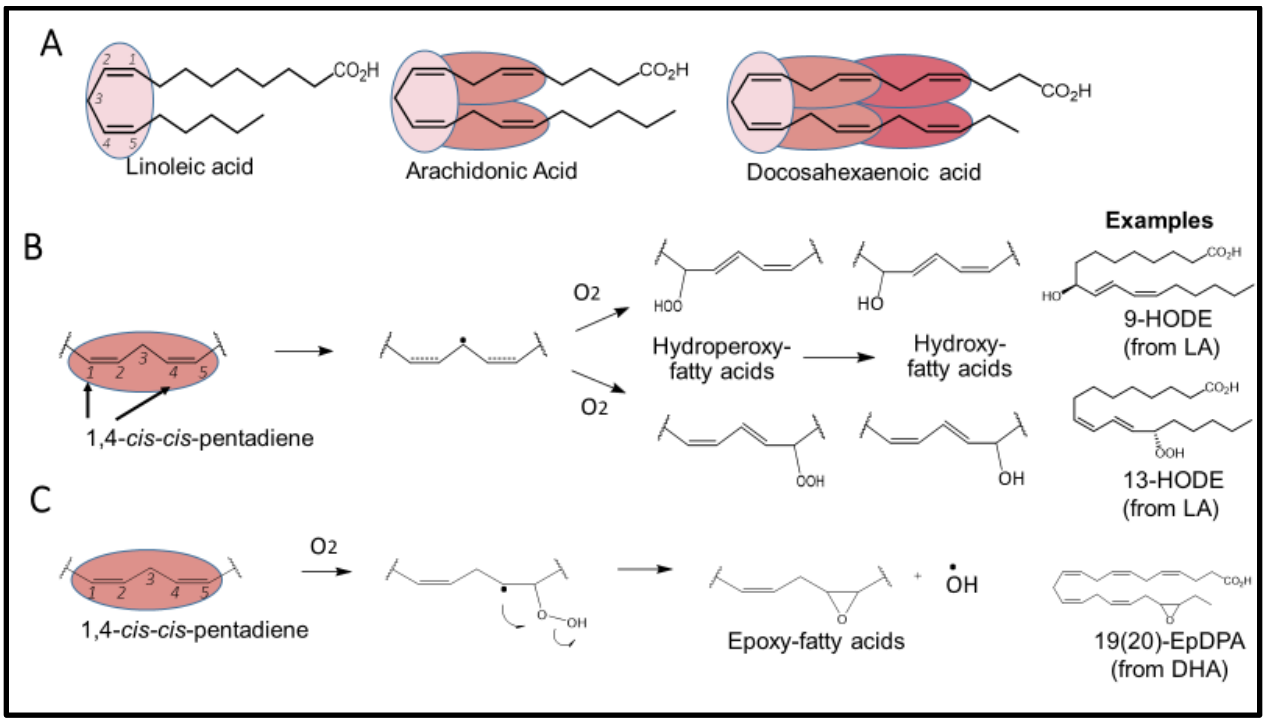


Figure 1. Oxidation of the 1,4-*cis-cis*-pentadiene structures in polyunsaturated fatty acids

(A) Linoleic, arachidonic and docosahexaenoic acid contain one, three and five 1,4-cis-cis-pentadiene structures, respectively (shown in blue). (B) 1,4-cis-cis-pentadiene structures are susceptible to enzymatic and free-radical mediated peroxidation, forming hydroperoxy-, hydroxy- and epoxy-fatty acids. Hydroperoxy-fatty acids are intermediates in the synthesis of numerous oxylipins, such as 9- and 13-hydroxy-octadecadienoic acids (9- and 13-HODEs) from linoleic acid. (C) Epoxy-fatty acids, such as the 19(20)-epoxy-docosapentaenoic acid derivative of DHA, contain one less carbon-carbon double bond than their parent fatty acid.

Detailed descriptions of the pathways involved in the biosynthesis of oxylipins derived from arachidonic acid (AA), docosahexaenoic acid (DHA), and linoleic acid (LA) are provided in Sections 1.1.1-1.1.3. Briefly, cyclooxygenase (COX) enzymes convert AA into endoperoxide prostaglandin PGG2, which is further converted to numerous prostanoids and thromboxanes (reviewed in Section 1.1.1). Lipoxygenase (LO) enzymes (5-LO, 12-LO, 15-LO) convert AA to AA-hydroperoxides, which can then be further metabolized to generate AA-monohydroxides, leukotrienes, lipoxins, and hepoxilins (23) (reviewed in Section 1.1.1). LO enzymes convert DHA to DHA-hydroperoxides, which can then be further metabolized to form monohydroxy-DHA derivatives, resolvins, protectins, and maresins (24) (reviewed in Section 1.1.2). LA is also a substrate for COX and LO-mediated enzymatic peroxidation, forming LA-hydroperoxides, which can be further metabolized to form a variety of bioactive oxylipins (reviewed in Section 1.1.3). AA, DHA, and LA are also substrates for CYP-epoxygenase enzymes that generate epoxy fatty acids, which can be then be converted to vicinal diols by soluble epoxide hydrolase (sEH) (25). Two-enzyme systems consisting of LO-mediated peroxidation followed by hydroperoxide isomerization (e.g. by e-LOX-3 or CYP2S1) can convert PUFAs to hydroxy-epoxides, which can be subsequently hydrolyzed to form tri-hydroxy PUFAs (25-27). PUFAs—including AA, DHA and LA—can also undergo non-enzymatic free-radical mediated peroxidation to generate the numerous additional families of oxylipins. Examples of free radical generated oxylipins include AA-derived isoprostanes, 8-isoPGF2α, and certain regioisomers and racemic mixtures of HETEs, DHA-derived neuroprostanes (28), certain regioisomers and racemic mixtures of HDHAs (29), and LA-derived racemic mixtures of HODEs (30). These oxylipins and their downstream chain-cleavage products can serve as markers for lipid peroxidation and “oxidative stress” (31, 32). Profiling of labile oxylipins and their more stable

pathway markers and inactivation products can provide insights into biochemical processes in tissues (1) and can potentially serve as predictive or pharmacodynamic biomarkers. Since humans lack the ability to synthesize PUFAs de novo, the levels of these compounds and their oxylipin derivatives can be altered by targeted dietary manipulation (19, 32, 33). These mechanistic insights and biomarker data could ultimately provide new targets and leads for the diagnosis, prevention and/or treatment of various diseases [reviewed in (1)].

1.1.1. Classic eicosanoids derived from arachidonic acid (AA)

AA is the precursor to dozens of oxylipins that have well-established biochemical and health effects in multiple organs and disease states (23). Bioactive oxylipins derived from AA are best known as potent mediators of nociception and parturition (e.g. 2-series prostaglandins) (16), vasodilation (e.g. prostacyclin, epoxyeicosatrienoic acids)(34), vasoconstriction and thrombosis (e.g. thromboxanes), immune activation (e.g. leukotriene B4) (35), inflammation-resolution (36) , allergy and atopy (e.g. leukotrienes C4/D4/E4) (37, 38)], and neuronal activation and pain (e.g. hepoxilins) (39, 40).

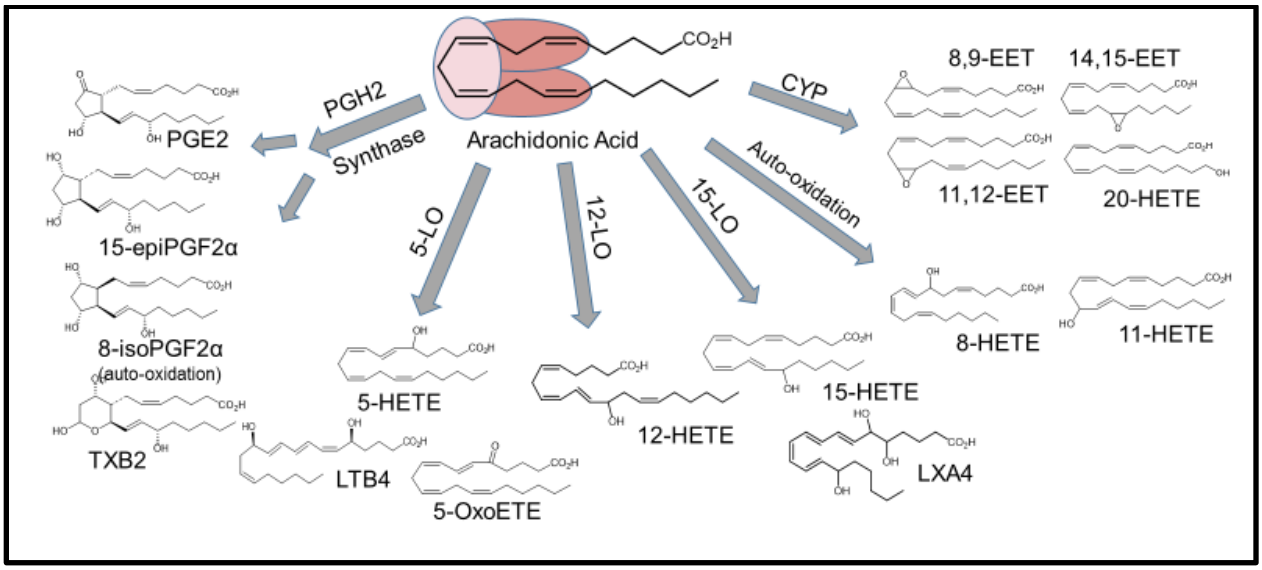


Figure 2: Molecular pathways involved in enzymatic and non-enzymatic conversion of arachidonic acid (AA) to representative AA-derived oxylipins. *AA contains three 1,4-cis-cis-pentadiene structures that are susceptible to peroxidation. AA is converted by prostaglandin (PG)H2 synthase, also known as cyclooxygenases (COX) with subsequent conversion to PGE2, 15-epiPGF2α, 8iso-PGF2α, or thromboxane (TX) A2 and its stable inactivation product TXB2. AA can be converted by 5-, 12-, and 15-lipoxygenases (LOs) to generate 5-HETE, 12-HETE or 15-HETE, respectively. LTB4 and 5-OxoETE are downstream products of the 5-LO pathway; lipoxin (LX)A4 is a downstream product of the 15-LO pathway. AA can be converted by auto-oxidation to generate 8- and 11-HETE, or can undergo cytochrome P450 (CYP) epoxygenase-mediated oxidation to generate multiple epoxyeicosatrienoic acids (EETs) or terminal hydroxylation to form 20-HETE.*

Well-established molecular pathways involved in the synthesis of bioactive AA-derived oxylipins include (**Fig. 2**): (i) COX-mediated enzymatic conversion of AA into endoperoxide prostaglandin (PGG2), which is further converted to numerous prostaglandins and thromboxanes; (ii) 5-LO-mediated conversion of AA to form 5-HPETE, with subsequent conversion to 5-HETE and 5-OxoETE or leukotrienes (LTs) B4, C4, D4 and E4; and (iii) CYP-mediated conversion of AA to form epoxyeicosatrienoic acids (EETs). These biosynthetic and signaling pathways for prostaglandins, thromboxanes, leukotrienes and d EETs are targets of many pharmacological interventions designed to prevent or treat conditions ranging from acute and chronic pain, to thrombosis, asthma, atopy, atherosclerosis, hypertension, and failure of labor progression (41-47).

1.1.2 Oxylipins derived from n-3 DHA

Emerging preclinical evidence suggests that oxylipins derived from n-3 docosahexaenoic acid (DHA), also known as docosanoids—including n-3 monohydroxides and epoxides, as well as resolvins, protectins, and maresins (collectively known as specialized pro-resolving mediators or SPMs) have potent anti-inflammatory and inflammation-resolution actions (24). Notable examples of SPMs exhibiting pro-resolving actions in preclinical models include E-series resolvins (48), D-series resolvins (RvD1-D6) (49), protectins (50), maresins (51) and related compounds derived from n-3 docosapentaenoic acid (52). The n-3 monoepoxides 13(14)-epoxy-DPA, 16(17)-epoxy-DPA, and 19(20)-EpDPA, and maresins, protectins and resolvins have also demonstrated anti-nociceptive effects in preclinical models (53-55). These promising preclinical findings have spurred the development of pharmacological, nutritional supplement, and dietary approaches for boosting the actions of these compounds as a strategy for treating inflammatory conditions and neuropathic pain (56-58).

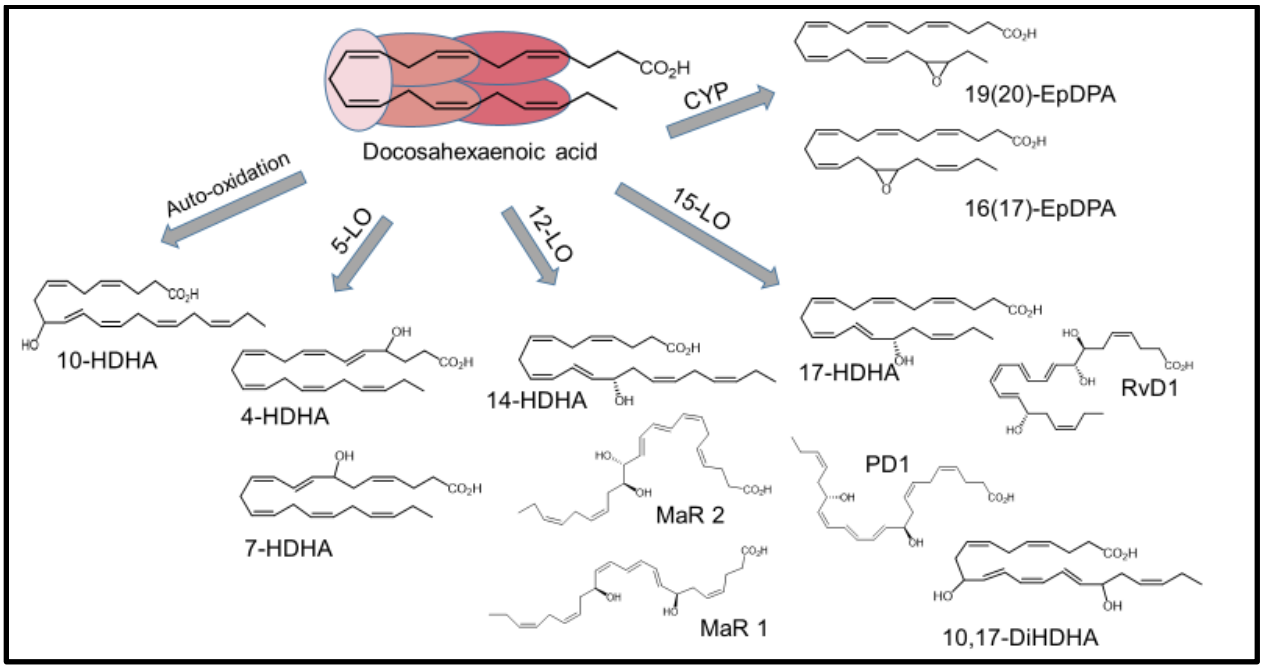


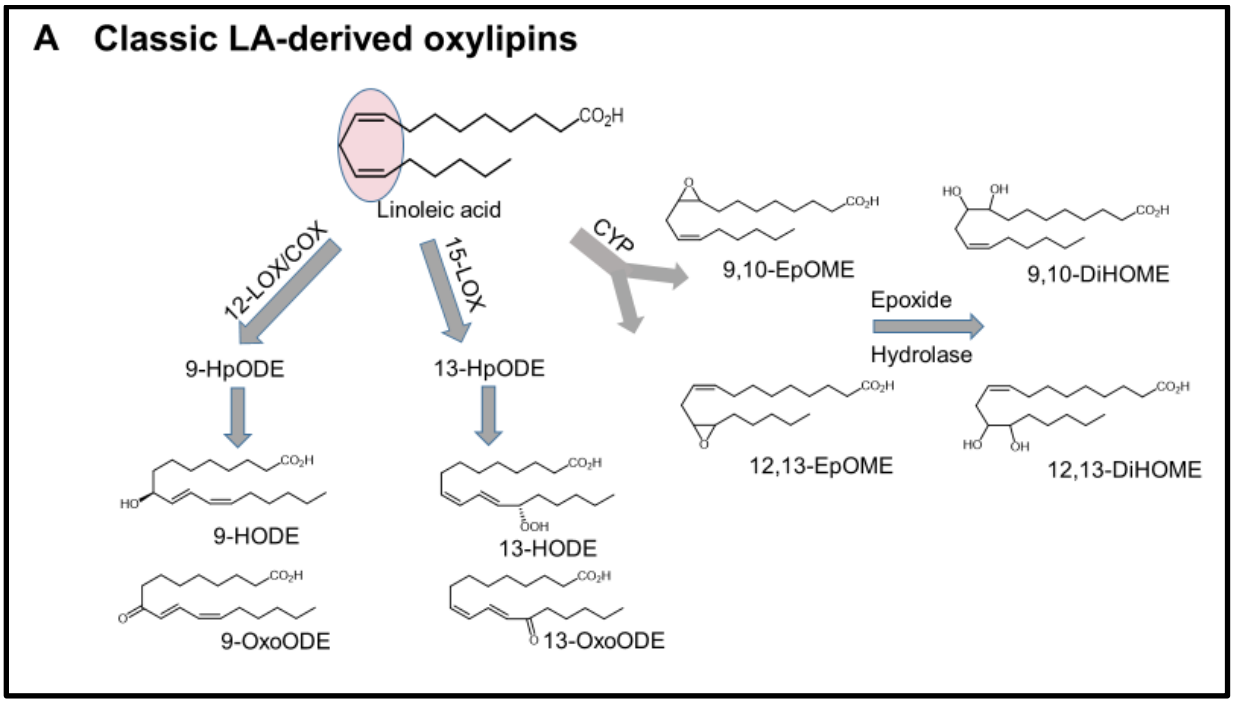
Figure 3: Molecular pathways involved in enzymatic and non-enzymatic conversion of docosahexaenoic acid (DHA) to representative DHA-derived oxylipins. *DHA contains five 1,4-cis-cis-pentadiene structures that are susceptible to peroxidation. DHA is converted by 5-, 12-, and 15-lipoxygenases (LOs) to generate 4-hydroxy-DHA (4-HDHA) and 7-HDHA, 14-HDHA, or 17-HDHA, respectively. 14-HDHA and 17-HDHA can serve as pathway precursors indicating activation of the 12-LO and 15-LO pathways that are used for production of bioactive maresins, and resolvins and protectins, respectively. Since humans lack an 8-lipoxygnease enzyme, 10-HDHA is generally considered to be an auto-oxidation product in humans.*

1.1.3 Oxidized linoleic acid metabolites (OXLAMs)

LA is the direct precursor to the classic oxidized linoleic acid metabolites, collectively known as OXLAMs, including hydroperoxy-octadecadienoates (HPODEs), hydroxy-octadecadienoates (HODEs), oxo-octadecadienoates (oxo-ODEs), epoxy-octadecenoates (EpOMEs), and dihydroxy-octadecenoates acids (DiHOMEs)(**Fig. 4A**), which have reasonably well-established actions and are linked to various diseases (reviewed in (59)). Several new families of oxidized LA derivatives have been recently described—including hydroxy-epoxide-octadecenoates, keto-epoxide-octadecenoates, and trihydroxy-octadecenoates (TriHOMEs)(26, 27, 60)—which have poorly understood functions (**Fig. 4B**).

Classic OXLAMs are enriched in the esterified pools of oxidized lipoproteins, and as free acids and in esterified lipid pools (such as phospholipids, triacyglycerols, and cholesteryl-esters) of tissues characterized by inflammation and/or oxidative stress (61). OXLAMs have been mechanistically linked to several pathological conditions including steatohepatitis (62, 63), atherosclerosis (64), chronic pain (9), and asthma (65). Accordingly, in preclinical models free HpODEs and HODEs have demonstrated pathological activities including endothelial cell activation, matrix degradation and remodelling (66), immune activation and foam cell formation (67, 68). OXLAMs may play beneficial roles in certain conditions (reviewed in (69)). For example, emerging evidence suggests that specific OXLAM regio- and stereoisomers play important structural roles in forming the epidermal water barrier (70, 71), which is required to prevent transepidermal water loss, and may protect against xerotic dermatoses (72). This function helps account for LAs designation as an “essential fatty acid”, since LA deficiency is implicated in the lack of a functional epidermal water barrier and scaly dermatitis (73, 74). Notably, only a very small amount of LA (about 0.5% of energy) in the diet appears necessary to

prevent manifestations of essential fatty acid deficiency (75). LA is enriched in the esterified lipid pools of skin (26, 71) and genetic variants coding for non-functional variants 12-R-LO and/or e-LOX-3 manifest as congenital ichthyoses and loss of water barrier integrity resembling experimental dietary “essential fatty acid deficiency”, suggesting that as of yet unidentified LA derivative(s) of these enzymes is critical for proper formation or function of the water barrier (71). Interestingly, several OXLAMs were recently reported to be abundant components of the human vernix caseosa, a cheesy layer that covers the skin of the fetus during pregnancy and at birth and is thought to help protect the newborn from external insult after birth (76). The putative protective roles of specific OXLAM species in the epidermal water barrier and vernix caseosa are a matter of intense inquiry (71, 76).



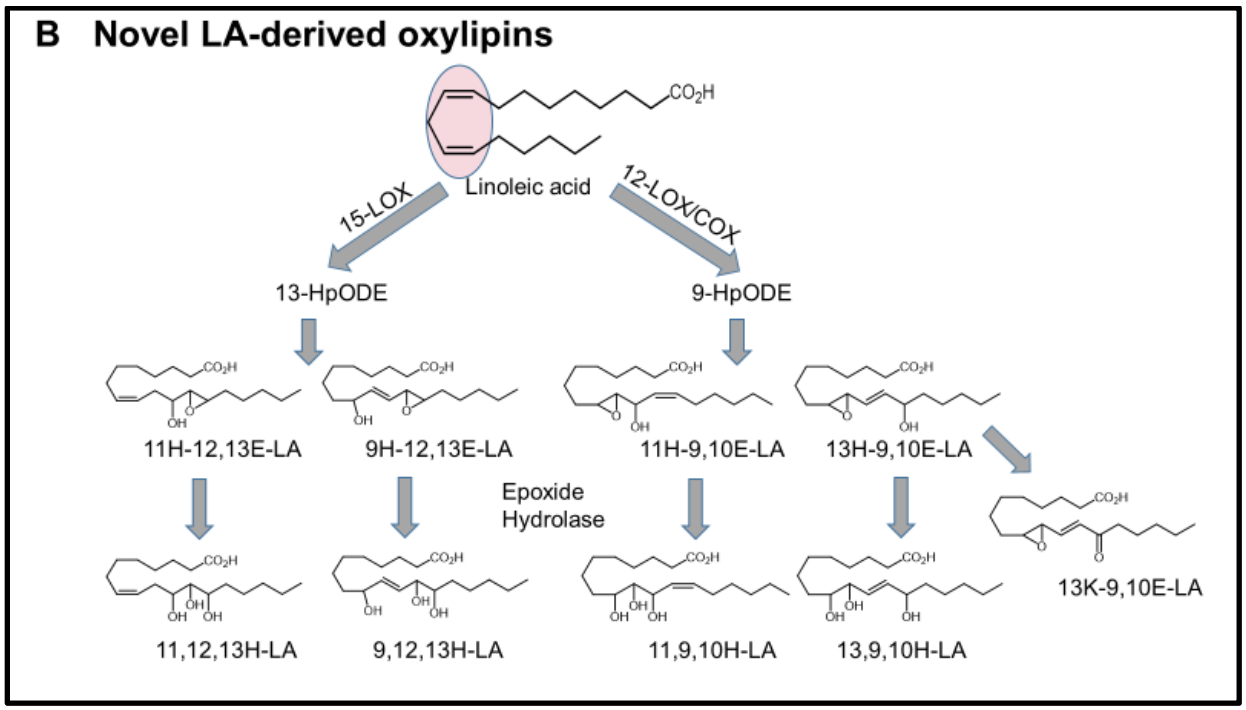


Figure 4: Molecular pathways involved in enzymatic and non-enzymatic conversion of linoleic acid (LA) to representative LA-derived oxylipins. *LA contains one 1,4-cis-cis-pentadiene structure that is susceptible to peroxidation. Panel A shows molecular pathways involved in enzymatic and non-enzymatic conversion of LA to classic LA-derived oxylipins including hydroxy-octadecadienoates (HODEs), oxo-octadecadienoates (oxo-ODEs), epoxy-octadecenoates (EpOMEs), and dihydroxy-octadecenoates acids (DiHOMEs). Panel B shows proposed pathways involved in conversion to emerging, less well-established oxylipins including hydroxy-epoxide-octadecenoates(H-E-LAs), keto-epoxide-octadecenoates (K-E-LAs), and trihydroxy-octadecenoates (TriHOMEs).*

1.2 Strategies for manipulating oxylipins by diet or supplementation

1.2.1 Seafood and/or n-3 supplements

Extensive evidence indicates that eating seafood or n-3 EPA and DHA supplementation increases the abundance of EPA and DHA and their n-3 derived oxylipins in both esterified and free lipid pools (77-80), in many human and mammalian tissues. This includes several EPA and DHA-derived epoxides and the precursors and pathway markers for E-series and D-series resolvins, maresins and protectins (19). There is also an extensive body of evidence indicating that dietary or supplemental n-3 EPA and DHA decreases the AA content of esterified lipid pools with subsequent reductions in AA-derived oxylipins including prostanoids, leukotrienes, and HETEs (19, 79, 81). Omega-3 derived oxylipins are reported to have beneficial effects in numerous preclinical studies (51, 53-55) and diet-induced changes n-3 fatty acids or derived oxylipins are associated with favorable outcomes in some but not all, human studies (81-85). A large, tightly controlled randomized trial testing the effect of DHA supplementation on the risk of preterm birth in pregnant Australian women (n=5,544) known as ORIP (Omega-3 fats to Reduce the Incidence of Prematurity) Trial, and led by my Thesis supervisors Professors Maria Makrides and Robert Gibson, was recently published in the *New England Journal of Medicine* (86). Makrides and Gibson had previously demonstrated that DHA supplementation provided from week 14 through delivery produced a shift the curve of birth, decreasing the risk of preterm birth, while increasing the risk of caesarean section (87). DHA supplementation is known to increase DHA and DHA-derived oxylipins with anti-inflammatory and pro-resolving properties in maternal tissues including placenta (88) and could hypothetically also decrease AA-derived oxylipins in maternal tissues, however these effects on AA remain to be demonstrated experimentally.

The ORIP study, which supplemented DHA through week 34 as a strategy for decreasing preterm birth without increasing the risk of C-section, found that DHA supplementation, in a combination of singleton and twin pregnancies, did not alter the overall risk of early preterm birth (primary endpoint)(86). However, in secondary analyses limited to singleton pregnancies, DHA supplementation did decrease the risk of preterm and early preterm birth compared to placebo (86). Beneficial effects were most evident in mothers with low baseline DHA status, measured as the total (esterified plus unesterified) DHA; however, an equally potent effect in the opposite direction was seen in mothers with high DHA status at baseline (86). These results suggest a complex relationship between DHA supplementation and the duration of gestation. Notably, in plasma fatty acids esterified in triacylglycerols, cholesteryl esters, and phospholipids account for the vast majority of total fatty acids, with unesterified fatty acids accounting for the remainder. Since unesterified fatty acids are thought to be labile and bioactive and to represent a more immediate oxylipin-precursor pool than their esterified counterparts (89), measurement of unesterified fatty acids may provide additional insights into metabolic status. Here, we used unique plasma samples from ORIP to test the hypothesis that DHA supplementation would increase unesterified DHA and DHA-derived oxylipins including HDHAs and EpDPAs, while decreasing unesterifted AA and AA-derived oxylipins including HETEs, prostaglandins and thromboxanes. The targeted LC-MS/MS oxylipin and unesterified fatty acid profiling completed as part of this Thesis is intended to provide unique insights into the potential roles unesterified precursor fatty acids and DHA-derived and AA-derived oxylipins in pregnancy and preterm birth. Our extensive biochemical investigation of ORIP plasma samples may provide insights that could help explain the complex ORIP results.

1.2.2 Rising intake of linoleic acid in modern industrial diets

Linoleic acid (LA) is the most abundant PUFA in modern human diets, currently accounting for about 7.2-7.5% of total energy intake and 89% of total PUFA intake in pregnant women and the general U.S. population (**Fig 5**). In Australia, LA accounts for about 4.5% of total energy intake and 79% of PUFA intake in reproductive age women and about 4% of total energy intake and 75% of PUFA the general population (90)(**Fig 6**). The higher LA intakes in the U.S. compared to Australia are due in part to higher use of LA-rich soybean oil in the U.S. (91), with higher use of canola oil in Australia. LA accounts for about 50% and 20% of energy in soybean and canola oils, respectively (**Fig 7 below**).

LA intakes have increased markedly in industrialized countries in the past half century, primarily due to increased use of liquid vegetable oils (concentrated sources of LA)(**Table 1 and Fig. 7A&B**) as ingredients in many packaged and processed foods, and in commonly used cooking and frying oils (reviewed in (59)).

High LA intakes of modern diets depend on industrial oil processing to create highly concentrated oils sources of LA. For example, LA accounts for 55% of the energy in corn oil versus <1% of energy in whole corn (**Fig. 7B**).

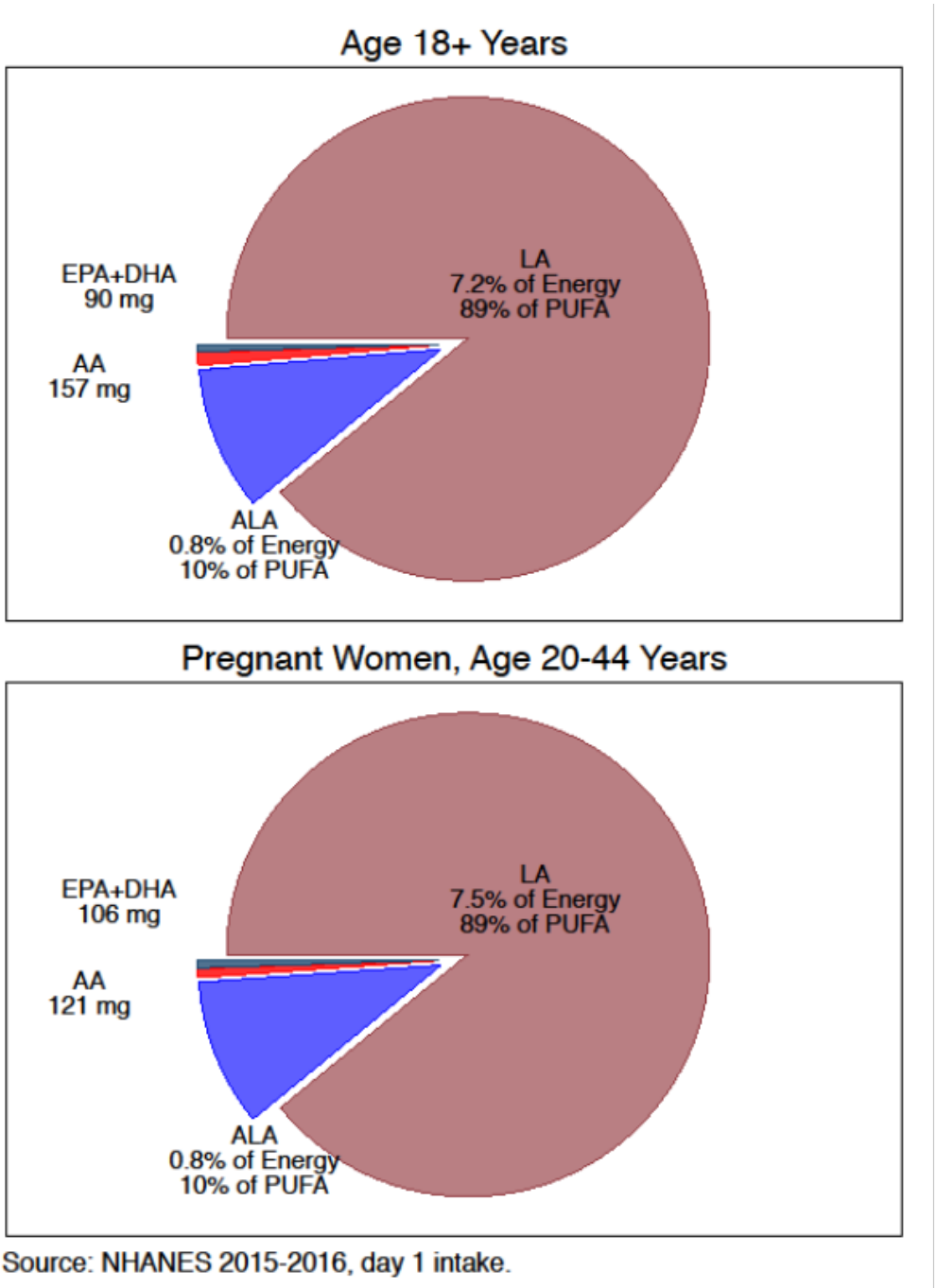


Fig. 5. Average polyunsaturated fatty acid intakes in U.S. adults and pregnant women

Abbreviations: EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; AA, arachidonic acid; LA, linoleic acid; ALA, alpha-linolenic acid; PUFA, polyunsaturated fatty acids.

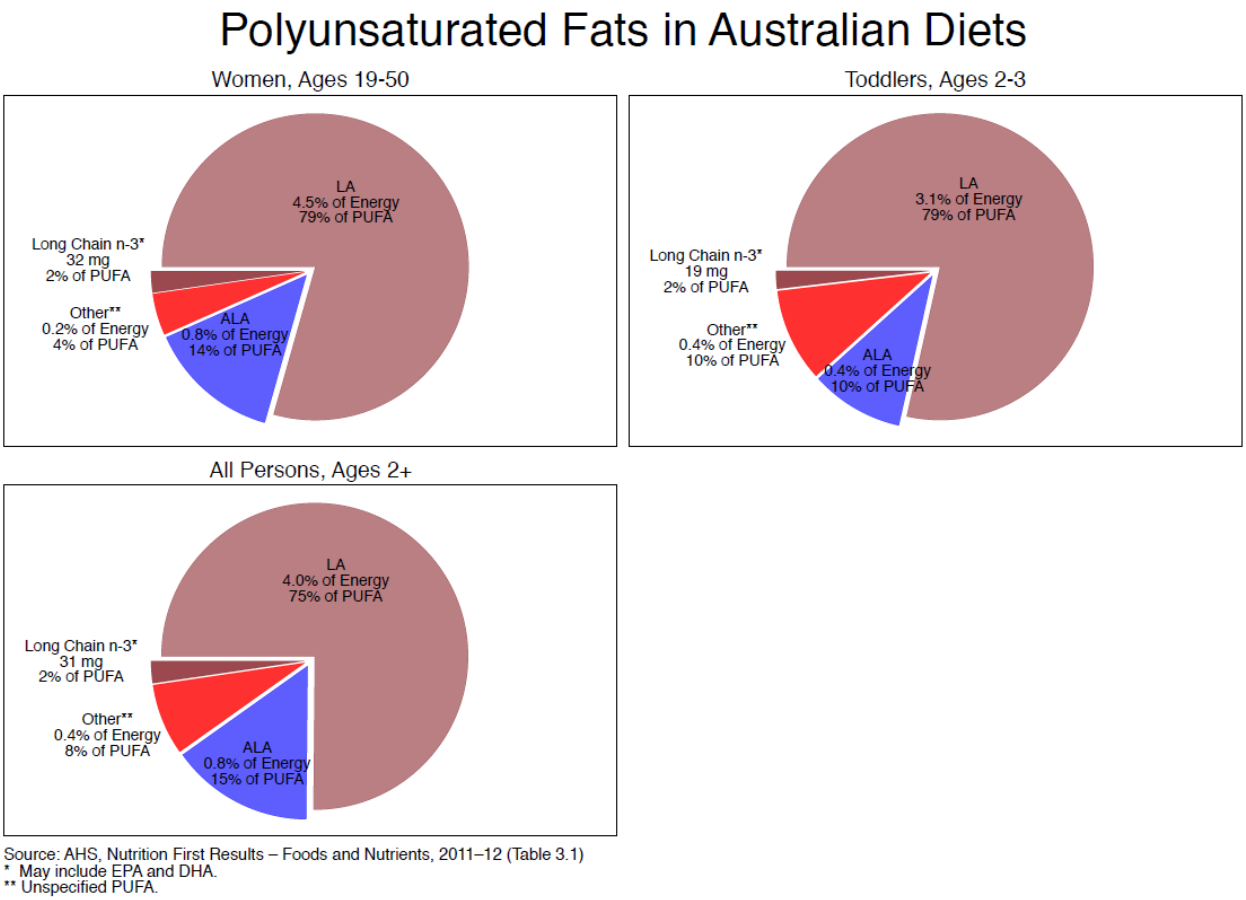


Fig. 6. Average intakes of polyunsaturated fatty acids in the Australian general population, toddlers and reproductive age women *(90)*

Abbreviations: LA, linoleic acid; ALA, alpha-linolenic acid; PUFA, polyunsaturated fatty acids.

Table 1. Main natural sources of ALA (adapted from the reference)

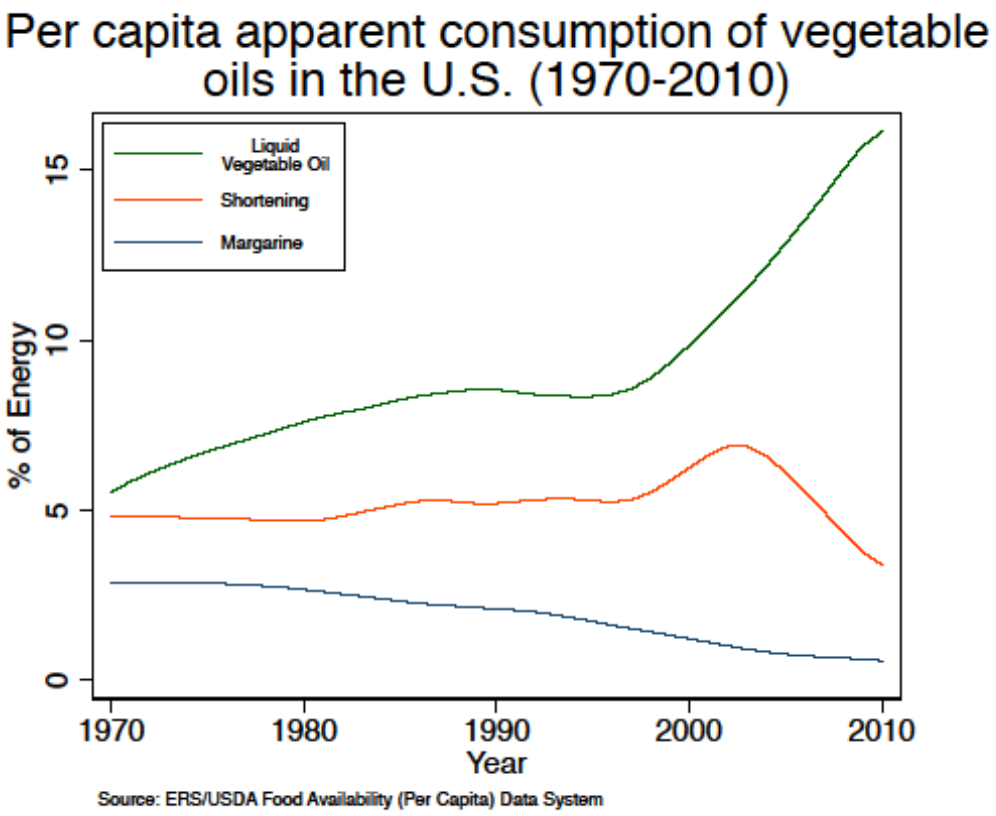
Fat source	Percentage LA of the total lipid (expressed as g/100g)
Safflower oil[ϕ]	75
Sunflower oil[ϕ]	66
Corn oil	54
Cottonseed oil	52
Soybean oil[ψ]	50
Canola oil[†]	19
Olive oil	10
Coconut oil	2
Butter	2

US Department of Agriculture, Agricultural Research Service. 2012. USDA National Nutrient Database for Standard Reference, Release 25. www.ars.usda.gov/nutrientdata (92).

[ϕ] high-oleic versions of these oils with lower linoleic acid (LA) are commercially available

[ψ] soybean oil is the most abundant liquid oil in the U.S. food supply

[†] canola oil is the most abundant liquid oil in the Australian food supply


Per capita apparent consumption of vegetable oils in the U.S. (1970-2010)
Liquid Vegetable Oil
Shortening
Margarine
% of Energy
0
5
10
15
1970
1980
1990
2000
2010
Year
Source: ERS/USDA Food Availability (Per Capita) Data System

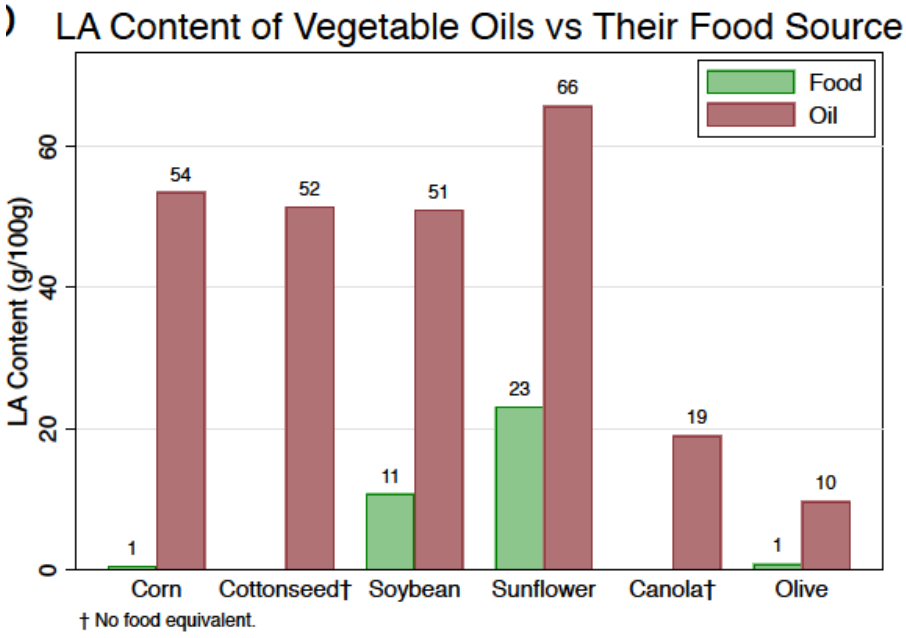

LA Content of Vegetable Oils vs Their Food Source
Food
Oil
LA Content (g/100g)
0
20
40
60
1
54
52
11
51
23
66
19
1
10
Corn
Cottonseed†
Soybean
Sunflower
Canola†
Olive
† No food equivalent.

Fig. 7. Consumption and composition of vegetable and seed oils that are highly concentrated sources of linoleic acid. *(A) Liquid vegetable oil consumption in the United States increased from about 5% to 15% of food energy between 1970 and 2010, while consumption of shortenings and margarine declined. (B) Liquid vegetable oils derived from corn, cottonseed, soybean and sunflower oils are far much more concentrated sources of LA than their unrefined precursor sources.*

Importantly, the LA exposures in pregnant women and the general population are highly variable, with daily intakes in U.S. women ranging from <3 to ≈20% of energy (**Fig. 8**); an estimated 25% of pregnant U.S. women get >10% of daily energy intake from LA.

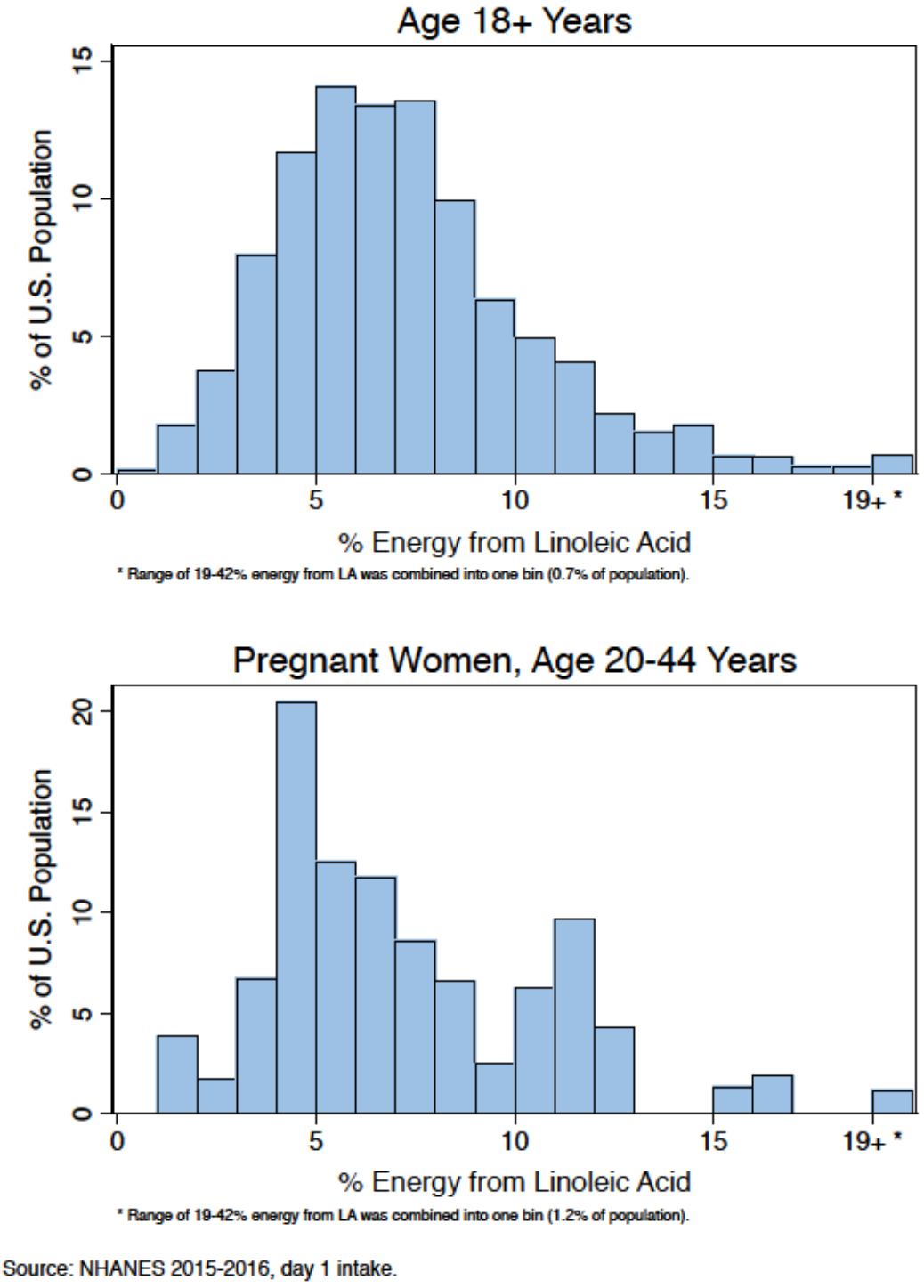


Fig. 8. Distributions of polyunsaturated fatty acid intakes in the United States general population and pregnant women. *LA intakes in the United States ranges are estimated to range from <3 to about 20% of food energy in the general population (A) and pregnant women (B).*

Despite this variability, modern intakes in the United States are uniformly higher than the historical norm of 2-3% of energy as LA provided by traditional diets, and by modern diets without added vegetable oils (reviewed in (59)). Thus, modern LA intakes in the United States are simply not possible without industrial food processing and widespread addition of concentrated vegetable oils. While current LA intakes in Australia (**Fig. 6**) are substantially lower than in the United States, intakes still tend to be higher than the historical norm of 2-3% of energy, especially in reproductive age women.

LA is classically recognized as having important health effects for three reasons: (1) for being an "essential fatty acid" because small amounts of LA (about 0.5% of energy) in the diet are required for integrity of the epidermal water barrier (73, 74); (2) for decreasing serum low density lipoprotein cholesterol when replacing dietary saturated fats (93); and (3) for being the precursor to arachidonic acid (AA, 20:4n-6) (94, 95), which in turn is the precursor to prostanoids, which have myriad effects ranging from promoting physical pain (96) to inducing parturition (14, 16). As replacing saturated fat with LA decreases low density lipoprotein (LDL) cholesterol, it has long been believed that high LA diets would be cardioprotective (93). Paradoxically, however, available evidence from randomized controlled trials indicates that replacement of saturated fat with vegetable oils rich in LA has neutral to unfavourable effect on the risks of coronary heart disease and deaths from all causes (59). Moreover, human controlled trials have not yet demonstrated that altering dietary LA has any effect on AA or prostaglandin production in *human* tissues (94). Thus, there is a need to understand more about the potential consequences of exposure to high LA intakes in humans.

1.2.3 Strategies for decreasing native and oxidized linoleic acid in the diet

One of the main reasons for potential concern about the increased abundance of LA in modern industrialized diets is due to the properties of the biologically active derivatives of this fatty acid. LA contains a 1,4-*cis,cis*-pentadiene structure, which is susceptible to enzymatic and non-enzymatic peroxidation (**Fig 1**). LA is the direct precursor to the oxidized linoleic acid metabolites known as OXLAMs (reviewed in **Section 1.1.2 and Fig 4**). OXLAMS can be created two ways. Relatively large quantities of these OXLAMs are formed when vegetable oils rich in LA are cooked or otherwise heated (97-99) , and published literature suggests that preformed OXLAMs may be either absorbed and incorporated into human tissues after consumption, or initiate peroxidation of endogenous LA (100, 101). Although human data are lacking, consumption of heated vegetable oils rich in LA has been linked to development of cerebellar ataxia in chicks (102, 103). And short term feeding of heated safflower oil to rats in early pregnancy had teratogenic effects (104) that were partially prevented by co-administration of tocopherols.

It is therefore potentially concerning that LA-enriched oils are ingredients in many food products that are fried or otherwise heated, including potato chips, french fries, cooking/frying oils, margarines, and shortenings. Because LA can be oxidized to generate several OXLAMs, it is likely that the levels of these OXLAMs, like LA, in modern diets have also increased markedly over the past few decades. However, there is currently virtually no information on the level of OXLAMs in frequently consumed foods in either the United States or Australia, and their relationship with OXLAM concentrations in humans.

In addition to being consumed preformed in foods, LA can also be endogenously converted into OXLAMs either via the actions of several mono-oxygenase enzymes, or non-

enzymatically via free radical-mediated oxidation (reviewed in **Fig 4**). Therefore, the abundance of OXLAMs in human tissues potentially reflects consumption of both preformed OXLAMs and those resulting from metabolism of their precursor LA in the body, and the higher intakes of LA are likely to be further contributing to increases in OXLAM concentrations in human tissues. Dietary LA lowering has been shown to decrease OXLAM concentrations in the total pool of human plasma (105). High LA diets appear to increase OXLAMs in rodents in a tissue dependent manner (10), suggesting the ability to reduce tissue OXLAMs by decreasing the amount of non-oxidized LA in the diet. At the time these Thesis projects were initiated, the effects of dietary OXLAMs on tissue OXLAMs, their precursor fatty acids and degradation products were largely unknown.

1.3 Complications of pregnancy and potential roles of oxylipins in pregnancy & preterm birth

Despite major advancements in clinical sciences, complications in pregnancy remain a major source of morbidity and mortality around the world [reviewed in (106)]. Important pregnancy-related complications include high blood pressure (preeclampsia), gestational diabetes, placental abruption, intrauterine growth restriction, preterm birth (before 37 weeks), very preterm birth (before 32 weeks) and perinatal mortality. In the United States and Australia, preterm birth affects about 10%, and 8% of infants born, respectively (107, 108). Preterm-related causes of death together account for about 35% of all infant deaths, more than any other single cause. Preterm birth is also a leading cause of long-term neurological disabilities in children (109, 110). The incidence of complications in pregnancy continues to rise around the world. While the reasons for this remain unclear, the significant shifts which have occurred in the composition of typical diets in industrialized countries over the past few decades, especially

the pronounced increase in concentrated vegetable and seed oils, has been suggested as a potential contributing factor.

1.3.1 Prostaglandins and leukotrienes in parturition

2-series prostaglandins (e.g. PGE2 and PGF2α) are well-known to play pivotal roles in initiation and progression of labor by promoting cervical maturation and myometrial contraction (14). Vaginal prostaglandins including PGE2 and PGF2α have been used for induction of labor since the 1960s (16). A 2014 Cochrane review of seventy randomized controlled trials concluded that vaginal application of prostaglandins at term probably reduces the likelihood of vaginal delivery not being achieved and the need for caesarean section (16). However vaginal prostaglandin treatment does increase the risk of uterine hyperstimulation with fetal heart rate changes (16). Inhibition of cyclooxygenase (COX)-mediated synthesis of 2-series prostaglandins via non-steroidal anti-inflammatory drugs (NSAIDs) can suppress preterm labor and prolong pregnancy in animal models and humans (14, 111) and aspirin during pregnancy appears to decrease risk of preeclampsia and hypoxia-related placental pathology in high risk patients (112, 113). However, there are concerns for important fetal side effects—including renal and cardiovascular toxicity—with aspirin and NSAID use during pregnancy. Prostaglandin receptor antagonists (111, 114) have shown promise in early stage clinical trials for preventing or delaying preterm birth without the side effects of NSAIDs.

The placenta in preterm birth is characterized by an inflammatory phenotype (115), including alterations in prostaglandin synthesis and catabolism (116). Human blood, placenta and fetal tissues are enriched in n-3 and n-6 fatty acids, and selected oxylipins derived from AA, DHA and LA have been measured in cord blood (15, 117) and aminiotic fluid (18, 118). 5-lipoxygenase derivatives of AA (e.g. 5-HETE and leukotriene B4) may also be involved in

parturition (2, 17, 18). Leukotriene B4 and 5-HETE has been shown to be elevated in amniotic fluid during normal parturition (18), and to be elevated in amniotic fluid of mothers with premature rupture of membranes in the presence of infection, labor, or especially both (2). 5-HETE and leukotriene B4 were elevated in amniotic fluid in pregnant women who had intra-amniotic infection, suggesting that these 5-lipoxygenase derivatives could serve as potential biomarkers of microbial invasion of the amniotic cavity, a well-established cause of preterm birth (118, 119). Thus, it is possible that in addition to prostaglandins, other bioactive oxylipins derived from n-6 AA (e.g. leukotrienes, HETEs), n-3 fatty acids, and/or linoleic acid (discussed below) could impact parturition and development.

1.3.2 Potential roles of DHA-derived oxylipins in pregnancy and development

DHA is well-known to be concentrated in fetal tissues (biomagnification) compared to maternal tissues and is a major component of the placenta and developing brain (120). DHA supplementation in pregnancy has been investigated as a strategy for enhancing neurodevelopment (121, 122) and for decreasing preterm birth (87, 123). Several plausible mechanisms exist whereby maternal DHA consumption could favorably influence pregnancy, parturition, and/or brain development. Notable example includes partial replacement of AA by DHA in oxylipin precursor pools following DHA supplementation, which has been reported to decrease PGE2 in multiple tissues (124). Such a reduction in placental AA would be expected to decrease uterine contractility and preterm birth (14, 111). DHA supplementation may also decrease inflammatory processes implicated in preterm birth by increasing the production of DHA-derived oxylipins with anti-inflammatory and pro-resolving properties in placenta or blood (88). ORIP study analyses may inform these hypotheses.

1.3.3 Potential roles of OXLAM exposure in pregnancy and development

OXLAMs have been mechanistically linked to several pathological conditions and consumption of heated vegetable oils rich in LA or infusion of LA-hydroperoxides formed from heating LA, have been linked to development of cerebellar ataxia in chicks, and teratogenicity in rats, suggesting that dietary and circulating OXLAMs could potentially have adverse effects on the developing brain. However, the effects of OXLAM exposures on neurodevelopment are largely unknown in mammals. OXLAMs are abundant in circulating lipoproteins—particularly in the oxidized low-density lipoprotein (OxLDL) particles of individuals with systemic disorders characterized by inflammation and/or oxidative stress (reviewed in (61)). Scavenger receptors, which readily bind OxLDL (e.g. CD36, Stabilin-1, and LOX-1), are strongly expressed in the human placenta (125-127), and increase during periods of oxidative stress (128) providing a potential mechanism for delivery of large amounts of OXLAMs and other oxidized lipids to placenta during conditions of inflammation or oxidative stress. Interestingly, OxLDL and its scavenger receptor LOX-1 have been described together in trophoblasts at the maternofetal interface (126), and OxLDL concentrations in placenta are linked to intrauterine growth restriction (129). Together, these observations provide an intriguing clue that OXLAM exposures in pregnancy could potentially affect parturition or development. Yet, despite major increases in LA intake, we currently have no data to assess OXLAM exposures in pregnant women, or to determine whether these exposures are associated with pregnancy and developmental outcomes.

In summary, there is accumulating evidence that oxylipins could affect the function of multiple organs, and that one subset of oxylipins (OXLAMs) can adversely affect the function of certain organs including the liver, while another subset (docosanoids) could have favorable effects. Classic oxylipins (prostaglandins) are known to play a critical role in parturition, and

there are plausible mechanisms whereby other oxylipins could have favorable or unfavorable effects on the placenta and developing brain (see above). However, we currently have only a very limited understanding of whether and how oxylipins influence biochemical and clinical endpoints in pregnancy and neurodevelopment. Despite these plausible mechanisms and the potential for public health impact, there are large gaps in our understanding of the biochemical effects, and any effects in humans are largely unknown.

1.4 Statement of Intent

The overall objective of this thesis is to lay the groundwork necessary for refining hypotheses, and for designing and conducting future, definitive studies that can determine the impacts of oxylipins on human pregnancy and neurodevelopment. The Aims of the Thesis are (1) To first establish proof of concept that the largest alteration in dietary PUFAs in modern times (increased consumption of linoleic acid and preformed OXLAMs from heated high LA oils) can alter brain biochemistry in a mammalian model; (2) Next, to apply the knowledge generated from the above to inform the development of targeted analytical tools needed to measure unesterified fatty acids and oxylipins in human samples from pregnant women; (3) To determine the temperature and time-dependent effects of delayed blood processing, which is typical of human studies, on oxylipin concentrations in human plasma; (4) To apply these targeted assays in the plasma collected from participants in the ORIP (Omega-3 fats to Reduce the Incidence of Prematurity) study to (a) determine the ranges of concentrations and trajectory of changes in specific oxylipins throughout pregnancy in Australian women; (b) determine the effects of DHA supplementation on plasma oxylipins concentrations; and (c) explore whether oxylipin concentrations measured in the first and second trimester of pregnancy can help predict risk of preterm birth in Australian women. The data and variance obtained from are intended to inform

the design and conduct of larger, definitive studies investigating the roles of unesterified fatty acids and oxylipins in pregnancy and development in humans.

Collectively, it is anticipated that this series of projects will help stimulate a new line of inquiry in humans, by taking initial steps needed before future research can definitively determine whether and how oxylipins in general, and OXLAMs and docosanoids in particular, affect pregnancy, parturition, and development. Such work could ultimately have important implications for human populations by informing strategies for manipulating oxylipins as in a manner to decrease the risk preterm birth and/or favorably impact developmental trajectory.

1.5 Structure of the thesis

1.5.1 Thesis body

This thesis is submitted as a Thesis by Publication in accordance with the University of Adelaide, Adelaide Graduate Center regulations. Christopher Ramsden was the first and primary author on the three publications included within this thesis. The published chapters with published manuscripts (Chapters 3-5) are included in the text with formatting and minor changes in wording made to conform to the rest of this thesis. These three published manuscripts are also included in Appendix 3.

The thesis begins with an Introduction and Context chapter (Chapter 1) comprising a detailed literature review of oxylipins synthesis, metabolism, and actions and potential roles in pregnancy and parturition and the Aims of my Thesis projects. This is followed by a chapter (Chapter 2) providing an overview of the methods applied in the Thesis. Next, three chapters (Chapters 3-5) containing the three manuscripts that are published or currently undergoing peer review. The final chapter (Chapter 6) provides overall context these Thesis project findings, describes ongoing project that grew out of this Thesis work, and suggests future directions to move this field forward.

1.5.2 Appendices

Appendix 1. Methods used for oxylipin analyses in Projects 2 and 3 (published methods paper). Yuan ZX, Majchrzak-Hong S, Keyes GS, Iadarola MJ, Mannes AJ, Ramsden CE. Lipidomic profiling of targeted oxylipins with ultra-performance liquid chromatography-tandem mass spectrometry. *Anal Bioanal Chem*. 2018;410(23):6009-29. doi: 10.1007/s00216-018-1222-4.

Appendix 2. De-identification agreement for sample analysis and exemption for analysis of de-identified plasma specimens is provided in Appendix 2.

Appendix 3. Published manuscripts and statements of authorship

The three published manuscripts are provided in Appendix 3. In accordance with thesis specifications of The University of Adelaide, Statements of Authorship demonstrating the contributions of all co-authors precede each published manuscript.

1.6 References (Chapter 1)

1. Yuan ZX, Majchrzak-Hong S, Keyes GS, Iadarola MJ, Mannes AJ, Ramsden CE. Lipidomic profiling of targeted oxylipins with ultra-performance liquid chromatography-tandem mass spectrometry. Anal Bioanal Chem. 2018;410(23):6009-29.

2. Romero R, Quintero R, Emamian M, Wan M, Grzyboski C, Hobbins JC, et al. Arachidonate lipoxygenase metabolites in amniotic fluid of women with intra-amniotic infection and preterm labor. Am J Obstet Gynecol. 1987;157(6):1454-60.

3. Romero R, Emamian M, Wan M, Quintero R, Hobbins JC, Mitchell MD. Prostaglandin concentrations in amniotic fluid of women with intra-amniotic infection and preterm labor. Am J Obstet Gynecol. 1987;157(6):1461-7.

4. Serhan CN. Pro-resolving lipid mediators are leads for resolution physiology. Nature. 2014;510(7503):92-101.

5. Nordgren TM, Anderson Berry A, Van Ormer M, Zoucha S, Elliott E, Johnson R, et al. Omega-3 Fatty Acid Supplementation, Pro-Resolving Mediators, and Clinical Outcomes in Maternal-Infant Pairs. Nutrients. 2019;11(1).

6. Shearer GC, Newman JW. Impact of circulating esterified eicosanoids and other oxylipins on endothelial function. Curr Atheroscler Rep. 2009;11(6):403-10.

7. Adili R, Hawley M, Holinstat M. Regulation of platelet function and thrombosis by omega-3 and omega-6 polyunsaturated fatty acids. Prostaglandins Other Lipid Mediat. 2018;139:10-8.

8. Ozen G, Norel X. Prostanoids in the pathophysiology of human coronary artery. Prostaglandins Other Lipid Mediat. 2017;133:20-8.

9. Hargreaves KM, Ruparel S. Role of Oxidized Lipids and TRP Channels in Orofacial Pain and Inflammation. J Dent Res. 2016;95(10):1117-23.

10. Ramsden CE, Ringel A, Majchrzak-Hong SF, Yang J, Blanchard H, Zamora D, et al. Dietary linoleic acid-induced alterations in pro- and anti-nociceptive lipid autacoids: Implications for idiopathic pain syndromes? Mol Pain. 2016;12.

11. Zhang LY, Liu ZH, Zhu Q, Wen S, Yang CX, Fu ZJ, et al. Resolvin D2 Relieving Radicular Pain is Associated with Regulation of Inflammatory Mediators, Akt/GSK-3β Signal Pathway and GPR18. Neurochem Res. 2018;43(12):2384-92.

12. Zhang L, Terrando N, Xu ZZ, Bang S, Jordt SE, Maixner W, et al. Distinct Analgesic Actions of DHA and DHA-Derived Specialized Pro-Resolving Mediators on Post-operative Pain After Bone Fracture in Mice. Front Pharmacol. 2018;9:412.

13. Park CK, Xu ZZ, Liu T, Lü N, Serhan CN, Ji RR. Resolvin D2 is a potent endogenous inhibitor for transient receptor potential subtype V1/A1, inflammatory pain, and spinal cord synaptic plasticity in mice: distinct roles of resolvin D1, D2, and E1. J Neurosci. 2011;31(50):18433-8.

14. Olson DM, Ammann C. Role of the prostaglandins in labour and prostaglandin receptor inhibitors in the prevention of preterm labour. Front Biosci. 2007;12:1329-43.

15. Gouveia-Figueira S, Martens DS, Nawrot TS, Nording ML. Cord blood eicosanoid signatures and newborn gestational age. Prostaglandins Other Lipid Mediat. 2017;133:123-7.

16. Thomas J, Fairclough A, Kavanagh J, Kelly AJ. Vaginal prostaglandin (PGE2 and PGF2a) for induction of labour at term. Cochrane Database Syst Rev. 2014(6):CD003101.

17. van der Elst CW, Lòpez Bernal A, Sinclair-Smith CC. The role of chorioamnionitis and prostaglandins in preterm labor. Obstet Gynecol. 1991;77(5):672-6.

18. Romero R, Emamian M, Wan M, Grzyboski C, Hobbins JC, Mitchell MD. Increased concentrations of arachidonic acid lipoxygenase metabolites in amniotic fluid during parturition. Obstet Gynecol. 1987;70(6):849-51.

19. Ramsden CE, Faurot KR, Zamora D, Suchindran CM, Macintosh BA, Gaylord S, et al. Targeted alteration of dietary n-3 and n-6 fatty acids for the treatment of chronic headaches: a randomized trial. Pain. 2013;154(11):2441-51.

20. Nayeem MA. Role of oxylipins in cardiovascular diseases. Acta Pharmacol Sin. 2018;39(7):1142-54.

21. Devassy JG, Leng S, Gabbs M, Monirujjaman M, Aukema HM. Omega-3 Polyunsaturated Fatty Acids and Oxylipins in Neuroinflammation and Management of Alzheimer Disease. Adv Nutr. 2016;7(5):905-16.

22. Niebyl JR, Blake DA, White RD, Kumor KM, Dubin NH, Robinson JC, et al. The inhibition of premature labor with indomethacin. Am J Obstet Gynecol. 1980;136(8):1014-9.

23. Martin SA, Brash AR, Murphy RC. The discovery and early structural studies of arachidonic acid. J Lipid Res. 2016;57(7):1126-32.

24. Spite M, Clària J, Serhan CN. Resolvins, specialized proresolving lipid mediators, and their potential roles in metabolic diseases. Cell Metab. 2014;19(1):21-36.

25. Yamanashi H, Boeglin WE, Morisseau C, Davis RW, Sulikowski GA, Hammock BD, et al. Catalytic activities of mammalian epoxide hydrolases with. J Lipid Res. 2018;59(4):684-95.

26. Ramsden CE, Domenichiello AF, Yuan ZX, Sapio MR, Keyes GS, Mishra SK, et al. A systems approach for discovering linoleic acid derivatives that potentially mediate pain and itch. Sci Signal. 2017;10(493).

27. Fuchs D, Hamberg M, Sköld CM, Wheelock Å, Wheelock CE. An LC-MS/MS workflow to characterize 16 regio- and stereoisomeric trihydroxyoctadecenoic acids. J Lipid Res. 2018;59(10):2025-33.

28. Gladine C, Laurie JC, Giulia C, Dominique B, Corinne C, Nathalie H, et al. Neuroprostanes, produced by free-radical mediated peroxidation of DHA, inhibit the inflammatory response of human macrophages. Free Radic Biol Med. 2014;75 Suppl 1:S15.

29. Reynaud D, Thickitt CP, Pace-Asciak CR. Facile preparation and structural determination of monohydroxy derivatives of docosahexaenoic acid (HDoHE) by alpha-tocopherol-directed autoxidation. Anal Biochem. 1993;214(1):165-70.

30. Santoro N, Caprio S, Feldstein AE. Oxidized metabolites of linoleic acid as biomarkers of liver injury in nonalcoholic steatohepatitis. Clin Lipidol. 2013;8(4):411-8.

31. Hauck AK, Bernlohr DA. Oxidative stress and lipotoxicity. J Lipid Res. 2016;57(11):1976-86.

32. Ayala A, Muñoz MF, Argüelles S. Lipid peroxidation: production, metabolism, and signaling mechanisms of malondialdehyde and 4-hydroxy-2-nonenal. Oxid Med Cell Longev. 2014;2014:360438.

33. Spector AA, Kim HY. Discovery of essential fatty acids. J Lipid Res. 2015;56(1):11-21.

34. Bihari D, Smithies M, Gimson A, Tinker J. The effects of vasodilation with prostacyclin on oxygen delivery and uptake in critically ill patients. N Engl J Med. 1987;317(7):397-403.

35. Hammarström S. Leukotrienes. Annu Rev Biochem. 1983;52:355-77.

36. Bhavsar PK, Levy BD, Hew MJ, Pfeffer MA, Kazani S, Israel E, et al. Corticosteroid suppression of lipoxin A4 and leukotriene B4 from alveolar macrophages in severe asthma. Respir Res. 2010;11:71.

37. Shirasaki H, Himi T. Role of Cysteinyl Leukotrienes in Allergic Rhinitis. Adv Otorhinolaryngol. 2016;77:40-5.

38. Christie PE, Schmitz-Schumann M, Spur BW, Lee TH. Airway responsiveness to leukotriene C4 (LTC4), leukotriene E4 (LTE4) and histamine in aspirin-sensitive asthmatic subjects. Eur Respir J. 1993;6(10):1468-73.

39. Carlen PL, Gurevich N, Zhang L, Wu PH, Reynaud D, Pace-Asciak CR. Formation and electrophysiological actions of the arachidonic acid metabolites, hepoxilins, at nanomolar concentrations in rat hippocampal slices. Neuroscience. 1994;58(3):493-502.

40. Pace-Asciak CR. Pathophysiology of the hepoxilins. Biochim Biophys Acta. 2015;1851(4):383-96.

41. Enthoven WT, Roelofs PD, Deyo RA, van Tulder MW, Koes BW. Non-steroidal anti-inflammatory drugs for chronic low back pain. Cochrane Database Syst Rev. 2016;2:CD012087.

42. McGill KA, Busse WW. Zileuton. Lancet. 1996;348(9026):519-24.

43. Yanes DA, Mosser-Goldfarb JL. Emerging therapies for atopic dermatitis: The prostaglandin/leukotriene pathway. J Am Acad Dermatol. 2018;78(3 Suppl 1):S71-S5.

44. Bhatt L, Roinestad K, Van T, Springman EB. Recent advances in clinical development of leukotriene B4 pathway drugs. Semin Immunol. 2017;33:65-73.

45. McLaughlin J, Devoe LD. Current Status of Prostaglandins for Cervical Ripening. J Reprod Med. 2017;62(5-6):221-8.

46. Maayah ZH, El-Kadi AO. The role of mid-chain hydroxyeicosatetraenoic acids in the pathogenesis of hypertension and cardiac hypertrophy. Arch Toxicol. 2016;90(1):119-36.

47. Rådmark O, Samuelsson B. 5-lipoxygenase: regulation and possible involvement in atherosclerosis. Prostaglandins Other Lipid Mediat. 2007;83(3):162-74.

48. Kantarci A, Aytan N, Palaska I, Stephens D, Crabtree L, Benincasa C, et al. Combined administration of resolvin E1 and lipoxin A4 resolves inflammation in a murine model of Alzheimer's disease. Exp Neurol. 2018;300:111-20.

49. Norling LV, Headland SE, Dalli J, Arnardottir HH, Haworth O, Jones HR, et al. Proresolving and cartilage-protective actions of resolvin D1 in inflammatory arthritis. JCI Insight. 2016;1(5):e85922.

50. Xu ZZ, Liu XJ, Berta T, Park CK, Lü N, Serhan CN, et al. Neuroprotectin/protectin D1 protects against neuropathic pain in mice after nerve trauma. Ann Neurol. 2013;74(3):490-5.

51. Serhan CN, Dalli J, Colas RA, Winkler JW, Chiang N. Protectins and maresins: New pro-resolving families of mediators in acute inflammation and resolution bioactive metabolome. Biochim Biophys Acta. 2015;1851(4):397-413.

52. Dalli J, Chiang N, Serhan CN. Elucidation of novel 13-series resolvins that increase with atorvastatin and clear infections. Nat Med. 2015;21(9):1071-5.

53. Morisseau C, Inceoglu B, Schmelzer K, Tsai HJ, Jinks SL, Hegedus CM, et al. Naturally occurring monoepoxides of eicosapentaenoic acid and docosahexaenoic acid are bioactive antihyperalgesic lipids. J Lipid Res. 2010;51(12):3481-90.

54. Wagner K, Inceoglu B, Hammock BD. Soluble epoxide hydrolase inhibition, epoxygenated fatty acids and nociception. Prostaglandins Other Lipid Mediat. 2011;96(1-4):76-83.

55. Wagner K, Lee KS, Yang J, Hammock BD. Epoxy fatty acids mediate analgesia in murine diabetic neuropathy. Eur J Pain. 2017;21(3):456-65.

56. Matsumoto N, Kataoka M, Hirosaki H, Morisseau C, Hammock BD, Suzuki E, et al. N-Substituted amino acid inhibitors of the phosphatase domain of the soluble epoxide hydrolase. Biochem Biophys Res Commun. 2019;515(1):248-53.

57. Orr SK, Colas RA, Dalli J, Chiang N, Serhan CN. Proresolving actions of a new resolvin D1 analog mimetic qualifies as an immunoresolvent. Am J Physiol Lung Cell Mol Physiol. 2015;308(9):L904-11.

58. Tang H, Liu Y, Yan C, Petasis NA, Serhan CN, Gao H. Protective actions of aspirin-triggered (17R) resolvin D1 and its analogue, 17R-hydroxy-19-para-fluorophenoxy-resolvin D1 methyl ester, in C5a-dependent IgG immune complex-induced inflammation and lung injury. J Immunol. 2014;193(7):3769-78.

59. Ramsden CE, Zamora D, Majchrzak-Hong S, Faurot KR, Broste SK, Frantz RP, et al. Re-evaluation of the traditional diet-heart hypothesis: analysis of recovered data from Minnesota Coronary Experiment (1968-73). BMJ. 2016;353:i1246.

60. Chiba T, Thomas CP, Calcutt MW, Boeglin WE, O'Donnell VB, Brash AR. The Precise Structures and Stereochemistry of Trihydroxy-linoleates Esterified in Human and Porcine Epidermis and Their Significance in Skin Barrier Function: IMPLICATION OF AN EPOXIDE HYDROLASE IN THE TRANSFORMATIONS OF LINOLEATE. J Biol Chem. 2016;291(28):14540-54.

61. Choi SH, Sviridov D, Miller YI. Oxidized cholesteryl esters and inflammation. Biochim Biophys Acta Mol Cell Biol Lipids. 2017;1862(4):393-7.

62. Warner DR, Liu H, Miller ME, Ramsden CE, Gao B, Feldstein AE, et al. Dietary Linoleic Acid and Its Oxidized Metabolites Exacerbate Liver Injury Caused by Ethanol via Induction of Hepatic Proinflammatory Response in Mice. Am J Pathol. 2017;187(10):2232-45.

63. Feldstein AE, Lopez R, Tamimi TA, Yerian L, Chung YM, Berk M, et al. Mass spectrometric profiling of oxidized lipid products in human nonalcoholic fatty liver disease and nonalcoholic steatohepatitis. J Lipid Res. 2010;51(10):3046-54.

64. Spiteller D, Spiteller G. Oxidation of Linoleic Acid in Low-Density Lipoprotein: An Important Event in Atherogenesis. Angew Chem Int Ed Engl. 2000;39(3):585-9.

65. Panda L, Gheware A, Rehman R, Yadav MK, Jayaraj BS, Madhunapantula SV, et al. Linoleic acid metabolite leads to steroid resistant asthma features partially through NF-κB. Sci Rep. 2017;7(1):9565.

66. Sasaguri Y, Kakita N, Murahashi N, Kato S, Hiraoka K, Morimatsu M, et al. Effect of linoleic acid hydroperoxide on production of matrix metalloproteinases by human aortic endothelial and smooth muscle cells. Atherosclerosis. 1993;100(2):189-96.

67. Vangaveti V, Baune BT, Kennedy RL. Hydroxyoctadecadienoic acids: novel regulators of macrophage differentiation and atherogenesis. Ther Adv Endocrinol Metab. 2010;1(2):51-60.

68. Lipton BA, Parthasarathy S, Ord VA, Clinton SK, Libby P, Rosenfeld ME. Components of the protein fraction of oxidized low density lipoprotein stimulate interleukin-1 alpha production by rabbit arterial macrophage-derived foam cells. J Lipid Res. 1995;36(10):2232-42.

69. Harris WS, Shearer GC. Omega-6 fatty acids and cardiovascular disease: friend, not foe? Circulation. 2014;130(18):1562-4.

70. Nugteren DH, Christ-Hazelhof E, van der Beek A, Houtsmuller UM. Metabolism of linoleic acid and other essential fatty acids in the epidermis of the rat. Biochim Biophys Acta. 1985;834(3):429-36.

71. Muñoz-Garcia A, Thomas CP, Keeney DS, Zheng Y, Brash AR. The importance of the lipoxygenase-hepoxilin pathway in the mammalian epidermal barrier. Biochim Biophys Acta. 2014;1841(3):401-8.

72. Yang Q, Liu M, Li X, Zheng J. The benefit of a ceramide-linoleic acid-containing moisturizer as an adjunctive therapy for a set of xerotic dermatoses. Dermatol Ther. 2019;32(4):e13017.

73. LOEB HG, BURR GO. A study of sex differences in the composition of rats, with emphasis on the lipid component; sex difference in susceptibility to essential fatty acid deficiency with high and low fat diets. J Nutr. 1947;33(5):541-51.

74. BARKI VM, NATH H. Production of essential fatty acid deficiency symptoms in the mature rat. Proc Soc Exp Biol Med. 1947;66(2):474-8.

75. Guesnet P, Lallemand SM, Alessandri JM, Jouin M, Cunnane SC. α-Linolenate reduces the dietary requirement for linoleate in the growing rat. Prostaglandins Leukot Essent Fatty Acids. 2011;85(6):353-60.

76. Checa A, Holm T, Sjödin MO, Reinke SN, Alm J, Scheynius A, et al. Lipid mediator profile in vernix caseosa reflects skin barrier development. Sci Rep. 2015;5:15740.

77. Schmöcker C, Zhang IW, Kiesler S, Kassner U, Ostermann AI, Steinhagen-Thiessen E, et al. Effect of Omega-3 Fatty Acid Supplementation on Oxylipins in a Routine Clinical Setting. Int J Mol Sci. 2018;19(1).

78. Ostermann AI, Schebb NH. Effects of omega-3 fatty acid supplementation on the pattern of oxylipins: a short review about the modulation of hydroxy-, dihydroxy-, and epoxy-fatty acids. Food Funct. 2017;8(7):2355-67.

79. Schuchardt JP, Ostermann AI, Stork L, Fritzsch S, Kohrs H, Greupner T, et al. Effect of DHA supplementation on oxylipin levels in plasma and immune cell stimulated blood. Prostaglandins Leukot Essent Fatty Acids. 2017;121:76-87.

80. Lundström SL, Yang J, Brannan JD, Haeggström JZ, Hammock BD, Nair P, et al. Lipid mediator serum profiles in asthmatics significantly shift following dietary supplementation with omega-3 fatty acids. Mol Nutr Food Res. 2013;57(8):1378-89.

81. Schebb NH, Ostermann AI, Yang J, Hammock BD, Hahn A, Schuchardt JP. Comparison of the effects of long-chain omega-3 fatty acid supplementation on plasma levels of free and esterified oxylipins. Prostaglandins Other Lipid Mediat. 2014;113-115:21-9.

82. Bisgaard H, Stokholm J, Chawes BL, Vissing NH, Bjarnadóttir E, Schoos AM, et al. Fish Oil-Derived Fatty Acids in Pregnancy and Wheeze and Asthma in Offspring. N Engl J Med. 2016;375(26):2530-9.

83. Wood K, Mantzioris E, Lingwood B, Couper J, Makrides M, Gibson RA, et al. The effect of maternal DHA supplementation on body fat mass in children at 7 years: follow-up of the DOMInO randomized controlled trial. Prostaglandins Leukot Essent Fatty Acids. 2018;139:49-54.

84. Best KP, Sullivan T, Palmer D, Gold M, Kennedy DJ, Martin J, et al. Prenatal Fish Oil Supplementation and Allergy: 6-Year Follow-up of a Randomized Controlled Trial. Pediatrics. 2016;137(6).

85. Chen B, Ji X, Zhang L, Hou Z, Li C, Tong Y. Fish Oil Supplementation does not Reduce Risks of Gestational Diabetes Mellitus, Pregnancy-Induced Hypertension, or Pre-Eclampsia: A Meta-Analysis of Randomized Controlled Trials. Med Sci Monit. 2015;21:2322-30.

86. Makrides M, Best K, Yelland L, McPhee A, Zhou S, Quinlivan J, et al. A Randomized Trial of Prenatal n-3 Fatty Acid Supplementation and Preterm Delivery. N Engl J Med. 2019;381(11):1035-45.

87. Makrides M, Gibson RA, McPhee AJ, Yelland L, Quinlivan J, Ryan P, et al. Effect of DHA supplementation during pregnancy on maternal depression and neurodevelopment of young children: a randomized controlled trial. JAMA. 2010;304(15):1675-83.

88. Keelan JA, Mas E, D'Vaz N, Dunstan JA, Li S, Barden AE, et al. Effects of maternal n-3 fatty acid supplementation on placental cytokines, pro-resolving lipid mediators and their precursors. Reproduction. 2015;149(2):171-8.

89. Balogun KA, Albert CJ, Ford DA, Brown RJ, Cheema SK. Dietary omega-3 polyunsaturated fatty acids alter the fatty acid composition of hepatic and plasma bioactive lipids in C57BL/6 mice: a lipidomic approach. PLoS One. 2013;8(11):e82399.

90. Australian Health Survey: Nutrition First Results – Food and Nutrients, Australian Bureau of Statistics [Internet]. 2011-12. Available from: https://www.abs.gov.au/ausstats/abs@.nsf/Lookup/4364.0.55.007main+features12011-12.

91. Blasbalg TL, Hibbeln JR, Ramsden CE, Majchrzak SF, Rawlings RR. Changes in consumption of omega-3 and omega-6 fatty acids in the United States during the 20th century. Am J Clin Nutr. 2011;93(5):950-62.

92. US Department of Agriculture, Agricultural Research Service. USDA National Nutrient Database for Standard Reference, Release 25. [Internet]. 2012. Available from: www.ars.usda.gov/nutrientdata.

93. KARPMAN HL. The Keys equation. Am Heart J. 1960;59:478-9.

94. Lankinen MA, Fauland A, Shimizu BI, Ågren J, Wheelock CE, Laakso M, et al. Inflammatory response to dietary linoleic acid depends on FADS1 genotype. Am J Clin Nutr. 2019;109(1):165-75.

95. Rett BS, Whelan J. Increasing dietary linoleic acid does not increase tissue arachidonic acid content in adults consuming Western-type diets: a systematic review. Nutr Metab (Lond). 2011;8:36.

96. Kawabata A. Prostaglandin E2 and pain--an update. Biol Pharm Bull. 2011;34(8):1170-3.

97. Pignitter M, Zaunschirm M, Lach J, Unterberger L, Kopic A, Keßler C, et al. Regioisomeric distribution of 9- and 13-hydroperoxy linoleic acid in vegetable oils during storage and heating. J Sci Food Agric. 2018;98(3):1240-7.

98. Haywood RM, Claxson AW, Hawkes GE, Richardson DP, Naughton DP, Coumbarides G, et al. Detection of aldehydes and their conjugated hydroperoxydiene precursors in thermally-stressed culinary oils and fats: investigations using high resolution proton NMR spectroscopy. Free Radic Res. 1995;22(5):441-82.

99. Claxson AW, Hawkes GE, Richardson DP, Naughton DP, Haywood RM, Chander CL, et al. Generation of lipid peroxidation products in culinary oils and fats during episodes of thermal stressing: a high field 1H NMR study. FEBS Lett. 1994;355(1):81-90.

100. R. Wilson KL, L. Smyth, C.E. Fernie, R.A. Riemersma. Dietary hydroxy fatty acids are absorbed in humans: implications for the measurement of 'oxidative stress' in vivo. Free radical biology & medicine. 2002;32:162-8.

101. C. Ferreiro-Vera FP-C, J.M. Mata-Granados, M.D. Luque de Castro. Short-term comparative study of the influence of fried edible oils intake on the metabolism of essential fatty acids in obese individuals Food chemistry. 2013;136:576-84.

102. Fischer VW, Nelson JS. Cerebrovascular changes in tocopherol-depleted chicks, fed linoleic acid. J Neuropathol Exp Neurol. 1973;32(3):474-83.

103. Budowski P, Bartov I, Dror Y, Frankel EN. Lipid oxidation products and chick nutritional encephalopathy. Lipids. 1979;14(9):768-72.

104. Indart A, Viana M, Grootveld MC, Silwood CJ, Sanchez-Vera I, Bonet B. Teratogenic actions of thermally-stressed culinary oils in rats. Free Radic Res. 2002;36(10):1051-8.

105. Ramsden CE, Ringel A, Feldstein AE, Taha AY, MacIntosh BA, Hibbeln JR, et al. Lowering dietary linoleic acid reduces bioactive oxidized linoleic acid metabolites in humans. Prostaglandins Leukot Essent Fatty Acids. 2012;87(4-5):135-41.

106. H. Blencowe ACL, S. Cousens, A. Bahalim, R. Narwal, N. Zhong, D. Chou, L. Say, N. Modi, J. Katz, T. Vos, N. Marlow, J.E. Lawn. Preterm birth-associated neurodevelopmental impairment estimates at regional and global levels for 2010. . Pediatr Res. 2013;74:17-34.

107. United States Center for Disease Control and Prevention. Preterm Birth [Internet]. Available from: www.cdc.gov/reproductivehealth/maternalinfanthealth/pretermbirth.htm.

108. Z. Li RZLH, E.A. Sullivan Australia's mothers and babies. Perinatal statistics series number 28, catalog number PER 59. Canberra: AIHW National Perinatal Epidemiology and Statistics Unit. 2011.

109. Keelan JA, Newnham JP. Recent advances in the prevention of preterm birth. F1000Res. 2017;6.

110. Govindaswami B, Jegatheesan P, Nudelman M, Narasimhan SR. Prevention of Prematurity: Advances and Opportunities. Clin Perinatol. 2018;45(3):579-95.

111. Olson DM. The promise of prostaglandins: have they fulfilled their potential as therapeutic targets for the delay of preterm birth? J Soc Gynecol Investig. 2005;12(7):466-78.

112. Rolnik DL, Wright D, Poon LC, O'Gorman N, Syngelaki A, de Paco Matallana C, et al. Aspirin versus Placebo in Pregnancies at High Risk for Preterm Preeclampsia. N Engl J Med. 2017;377(7):613-22.

113. Ye J, Chen Y, Zhu J, Chen C, Zhu X, Feng L, et al. Aspirin use during pregnancy and hypoxia-related placental pathology. Pregnancy Hypertens. 2018;14:177-88.

114. Pohl O, Chollet A, Kim SH, Riaposova L, Spézia F, Gervais F, et al. OBE022, an Oral and Selective Prostaglandin F. J Pharmacol Exp Ther. 2018;366(2):349-64.

115. Boyle AK, Rinaldi SF, Norman JE, Stock SJ. Preterm birth: Inflammation, fetal injury and treatment strategies. J Reprod Immunol. 2017;119:62-6.

116. Christiaens I, Zaragoza DB, Guilbert L, Robertson SA, Mitchell BF, Olson DM. Inflammatory processes in preterm and term parturition. J Reprod Immunol. 2008;79(1):50-7.

117. Martens DS, Gouveia S, Madhloum N, Janssen BG, Plusquin M, Vanpoucke C, et al. Neonatal Cord Blood Oxylipins and Exposure to Particulate Matter in the Early-Life Environment: An ENVIR. Environ Health Perspect. 2017;125(4):691-8.

118. Maddipati KR, Romero R, Chaiworapongsa T, Chaemsaithong P, Zhou SL, Xu Z, et al. Lipidomic analysis of patients with microbial invasion of the amniotic cavity reveals up-regulation of leukotriene B4. FASEB J. 2016;30(10):3296-307.

119. Peng CC, Chang JH, Lin HY, Cheng PJ, Su BH. Intrauterine inflammation, infection, or both (Triple I): A new concept for chorioamnionitis. Pediatr Neonatol. 2018;59(3):231-7.

120. Kuipers RS, Luxwolda MF, Janneke Dijck-Brouwer DA, Muskiet FA. Intrauterine, postpartum and adult relationships between arachidonic acid (AA) and docosahexaenoic acid (DHA). Prostaglandins Leukot Essent Fatty Acids. 2011;85(5):245-52.

121. Meldrum S, Simmer K. Docosahexaenoic Acid and Neurodevelopmental Outcomes of Term Infants. Ann Nutr Metab. 2016;69 Suppl 1:22-8.

122. Gould JF, Treyvaud K, Yelland LN, Anderson PJ, Smithers LG, Gibson RA, et al. Does n-3 LCPUFA supplementation during pregnancy increase the IQ of children at school age? Follow-up of a randomised controlled trial. BMJ Open. 2016;6(5):e011465.

123. Middleton P, Gomersall JC, Gould JF, Shepherd E, Olsen SF, Makrides M. Omega-3 fatty acid addition during pregnancy. Cochrane Database Syst Rev. 2018;11:CD003402.

124. Calder PC. Omega-3 fatty acids and inflammatory processes: from molecules to man. Biochem Soc Trans. 2017;45(5):1105-15.

125. Dye JF, Jablenska R, Donnelly JL, Lawrence L, Leach L, Clark P, et al. Phenotype of the endothelium in the human term placenta. Placenta. 2001;22(1):32-43.

126. Fournier T, Handschuh K, Tsatsaris V, Evain-Brion D. Involvement of PPARgamma in human trophoblast invasion. Placenta. 2007;28 Suppl A:S76-81.

127. Palani S, Maksimow M, Miiluniemi M, Auvinen K, Jalkanen S, Salmi M. Stabilin-1/CLEVER-1, a type 2 macrophage marker, is an adhesion and scavenging molecule on human placental macrophages. Eur J Immunol. 2011;41(7):2052-63.

128. Satoh H, Kiyota E, Terasaki Y, Sawamura T, Takagi K, Mizuta H, et al. Expression and localization of lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) in murine and human placentas. J Histochem Cytochem. 2008;56(8):773-84.

129. Pecks U, Rath W, Caspers R, Sosnowsky K, Ziems B, Thiesen HJ, et al. Oxidatively modified LDL particles in the human placenta in early and late onset intrauterine growth restriction. Placenta. 2013;34(12):1142-9.

CHAPTER 2: Materials and Methods (detailed methods integrated into thesis papers).

2.1 Introduction: Quantitation of oxylipins and precursor fatty acids

2.1.1 Specimens

Detailed descriptions of the methods used for collection and processing of mouse brain tissues (Chapter 3), human plasma samples from the delayed processing study (Chapter 4), and human plasma from the ORIP plasma substudy (Chapter 5) are included in the respective sections and appended manuscripts.

2.1.2 Targeted oxylipin profiling with liquid chromatography tandem mass spectrometry

Targeted oxylipin profiling of solid mouse brain tissue (Chapter 3) was completed in the Taha laboratory at the University of California-Davis using methods adapted from Hammock et al (1). Oxylipin profiling of plasma samples, which provided data presented in the second and third manuscripts (Chapters 4 and 5), was completed in the NIH Clinical Center Department of Perioperative Medicine, led by analytical chemist and mass spectrometry operator Zhi-Xin Yuan, Ph.D. under my supervision. The full methods and their validation are provided in the manuscript entitled “Lipidomic profiling of targeted oxylipins with ultra-performance liquid chromatography-tandem mass spectrometry” (2) (see published manuscript in Appendix 1). Briefly, we developed and validated a sensitive liquid chromatography-tandem mass spectrometry (LC-MS/MS) method for simultaneous profiling of up to 57 targeted oxylipins derived from the oxylipin precursors LA, AA, and DHA. The assay includes oxylipins generated from the cyclooxygenase, lipoxygenase, CYP-epoxygenase and hydroxylase pathways, and from free radical mediated peroxidation. The method comprises protein precipitation and solid-phase

extraction, followed by LC-MS/MS with isotope dilution for quantitation (2). The separation was performed on a reverse phase ZorBAX RRHD Eclipse Plus C18 column using gradient programs consisting of 0.02% acetic acid and ammonium acetate buffer in water and acetonitrile mobile phases. Detection was achieved using the Qtrap 5500 system in electrospray negative ion mode with scheduled multiple reaction monitoring (sMRM)(2). For LC-MS/MS profiling in the second and third manuscripts (Chapters 4 and 5) we selected a subset of oxylipins that are generally quantifiable in plasma and/or that have plausible links to pregnancy and parturition-related outcomes based on the literature review, including prostaglandins and 5-lipoxygenase derivatives (reviewed in Chapter 1). Additional considerations for selecting analytes included commercial availability and cost of internal and authentic standards. The full list of profiled analytes and limits of quantitation are provided in **Table 1.**

2.1.3 Targeted precursor fatty acid profiling

During the course of this project, Professors Gibson and Makrides suggested that the abundance of DHA, AA, LA and other PUFAs as free fatty acids (potential immediate precursors to some of the oxylipins described in 2.1 above) in maternal and fetal tissues might play key roles in mediating pregnancy and neurodevelopmental outcomes. We therefore sought to expand our oxylipin profiling assay for simultaneous quantitation of oxylipins, their precursor n-3 and n-6 fatty acids, and other selected unesterified fatty acids in a single same sample and single run. Development and validation of this assay was performed by Dr. Zhi-Xin Yuan under my supervision. This assay was used to quantify free fatty acids alongside oxylipins in ORIP samples (see Chapter 5 manuscript). The detailed methods used in the unesterified fatty acid assay will be published in another methods paper led by Dr. Yuan.

Table 1: Oxylipins and unesterified fatty acids included in LC-MS/MS assays

Oxylipins	LOQ (ng/mL)
9-HODE	0.2
13-HODE	0.2
9,10-EpOME**	0.5
9,10-DiHOME	0.1
9,10,13-TriHOME	0.1
9,12,13-TriHOME	0.1
PGE2*	N/A
PGF2a*	N/A
8-isoPGF2a*	N/A
TxB2	0.02
5-HETE	0.02
12-HETE	0.1
15-HETE	0.05
4-HDHA	0.02
10-HDHA	0.02
14-HDHA	0.05
17-HDHA	0.1
19,20-EpDPA	0.02
9,10,13-TriHOME**	0.05
9,12,13-TriHOME	0.1
Fatty acids* **	**LOQ (ug/mL)**
ALA	0.02
GLA	0.01
EPA	0.02
DHA	0.04
LA	0.40
DGLA	0.01
AA	0.01
ETA (20:4n-3)	0.004
DTA	0.01
Mead	0.002
DPAn-3	0.004
DPAn-6	0.004
SDA (18:4 n-3)	0.002

N/A, not applicable

* indicates below the limit of detection.

** indicates that 9,10-EpOME & 9,10,13-TriHOME had >50% values below limit of quantitation (LOQ).

***Unesterified fatty acids were profiled in Project 3 (Thesis Chapter 5).

2.1.4 References (Chapter 2)

1. Yang J, Schmelzer K, Georgi K, Hammock BD. Quantitative profiling method for oxylipin metabolome by liquid chromatography electrospray ionization tandem mass spectrometry. Anal Chem. 2009;81(19):8085-93.

2. Yuan ZX, Majchrzak-Hong S, Keyes GS, Iadarola MJ, Mannes AJ, Ramsden CE. Lipidomic profiling of targeted oxylipins with ultra-performance liquid chromatography-tandem mass spectrometry. Anal Bioanal Chem. 2018;410(23):6009-29.

CHAPTER 3 (Manuscript 1): Effects of diets enriched in linoleic acid and its peroxidation products on brain fatty acids, oxylipins, and aldehydes in mice

Effects of diets enriched in linoleic acid and its peroxidation products on brain fatty acids, oxylipins, and aldehydes in mice

Published in *Biochim Biphys Acta (BBA) - Molecular and Cell Biology of Lipids*. 2018 Oct; 1863(10):1206-1213 (Doi:10.1016/j.bbalip. 2018.07.007). Published online 25 July 2018.

Statements of Authorship are provided in Appendix 3.1.

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3.1 Highlights

- Linoleic acid (LA) in the diet increased LA derived oxylipins in mouse brain
- Oxidized LA in the diet decreased brain polyunsaturated fats but had no effect on oxylipins
- Neither LA nor oxidized LA in the diet altered brain 4-hydroxy-2-nonenal adducts

3.2 Abstract

Background: Linoleic acid (LA) is abundant in modern industrialized diets. Oxidized LA metabolites (OXLAMs) and reactive aldehydes, such as 4-hydroxy-2-nonenal (4-HNE), are present in heated vegetable oils and can be endogenously synthesized following consumption of dietary LA. OXLAMs have been implicated in cerebellar degeneration in chicks; 4-HNE is linked to neurodegenerative conditions in mammals. It unknown whether increasing dietary LA or OXLAMs alters the levels of oxidized fatty acids (oxylipins), precursor fatty acids, or 4-HNE in mammalian brain.

Objectives: To determine the effects of increases in dietary OXLAMs and dietary LA, on levels of fatty acids, oxylipins, and 4-HNE in mouse brain tissues.

Methods: Mice (n=8 per group) were fed one of three controlled diets for 8 weeks: (1) a low LA diet, (2) a high LA diet, or (3) the low LA diet with added OXLAMs. Brain fatty acids, oxylipins, and 4-HNE were quantified in mouse cerebellum and cerebral cortex by gas chromatography-flame ionization detection, liquid chromatography-tandem mass spectrometry, and immunoblot, respectively.

Results: Increasing dietary LA significantly increased omega-6 fatty acids, decreased omega-3 fatty acids, and increased OXLAMs in brain. Dietary OXLAMs had minimal effect on oxidized lipids but did decrease both omega-6 and omega-3 fatty acids. Neither dietary LA nor OXLAMs altered 4-HNE levels.

Conclusion: Brain fatty acids are modulated by both dietary LA and OXLAMs, while brain OXLAMs are regulated by endogenous synthesis from LA, rather than incorporation of preformed OXLAMs.

Key words: Oxylipins, linoleic acid, OXLAMs, cerebrum, cerebellum, peroxidation

3.3 Introduction

Linoleic acid (LA) is the most abundant polyunsaturated fatty acid in modern industrialized diets, accounting for approximately 3 to >17% percent of energy (%E) intake in individuals worldwide (1-3). Current LA intakes in industrialized populations are higher than historical and evolutionary norms of 2-3%E. The importance of LA to human health has, classically, been attributed to three primary functions: (i) its role as an "essential fatty acid" because small amounts of LA (about 0.5%E) in the diet are required for integrity of the epidermal water barrier (4); (ii) its ability to reduce serum low density lipoprotein cholesterol when replacing dietary saturated fats (3,5); and (iii) for being the precursor to arachidonic acid (AA); AA is enzymatically converted to peroxidation products with well-established bioactivities, including prostanoids and leukotrienes (reviewed in (3) and (6)).

Like AA, docosahexaenoic acid (DHA) and other polyunsaturated fatty acids, LA contains a 1,4 cis-cis pentadiene system and thus can serve as the substrate for enzymatic peroxidation to synthesize biologically active oxygenated derivatives, collectively known as oxylipins. The subset of oxylipins derived from LA, which are known as oxidized linoleic acid metabolites (OXLAMs), include hydroperoxy-octadecadienoates (HpODEs), hydroxy-octadecadienoates (HODEs), oxo-octadecadienoates (oxo-ODEs), epoxy-octadecenoates (EpOMEs), dihydroxy-octadecenoates acids (DiHOMEs), hydroxy-epoxides(6), keto-epoxides(6), and trihydroxy-octadecenoates (TriHOMEs)(2). HpODEs and lipid peroxides derived from other omega-6 fatty acids are also precursors for α,β unsaturated reactive aldehyde degradation products including 4-hydroxy-2-nonenal (4-HNE)(7). Since mammals cannot synthesize LA *de novo*, the LA content of diet is likely to be a critical determinant of accumulation of LA, OXLAMs, and 4-HNE in many tissues (2,8). HpODEs and other OXLAMs

can also be formed non-enzymatically when vegetable oils rich in LA are cooked or otherwise heated (9-11). A substantial portion of vegetable oils in industrialized populations, including those used in many processed and packaged foods, is heated prior to consumption; and these preformed OXLAMs could potentially be absorbed and incorporated into certain tissues including brain after consumption (12-14). Therefore, the abundance of OXLAMs and 4-HNE in human and other mammalian tissues could potentially be affected by both consumption of non-oxidized LA, with subsequent conversion to OXLAMs in the body, and by consumption of preformed OXLAMs.

OXLAMs and 4-HNE have been mechanistically linked to several pathological conditions including cardiovascular disease (15), steatohepatitis (16,17), neurodegenerative diseases (18), and chronic pain (6,19,20), reviewed in (3,15,21). Consumption of heated vegetable oils rich in LA (22,23), or intravenous administration of HpODEs (24), produces cerebellar necrosis and ataxia in chicks without damaging the cerebral cortex, indicating that OXLAMs could potentially have brain-region specific neurotoxic effects in some species. Plausible mechanisms exist whereby high exposure to OXLAMs could have neurotoxic effects in humans, include endothelial cell activation (25), generalized lipid and membrane peroxidation (26,27), mitochondrial dysfunction (28), and microglial activation (29-31). 4-HNE, which forms chemical bonds with cysteine, lysine, and histidine residues (32) and has been implicated in protein misfolding and aggregation (33), is linked to the development or progression of neurofibrillary tangles and amyloid plaques that are characteristic of Alzheimer's disease (33,34). However, despite these plausible mechanisms, there is a lack of data to assess the effects of increasing dietary LA and dietary OXLAMs on the fatty acid, oxylipin, and aldehyde compositions in mammalian brain.

In the present paper, we examine if high intakes of LA (from unheated corn oil), or exogenously produced OXLAMs (from heated corn oil), both characteristic of modern industrialized diets, impact mammalian brain biochemistry. Findings support the hypothesis that diets enriched in LA and OXLAMs alter cerebral and cerebellar lipid accumulation and peroxidation in mammals.

3.4 Materials and Methods

Wild type male C57BL/6 mice (n=8 per group) were fed ad-libitum one of three controlled diets designed to contain 40% fat by weight in g/kg, for 8 weeks: (i) a low LA diet designed to contain 4%E as LA, (ii) a high LA diet designed to contain 17%E as LA or (iii) the low 4% E LA diet enriched with dietary OXLAMs from thermally-stressed corn oil. The three study diets were prepared by Dyets Inc. (Bethlehem, PA) using unheated and thermally stressed oils of known fatty acid composition. Despite being designed to contain the same amount of LA as the Low LA diet, the Low LA + OXLAMs diet was observed to contain substantially less LA on gas chromatography analysis. Fatty acid and OXLAM concentrations of the three diets are shown in Table 1. Fatty acid percent compositions are shown in Table S1. This animal protocol followed the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 80-23) and was approved by the University of California, San Diego Institutional Animal Care and Use Committee (protocol number S11200).

3.4.1 Preparation of heated corn oil

Thermally stressed corn oil was prepared by heating 2 kg of Crisco^{TM} brand corn oil purchased from a local grocery store in a shallow cast iron pan in an oven at 115°C for approximately 4 weeks, with daily stirring of the oil. Progress was monitored by ^{1}H NMR (400MHz) in deuterated chloroform by comparing the decrease in integration of the bis-allylic protons (centered around 2.76ppm) of the heated oil with the glyceryl methylene protons (multiplets at 4.13ppm and 4.28ppm) which remained unchanged (referenced to a sample taken from the corn oil immediately upon opening). Lipid peroxide composition was assessed by estimating the integration of newly formed peaks in regions that were sufficiently separated from potential interference of peaks

arising from the vitamin E in the corn oil. These corresponded to the conjugated *cis, trans,* and *trans, trans* dienes of HpODEs (5.40-5.55ppm, 5.86-6.11ppm, smaller multiplets at 6.12-6.32ppm) as described by Guillen et al (35). Based on LA composition of corn oil as 55%, in the 2KG sample there were 192g of HpODEs present in the total oil mixture at reaction end as calculated from the NMR integration results. Further oxidation products were also observed as minor aldehyde peaks (9.40-9.56ppm).

3.4.2 Fatty acid and oxylipin analysis of study diets

Dietary fatty acids in the food pellets were analyzed by gas chromatography (GC) with a flame ionization detector (FID) as previously reported (36). Briefly, food pellets were crushed with pestle and mortar. 0.4 mL toluene, 3 mL methanol and 0.6 mL 8% HCl in methanol were added to 30 mg of powder after adding 1,2-diheptadecanoyl-sn-glycero-3-phosphocholine as an internal standard. Samples were heated for 1h at 90°C. One mL of hexane and 1 mL water were added, and the samples were allowed to sit at room temperature for a few minutes. The upper hexane layer containing the fatty acid methyl esters was transferred to a new tube containing 0.45 mL water, vortexed and centrifuged for 2 min at 13,000 rpm. The hexane layer was dried under nitrogen, reconstituted in 0.2 mL hexane and subjected to gas-chromatography analysis. Fatty acid methyl esters were analyzed with a Clarus 500 GC system equipped with FID (Perkin Elmer, CA, USA) and a fused silica capillary column (DB-FFAP, 30m, 0.25mm i.d, 0.25 μm film thickness, Agilent, Santa Clara, CA, USA). The injector and detector temperatures were set to 240°C and 300°C, respectively. The oven temperature program was set at 80°C for 2min, increased to 185°C at the rate of 10°C/min, then to 240°C at the rate of 5°C/min, and held at 240°C for 13 min. Helium was the carrier gas and was maintained at a flow rate of 1.3mL/min.

Diet OXLAM concentrations were measured in 30 mg of a crushed food pellet dissolved in 200 µL ice-cold methanol containing 0.1% acetic acid and 0.1% BHT and spiked with 10 µL antioxidant mix and 10 µL surrogate standard containing 5 pmol of 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 dissolved in methanol. The antioxidant solution contained three antioxidants mixed at a 1:1:1 ratio (v/v/v) consisting of 0.6 mg/ml ethylenediaminetetraacetic acid in water, 0.6 mg/ml BHT in methanol, and 0.6 mg/ml triphenylphosphine in water:methanol (1:1; v/v) and filtered through a Millipore filter to remove solid particles. Samples were hydrolyzed and oxylipins extracted with solid phase extraction and analyzed by LC-MS/MS as described below.

Table 1: Concentrations of fatty acids and oxidized linoleic acid metabolites (OXLAMs) in study diets

	Low LA diet	High LA diet$^{\phi}$	Low LA + OXLAM diet$^{\phi}$
Fatty acids (mg/g)			
LA	28.0 ± 3.9	92.0 ± 5.5	16.5 ± 0.3
ALA	10.6 ± 1.5	11.3 ± 0.6	9.1 ± 0.3
SFA	122.6 ± 13.1	42.7 ± 2.1	91.0 ± 3.7
MUFA	16.0 ± 2.2	49.5 ± 12.5	18.6 ± 0.7
OXLAMs (nmol/g)			
Total OXLAMs	20.2 ± 4.7	33.4 ± 2.0	259.6 ± 21.6
9-HODE	6.8 ± 1.6	7.9 ± 0.5	20.0 ± 4.1
9-oxo-ODE	0.4 ± 0.1	0.5 ± 0.1	3.6 ± 0.8
9(10)-EpOME	0.9 ± 0.2	1.0 ± 0.1	51.8 ± 6.0
9,10-DiHOME	1.9 ± 0.3	8.0 ± 0.4	2.2 ± 0.2
13-HODE	7.1 ± 2.5	8.8 ± 1.0	67.5 ± 12.8
13-oxo-ODE	0.0 ± 0.0	0.3 ± 0.1	1.9 ± 0.7
12(13)-EpOME	1.5 ± 0.4	2.0 ± 0.4	109.3 ± 7.5
12,13-DiHOME	1.5 ± 0.2	4.9 ± 0.1	3.4 ± 0.2

Data are mean ± standard deviation. LA, linoleic acid; ALA, alpha-linolenic acid; SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; HODE, hydroxy-octadecadienoic acid; oxo-ODE, oxo-octadecadienoic acid; EpOME, epoxy-octadecenoic acid; DiHOME, dihydroxy-octadecenoic acid. $^{\phi}$indicates that the Low LA plus OXLAM diet had substantially less non-oxidized LA on GC analysis. Diets did not contain arachidonic acid, eicosapentaenoic acid, or docosahexaenoic acid.

3.4.3 Mouse tissue collection and fatty acid, oxylipin, and 4-HNE analyses

Mice were killed by CO2 overdose. Tissue samples were immediately collected, frozen on dry-ice chilled isobutane, and stored at -80C. Tissue oxylipins were analyzed as previously described (37). Briefly, two hundred microliters of ice-cold methanol containing 0.1% acetic acid and 0.1% BHT was added to approximately 30-50 mg of frozen tissue, following the addition of 10 µL of the antioxidant mix and 10 µL surrogate standards as described above. The brain samples containing the extraction solvent, antioxidant mix, and surrogate standards were cooled in -80°C freezer for 30 min and then homogenized for 2 min using a bead homogenizer. The homogenized samples were stored for 30 min at -80C freezer, followed by centrifugation at 13,000 rpm (15,870 g) in a 5424R microcentrifuge (Eppendorf) for 10 min at 0°C. The supernatant was transferred to a new tube for the oxylipin analysis, while the precipitate was kept at -80°C for fatty acid analysis. The supernatant was hydrolyzed in equal volumes of 0.5 M sodium carbonate solution (26.5 mg per ml of 1:1 v/v methanol/water) at 60°C for 30 min. The samples were acidified to pH 4 to 6 with 25 µL acetic acid and 1575 µL water was added to dissolve the resulting salts. The solution containing hydrolyzed oxidized lipids (oxylipins) was subjected to solid phase extraction (SPE). Samples were poured onto 60 mg Waters Oasis HLB 3cc cartridges (Waters, Milford, MA, USA) pre-rinsed with one volume ethyl acetate and two volumes methanol and conditioned with two volumes of SPE buffer (5% methanol and 0.1% acetic acid in ultrapure water). The columns were rinsed twice with SPE buffer and subjected to a vacuum (≈15-20 psi) for 20 min. Oxylipins were eluted with 0.5 mL of methanol and 1.5 mL ethyl acetate, dried under nitrogen, reconstituted in 100 µL methanol and filtered by centrifugation in a Ultrafree-MC-VV centrifugal filter (0.1 µm; Millipore Sigma, MA, USA). Oxylipins were quantified on an Agilent 1290 Infinity UHPLC system coupled to a 6460 triple-quadrupole

tandem mass spectrometer with electrospray ionization (Agilent Corporation, Palo Alto, CA, USA), as previously described (38). The precipitate kept for fatty acid analyses was reconstituted in 0.4 mL toluene and subjected to direct transesterification with methanolic HCl, using the same protocol than described above for the diet analysis (36). Samples were analyzed by GC using the column and temperature program described above. The DHA peak co-eluted with nervonic acid, based on the retention times of the authentication standards. However, nervonic acid is negligible in brain tissue (39).

4-HNE-modified protein adducts in one hemisphere of cerebral cortex were analyzed by immunoblot using Anti-4-HNE (1:1000, Abcam, Cambridge, UK) and the intensity of bands were quantified using ImageJ. Briefly, cortex tissues were homogenized in RIPA buffer (Cell Signaling, Danvers, MA, USA) containing protease and phosphatase inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA). For immunoblot analysis 40ug of protein lysate was resolved on Any kD™ Mini-PROTEAN® TGX™ Precast polyacrylamide gels (Biorad, Hercules, CA, USA), transferred to nitrocellulose membrane, blocked in 5% Blotting-grade Blocker (Biorad, Hercules, CA, USA), and incubated with anti-4-HNE antibody (1:1000, Abcam, Cambridge, UK) and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (1:10000, Genetex, Irvine, CA, USA) for normalization, followed by incubation with peroxidase-conjugated secondary antibody (Cell Signaling, Danvers, MA, USA). Protein bands were visualized with enhanced chemiluminescence reagent and digitized using a CCD camera (ChemiDoc®, Biorad, Hercules, CA, USA). Densitometric analysis was performed with ImageJ after background subtraction and normalization to GAPDH (1:10,000, Genetex, Irvine, CA, USA).

3.4.4 Data analysis and graphical representation

Statistical analyses were performed using Stata version 13.1. Nonparametric analyses were employed due to the presence of non-normal distributions. A Kruskal–Wallis test was used for between-group comparisons, and the Dunn's test of multiple comparisons was used to compare the high LA group and Low LA + OXLAM group to the Low LA reference group. P-values for the multiple comparisons were Sidak-adjusted. Diet-induced changes in selected oxylipins and their precursor n-3 and n-6 fatty acids were graphed using boxplots with medians and interquartile ranges.

3.5 Results

Body weight in the Low LA + OXLAM diet group was significantly lower than the other two groups (median weights in grams were 39.2g (IQR 34.1- 42.2g), 40.3g (IQR 33.5- 42.5g), and 30.0g (IQR 27.5- 34.2g) for the Low LA, High LA, and Low LA + OXLAMs diets, respectively; $p<0.01$).

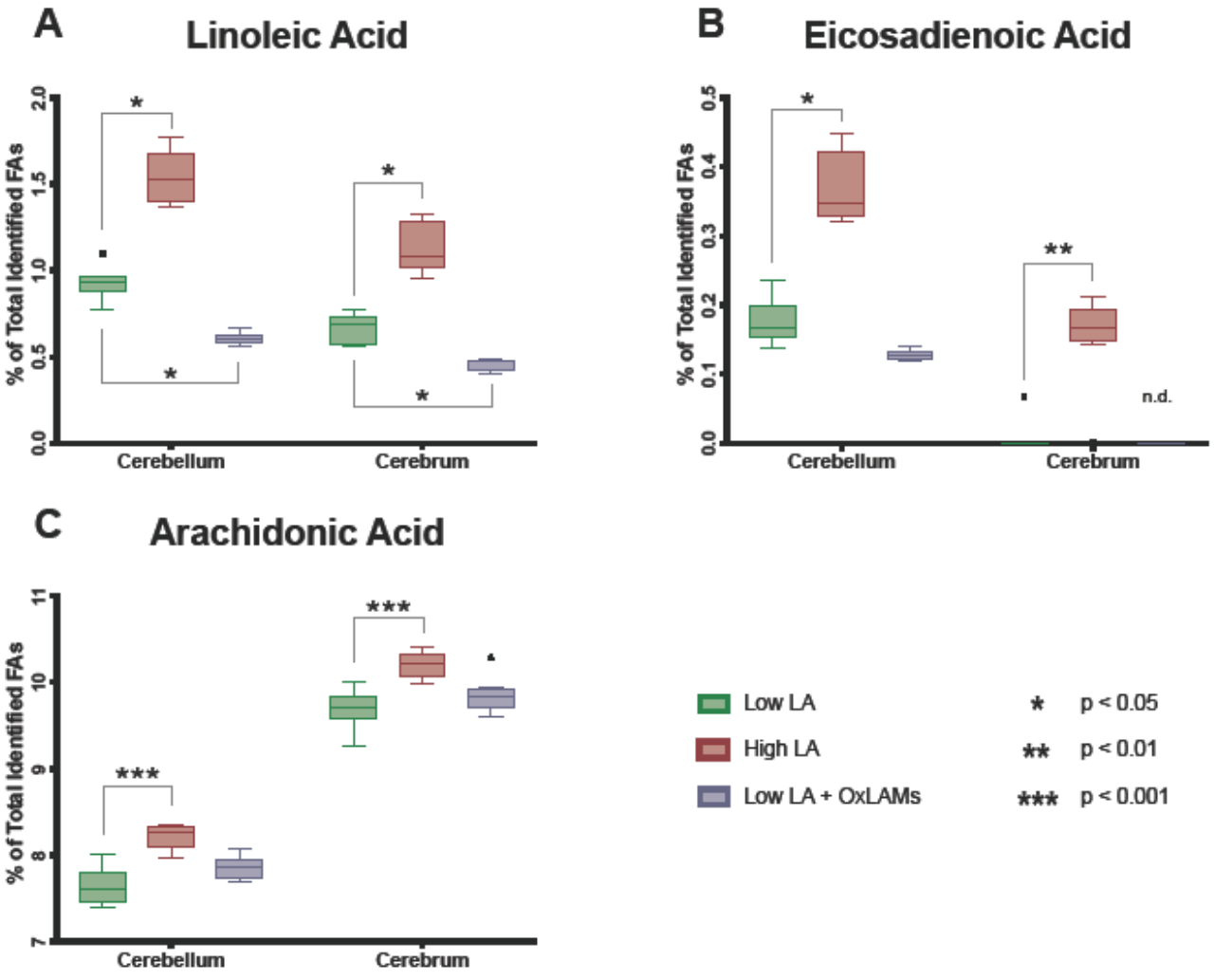

Fig. 1. Dietary LA and OXLAM-induced changes in n-6 fatty acid content of cerebellum and cerebrum

N=8 tissue samples per group. Y-axis ranges differ in each graph. Box plots are based on the Tukey method. P-values are derived from Dunn's test of multiple comparisons using rank sums, comparing the Low LA reference group vs each of the other two groups. P-values are Sidak-adjusted. FAs, Fatty Acids; LA, Linoleic Acid; OXLAM, Oxidized Linoleic Acid Metabolite.

3.5.1 Effects of dietary LA and OXLAMs on brain n-6 fatty acids

Increasing dietary LA significantly increased the abundance of LA (18:2n-6), the LA elongation product eicosadienoic acid (EDA, 20:2n-6), and the LA elongation and desaturation product arachidonic acid (AA, 20:4n-6), in both cerebellum and cerebrum (**fig. 1**)(Tables S2-S3). The Low LA + OXLAM diet group had lower abundance of LA and EDA in cerebellum and decreased LA in cerebral cortex but had no effect on arachidonic acid in either tissue.

3.5.2 Effects of dietary LA and OXLAMs on brain n-3 fatty acids

Increasing dietary LA significantly decreased the abundance of both eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6-3) in cerebellum, and decreased the abundance of EPA but had no effect on DHA in cerebrum (**fig. 2**)(Tables S2-S3). The Low LA + OXLAM diet group had lower abundance of EPA in both cerebellum and cerebrum, and decreased DHA in cerebral cortex, but had no effect on DHA in cerebellum.

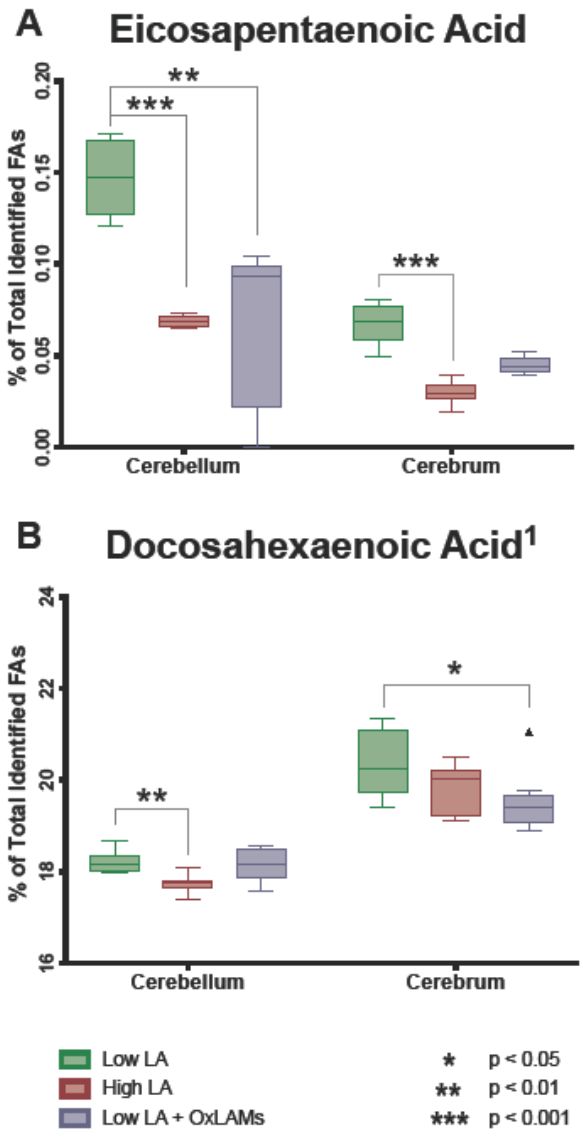

Fig 2. Dietary LA and OXLAM-induced changes in n-3 fatty acid content of cerebellum and cerebrum

N=8 tissue samples per group. Y-axis ranges differ in each graph. Box plots are based on the Tukey method. P-values are derived from Dunn's test of multiple comparisons using rank sums, reporting the Low LA reference group vs each of the other two groups. P-values are Sidak adjusted. LA, linoleic acid; OXLAM, Oxidized Linoleic Acid Metabolite.

3.5.3 Effects of dietary LA and OXLAMs on brain oxylipins

Increasing dietary LA significantly increased the abundance of OXLAMs, including epoxy- and hydroxy-LA derivatives, in both cerebellum (**fig. 3**) and cerebral cortex (**fig. 4**), but had comparatively minor effects on oxylipins derived from AA, EPA or DHA. Increasing dietary OXLAMs decreased the abundance of 12-HETE in cerebellum and cerebral cortex, and decreased TxA2 in cerebral cortex, without altering any other oxylipins (**fig 4**)(Tables S4-S5).

Cerebellum

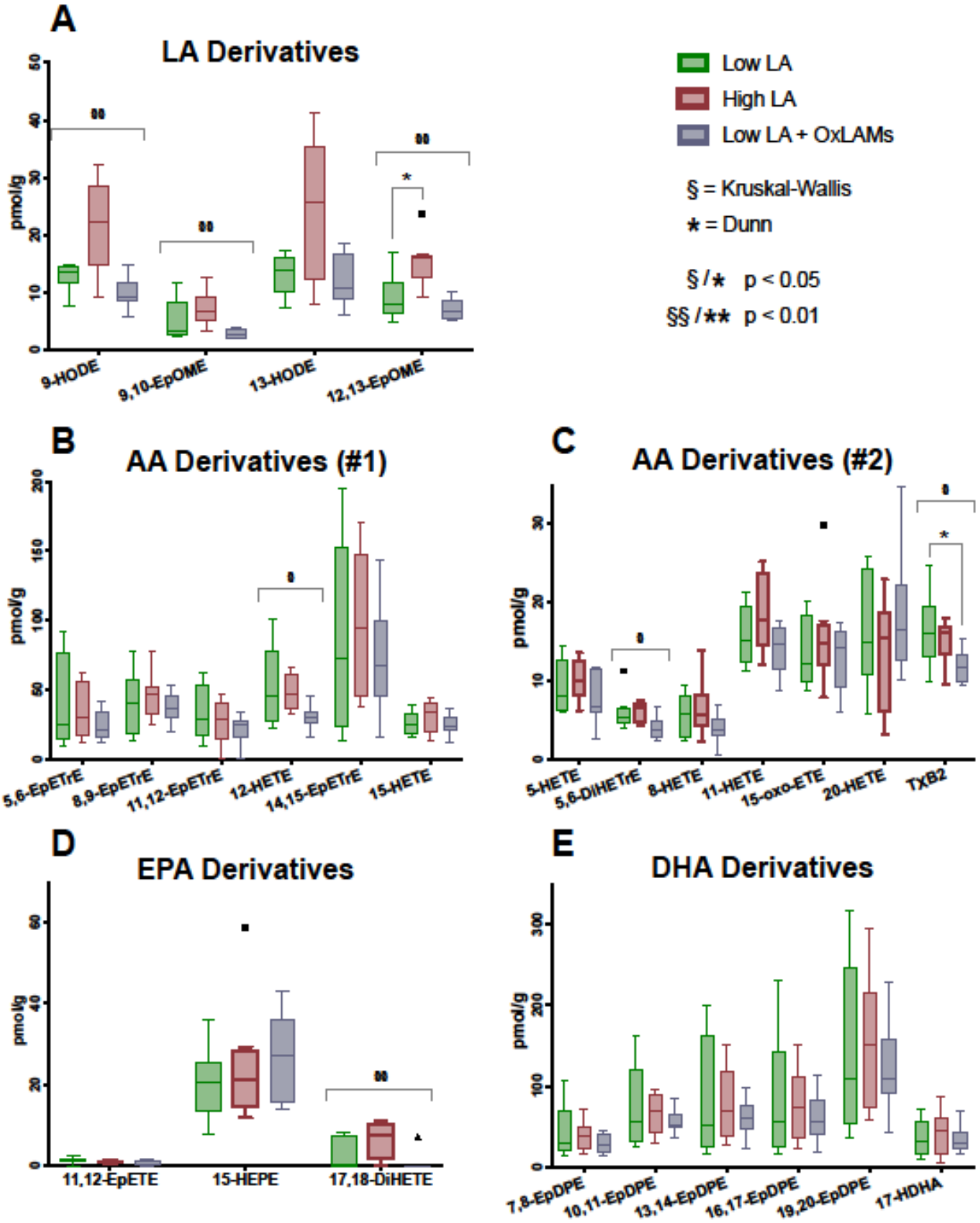
A
LA Derivatives
pmol/g
9-HODE
9,10-EpOME
13-HODE
12,13-EpOME
Low LA
High LA
Low LA + OxLAMs
§ = Kruskal-Wallis
* = Dunn
§ / * p < 0.05
§§ / ** p < 0.01
B
AA Derivatives (#1)
5,6-EpETrE
8,9-EpETrE
11,12-EpETrE
12-HETE
14,15-EpETrE
15-HETE
C
AA Derivatives (#2)
5-HETE
5,6-DiHETrE
8-HETE
11-HETE
15-oxo-ETE
20-HETE
TXB2
D
EPA Derivatives
11,12-EpETE
15-HEPE
17,18-DiHETE
E
DHA Derivatives
7,8-EpDPE
10,11-EpDPE
13,14-EpDPE
16,17-EpDPE
19,20-EpDPE
17-HDHA

Fig. 3. Effects of increases in dietary LA and dietary OXLAMs on cerebellar oxylipins

N=8 tissue samples per group. Y-axis ranges differ in each graph. Box plots are based on the Tukey method. P-values with asterisks are derived from Dunn's test of multiple comparisons using rank sums, comparing the Low LA reference group vs each of the other two groups. These P-values are also Sidak-adjusted. P-values with section signs are derived from the Kruskal-Wallis nonparametric rank-sum test across all three groups. LA, linoleic acid; AA, arachidonic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid.

Cerebrum

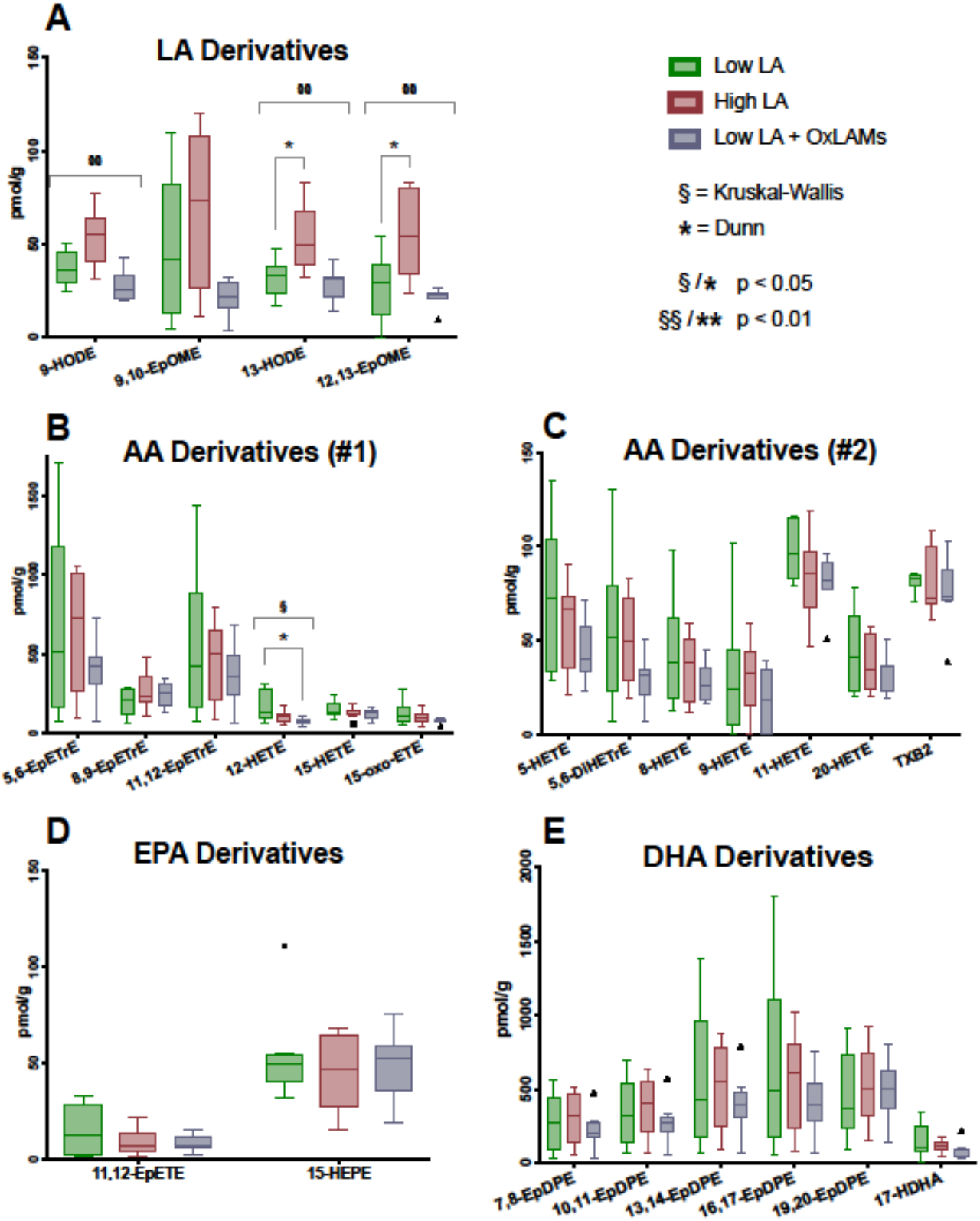
A
LA Derivatives
pmol/g
9-HODE
9,10-EpOME
13-HODE
12,13-EpOME
Low LA
High LA
Low LA + OxLAMs
§ = Kruskal-Wallis
* = Dunn
§ /* p < 0.05
§§ /** p < 0.01
B
AA Derivatives (#1)
5,6-EpETrE
8,9-EpETrE
11,12-EpETrE
12-HETE
15-HETE
15-oxo-ETE
C
AA Derivatives (#2)
5-HETE
5,6-DiHETrE
8-HETE
9-HETE
11-HETE
20-HETE
TXB2
D
EPA Derivatives
11,12-EpETE
15-HEPE
E
DHA Derivatives
7,8-EpDPE
10,11-EpDPE
13,14-EpDPE
16,17-EpDPE
19,20-EpDPE
17-HDHA

Fig. 4. Effects of increases in dietary LA and dietary OXLAMs on cerebral cortex oxylipins

N=8 tissue samples per group. Y-axis scales differ in each graph. Box plots are based on the Tukey method. P-values are derived from Dunn's test of multiple comparisons using rank sums, reporting the Low LA reference group vs each of the other two groups. P-values are Sidak adjusted. LA, linoleic acid; AA, arachidonic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid.

3.5.4 Effects of dietary LA and OXLAMs on brain 4-HNE

4-HNE, a secondary product of fatty acid peroxidation, was not significantly altered in cerebral cortex by increases in dietary LA or dietary OXLAMs (**fig. 5**).

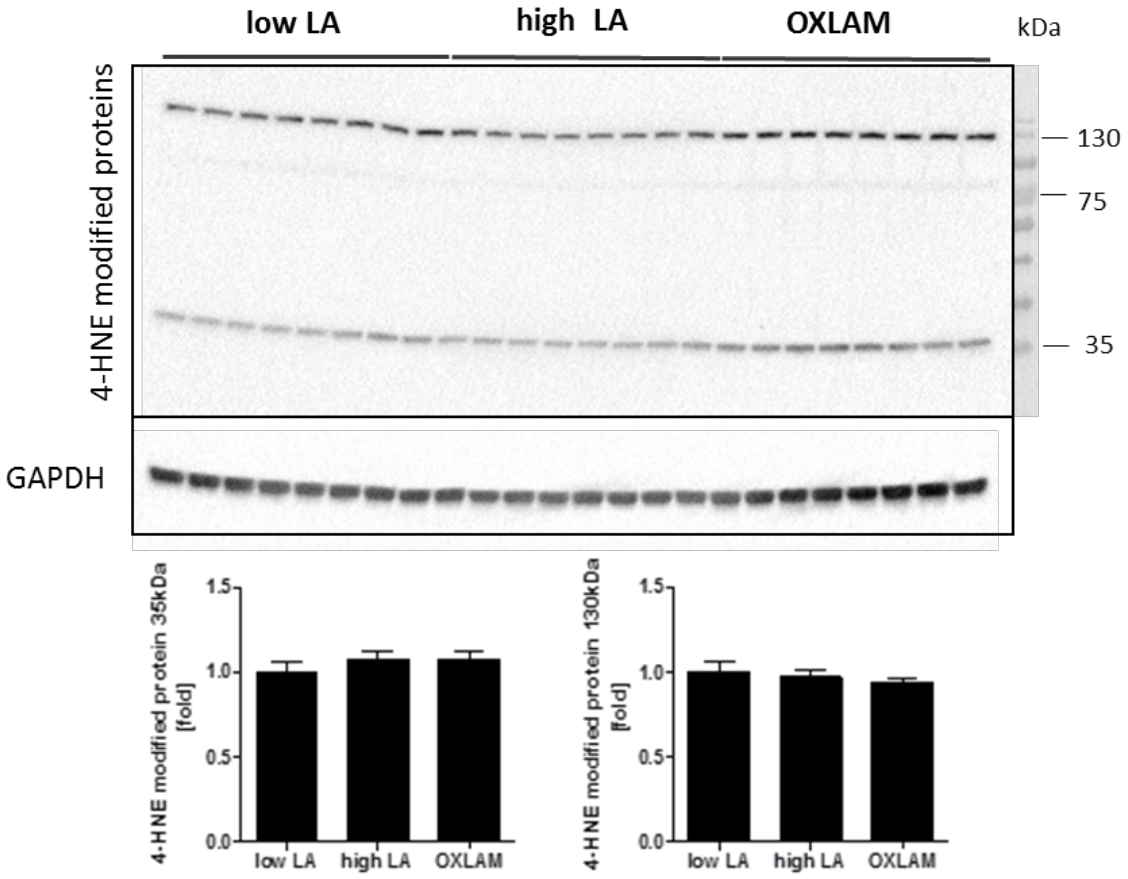

Fig. 5. Effects of increases in dietary LA and dietary OXLAMs on 4-HNE in mouse cerebral cortex

N=8 tissue samples per group. Immunoblot analysis of cerebral cortex protein lysates for the detection of 4-HNE-modified proteins. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a loading control. Densitometric analysis was performed on background-subtracted blots and normalized to GAPDH. The Low LA group was used as a reference control and set to 1.

3.6 Discussion

Per capita mean dietary LA has increased markedly in industrialized populations over the past century. Current LA intakes are much higher than historical and evolutionary norms of 2-3%E and depend on industrial oil processing to create concentrated oils, which scarcely resemble their unprocessed food sources. For example, LA accounts for 55% of calories in corn oil versus 0.5% of calories in whole corn (40). The LA in concentrated oil is susceptible to exogenous oxidation when oils are thermally stressed from frying, cooking, during deodorization and refinement of liquid vegetable oils, or when processed foods containing high LA oils are heated (9,41). Because a substantial portion of the dietary LA in industrialized populations is heated prior to consumption, modern populations have substantially increased intakes of both LA and preformed LA peroxidation products. Consumption of heated oils rich in LA without adequate vitamin E (22,23), or intravenous administration of HPODEs (24), can produce cerebellar necrosis and ataxia in chicks, suggesting that dietary OXLAMs might potentially have adverse effects in CNS tissues. However, it is not yet known whether eating non-oxidized or heated vegetable oils has biochemical consequences in mammalian brain tissues.

Here we showed that increasing dietary LA specifically increased LA peroxidation products in cerebellum and cerebrum, without substantially affecting oxylipins derived from AA, EPA or DHA. By contrast, the consumption of preformed LA peroxidation products (OXLAMs) had minimal effect on the concentrations of brain oxylipins. Neither dietary LA or dietary OXLAMs altered cerebral 4-HNE levels. These collective findings suggest that the altered mammalian brain lipid oxidation observed after LA feeding is driven by either endogenous enzymatic LA peroxidation, or endogenous free-radical mediated LA peroxidation, rather than by absorption of preformed LA oxidation products present in the diet. The specific LA

peroxidation products that were increased by high LA diets in the brain in the present study—including HODEs, EpOMEs, and DiHOMEs—have shown diverse bioactivities in preclinical models, and have been implicated in the pathogenesis of conditions outside of the CNS that are characterized by inflammation and oxidative stress, including cardiovascular disease (3,15), non-alcoholic steatohepatitis (16,17), acute respiratory distress syndrome (42,43) asthma (45,46), and chronic pain (6,19,20), reviewed in (3,15,21). The present finding that the body weight of the Low LA + OXLAM diet group was significantly lower than the other two groups, suggests that dietary OXLAMs could potentially have unfavorable effects on growth in mammals. However, future studies are needed to determine whether weight reducing effects of heated oils are due to decreased food intake, partial displacement of the energy provided by fatty acids with non-caloric oxidized lipids, or to other effects on metabolism.

Consistent with previous reports (8,19,47), results of the present study indicate that increasing dietary non-oxidized LA shifts the balance of brain precursor polyunsaturated fatty acids away from the n-3 family (EPA and DHA) towards the n-6 family (increases in LA, eicosadienoic acid, and AA). However, unlike LA, the observed diet-induced changes in EPA, DHA, and AA did not translate to changes in their oxylipin derivatives. Interestingly, the Low LA + OXLAMs diet appeared to significantly decrease the abundance of n-3 DHA and EPA in CNS tissues, despite containing less dietary LA than the Low LA diet alone. DHA and EPA are proposed to play critical structural and functional roles in CNS tissues (48,49). Thus, future studies are warranted to determine the effects of high intakes of thermally stressed high LA oils on developmental, behavioral, or cognitive endpoints.

Since heated oils are major source of dietary OXLAMs in modern diets, the use of heated corn oil is a strength of the present study. However, to gain a better understanding of the effects

of specific components in heated oils, future studies should consider adding specific oxidation products (e.g. HpODEs) and degradation products (e.g. aldehydes) that are present in heated high LA oils to study diets. Since inclusion of oxidized oils may also alter the taste and texture of food pellets to influence food consumption, future studies should consider gavaging study oils to optimize the controlled nature of study diets. Future dietary OXLAM studies should consider using more tightly controlled diets that isolate OXLAMs as a controlled variable (keeping non-oxidized LA constant), to isolate the specific effects of individual OXLAMs.

In summary, the present study fills an important gap by establishing proof of principle in a mammalian model that diets enriched in LA and OXLAMs alter cerebral and cerebellar lipid accumulation and peroxidation. Mammalian brain OXLAM concentrations appear to be regulated by endogenous synthesis from dietary LA, rather than direct incorporation of preformed OXLAMs that are in the diet. Future studies are warranted to investigate whether and how diets enriched in LA and OXLAMs impact behavior, neurodevelopment, or neurodegeneration.

Acknowledgments

This study was supported by the Intramural Programs of the National Institute on Aging and the National Institute on Alcohol Abuse and Alcoholism (C.E.R), U01 AA022489-01A1 from the National Institute on Alcohol Abuse and Alcoholism (A.E.F.), the University of Adelaide (R.A.G), and the German Research Foundation (SCHU3146/1-2 to S.S.). The authors acknowledge the intellectual contributions of Duong Nguyen.

3. 7 References (Chapter 3)

1. Blasbalg TL, Hibbeln JR, Ramsden CE, Majchrzak SF, Rawlings RR. Changes in consumption of omega-3 and omega-6 fatty acids in the United States during the 20th century. Am J Clin Nutr. 2011;93(5):950-62.

2. Ramsden CE, Ringel A, Feldstein AE, Taha AY, MacIntosh BA, Hibbeln JR, et al. Lowering dietary linoleic acid reduces bioactive oxidized linoleic acid metabolites in humans. Prostaglandins, leukotrienes, and essential fatty acids. 2012;87(4-5):135-41.

3. Ramsden CE, Zamora D, Majchrzak-Hong S, Faurot KR, Broste SK, Frantz RP, et al. Re-evaluation of the traditional diet-heart hypothesis: analysis of recovered data from Minnesota Coronary Experiment (1968-73). Bmj. 2016;353:i1246.

4. Hansen AE, Haggard ME, Boelsche AN, Adam DJ, Wiese HF. Essential fatty acids in infant nutrition. III. Clinical manifestations of linoleic acid deficiency. The Journal of nutrition. 1958;66(4):565-76.

5. Mensink RP, Zock PL, Kester AD, Katan MB. Effects of dietary fatty acids and carbohydrates on the ratio of serum total to HDL cholesterol and on serum lipids and apolipoproteins: a meta-analysis of 60 controlled trials. Am J Clin Nutr. 2003;77(5):1146-55.

6. Ramsden CE, Domenichiello AF, Yuan ZX, Sapio MR, Keyes GS, Mishra SK, et al. A systems approach for discovering linoleic acid derivatives that potentially mediate pain and itch. Sci Signal. 2017;10(493).

7. Schneider C, Porter NA, Brash AR. Autoxidative transformation of chiral omega6 hydroxy linoleic and arachidonic acids to chiral 4-hydroxy-2E-nonenal. Chem Res Toxicol. 2004;17(7):937-41.

8. Taha AY, Hennebelle M, Yang J, Zamora D, Rapoport SI, Hammock BD, et al. Regulation of rat plasma and cerebral cortex oxylipin concentrations with increasing levels of dietary linoleic acid. Prostaglandins, leukotrienes, and essential fatty acids. 2016.

9. Morales A, Marmesat S, Dobarganes MC, Marquez-Ruiz G, Velasco J. Quantitative analysis of hydroperoxy-, keto- and hydroxy-dienes in refined vegetable oils. J Chromatogr A. 2012;1229:190-7.

10. Marmesat S, Velasco J, Dobarganes MC. Quantitative determination of epoxy acids, keto acids and hydroxy acids formed in fats and oils at frying temperatures. J Chromatogr A. 2008;1211(1-2):129-34.

11. Marmesat S, Morales A, Velasco J, Carmen Dobarganes M. Influence of fatty acid composition on chemical changes in blends of sunflower oils during thermoxidation and frying. Food chemistry. 2012;135(4):2333-9.

12. Wilson R, Lyall K, Smyth L, Fernie CE, Riemersma RA. Dietary hydroxy fatty acids are absorbed in humans: implications for the measurement of 'oxidative stress' in vivo. Free radical biology & medicine. 2002;32(2):162-8.

13. Ferreiro-Vera C, Priego-Capote F, Mata-Granados JM, Luque de Castro MD. Short-term comparative study of the influence of fried edible oils intake on the metabolism of essential fatty acids in obese individuals. Food chemistry. 2013;136(2):576-84.

14. Wilson R, Fernie CE, Scrimgeour CM, Lyall K, Smyth L, Riemersma RA. Dietary epoxy fatty acids are absorbed in healthy women. Eur J Clin Invest. 2002;32(2):79-83.

15. Ramsden CE, Zamora D, Leelarthaepin B, Majchrzak-Hong SF, Faurot KR, Suchindran CM, et al. Use of dietary linoleic acid for secondary prevention of coronary heart disease and death: evaluation of recovered data from the Sydney Diet Heart Study and updated meta-analysis. Bmj. 2013;346:e8707.

16. Liu H, Beier JI, Arteel GE, Ramsden CE, Feldstein AE, McClain CJ, et al. Transient receptor potential vanilloid 1 gene deficiency ameliorates hepatic injury in a mouse model of chronic binge alcohol-induced alcoholic liver disease. Am J Pathol. 2015;185(1):43-54.

17. Feldstein AE, Lopez R, Tamimi TA, Yerian L, Chung YM, Berk M, et al. Mass spectrometric profiling of oxidized lipid products in human nonalcoholic fatty liver disease and nonalcoholic steatohepatitis. Journal of lipid research. 2010;51(10):3046-54.

18. Di Domenico F, Tramutola A, Butterfield DA. Role of 4-hydroxy-2-nonenal (HNE) in the pathogenesis of alzheimer disease and other selected age-related neurodegenerative disorders. Free Radic Biol Med. 2017;111:253-61.

19. Ramsden CE, Ringel A, Majchrzak-Hong SF, Yang J, Blanchard H, Zamora D, et al. Dietary linoleic acid-induced alterations in pro- and anti-nociceptive lipid autacoids: Implications for idiopathic pain syndromes? Molecular pain. 2016;12.

20. Ramsden CE, Faurot KR, Zamora D, Suchindran CM, Macintosh BA, Gaylord S, et al. Targeted alteration of dietary n-3 and n-6 fatty acids for the treatment of chronic headaches: A randomized trial. Pain. 2013.

21. Budowski P, Bartov I, Dror Y, Frankel EN. Lipid oxidation products and chick nutritional encephalopathy. Lipids. 1979;14(9):768-72.

22. Fischer VW, Nelson JS. Cerebrovascular changes in tocopherol-depleted chicks, fed linoleic acid. J Neuropathol Exp Neurol. 1973;32(3):474-83.

23. Dror Y, Budowski P. Lysosomal acid phosphatase decrease in nutritional encephalopathy in chicks. Nutr Metab. 1980;24(3):154-60.

24. Nishida T, Tsuchiyama H, Inoue M, Kummerow FA. Effect of intravenous injection of oxidized methyl esters of unsaturated fatty acids on chick encephalomalacia. Proc Soc Exp Biol Med. 1960;105:308-12.

25. Pacifici EH, McLeod LL, Peterson H, Sevanian A. Linoleic acid hydroperoxide-induced peroxidation of endothelial cell phospholipids and cytotoxicity. Free radical biology & medicine. 1994;17(4):285-95.

26. Polivoda BI. [Damage of cell membranes by linoleic acid hydroperoxide]. Biofizika. 1986;31(3):453-5.

27. Nakagawa K, Kato S, Miyazawa T. Determination of Phosphatidylcholine Hydroperoxide (PCOOH) as a Marker of Membrane Lipid Peroxidation. J Nutr Sci Vitaminol (Tokyo). 2015;61 Suppl:S78-80.

28. Yin H, Zhu M. Free radical oxidation of cardiolipin: chemical mechanisms, detection and implication in apoptosis, mitochondrial dysfunction and human diseases. Free Radic Res. 2012;46(8):959-74.

29. Obinata H, Hattori T, Nakane S, Tatei K, Izumi T. Identification of 9-hydroxyoctadecadienoic acid and other oxidized free fatty acids as ligands of the G protein-coupled receptor G2A. The Journal of biological chemistry. 2005;280(49):40676-83.

30. Obinata H, Izumi T. G2A as a receptor for oxidized free fatty acids. Prostaglandins Other Lipid Mediat. 2009;89(3-4):66-72.

31. Sheikh AM, Nagai A, Ryu JK, McLarnon JG, Kim SU, Masuda J. Lysophosphatidylcholine induces glial cell activation: role of rho kinase. Glia. 2009;57(8):898-907.

32. Zarkovic K, Jakovcevic A, Zarkovic N. Contribution of the HNE-immunohistochemistry to modern pathological concepts of major human diseases. Free Radic Biol Med. 2017;111:110-26.

33. Nieva J, Shafton A, Altobell LJ, 3rd, Tripuraneni S, Rogel JK, Wentworth AD, et al. Lipid-derived aldehydes accelerate light chain amyloid and amorphous aggregation. Biochemistry. 2008;47(29):7695-705.

34. Montine KS, Olson SJ, Amarnath V, Whetsell WO, Jr., Graham DG, Montine TJ. Immunohistochemical detection of 4-hydroxy-2-nonenal adducts in Alzheimer's disease is associated with inheritance of APOE4. Am J Pathol. 1997;150(2):437-43.

35. Guillen MD, Goicoechea E. Oxidation of corn oil at room temperature: Primary and secondary oxidation products and determination of their concentration in the oil liquid matrix from 1H nuclear magnetic resonance data. Food Chemistry. 2009;116(1):183-192.

36. Zhang Z, Richardson CE, Hennebelle M, Taha AY. Validation of a One-Step Method for Extracting Fatty Acids from Salmon, Chicken and Beef Samples. J Food Sci. 2017;82(10):2291-7.

37. Hennebelle M, Zhang Z, Metherel AH, Kitson AP, Otoki Y, Richardson CE, et al. Linoleic acid participates in the response to ischemic brain injury through oxidized metabolites that regulate neurotransmission. Scientific reports. 2017;7(1):4342.

38. Yang J, Schmelzer K, Georgi K, Hammock BD. Quantitative profiling method for oxylipin metabolome by liquid chromatography electrospray ionization tandem mass spectrometry. Anal Chem. 2009;81(19):8085-93.

39. Taha AY, Basselin M, Ramadan E, Modi HR, Rapoport SI, Cheon Y. Altered lipid concentrations of liver, heart and plasma but not brain in HIV-1 transgenic rats. Prostaglandins Leukot Essent Fatty Acids. 2012; 87(4-5):91-101.

40. US Department of Agriculture, Agricultural Research Service, Nutrient Data Laboratory. USDA National Nutrient Database for Standard Reference, Release 28. Internet: /nea/bhnrc/ndl.

41. Dobarganes C, Marquez-Ruiz G. Possible adverse effects of frying with vegetable oils. The British journal of nutrition. 2015;113 Suppl 2:S49-57.

42. Zheng J, Plopper CG, Lakritz J, Storms DH, Hammock BD. Leukotoxin-diol: a putative toxic mediator involved in acute respiratory distress syndrome. Am J Respir Cell Mol Biol. 2001;25(4):434-8.

43. Ozawa T, Sugiyama S, Hayakawa M, Satake T, Taki F, Iwata M, et al. Existence of leukotoxin 9,10-epoxy-12-octadecenoate in lung lavages from rats breathing pure oxygen and from patients with the adult respiratory distress syndrome. Am Rev Respir Dis. 1988;137(3):535-40.

44. Hu JN, Taki F, Sugiyama S, Asai J, Izawa Y, Satake T, et al. Neutrophil-derived epoxide, 9,10-epoxy-12-octadecenoate, induces pulmonary edema. Lung. 1988;166(6):327-37.

45. Mabalirajan U, Rehman R, Ahmad T, Kumar S, Singh S, Leishangthem GD, et al. Linoleic acid metabolite drives severe asthma by causing airway epithelial injury. Scientific reports. 2013;3:1349.

46. Panda L, Gheware A, Rehman R, Yadav MK, Jayaraj BS, Madhunapantula SV, et al. Linoleic acid metabolite leads to steroid resistant asthma features partially through NF-kappaB. Scientific reports. 2017;7(1):9565.

47. Sakayori N, Kikkawa T, Tokuda H, Kiryu E, Yoshizaki K, Kawashima H, et al. Maternal dietary imbalance between omega-6 and omega-3 polyunsaturated fatty acids impairs neocortical development via epoxy metabolites. Stem Cells. 2016;34(2):470-82.

48. Lee JW, Huang BX, Kwon H, Rashid MA, Kharebava G, Desai A, et al. Orphan GPR110 (ADGRF1) targeted by N-docosahexaenoylethanolamine in development of neurons and cognitive function. Nat Commun. 2016;7:13123.

49. Matsuoka YJ, Sawada N, Mimura M, Shikimoto R, Nozaki S, Hamazaki K, et al. Dietary fish, n-3 polyunsaturated fatty acid consumption, and depression risk in Japan: a population-based prospective cohort study. Transl Psychiatry. 2017;7(9):e1242.

3.8 Supplementary Appendices

Table S1: Fatty acid composition of study diets (% of total fatty acids)

Table S2: Effects of the study diets on fatty acid composition of cerebellum (% of total fatty acids)

Table S3: Effects of the study diets on fatty acid composition of cerebral cortex (% of total fatty acids)

Table S4: Effects of the study diets on fatty acid composition of cerebellum (% of total fatty acids)

Table S5: Effects of the study diets on oxylipins in cerebral cortex (pmol/g)*

Table S1: Fatty acid composition of study diets (% of total fatty acids)

	LA (% FA)	ALA (% FA)	SFA (% FA)	MUFA (% FA)
Diet group				
Low LA	15.8 ± 0.7	6.0 ± 0.2	69.2 ± 1.2	9.0 ± 0.3
High LA	46.9 ± 0.3	5.8 ± 0.02	21.8 ± 0.2	25.2 ± 0.1
Low LA + OXLAM	12.2 ± 0.3	6.7 ± 0.06	67.2 ± 0.2	13.9 ± 0.2

Data are mean ± standard deviation. LA, linoleic acid; ALA, alpha-linolenic acid; SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; FA, fatty acid.

Table S2: Effects of the Study Diets on Fatty Acids (% of Total Fatty Acids) in Cerebellum

	Low LA		High LA		Low LA + OxLAMs		Kruskal-Wallis
	Median	IQR	Median	IQR	Median	IQR	p-value
Myristic Acid	0.29	(0.28, 0.32)	0.16	(0.16, 0.17)	0.21	(0.20, 0.21)	0.000
Myristoleic Acid	0.05	(0.04, 0.06)	0.04	(0.04, 0.04)	0.05	(0.04, 0.05)	0.221
Palmitic Acid	18.88	(18.77, 19.11)	19.15	(19.08, 19.28)	19.15	(18.95, 19.23)	0.041
Palmitoleic Acid	0.28	(0.00, 0.57)	0.38	(0.33, 0.39)	0.44	(0.43, 0.46)	0.064
Heptadecanoic Acid	0.16	(0.15, 0.17)	0.17	(0.16, 0.18)	0.16	(0.15, 0.17)	0.298
Stearic Acid/Octadecanoic Acid	22.06	(21.78, 22.28)	21.74	(21.58, 21.94)	21.65	(21.55, 21.75)	0.018
Oleic Acid +Elaidic Acid	19.79	(19.67, 19.94)	19.28	(19.16, 19.41)	20.34	(19.96, 20.54)	0.000
Vaccenic Acid	4.47	(3.99, 4.60)	3.98	(3.80, 4.08)	4.35	(4.04, 4.52)	0.076
Linoleic Acid	0.93	(0.89, 0.96)	1.52	(1.40, 1.64)	0.61	(0.58, 0.63)	0.000
Linolenic Acid	0.03	(0.03, 0.04)	0.03	(0.02, 0.03)	0.02	(0.02, 0.02)	0.000
Gamma-Linolenic Acid	0.04	(0.04, 0.05)	0.05	(0.05, 0.06)	0.04	(0.04, 0.05)	0.126
Arachidic Acid	0.45	(0.44, 0.47)	0.46	(0.45, 0.46)	0.44	(0.43, 0.45)	0.230
Gondoic Acid	2.87	(2.80, 2.99)	2.91	(2.85, 2.94)	2.95	(2.78, 2.99)	0.912
Eicosadienoic Acid	0.17	(0.16, 0.20)	0.35	(0.33, 0.41)	0.13	(0.12, 0.13)	0.000
Dihomo-gamma-linolenic acid	0.59	(0.55, 0.61)	0.59	(0.52, 0.62)	0.67	(0.60, 0.70)	0.041
Eicosatrienoic Acid	0.02	(0.01, 0.03)	0.02	(0.02, 0.02)	0.01	(0.01, 0.02)	0.002
Arachidonic Acid	7.61	(7.45, 7.77)	8.27	(8.10, 8.33)	7.85	(7.75, 7.95)	0.000
Eicosapentaenoic Acid	0.15	(0.13, 0.17)	0.07	(0.07, 0.07)	0.09	(0.04, 0.10)	0.000
Behenic Acid	0.22	(0.21, 0.23)	0.19	(0.19, 0.20)	0.20	(0.19, 0.25)	0.013
Docosahexaenoic Acid	18.17	(18.02, 18.31)	17.76	(17.62, 17.82)	18.15	(17.85, 18.48)	0.008

Table S3: Effects of the Study Diets on Fatty Acids (% of Total Fatty Acids) in Cerebral Cortex

	Low LA		High LA		Low LA + OxLAMs		Kruskal-Wallis
	Median	IQR	Median	IQR	Median	IQR	p-value
Myristic Acid	0.24	(0.21, 0.26)	0.12	(0.11, 0.14)	0.18	(0.18, 0.19)	0.000
Myristoleic Acid	0.08	(0.05, 0.10)	0.08	(0.05, 0.10)	0.05	(0.04, 0.06)	0.185
Palmitic Acid	22.49	(21.70, 22.60)	22.66	(22.38, 23.02)	22.87	(22.50, 23.15)	0.151
Palmitoleic Acid	0.50	(0.45, 0.54)	0.38	(0.35, 0.40)	0.44	(0.43, 0.45)	0.005
Heptadecanoic Acid	0.00	(0.00, 0.17)	0.09	(0.00, 0.21)	0.16	(0.00, 0.20)	0.478
Stearic Acid	23.73	(23.42, 24.24)	23.36	(23.18, 23.54)	23.18	(22.90, 23.47)	0.076
Oleic Acid	14.08	(13.47, 14.41)	13.65	(13.28, 14.42)	15.08	(14.74, 15.27)	0.028
Vaccenic Acid	2.92	(2.64, 3.05)	2.81	(2.74, 2.92)	3.19	(3.05, 3.39)	0.007
Linoleic Acid	0.69	(0.58, 0.73)	1.09	(1.02, 1.28)	0.43	(0.41, 0.47)	0.000
Linolenic Acid	0.03	(0.02, 0.03)	0.02	(0.02, 0.03)	0.02	(0.01, 0.02)	0.249
Gamma-linolenic Acid	0.03	(0.02, 0.03)	0.03	(0.03, 0.03)	0.02	(0.02, 0.02)	0.017
Arachidic Acid	0.18	(0.18, 0.21)	0.19	(0.15, 0.20)	0.18	(0.17, 0.20)	0.954
Gondoic Acid	1.04	(0.99, 1.14)	1.04	(0.88, 1.13)	1.07	(1.00, 1.13)	0.846
Eicosadienoic Acid	0.00	(0.00, 0.00)	0.17	(0.15, 0.19)	0.00	(0.00, 0.00)	0.000
Dihomo-gamma-linolenic acid	0.48	(0.44, 0.51)	0.48	(0.45, 0.49)	0.55	(0.53, 0.57)	0.003
Eicosatrienoic Acid	0.00	(0.00, 0.00)	0.00	(0.00, 0.00)	0.00	(0.00, 0.00)	
Arachidonic Acid	9.70	(9.60, 9.81)	10.22	(10.06, 10.33)	9.83	(9.71, 9.93)	0.001
Eicosapentaenoic Acid	0.07	(0.06, 0.08)	0.03	(0.03, 0.03)	0.04	(0.04, 0.05)	0.000
Behenic Acid	0.12	(0.11, 0.13)	0.10	(0.09, 0.11)	0.11	(0.09, 0.11)	0.025
Docosahexaenoic Acid	20.24	(19.85, 21.02)	20.01	(19.30, 20.20)	19.42	(19.13, 19.62)	0.038

Table S4: Effects of the Study Diets on Oxylipins (pmol/g) in Cerebellum

	Low LA			High LA			Low LA + OxLAMs			Kruskal-Wallis
	Median	IQR		Median	IQR		Median	IQR		p-value
LA-derived										
9-HODE	13.69	(12.24,	14.41)	22.24	(16.14,	28.39)	9.28	(8.33,	11.82)	0.006
9-oxo-ODE	0.38	(0.10,	0.44)	0.62	(0.43,	0.82)	0.00	(0.00,	0.18)	0.003
9,10-EpOME	3.36	(2.60,	7.12)	6.75	(5.35,	9.20)	2.84	(2.04,	3.76)	0.010
9,10-DiHOME	0.14	(0.00,	0.64)	0.57	(0.45,	0.82)	0.76	(0.62,	0.90)	0.067
13-HODE	13.77	(10.50,	16.19)	25.80	(13.19,	35.52)	10.62	(8.86,	15.07)	0.113
12,13-EpOME	7.92	(6.44,	10.81)	16.11	(12.68,	16.61)	6.75	(5.40,	8.61)	0.003
12,13-DiHOME	0.82	(0.41,	1.69)	0.80	(0.62,	1.59)	1.24	(0.98,	1.57)	0.365
DGLA-derived										
15-HETrE	2.51	(1.85,	3.76)	2.88	(1.42,	3.62)	2.58	(2.15,	3.57)	0.907
AA-derived										
5-HETE	7.98	(6.13,	11.97)	9.87	(8.13,	12.28)	6.78	(6.02,	11.51)	0.418
5,6-EpETrE	24.60	(14.13,	66.43)	29.81	(18.58,	51.18)	20.67	(15.67,	32.80)	0.636
5,6-DiHETrE	5.27	(4.44,	6.40)	6.58	(4.69,	7.20)	3.70	(2.77,	4.86)	0.037
8-HETE	5.69	(2.77,	7.99)	5.72	(4.32,	8.28)	3.74	(3.19,	5.13)	0.279
8,9-EpETrE	39.61	(21.90,	55.14)	46.62	(35.37,	52.34)	36.07	(31.05,	43.20)	0.623
11-HETE	15.19	(12.65,	18.97)	17.75	(15.07,	22.57)	14.72	(12.08,	16.89)	0.203
11,12-EpETrE	28.87	(16.89,	49.41)	28.23	(14.30,	37.56)	24.14	(16.87,	27.40)	0.595
12-HETE	45.60	(29.62,	71.44)	46.26	(36.58,	60.66)	29.41	(25.98,	33.60)	0.042
14,15-EpETrE	72.06	(30.23,	137.01)	94.18	(50.88,	139.15)	67.31	(45.80,	94.49)	0.756
14,15-DiHETrE	0.30	(0.00,	0.40)	0.45	(0.23,	0.64)	0.31	(0.14,	0.38)	0.288
15-HETE	24.35	(17.97,	32.77)	34.30	(19.54,	39.10)	23.83	(19.77,	29.85)	0.462
15-oxo-ETE	12.06	(9.82,	17.47)	14.83	(12.49,	16.78)	14.23	(10.06,	15.78)	0.691
20-HETE	14.89	(10.96,	23.20)	15.35	(6.69,	18.46)	16.35	(13.21,	20.89)	0.539
TXB2	15.96	(13.26,	19.21)	16.11	(13.41,	17.05)	11.66	(9.96,	13.19)	0.037
EPA-derived										
11,12-EpETE	1.22	(0.70,	1.76)	0.89	(0.66,	1.11)	0.95	(0.31,	1.51)	0.604
15-HEPE	20.46	(14.78,	24.39)	21.11	(15.87,	27.58)	27.24	(16.38,	34.34)	0.505
17,18-DiHETE	0.00	(0.00,	7.61)	7.55	(2.50,	10.11)	0.00	(0.00,	0.00)	0.027
DHA-derived										
7,8-EpDPE	30.38	(20.98,	58.91)	38.94	(23.62,	51.04)	27.27	(20.76,	39.05)	0.442
10,11-EpDPE	54.99	(35.39,	103.25)	69.09	(47.59,	90.23)	50.96	(47.52,	63.77)	0.564
13,14-EpDPE	52.32	(30.11,	141.92)	69.35	(43.74,	117.58)	60.31	(47.99,	73.31)	0.728
16,17-EpDPE	55.69	(29.57,	129.19)	74.65	(39.29,	108.58)	56.39	(41.34,	76.96)	0.773
19,20-EpDPE	108.62	(63.99,	230.52)	150.08	(87.21,	204.28)	109.41	(91.85,	153.39)	0.724
17-HDHA	32.49	(16.29,	55.13)	43.85	(20.44,	61.15)	29.31	(21.50,	42.45)	0.827

Table S5: Effects of the Study Diets on Oxylipins (pmol/g) in Cerebrum

	Low LA		High LA		Low LA + OxLAMs		Kruskal-Wallis
	Median	IQR	Median	IQR	Median	IQR	p-value
LA-derived							
9-HODE	35.78	(29.66, 42.79)	54.63	(42.32, 63.32)	25.63	(19.96, 32.98)	0.003
9-oxo-ODE	1.03	(0.66, 1.65)	1.50	(0.98, 1.93)	0.78	(0.00, 0.89)	0.044
9,10-EpOME	41.73	(13.06, 77.15)	73.24	(29.35, 104.69)	21.58	(16.26, 28.94)	0.093
9,10-DiHOME	1.01	(0.24, 1.56)	1.20	(1.03, 2.00)	1.56	(1.33, 1.69)	0.349
13-HODE	33.06	(24.02, 37.28)	49.75	(38.35, 65.79)	31.30	(21.92, 32.34)	0.006
12,13-EpOME	28.94	(12.43, 38.58)	53.77	(35.11, 79.66)	22.17	(20.46, 24.03)	0.009
12,13-DiHOME	1.52	(0.86, 2.92)	2.28	(0.00, 2.85)	2.04	(1.47, 2.45)	0.933
DGLA-derived							
15-HETrE	8.19	(6.13, 11.79)	6.87	(5.31, 8.68)	7.76	(5.52, 8.83)	0.505
AA-derived							
5-HETE	72.21	(33.47, 99.21)	67.10	(37.40, 72.66)	40.15	(34.63, 54.18)	0.334
5,6-EpETrE	517.77	(160.91, 1034.75)	724.79	(263.89, 995.52)	418.28	(311.79, 475.78)	0.633
5,6-DiHETrE	51.88	(24.89, 71.99)	49.56	(30.37, 66.40)	31.63	(22.20, 34.90)	0.281
8-HETE	38.58	(19.05, 59.78)	38.44	(18.07, 49.87)	26.16	(18.07, 35.24)	0.344
8,9-EpETrE	205.04	(119.60, 276.12)	231.52	(200.55, 350.60)	251.94	(172.04, 310.41)	0.500
9-HETE	23.89	(8.20, 44.08)	32.88	(19.93, 42.33)	17.87	(0.00, 34.03)	0.458
11-HETE	95.73	(84.28, 114.13)	86.10	(69.14, 95.06)	82.26	(77.10, 91.00)	0.123
11,12-EpETrE	425.87	(170.58, 778.05)	502.42	(202.98, 644.65)	354.71	(247.69, 471.50)	0.833
11,12-DiHETrE	1.47	(0.94, 2.03)	1.40	(0.85, 1.76)	0.92	(0.45, 1.25)	0.127
12-HETE	126.85	(93.09, 275.37)	102.44	(69.76, 125.08)	69.65	(60.67, 84.48)	0.032
12-oxo-ETE	0.00	(0.00, 5.16)	4.00	(1.07, 6.23)	0.00	(0.00, 1.04)	0.105
14,15-EpETrE	591.40	(313.77, 1389.31)	852.06	(368.64, 1409.01)	793.71	(542.61, 947.26)	0.935
14,15-DiHETrE	2.22	(1.56, 2.98)	1.82	(1.61, 2.23)	1.36	(1.06, 2.08)	0.251
15-HETE	125.89	(111.89, 186.62)	141.50	(122.77, 147.65)	125.93	(90.92, 145.61)	0.619
15-oxo-ETE	111.14	(68.30, 156.82)	95.58	(74.51, 121.70)	79.57	(70.02, 88.50)	0.242
20-HETE	40.80	(24.34, 59.44)	34.11	(24.20, 52.49)	35.98	(24.21, 36.70)	0.566
TXB2	82.56	(79.19, 84.94)	72.56	(69.42, 93.17)	73.63	(70.81, 84.06)	0.369
EPA-derived							
11,12-EpETE	12.64	(2.94, 24.64)	6.83	(3.70, 13.36)	7.14	(5.72, 12.41)	0.821
15-HEPE	49.38	(41.56, 54.01)	46.88	(31.21, 64.40)	52.55	(39.56, 59.00)	0.925
DHA-derived							
7,8-EpDPE	274.22	(86.38, 436.47)	318.44	(142.60, 456.18)	196.58	(169.13, 260.23)	0.773
10,11-EpDPE	321.99	(132.69, 526.21)	401.11	(202.09, 526.99)	276.50	(221.40, 311.23)	0.675
13,14-EpDPE	423.61	(171.58, 905.71)	545.31	(247.31, 783.64)	395.04	(321.79, 464.88)	0.925
16,17-EpDPE	482.88	(184.88, 1054.71)	603.02	(245.08, 796.34)	392.47	(298.31, 527.46)	0.756
19,20-EpDPE	363.67	(239.28, 695.69)	499.39	(317.56, 753.00)	501.09	(367.97, 620.51)	0.746
17-HDHA	104.28	(72.88, 227.18)	119.49	(89.96, 149.53)	91.70	(40.58, 100.11)	0.400

CHAPTER 4 (Manuscript 2): Temperature and time-dependent effects of delayed blood processing on oxylipin concentrations in human plasma

Temperature and time-dependent effects of delayed blood processing on oxylipin concentrations in human plasma

Published in *Prostaglandins, Leukotrienes Essential Fatty Acids*. 2019; 150; 31-37; 4 Sept 2019.

https://doi.org/10.1016/j.plefa.2019.09.001

Statements of Authorship are provided in Appendix 3.2.

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4.1 Highlights

• Concentrations of certain oxylipins measured in human plasma in potassium-EDTA tubes are reasonably stable when stored on wet ice for up to 2 hours prior to processing

• Substantial time-dependent increases in the 12-lipoxygenase-derived and platelet-derived oxylipins were observed when whole blood stored at room temperature prior to processing

• Future studies are needed to determine the impacts of other pre-analytical variables on plasma oxylipin concentrations

4.2 Abstract

BACKGROUND:

Oxidized derivatives of polyunsaturated fatty acids, collectively known as oxylipins, are labile bioactive mediators with diverse roles in human physiology and pathology. Oxylipins are increasingly being measured in plasma collected in clinical studies to investigate biochemical mechanisms mediating disease and to assess for pharmacodynamic biomarkers in nutrient-based and drug-based intervention studies. Whole blood is generally stored either on ice or at room temperature prior to processing. However, the potential impacts of delays in processing, and of temperature prior to processing, on oxylipin concentrations are incompletely understood.

OBJECTIVE:

To evaluate the effects of delayed processing of blood samples in a timeframe that is typical of a clinical laboratory setting, using typical storage temperatures, on concentrations of representative unesterified oxylipins measured by liquid chromatography-tandem mass spectrometry.

DESIGN:

Whole blood (drawn on three separate occasions from a single person) was collected into 5 mL purple-top potassium-EDTA tubes and stored for 0, 10, 20, 30, 60 or 120 minutes at room temperature or on wet ice, followed by centrifugation at 4°C and 1880g for 10 minutes with plasma collection. Each sample was run in duplicate, therefore there were six tubes and up to six data points at each time point for each oxylipin at each condition (wet ice/room temperature). Seventeen representative oxylipins derived from arachidonic acid (AA), docosahexaenoic acid (DHA) and linoleic acid (LA) were quantified by liquid chromatography tandem mass spectrometry. Longitudinal models were used to estimate differences between temperature groups two hours after blood draw.

RESULTS:

We found that most oxylipins measured in human plasma in traditional potassium-EDTA tubes are reasonably stable when stored on wet ice for up to 2 hours prior to processing, with little evidence of auto-oxidation products in either condition. By contrast, in whole blood stored at room temperature, substantial time-dependent increases in the 12-lipoxygenase-derived (12-HETE, 14-HDHA) and platelet-derived (thromboxane B2) oxylipins were observed.

CONCLUSION:

These findings suggest that certain plasma oxylipins can be measured with reasonable accuracy despite delayed processing for up to two hours following blood collection, as long as blood is stored on ice prior to centrifugation. 12-lipoxygenase- and platelet-derived oxylipins may be particularly sensitive to post-collection artifact with delayed processing at room temperature. Future studies are needed to determine the impacts of duration and temperature of centrifugation on oxylipin concentrations.

Keywords: oxylipins, plasma, stability, blood processing, peroxidation

4.3 Introduction

Oxidized derivatives of 18, 20, and 22 carbon polyunsaturated fatty acids, collectively known as oxylipins, are increasingly recognized as bioactive mediators with diverse roles in human physiology and pathology [1-3]. Oxylipins can be divided into three general functional classes: (1) autacoids are labile signaling molecules that act locally and are rapidly inactivated or degraded; (2) pathway precursors are intermediates in the biosynthetic pathway leading to generation of more labile, bioactive autacoids; and (3) inactivation products are downstream metabolic derivatives that are often more stable than their autacoid precursors [4]. Oxylipins in these three categories are increasingly being measured in plasma collected in clinical studies to investigate biochemical mechanisms mediating disease [5-8], and to assess for pharmacodynamic biomarkers of nutrient-based and drug-based [9-11] interventions.

Ideally, plasma oxylipin measurements provide an accurate representation of *in vivo* intravascular levels. However, the labile nature of oxylipins and related compounds presents unique challenges for accurate identification, quantitation, and interpretation of concentrations in human plasma and other tissues [12-15]. There is potential to generate artifact during each step of collection, transport, pre-processing delay, processing, storage, and/or analysis due to: (1) degradation, inactivation, or metabolic conversion, and (2) enzymatic and/or non-enzymatic biosynthesis from precursor fatty acids present in the specimen. The potential for post-collection oxidation may be especially pronounced in polyunsaturated fatty acids containing multiple 1,4-*cis,cis*-pentadiene structures, such as arachidonic acid (AA) and docosahexaenoic acid (DHA)(**Fig. 1**).

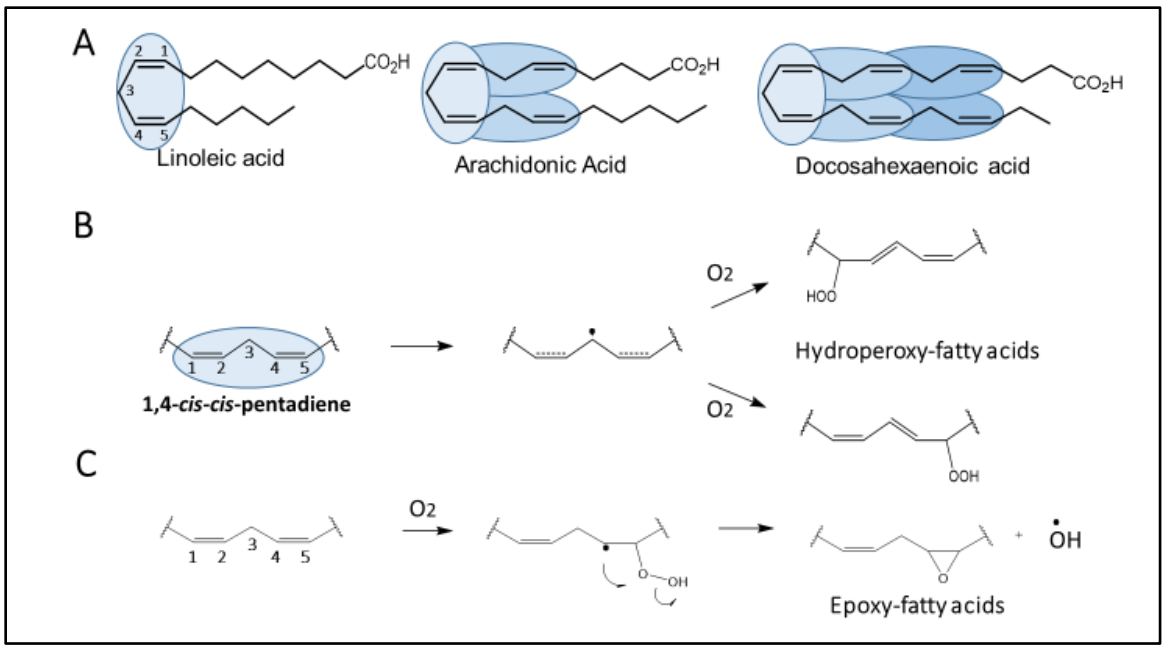


Fig 1. Oxidation of the 1,4-cis-cis-pentadiene structures in polyunsaturated fatty acids

(A) Linoleic, arachidonic and docosahexaenoic acid contain one, three and five 1,4-cis-cis-pentadiene structures, respectively. (B) 1,4-cis-cis-pentadiene structures are susceptible to enzymatic and free-radical mediated peroxidation, forming hydroperoxy-fatty acids, and (C) epoxy-fatty acids. Hydroperoxy-fatty acids are intermediates in the synthesis of numerous oxylipins.

Post-collection artifact could likely be minimized by centrifuging immediately after the collection of whole blood, followed by immediate pipetting, and storing at -80˚C until analysis. Ideally, the storage duration will not be excessive and will be uniform among all samples. However, this ideal approach can be challenging to achieve in a clinical setting, where logistical factors often lead to substantial delays before processing and variations in the duration of storage at -80˚C before analysis. Following collection, whole blood can be cooled or maintained at room temperature prior to processing, sometimes for periods of several hours. Furthermore, details regarding the time interval between sample collection and processing are rarely reported in manuscripts or discussed as a limitation in papers.

The goal of this pilot project is to evaluate the effects of delayed processing of blood samples in a timeframe that is typical of a clinical setting, using a range of storage temperatures, on concentrations of representative oxylipins, pathway precursors, and inactivation products measured by liquid chromatography tandem mass spectrometry.

4.4 Materials and methods

The study compares plasma levels of oxylipins in samples that were processed immediately after collection to those that were processed after up to 120 minutes of storage either on wet ice or at room temperature prior to centrifugation. The protocol was approved by NIH (#03-AG-N322) and written informed consent was obtained for all blood collection procedures. Following an overnight fast, venipuncture of the median cubital vein in the antecubital fossa was performed with an 18-gauge butterfly needle, with venous whole blood collected into 5 mL 'purple top' potassium-EDTA tubes. Whole blood was stored for 0, 10, 20, 30, 60 or 120 minutes at room temperature or on wet ice (**Fig. 2**), followed by centrifugation at 4°C and 1880g for 10 minutes.

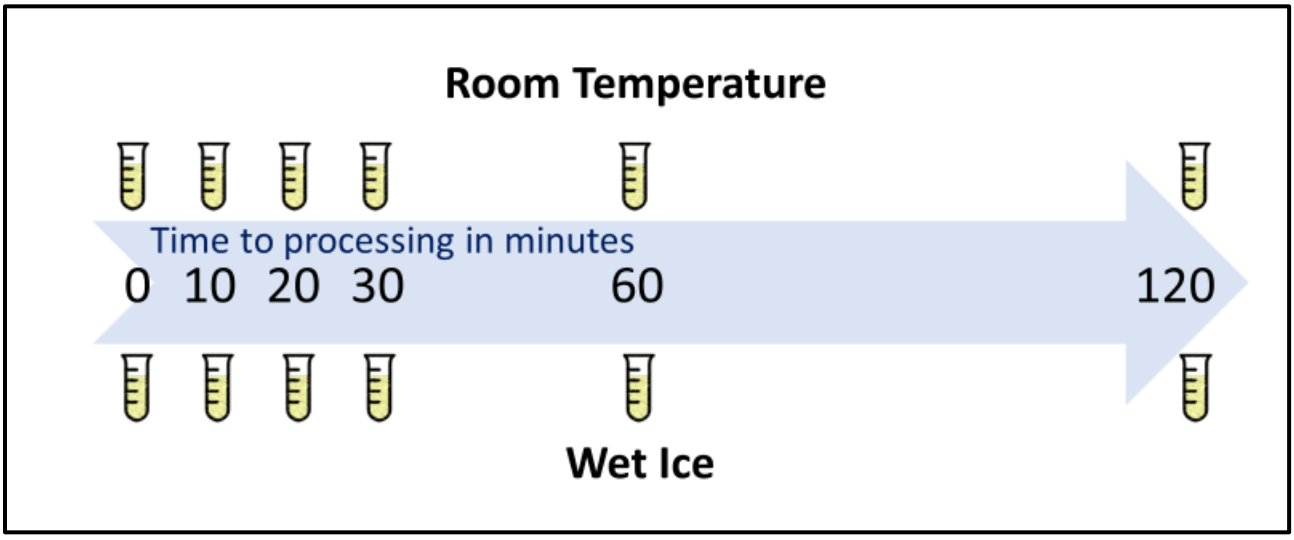


Figure 2: Sampling timeline used to assess temperature and time-dependent effects of delayed processing on oxylipin concentrations in human plasma.

4.4.1 Sample collection

Following centrifugation, plasma was carefully pipetted into an empty tube with care taken not to disturb the buffy coat. The entire experiment was repeated in three separate rounds on one subject, on three different dates. On each of the three experimental days, each sample was run in

duplicate, therefore there were six tubes and up to six data points at each time point for each oxylipin at each condition (wet ice/room temperature).

4.4.2 Quantitation of oxylipins in plasma

To quantify concentrations of lipid mediators in plasma, lipid extracts were purified using solid phase extraction (SPE) and quantified using liquid chromatography-tandem mass spectrometry (LC-MS/MS) as previously described [3]. Briefly, SPE of bioactive lipids from biological matrices was performed using Strata X cartridges (33 u, 200 mg/6 mL, Phenomenex, PA). The cartridges were conditioned with 6 mL of methanol, followed by 6 mL of water before samples were extracted. Samples were washed with 6 mL of 10% methanol. The lipids were eluted with 6 mL of methanol into a glass tube containing 10 µL of 30% glycerol in methanol. The eluate was evaporated to dryness under a stream of nitrogen and reconstituted with 40 µL of methanol, and an aliquot (10 µL) was injected into a UPLC (Shimadzu Scientific Instruments, Columbia, MD) system coupled with a Qtrap 5500 (AB SCIEX, USA) for quantitative analysis. Briefly, separation was performed on a ZorBAX RRHD Eclipse Plus C18 column (100 mm x 4 mm; 1.8 µm) (Agilent Corporation, Palo Alto, CA) consisting of (A) 12 mM ammonium acetate solution and acetic acid (100:0.02 v/v) and (B) 12 mM ammonium acetate and was composed of acetonitrile / water / acetic acid (90:10:0.02, v/v/v). The flow rate was 0.5 mL/min. The column oven temperature was set at 30 °C. The elution gradient conditions were as follows: 25-40% B from 0-2.0 min, 40–46% B from 2 to 8 min, 46–57% B from 8 to 9 min, 57–66% B from 9 to 20 min, 66–76% B from 20 to 22 min, 76–100% B from 22 to 27 min, held at 100% B from 27 to 33 min, 100–25% B from 33.1 to 35 min. The mass spectrometer was operated in electrospray negative ionization mode using scheduled multiple reaction monitoring (sMRM) acquiring MRM

data for each analyte within a retention time window of 90s. The source parameters were set as follows: ion spray voltage, −4500 V; nebulizer gas (GS1), 65 psi; turbo-gas (GS2), 70 psi; and the turbo ion spray source temperature (TEM), 500 °C. The analytes were quantified using MRM, as previously described [3].

4.4.3 Data analysis

To explore and graphically depict the effects of temperature and time on oxylipin concentrations, we plotted individual data points separately by temperature group, for oxylipins that were above the limit of quantitation in at least half of the samples. The limit of quantitation for each oxylipin was defined as a signal-to-noise ratio of >5. Oxylipin values that were below the limit of quantitation were imputed by substituting one half of the limit of quantitation value for each respective oxylipin. We hypothesized that TXB2, as a platelet derived mediator generated from TXA2, could be particularly sensitive blood clotting with delayed processing. TXB2 values were above the LOQ in only 33% of samples, and had peaks present in only 53% of samples. Despite this, we included TXB2 in these graphs and our analysis in an exploratory manner. To determine the between-group difference at minute 120, we used longitudinal mixed models for repeated measures controlling for the time, time by group interaction, and measurement round.

4.5 Results

Temperature and time-dependent effects of delayed processing on the concentrations of sixteen oxylipins derived from AA, DHA and LA that were above the limit of quantitation in half or more of the study samples, plus TXB2, are shown in **Figs. 3-5**, respectively. Model predicted oxylipin estimates after 120 minutes of exposure to either wet ice or room temperature and between-group comparisons of wet ice to room temperature are shown in **Table 1**.

4.5.1 AA-derived oxylipins

Biosynthetic pathways for production of six AA-derived oxylipins (PGE2, 8-isoPGF2α, 5-HETE, 12-HETE, 15-HETE, TXB2) are shown in **Figure 3A.** Prostaglandin E2 and 8-isoPGF2α were below the limit of quantitation in all samples. Since 8-isoPGF2α is generally considered to be an auto-oxidation product in humans [20], the finding that PGE2 and 8-isoPGF2α remained below the limit of quantitation and did not differ between ice and room temperature, suggests that auto-oxidation likely did not occur to a substantial extent in the conditions of this study. Concentrations of 12-HETE increased substantially over time in samples that had been stored at room temperature prior to processing (**Fig. 3A**), however these effects were not evident with delayed processing on wet ice. Since 12-HETE can be synthesized by platelet-derived 12-lipoxygenase, this increase suggests that alterations in platelet activation and the coagulation cascade occurred with delayed processing at room temperature [21-24]. Concentrations of TXB2, a more stable inactivation product of labile, bioactive platelet precursor TXA2 [4] (**Fig. 3A**), also increased over time in samples that had been stored at room temperature prior to processing, however since TXB2 was above the limit of quantitation in only 33% of samples, this finding should be interpreted with some caution. The concentrations of the other two measured AA-derived oxylipins biosynthesized by 5-lipoxygenase (5-HETE) and 15-

lipoxygenase (15-HETE), did not change substantially over time and did not differ between the ice and room temperature groups.

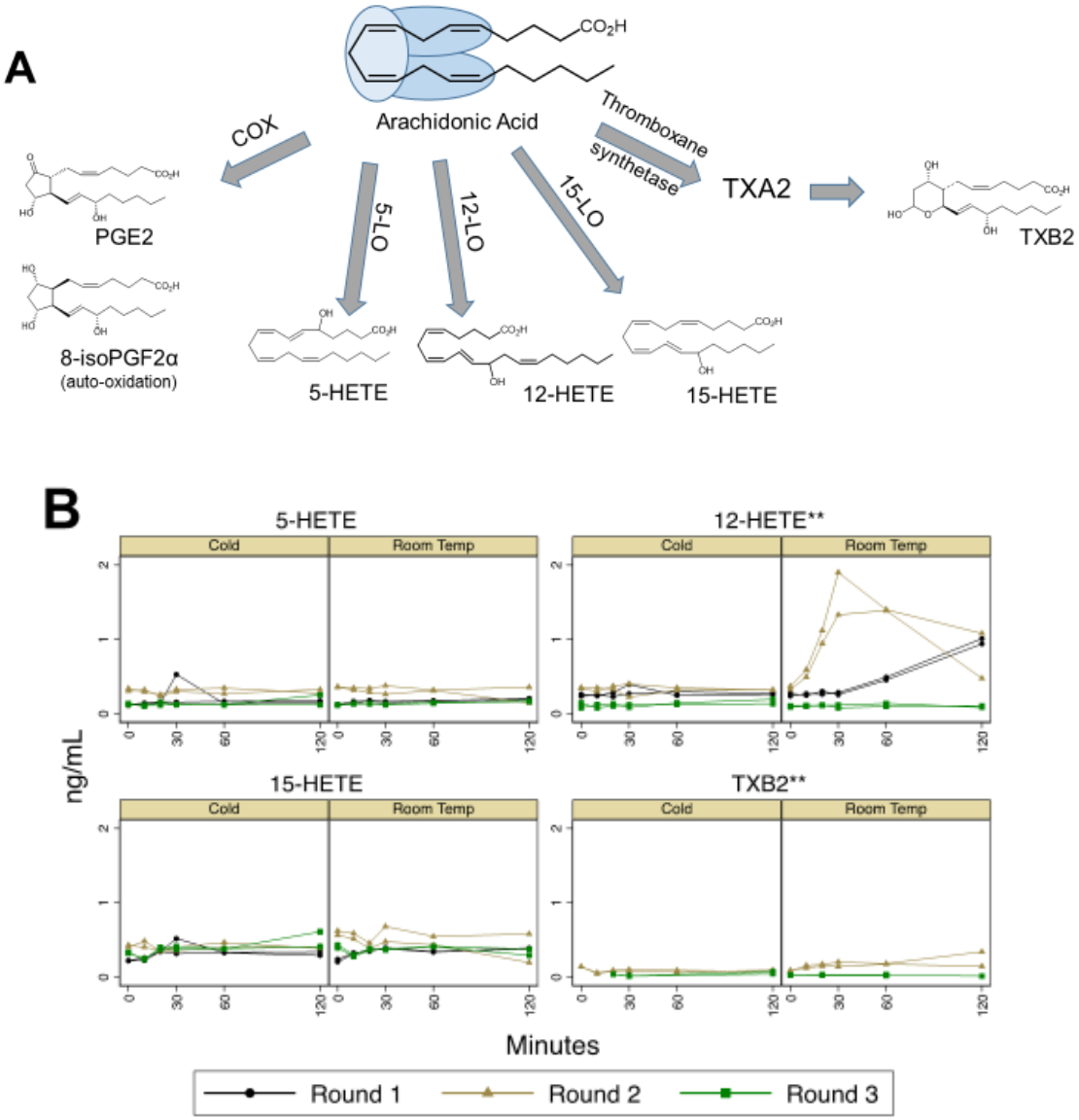


Fig 3: Arachidonic acid-derived oxylipins: Molecular pathways for synthesis and change from baseline by time and temperature. *Panel A shows the molecular pathways involved in enzymatic and non-enzymatic conversion of arachidonic acid (AA) to AA-derived oxylipins. Panel B shows individual data points for plasma concentrations of each of the AA-derived oxylipin measured at baseline and following delayed processing of up to 120 minutes on wet ice*

*or at room temperature. One half of the LOQ was imputed for values below LOQ. Differences between cold and room temperature at minute 120 minute are based on longitudinal mixed models with repeated observations controlling for the data collection round. P-values: *<0.05, **<0.01, ***<0.001.*

4.5.2 DHA-derived oxylipins

Biosynthetic pathways for production of five DHA-derived oxylipins (4-hydroxy-docohexaenoic acid (4-HDHA), 10-HDHA, 14-HDHA, 17-HDHA and 19,20-epoxy-docosapentaenoic acid (19,20-EpDPA)) are shown in **Figure 4A**. Three of these oxylipins (4-HDHA, 10-HDHA, 19,20-EpDPA) appeared stable over time when stored on wet ice or at room temperature. 10-HDHA is generally considered to be an auto-oxidation product in humans because unlike rodents, humans lack 8-lipoxygenase [25]. Thus, the observation that plasma 10-HDHA did not change over time or differ between ice and room temperature, confirms a lack of substantial auto-oxidation in the conditions of this study. 14-HDHA, a 12-lipoxygenase generated product of DHA, increased substantially in the room temperature samples compared to wet ice, in a similar manner as observed for 12-HETE, suggesting that 12-lipoxygenase activation during clotting is not specific to one substrate. 17-HDHA values varied widely and appeared to be independent of time and temperature.

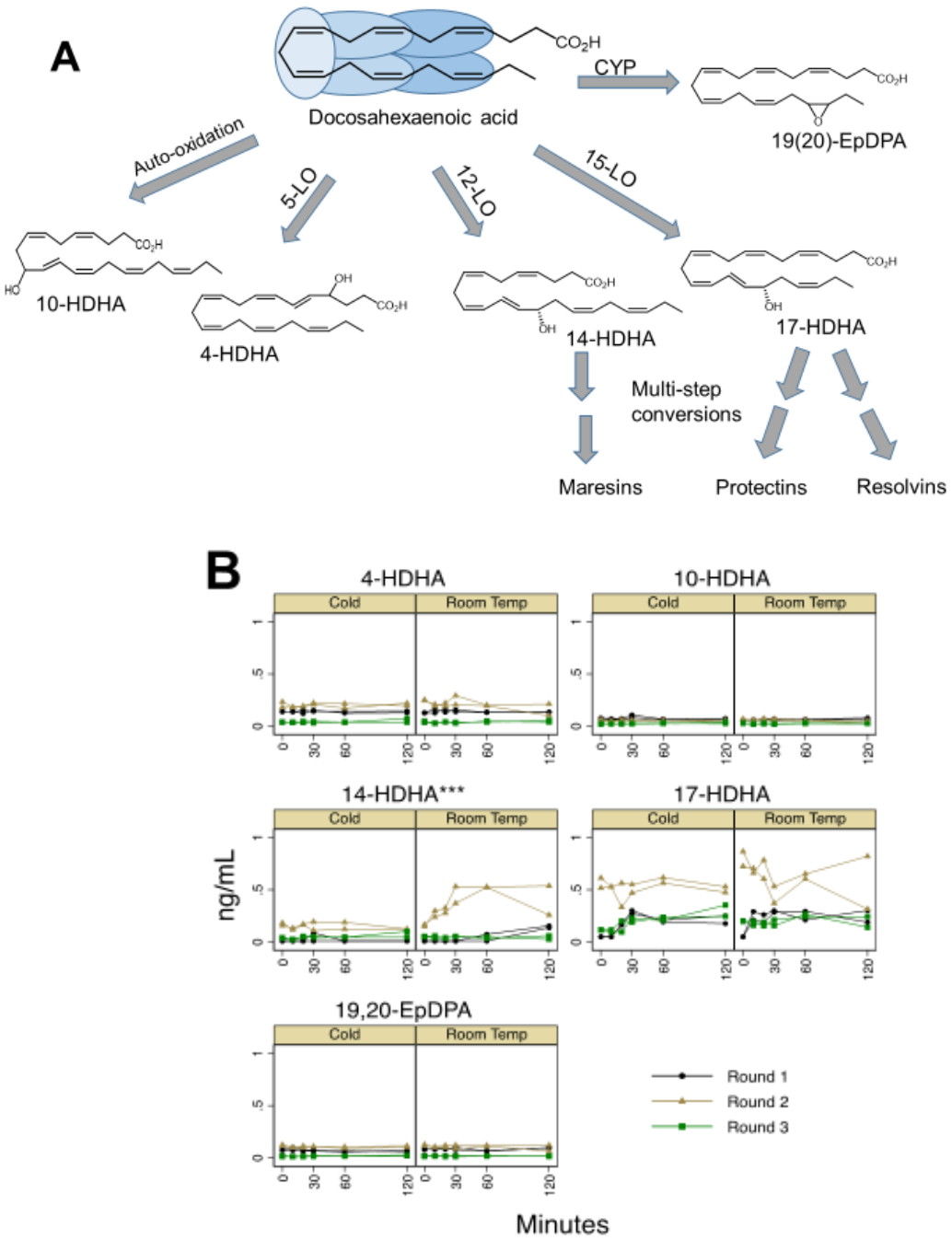
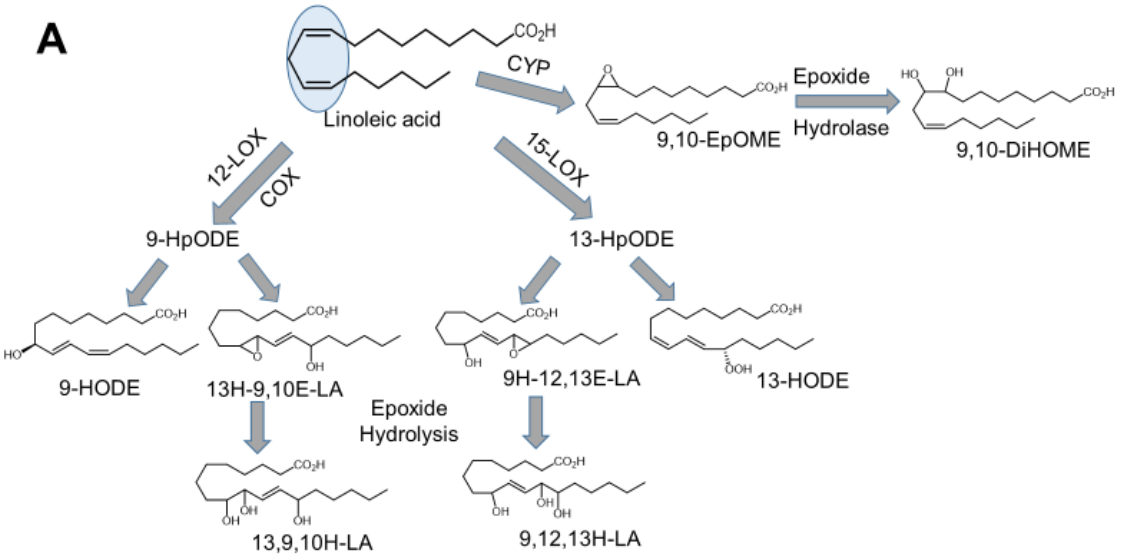


Fig 4: Docosahexaenoic acid-derived oxylipins: Molecular pathways for synthesis and change from baseline by time and temperature. *Panel A shows the molecular pathways involved in enzymatic and non-enzymatic conversion of docosahexaenoic acid (DHA) to DHA-derived oxylipins. Panel B shows individual data points for plasma concentrations of each of the DHA-derived oxylipin measured at baseline and following delayed processing of up to 120 minutes on*

*wet ice or at room temperature. One half of the LOQ was imputed for values below LOQ. Differences between cold and room temperature at minute 120 minute are based on longitudinal mixed models with repeated observations controlling for the data collection round. P-values: *<0.05, **<0.01, ***<0.001.*



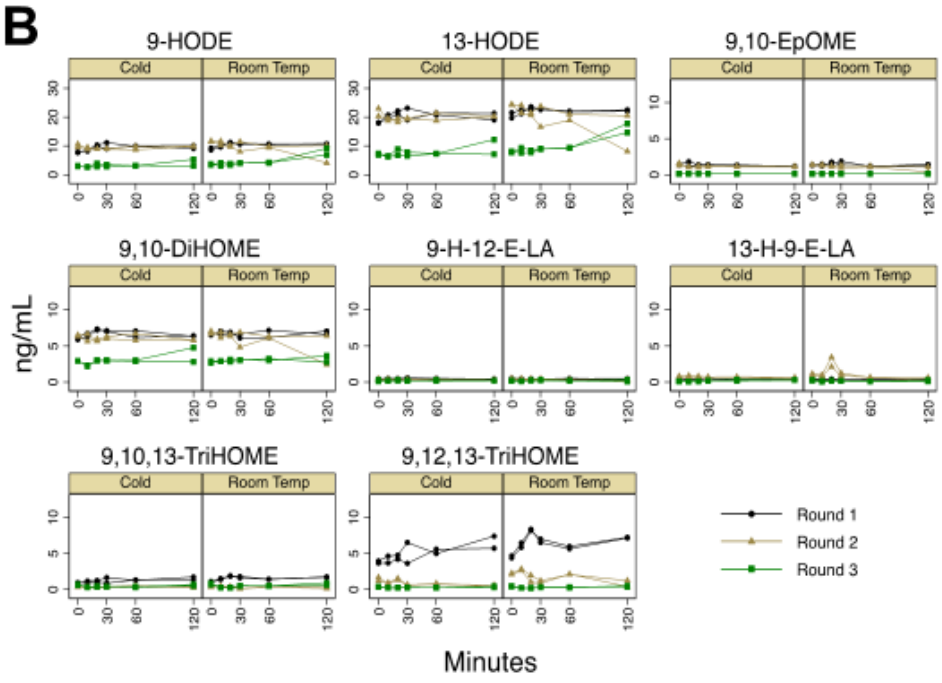


Fig 5: Linoleic acid-derived oxylipins: Molecular pathways for synthesis and change from baseline by time and temperature. *Panel A shows the molecular pathways involved in enzymatic and non-enzymatic conversion of linoleic acid (LA) to LA-derived oxylipins. Panel B shows individual data points for plasma concentrations of each of the DHA-derived oxylipin measured at baseline and following delayed processing of up to 120 minutes on wet ice or at room temperature. One half of the LOQ was imputed for values below LOQ. Differences between cold and room temperature at minute 120 minute are based on longitudinal mixed models with repeated observations controlling for the data collection round. P-values: *<0.05, **<0.01, ***<0.001.*

4.5.3 LA-derived oxylipins

Proposed biosynthetic pathways for production of eight LA-derived oxylipins are shown in **Figure 5A.** Like 12-HETE (derived from AA) and 14-HDHA (derived form DHA), 9-HODE is reported to be a 12-lipoxygenase derivative of LA [26]. However, unlike 12-HETE and 14-HDHA, plasma 9-HODE concentrations were not significantly higher when whole blood was stored at room temperature compared to ice, for up to 2 hours. Potential explanations for this discrepancy include that 9-HODE can be synthesized via several other enzymatic and non-enzymatic sources including cyclooxygenases [27,28], 12-lipoxygenase (non-platelet), and free-radical mediated oxidation [29]. Moreover, AA and DHA contain three and five 1,4-*cis,cis*-pentadiene structures respectively (**Figure 1**), and therefore are considered to be more susceptible to oxidation compared to LA which contains a single 1,4-*cis,cis*-pentadiene structure.

Table 1. Model-predicted oxylipin estimates after 120 minutes of exposure to room temperature or wet ice (ng/ml)

	Wet ice	Room temperature	Difference (Room-Wet Ice)*	
	Estimate (95% CI)	Estimate (95% CI)	Estimate (95% CI)	p-value
AA				
5-HETE	0.22 (0.18, 0.26)	0.21 (0.16, 0.25)	-0.01 (-0.07, 0.05)	0.69
12-HETE	0.25 (0.07, 0.43)	0.62 (0.43, 0.80)	0.37 (0.11, 0.62)	0.005
15-HETE	0.41 (0.35, 0.48)	0.36 (0.30, 0.43)	-0.05 (-0.14, 0.04)	0.246
TXB2**	0.05 (0.03, 0.08)	0.10 (0.07, 0.12)	0.05 (0.01, 0.08)	0.009
DHA				
4-HDHA	0.13 (0.12, 0.15)	0.11 (0.10, 0.13)	-0.02 (-0.04, 0.00)	0.069
10-HDHA	0.05 (0.04, 0.06)	0.05 (0.04, 0.05)	-0.00 (-0.01, 0.01)	0.466
14-HDHA**	0.07 (0.02, 0.12)	0.20 (0.14, 0.25)	0.13 (0.06, 0.20)	0.001
17-HDHA**	0.34 (0.27, 0.42)	0.33 (0.26, 0.40)	-0.01 (-0.12, 0.09)	0.787
19,20-EpDPA	0.07 (0.06, 0.07)	0.07 (0.06, 0.08)	0.00 (-0.01, 0.01)	0.713
LA				
9-HODE	7.86 (6.87, 8.86)	8.58 (7.59, 9.57)	0.72 (-0.68, 2.12)	0.316
13-HODE	16.66 (14.70, 18.62)	17.58 (15.63, 19.54)	0.93 (-1.85, 3.70)	0.513
9,10-EpOME**	0.79 (0.67, 0.91)	0.72 (0.61, 0.84)	-0.07 (-0.23, 0.10)	0.428
9,10-DiHOME	5.33 (4.85, 5.82)	4.83 (4.34, 5.31)	-0.51 (-1.19, 0.18)	0.149
9-H-12-E-LA	0.30 (0.25, 0.35)	0.28 (0.23, 0.33)	-0.02 (-0.09, 0.04)	0.479
13-H-9-E-LA**	0.39 (0.14, 0.63)	0.32 (0.08, 0.57)	-0.07 (-0.41, 0.28)	0.714
9,10,13-TriHOME	0.83 (0.67, 0.99)	0.92 (0.76, 1.08)	0.09 (-0.14, 0.31)	0.453
9,12,13-TriHOME	2.49 (1.86, 3.12)	2.79 (2.16, 3.41)	0.30 (-0.59, 1.18)	0.513

* Based on longitudinal mixed models for repeated measures controlling for the data collection round.

** Some observations were below the limit of quantitation (LOQ) and were imputed with ½ of the LOQ.

4.6 Discussion

Oxylipins hold promise as biomarkers for predicting diseases in observational settings and as pharmacodynamic markers for targeting diet or drug interventions [9,10]. Since plasma oxylipins are derived from more abundant polyunsaturated fatty acids containing 1,4-*cis,cis*-pentadiene structures that are highly susceptible to oxidation, any pre-analytical variable that alters synthesis or degradation could potentially create post-collection artifact in measured metabolites [12, 16-18, 30], including oxylipins.

In the present study we sought to determine the effect of delayed processing of whole blood samples in a timeframe that is typical of a clinical setting, using typical storage temperatures on concentrations of unesterified oxylipins including pathway precursors, and inactivation products measured by LC-MS/MS. Findings suggest that the concentrations of many oxylipins measured in human plasma using traditional potassium-EDTA tubes are reasonably stable when stored for up to 2 hours prior to processing, particularly when stored on wet ice, with little evidence of auto-oxidation products in either condition. The substantial increases observed for 12-lipoxygenase-derived (12-HETE, 14-HDHA) and platelet-derived (TXB2) oxylipins with delayed processing at room temperature indicate that these compounds may be particularly sensitive to post-collection artifact. These findings suggest that certain plasma oxylipins can be measured with reasonable accuracy despite delayed processing for up to two hours following blood collection, as long as blood is stored on ice prior to centrifugation. The pronounced post-collection artifact in 12-lipoxygenase and platelet-derived oxylipins with delayed processing at room temperature could potentially be due to activation of clotting cascade [21,23,24].

Factors that limit the generalizability of this study include the small sample size, non-independent samples, and the substantial number of samples below the limits of quantitation. Experiments used standard clinical "purple top" potassium EDTA tubes for blood collection and therefore findings are not necessarily generalizable to blood collected using other tubes (i.e. plasma citrate, heparin) or for serum or other blood fractions. Use of heparin tubes has previously been reported to markedly increase oxylipin concentrations [31]. Since serum is generated via activation of the clotting cascade, one could speculate that use of serum separator tubes might alter the production of oxylipins derived from platelets (e.g. 12-HETE, 14-HDHA, thromboxanes) in a similar manner as observed with delayed processing at room temperature in the present study, however this requires experimental confirmation. The finding that oxylipins that were below the limit of quantitation with immediate processing were not increased with delayed processing suggests that the oxylipins profiled in the present study were not synthesized *ex vivo*. Importantly, some oxylipins have potent bioactions at very low concentrations, and it is possible that certain labile oxylipins were already degraded even with immediate processing. Future studies should examine whether more rapid centrifugation (e.g. 1-2 min) impacts results.

Summary and conclusions

Plasma samples derived from whole blood that was kept on ice for up to 2 hours prior to centrifugation appear suitable for measuring many oxylipins with the caveats noted above. 12-lipoxygenase and platelet-derived oxylipins are sensitive to post-collection artifact with delayed processing at room temperature. Results may have implications for design, implementation, and interpretation of clinical studies measuring oxylipins in plasma. Future studies are needed to determine the impacts of other pre-analytical variables including venipuncture location, needle

gauge, blood collection tube type, duration and temperature of centrifugation, and post-processing storage conditions.

Acknowledgements

Supported by the intramural programs of the National Institute on Aging and National Institute on Alcohol Abuse and Alcoholism National Institutes of Health, the University of Adelaide, the South Australian Health and Medical Research Institute, and the College of Agriculture and Environmental Sciences, University of California at Davis.

4.7 References (Chapter 4)

1. Caligiuri SPB, Parikh M, Stamenkovic A, Pierce GN, Aukema HM. Dietary modulation of oxylipins in cardiovascular disease and aging. Am J Physiol Heart Circ Physiol. 2017;313(5):H903-H18.

2. Pazderka CW, Oliver B, Murray M, Rawling T. Omega-3 Polyunsaturated Fatty Acid Derived Lipid Mediators and Their Application in Drug Discovery. Curr Med Chem. 2018.

3. Yuan ZX, Majchrzak-Hong S, Keyes GS, Iadarola MJ, Mannes AJ, Ramsden CE. Lipidomic profiling of targeted oxylipins with ultra-performance liquid chromatography-tandem mass spectrometry. Anal Bioanal Chem. 2018;410(23):6009-29.

4. Catella F, Healy D, Lawson JA, FitzGerald GA. 11-Dehydrothromboxane B2: a quantitative index of thromboxane A2 formation in the human circulation. Proc Natl Acad Sci U S A. 1986;83(16):5861-5.

5. Lundström SL, Yang J, Källberg HJ, Thunberg S, Gafvelin G, Haeggström JZ, et al. Allergic asthmatics show divergent lipid mediator profiles from healthy controls both at baseline and following birch pollen provocation. PLoS One. 2012;7(3):e33780.

6. Ramsden CE, Domenichiello AF, Yuan ZX, Sapio MR, Keyes GS, Mishra SK, et al. A systems approach for discovering linoleic acid derivatives that potentially mediate pain and itch. Sci Signal. 2017;10(493).

7. Sorokin AV, Domenichiello AF, Dey AK, Yuan ZX, Goyal A, Rose SM, et al. Bioactive Lipid Mediator Profiles in Human Psoriasis Skin and Blood. J Invest Dermatol. 2018;138(7):1518-28.

8. Grapov D, Adams SH, Pedersen TL, Garvey WT, Newman JW. Type 2 diabetes associated changes in the plasma non-esterified fatty acids, oxylipins and endocannabinoids. PLoS One. 2012;7(11):e48852.

9. Lundström SL, Yang J, Brannan JD, Haeggström JZ, Hammock BD, Nair P, et al. Lipid mediator serum profiles in asthmatics significantly shift following dietary supplementation with omega-3 fatty acids. Mol Nutr Food Res. 2013;57(8):1378-89.

10. Ramsden CE, Faurot KR, Zamora D, Suchindran CM, Macintosh BA, Gaylord S, et al. Targeted alteration of dietary n-3 and n-6 fatty acids for the treatment of chronic headaches: a randomized trial. Pain. 2013;154(11):2441-51.

11. Ramsden CE, Ringel A, Feldstein AE, Taha AY, MacIntosh BA, Hibbeln JR, et al. Lowering dietary linoleic acid reduces bioactive oxidized linoleic acid metabolites in humans. Prostaglandins Leukot Essent Fatty Acids. 2012;87(4-5):135-41.

12. Golovko MY, Murphy EJ. An improved LC-MS/MS procedure for brain prostanoid analysis using brain fixation with head-focused microwave irradiation and liquid-liquid extraction. J Lipid Res. 2008;49(4):893-902.

13. Hennebelle M, Metherel AH, Kitson AP, Otoki Y, Yang J, Lee KSS, et al. Brain oxylipin concentrations following hypercapnia/ischemia: effects of brain dissection and dissection time. J Lipid Res. 2019;60(3):671-82.

14. Liakh I, Pakiet A, Sledzinski T, Mika A. Modern Methods of Sample Preparation for the Analysis of Oxylipins in Biological Samples. Molecules. 2019;24(8).

15. Gladine C, Ostermann AI, Newman JW, Schebb NH. MS-based targeted metabolomics of eicosanoids and other oxylipins: Analytical and inter-individual variabilities. Free Radic Biol Med. 2019.

16. Kamlage B, Neuber S, Bethan B, González Maldonado S, Wagner-Golbs A, Peter E, et al. Impact of Prolonged Blood Incubation and Extended Serum Storage at Room Temperature on the Human Serum Metabolome. Metabolites. 2018;8(1).

17. Metherel AH, Stark KD. The stability of blood fatty acids during storage and potential mechanisms of degradation: A review. Prostaglandins Leukot Essent Fatty Acids. 2016;104:33-43.

18. Thiara AS, Cundliffe E. Cloning and characterization of a DNA gyrase B gene from Streptomyces sphaeroides that confers resistance to novobiocin. EMBO J. 1988;7(7):2255-9.

19. Wagner-Golbs A, Neuber S, Kamlage B, Christiansen N, Bethan B, Rennefahrt U, et al. Effects of Long-Term Storage at -80 °C on the Human Plasma Metabolome. Metabolites. 2019;9(5).

20. van 't Erve TJ, Lih FB, Kadiiska MB, Deterding LJ, Eling TE, Mason RP. Reinterpreting the best biomarker of oxidative stress: The 8-iso-PGF(2alpha)/PGF(2alpha) ratio distinguishes chemical from enzymatic lipid peroxidation. Free Radic Biol Med. 2015;83:245-51.20

21. Herbertsson H, Kühme T, Hammarström S. The 650-kDa 12(S)-hydroxyeicosatetraenoic acid binding complex: occurrence in human platelets, identification of hsp90 as a constituent, and binding properties of its 50-kDa subunit. Arch Biochem Biophys. 1999;367(1):33-8.

22. Marcus AJ, Broekman MJ, Safier LB, Ullman HL, Islam N, Serhan CN, et al. Production of arachidonic acid lipoxygenase products during platelet-neutrophil interactions. Clin Physiol Biochem. 1984;2(2-3):78-83.

23. Porro B, Songia P, Squellerio I, Tremoli E, Cavalca V. Analysis, physiological and clinical significance of 12-HETE: a neglected platelet-derived 12-lipoxygenase product. J Chromatogr B Analyt Technol Biomed Life Sci. 2014;964:26-40.

24. Squellerio I, Porro B, Songia P, Veglia F, Caruso D, Tremoli E, et al. Liquid chromatography-tandem mass spectrometry for simultaneous measurement of thromboxane B2 and 12(S)-hydroxyeicosatetraenoic acid in serum. J Pharm Biomed Anal. 2014;96:256-62.

25. Schneider C, Strayhorn WD, Brantley DM, Nanney LB, Yull FE, Brash AR. Upregulation of 8-lipoxygenase in the dermatitis of IkappaB-alpha-deficient mice. J Invest Dermatol. 2004;122(3):691-8.

26. Bürger F, Krieg P, Marks F, Fürstenberger G. Positional- and stereo-selectivity of fatty acid oxygenation catalysed by mouse (12S)-lipoxygenase isoenzymes. Biochem J. 2000;348 Pt 2:329-35.

27. Kaduce TL, Figard PH, Leifur R, Spector AA. Formation of 9-hydroxyoctadecadienoic acid from linoleic acid in endothelial cells. J Biol Chem. 1989;264(12):6823-30.

28. Godessart N, Camacho M, López-Belmonte J, Anton R, García M, de Moragas JM, et al. Prostaglandin H-synthase-2 is the main enzyme involved in the biosynthesis of octadecanoids from linoleic acid in human dermal fibroblasts stimulated with interleukin-1beta. J Invest Dermatol. 1996;107(5):726-32.

29. Inouye M, Mio T, Sumino K. Formation of 9-hydroxy linoleic acid as a product of phospholipid peroxidation in diabetic erythrocyte membranes. Biochim Biophys Acta. 1999;1438(2):204-12.

30. Wagner-Golbs A, Neuber S, Kamlage B, Christiansen N, Bethan B, Rennefahrt U, et al. Effects of Long-Term Storage at -80 °C on the Human Plasma Metabolome. Metabolites. 2019;9(5).

31. Goodfriend TL, Pedersen TL, Grekin RJ, Hammock BD, Ball DL, Vollmer A. Heparin, lipoproteins, and oxygenated fatty acids in blood: a cautionary note. Prostaglandins Leukot Essent Fatty Acids. 2007;77(5-6):363-6.

4.8 Supplementary Appendices (additional exploratory data analysis graphs and graphs that were not submitted with manuscript)

Exploratory data analysis

In an additional exploratory analysis, we explored and graphically depicted the effects of temperature and time on oxylipin concentrations by plotting the *median percent change from baseline* over time for each temperature group. The limit of quantitation for each oxylipin was defined as a signal-to-noise ratio of >5. Oxylipin values that were below the limit of quantitation were imputed by substituting one half of the limit of quantitation value for each respective oxylipin. To determine the between-group difference at minute 120, we used longitudinal mixed models with repeated observations per blood samples, time by group interaction terms, and adjustment for measurement round. The results of this analyses are shown in **Table S1** and graphically depicted in **Figures S1-S3** for AA-derived, DHA-derived, and LA-derived oxylipins, respectively. **Table S2** shows the percentage of samples that were below the limit of quantitation.

Table S1. Crude and model-predicted oxylipin estimates after 120 minutes of exposure to room and cold temperatures

	Median		Adjusted Difference of Room Temperature Minus Cold Group*				
	Cold (n=6)	Room (n=6)	Coef	z	p-value	95% CI	
5-HETE	0.21	0.18	-0.01	-0.40	0.69	-0.07	0.05
12-HETE	0.27	0.71	0.37	2.82	0.005	0.11	0.62
15-HETE	0.40	0.38	-0.05	-1.16	0.25	-0.14	0.04
TXB2	0.06	0.03	0.05	2.60	0.009	0.01	0.08
10-HDHA	0.05	0.04	0.00	-0.73	0.47	-0.01	0.01
14-HDHA	0.07	0.15	0.13	3.47	0.001	0.06	0.20
17-HDHA	0.30	0.27	-0.01	-0.27	0.79	-0.12	0.09
19,20-EpDPA	0.07	0.08	0.002	0.37	0.71	-0.01	0.01
9-HODE	9.42	9.60	0.72	1.00	0.32	-0.68	2.12
13-HODE	19.6	19.1	0.92	0.65	0.51	-1.85	3.70
9,10-EpOME	1.11	0.73	-0.07	-0.79	0.43	-0.23	0.10
9,10-DiHOME	5.8	5.0	-0.51	-1.44	0.15	-1.19	0.18
9-H-12-E-LA	0.28	0.32	-0.02	-0.71	0.48	-0.09	0.04
13-H-9-E-LA	0.35	0.30	-0.07	-0.37	0.71	-0.41	0.28
9,10,13-TriHOME	0.60	0.77	0.09	0.75	0.45	-0.14	0.31
9,12,13-TriHOME	0.52	0.86	0.30	0.65	0.51	-0.59	1.18

* Based on longitudinal mixed models for repeated measures controlling for th
data collection round.

This exploratory (change from baseline) analysis produced comparable results as the main longitudinal analyses that did not convert to change from baseline, with the 12-lipoxygenase derivatives 12-HETE and 14-HDHA significantly increased over time in the room temperature group compared to the ice group. The exception was for TXB2, which in these change from baseline models produced paradoxical results, likely due to the difficulty of calculating change from baseline for variables with missing data (TXB2 had values above the limit of quantitation

in 33% of samples). These exploratory results contributed to our interpretation that TXB2 findings should be interpreted cautiously.

Table S2. Percentage of samples below the limit of quantitation

Mediator	Percent	
	Cold	Room
5-HETE	0	0
12-HETE	0	0
15-HETE	0	0
8-IsoPGF2a	100	100
PGE2	100	100
PGF2a	100	100
TXB2	47	47
4-HDHA	0	0
10-HDHA	0	0
14-HDHA	31	25
17-HDHA	11	6
19,20-EpDPA	0	0
9-HODE	0	0
13-HODE	0	0
9,10-EpOME	33	33
9,10-DiHOME	0	0
9-H-12-E-LA	0	0
13-H-9-E-LA	0	3
9,10,13-TriHOME	0	0
9,12,13-TriHOME	0	0

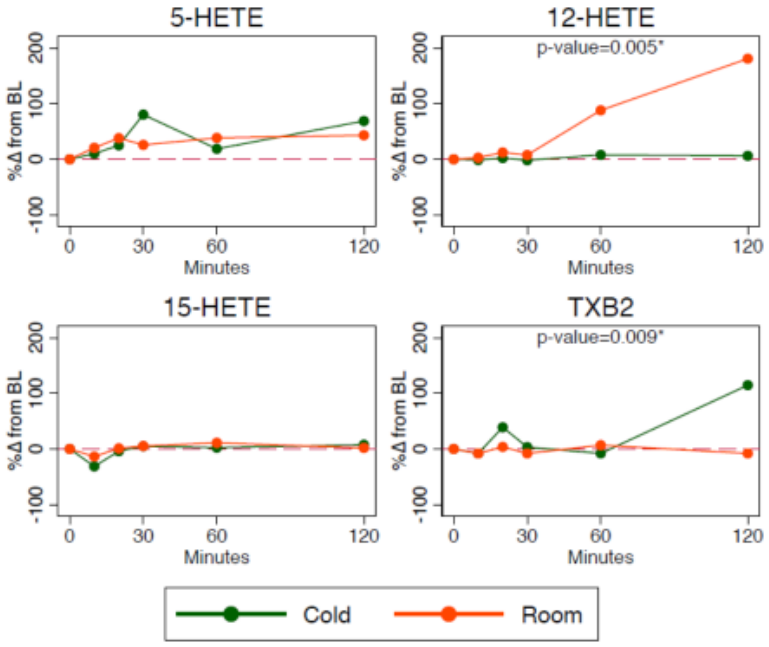


Fig S1: Arachidonic acid-derived oxylipins as percent change from baseline by time and temperature. *Percent changes in median plasma concentrations of AA-derived oxylipins measured at baseline and following delayed processing of up to 120 minutes on wet ice or at room temperature. Percent change from baseline defined as [(median at each time point / median at baseline - 1) * 100%]. One half of the limit of quantitation (LOQ) was imputed for values below LOQ. * P-values are for the between-group difference at minute 120, which were derived from longitudinal mixed models with repeated observations per blood sampling time, time by group interaction terms, and adjustment for measurement round. Only p-values ≤ 0.05 are shown.*

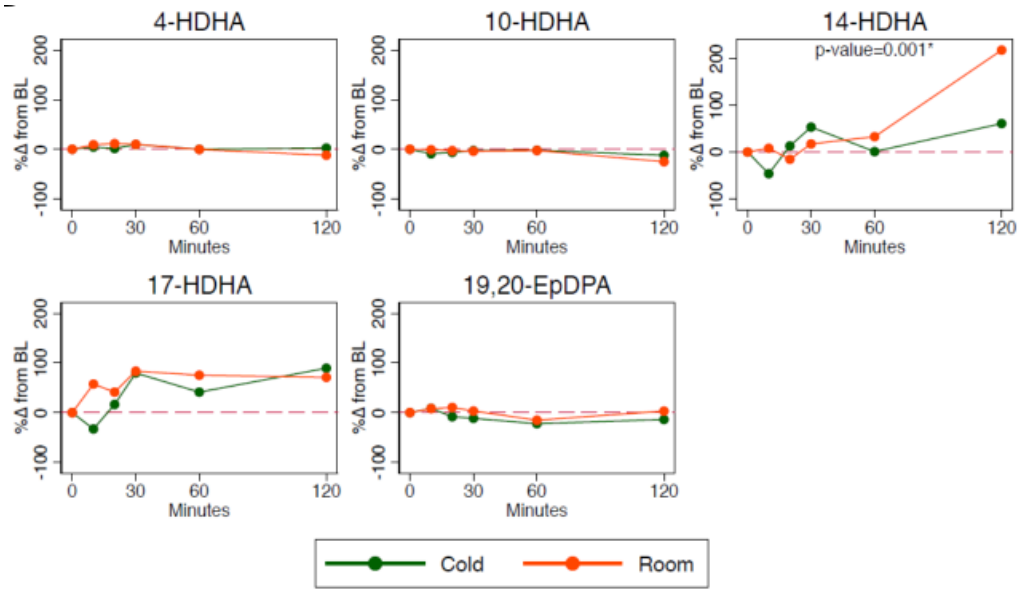


Fig S2: Docosahexaenoic acid-derived oxylipins: percent change from baseline by time and temperature. *Percent changes in median plasma concentrations of each of the DHA-derived oxylipins measured at baseline and following delayed processing of up to 120 minutes on wet ice or at room temperature. Percent change from baseline defined as [(median at each time point / median at baseline - 1) * 100%]. One half of the limit of quantitation (LOQ) was imputed for values below LOQ. * P-values are for the between-group difference at minute 120, which were derived from longitudinal mixed models with repeated observations per blood sampling time, time by group interaction terms, and adjustment for measurement round. Only p-values ≤ 0.05 are shown.*

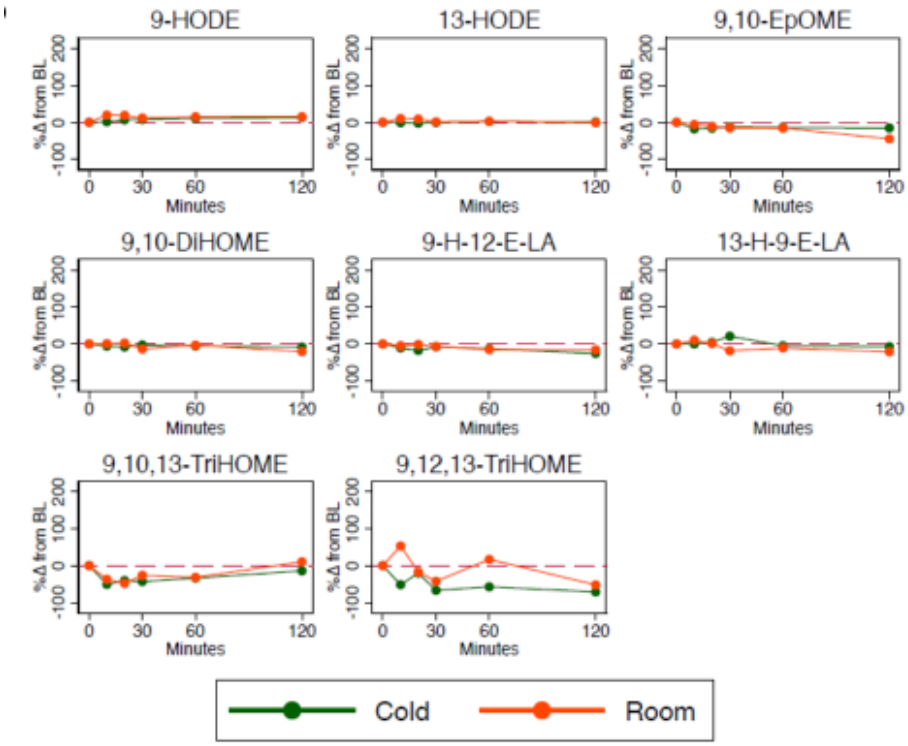


Fig S3: Linoleic acid-derived oxylipins: percent change from baseline by time and temperature. *Percent changes in median plasma concentrations of each oxylipin at baseline following delayed processing of up to 120 minutes on wet ice or at room temperature. Percent change from baseline defined as [(median at each time point / median at baseline - 1) * 100%]. One half of the limit of quantitation (LOQ) was imputed for values below LOQ. * P-values are for the between-group difference at minute 120, which were derived from longitudinal mixed models with repeated observations per blood sampling time, time by group interaction terms, and adjustment for measurement round. Only p-values ≤ 0.05 are shown.*

CHAPTER 5 (Manuscript 3): Plasma oxylipins and unesterified precursor fatty acids are altered by DHA supplementation in pregnancy: Can they help predict risk of preterm birth?

Plasma oxylipins and unesterified precursor fatty acids are altered by DHA supplementation in pregnancy: Can they help predict risk of preterm birth?

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5.1 Highlights

- DHA supplementation increased plasma unesterified DHA and several DHA-derived oxylipins in plasma
- Participants with higher concentrations of 5-lipoxygenase derived oxylipins at 14 weeks, or unesterified AA at 24 weeks had higher risk of spontaneous preterm birth in exploratory analysis
- The hypothesis that 5-lipoxygenase-derived oxylipins and/or unesterified AA could serve as biomarkers for spontaneous preterm birth should be evaluated in larger, adequately powered studies

5.2 Abstract

Oxidized lipids derived from omega-6 (n-6) and omega-3 (n-3) polyunsaturated fatty acids, collectively known as oxylipins, are bioactive signaling molecules that play diverse roles in human health and disease. Supplementation with n-3 docosahexaenoic acid (DHA) during pregnancy has been reported to decrease the risk of preterm birth in singleton pregnancies, which may be due to effects of DHA supplementation on oxylipins or their precursor n-6 and n-3 fatty acids. There is only limited understanding of the levels and trajectory of changes in plasma oxylipins during pregnancy, effects of DHA supplementation on oxylipins and unesterified fatty acids, and whether and how oxylipins and their unesterified precursor fatty acids influence preterm birth. In the present study we used liquid chromatography-tandem mass spectrometry to profile oxylipins and their precursor fatty acids in the unesterified pool using plasma samples collected from a subset of pregnant Australian women who participated in the ORIP (Omega-3 fats to Reduce the Incidence of Prematurity) study. ORIP is a large randomized controlled trial testing whether daily supplementation with n-3 DHA can reduce the incidence of early preterm birth compared to control. Plasma was collected at study entry (≈pregnancy week 14) and again at ≈week 24, in a subgroup of 48 ORIP participants—12 cases with spontaneous preterm (<37 weeks) birth and 36 matched controls with spontaneous term (≥40 weeks) birth. In the combined preterm and term pregnancies, we observed that in the control group without DHA supplementation unesterified AA and AA-derived oxylipins 12-HETE, 15-HETE and TXB2 declined between weeks 14-24 of pregnancy. Compared to control, DHA supplementation increased unesterified DHA, EPA, and AA, DHA-derived 4-HDHA, 10-HDHA and 19,20-EpDPA, and AA-derived 12-HETE at 24 weeks. In exploratory analysis independent of DHA supplementation, participants with concentrations above the median for 5-lipoxygenase

derivatives of AA (5-HETE, Odds Ratio (OR) 8.2; p=0.014) or DHA (4-HDHA, OR 8.0; p=0.015) at 14 weeks, or unesterified AA (OR 5.1; p=0.038) at 24 weeks had higher risk of spontaneous preterm birth. The hypothesis that 5-lipoxygenase-derived oxylipins and unesterified AA could serve as mechanism-based biomarkers predicting spontaneous preterm birth should be evaluated in larger, adequately powered studies.

Keywords: oxylipins, plasma, docosahexaenoic, arachidonic, linoleic, preterm, development

5.3 Introduction

Oxidized lipids derived from polyunsaturated fatty acids (PUFAs), collectively known as oxylipins, comprise a large, heterogeneous family of labile, bioactive signaling molecules that play diverse roles in human health and disease (1). Oxylipins derived from arachidonic acid (AA), docosahexaenoic acid (DHA) and linoleic acid (LA) are reported to play pivotal regulatory roles in physiological processes that are relevant for pregnancy—including immune activation (2, 3), resolution of inflammation (4, 5), endothelial cell activation (6), and coagulation (7)—and have been proposed to play a role in gestational age at birth (8). Most notably, 2-series prostaglandins (e.g. PGE2 and PGF2α) are well-known to play pivotal roles in initiation and progression of labor by promoting cervical maturation and myometrial contraction (9, 10).

The placenta in preterm birth is characterized by an inflammatory phenotype (11), including alterations in prostanoid synthesis and catabolism (12), and women who delivered preterm had higher AA in maternal red blood cells and trophoblast tissue than women who delivered at term (13). Human blood, placenta and fetal tissues are enriched in n-3 and n-6 fatty acids, and selected oxylipins derived from AA, DHA, and LA have been measured in cord blood (8, 14). Moreover, gene variants in fatty acid desaturase (FADS) enzymes involved in AA and DHA synthesis are associated with gestation duration among women with spontaneous labor (15).

Thus, it is possible that in addition to prostanoids, other bioactive oxylipins derived from AA (e.g. leukotrienes) (3, 16, 17), n-3 fatty acids, and/or LA could impact parturition; however, this line of inquiry has not been fully investigated. DHA is well-known to be concentrated in fetal tissues and the developing brain (18) and DHA supplementation in pregnancy has been investigated as a strategy for enhancing neurodevelopment (19, 20) and decreasing the risk of

preterm birth (21, 22). Several, but not all, studies reported that DHA supplementation decreased the risk of preterm birth and/or increased the risk of post-term births requiring obstetric intervention (induction or caesarean section) (19, 21, 23). Such a shift in the curve of gestation length may be due to partial replacement of AA by DHA in placental oxylipin precursor pools (24-26), with subsequent reduction in PGE2 and uterine contractility. However, the effect of DHA supplementation on unesterified AA or PGE2 has not yet been demonstrated in pregnant women. DHA supplementation may also decrease inflammatory processes implicated in preterm birth by increasing the production of DHA-derived oxylipins with anti-inflammatory and pro-resolving properties in placenta or blood (27).

Most studies have focused on the role of AA, DHA and their metabolites during pregnancy. Little is known about LA or its metabolites (oxidized linoleic acid metabolites; OXLAMs), which have been mechanistically linked to several pathological conditions (reviewed in (28)). The combination of relatively high concentrations of OXLAMs in lipoproteins and strong expression of scavenger receptors in human placenta (29-31), provides a mechanism for delivery of OXLAMs to placenta (31, 32), particularly during periods of inflammation and/or oxidative stress (reviewed in (33)) Feeding heated safflower oil (source of OXLAMs) to rats in early pregnancy had teratogenic effects (34). However, the effects of OXLAM exposures on pregnancy are understudied in mammals (35).

Together, these observations indicate that exposures to oxylipins derived from DHA, AA, and LA in pregnancy could potentially play an important role in pregnancy. However, there is currently only limited understanding of whether and how oxylipins influence biochemical and clinical endpoints in pregnancy. Here we take an initial step toward filling these gaps by profiling oxylipins and their precursor fatty acids using plasma samples collected from a subset

of pregnant Australian women enrolled in the ORIP (Omega-3 fats to Reduce the Incidence of Prematurity) study, a large randomized controlled trial testing whether daily supplementation with n-3 DHA can reduce the incidence of early preterm birth in Australian women. The ORIP team collected plasma from a subgroup of participants at study entry (<20 weeks of gestation, median 14 weeks) and again at pregnancy week 24, providing a unique opportunity to: (1) describe the levels and trajectory of changes in plasma unesterified oxylipins and precursor fatty acids in early pregnancy and near the threshold for viability of pregnancy; (2) determine the impact of DHA supplementation on oxylipins and precursor fatty acids; and (3) explore whether differences in oxylipins and precursor fatty acids at pregnancy weeks 14 and 24 hold promise as biomarkers for predicting risk of spontaneous preterm birth. This analysis is restricted to spontaneous births because obstetrical interventions (i.e. induced births, Caesarean sections) may occur for multiple reasons, some of which may be unrelated to endogenous biochemical mediators. Given the small size of the ORIP plasma subgroup, the latter exploration is anticipated to inform sample size calculations for larger, more adequately powered studies testing associations between these variables and spontaneous preterm birth.

5.4 Materials and methods

5.4.1 ORIP Study design

A protocol paper (22) and primary outcome (23) detailing the main ORIP study have been previously published. Briefly, ORIP is a large randomized controlled trial testing whether daily supplementation with long-chain omega-3 (n-3) fatty acids (docosahexaenoic acid (DHA) 800mg plus eicosapentaenoic acid (EPA) 100mg) provided from trial entry (<20 weeks of gestation) until 34 weeks or delivery (whichever occurred first), can reduce the incidence of early preterm (<34 weeks) and preterm (<37 weeks) birth and other secondary maternal and fetal endpoints, compared to control. The baseline and demographic characteristics of the plasma subgroup (n=48) analyzed here and the full ORIP cohort of randomized participants (n=5,544) are shown in **Table 1**. Compared to the full ORIP cohort, the plasma subgroup tended to be slightly older, less racially diverse, more likely to drink alcohol, less likely to be primiparous, and more likely to have a previous preterm delivery. The ORIP study (23) showed that DHA supplementation did not decrease risk of early preterm birth (primary endpoint) or preterm birth (key secondary endpoint) in the total population. However, there was a significant reduction in the risk of preterm birth in singleton pregnancies (a prespecified subgroup analysis).

Table 1: Baseline characteristics of pregnancies in the plasma subgroup and the full ORIP trial

	Plasma Subgroup					ORIP Trial	
Characteristic	**Case (N=12)**	**Control (N=36)**	**n-3 Group (N=27)**	**Control Group (N=21)**	**Total (N=48)**	**n-3 Group (N=2770)**	**Control Group (N=2774)**
Age (yr): Median (IQR)	33.0 (31.0-34.2)	33.0 (30.0-33.2)	33.0 (30.0-34.0)	33.0 (31.0-34.0)	33.0 (30.8-34.0)	30.0 (26.0-34.0)	30.0 (27.0-33.0)
Gestation (wk): Median (IQR)	13.4 (12.4-15.1)	13.4 (12.7-14.6)	13.4 (12.6-15.1)	13.1 (12.4-14.6)	13.4 (12.5-14.8)	14.1 (12.7-16.4)	14.1 (12.7-16.6)
Primiparous: n/N (%)	3/12 (25.0)	9/36 (25.0)	6/27 (22.2)	6/21 (28.6)	12/48 (25.0)	1223/2754 (44.4)	1209/2765 (43.7)
Pregnancy with multiple fetuses: n/N (%)	1/12 (8.3)	0/36 (0.0)	0/27 (0.0)	1/21 (4.8)	1/48 (2.1)	52/2698 (1.9)	48/2721 (1.8)
Weight (kg): Median (IQR)	67.2 (63.6-71.6)	67.5 (57.8-73.8)	70.6 (57.6-73.5)	66.9 (60.1-73.0)	67.2 (58.0-73.4)	69.7 (60.9-81.5)	69.0 (60.9-81.0)
Caucasian race: n/N (%)	10/12 (83.3)	32/36 (88.9)	24/27 (88.9)	18/21 (85.7)	42/48 (87.5)	2057/2764 (74.4)	2041/2767 (73.8)
Completed a high-school education: n/N (%)	11/12 (91.7)	31/36 (86.1)	23/27 (85.2)	19/21 (90.5)	42/48 (87.5)	2246/2756 (81.5)	2194/2764 (79.4)
Smoked cigarettes at trial entry or leading up to pregnancy: n/N (%)	0/12 (0.0)	7/36 (19.4)	4/27 (14.8)	3/21 (14.3)	7/48 (14.6)	435/2755 (15.8)	430/2766 (15.5)
Drank alcohol at trial entry or leading up to pregnancy: n/N (%)	10/12 (83.3)	26/36 (72.2)	20/27 (74.1)	16/21 (76.2)	36/48 (75.0)	1513/2755 (54.9)	1562/2764 (56.5)
Previous preterm birth <37 weeks of gestation: n/N (%)	3/12 (25.0)	2/36 (5.6)	4/27 (14.8)	1/21 (4.8)	5/48 (10.4)	185/2754 (6.7)	184/2766 (6.7)
Consumed dietary supplements containing n-3 long-chain polyunsaturated fatty acid in the previous 3 months: n/N (%)	4/12 (33.3)	3/36 (8.3)	2/27 (7.4)	5/21 (23.8)	7/48 (14.6)	374/2770 (13.5)	368/2774 (13.3)
DHA level (% of total fatty acids in capillary whole blood): Median (IQR)	2.8 (2.1-3.2)	2.7 (2.5-3.0)	2.7 (2.5-3.0)	2.7 (2.4-3.1)	2.7 (2.4-3.1)	2.7 (2.3-3.1)	2.7 (2.3-3.1)
Male fetus: n/N(%)*	9/13 (69.2)	19/36 (52.8)	15/27 (55.6)	13/22 (59.1)	28/49 (57.1)	1395/2758 (50.6)	1409/2770 (50.9)

DHA denotes docosahexaenoic acid, IQR denotes interquartile range. N is the total number of pregnancies unless otherwise indicated.
* N is the total number of fetuses.

5.4.2 Sample collection, processing and selection

The protocol was approved by the Institutional Review Board of the Women's and Children's Health Network and written informed consent was obtained for all blood collection procedures. Following an overnight fast, venipuncture of the median cubital vein in the antecubital fossa was performed with an 18-gauge butterfly needle, with venous whole blood collected into 5 mL 'purple top' potassium-EDTA tubes. Whole blood was stored for up to 120 minutes at room temperature, followed by centrifugation. Plasma was carefully pipetted into each aliquot with care taken not to disturb the buffy coat. Samples were stored at -80°C in Adelaide and shipped with ample dry ice and tracking information for analysis in the National Institutes of Health in Bethesda, Maryland, USA. Samples were analyzed in a de-identified, blinded manner, without knowledge of treatment group or case versus control status. Since each of these participants provided two samples (≈14 and 24 weeks), a total of 96 samples were analyzed.

5.4.3 Quantitation of oxylipins and precursor fatty acids in plasma

Liquid chromatography tandem mass spectrometry oxylipin assays were based on our previously published methodology (1), adapted to include free fatty acids. In this method, human plasma samples were deproteinized with ice-cold methanol and extracted with solid-phase extraction. The separation was performed on a reverse phase ZorBAX RRHD Eclipse Plus C18 column using gradient programs consisting of 0.02% acetic acid and ammonium acetate buffer in water and acetonitrile mobile phases. Detection was achieved using the Qtrap 5500 system in electrospray negative ion mode with scheduled multiple reaction monitoring (sMRM).

5.4.4 Experimental design and matching strategy

To explore the hypothesis that an imbalance between unesterified LA, AA, and DHA and their respective oxylipin derivatives could create a biochemical predisposition to preterm delivery we matched the 12 participants who provided plasma at both of these time points and went on to have spontaneous preterm (<37 weeks) birth with 36 controls who went on to have spontaneous term (40+0-40+6 weeks) birth. The two groups were matched on maternal age and parity. Treatment group was not considered in the matching process, as the trial was still blinded at the time of selection for the plasma subgroup. Exact matching was performed first on parity and then age was matched using the nearest neighbor approach with Mahalanobis distance allowing for 1.5 SD of distance (36), resulting in a matched age range within 3 years.

5.4.5 Data analysis

Compounds were only reported if values were above the limit of quantitation (LOQ) in at least half of the samples with LOQ defined as a signal-to-noise ratio of >5. LOQ for profiled oxylipins are provided in **Table S1**. In the main analysis, we use only the oxylipin samples that are not below LOQ. In the sensitivity analysis reported in the supplementary appendix, we impute the values below the LOQ by substituting one half of the LOQ value for each respective plasma oxylipin. For the precursor unesterified fatty acids, all values were above the LOQ.

The aim of the first analysis was to determine if there was a significant change in the plasma fatty acid concentrations between weeks 14 and 24 separately in the control group and DHA group through a Wilcoxon matched-pairs signed-ranks test. The aim of the second analysis was to determine the difference in plasma fatty acid concentrations between the DHA and control groups at week 24 through a nonparametric local-linear regression controlling for the

plasma fatty acid concentration at week 14. Each regression had 200 bootstrap replications. The aim of the third analysis was to explore if the plasma unesterified fatty acid or oxylipin concentrations could be biomarkers for spontaneous preterm birth. To account for non-linear associations, this analysis was performed conservatively by analyzing the sample according to groups of below and above median concentrations at each time point. Odds ratios of the above median group versus the below median group were calculated for each plasma oxylipin or fatty acid at each time point using logistic regressions adjusted for age and parity. Analyses were performed using Stata 15. Statistical significance was assessed at the $P<0.05$ level with no adjustment for multiple comparisons due to the exploratory nature of the study.

5.5 Results

We investigated the levels and trajectory of changes of plasma oxylipins and precursor (unesterified) fatty acids during pregnancy, by measuring concentrations of these variables at 14 weeks and 24 weeks (shortly before the gestational limit for viable pregnancy) in the participants (n=21) from the control group without DHA supplementation. Within-group changes in precursor fatty acids and oxylipin concentrations in the control group are shown in **Table 2**. Without DHA supplementation, we observed that unesterified concentrations of three n-6 fatty acids (AA, docosatetraenoic acid, and gamma-linolenic acid) and one n-9 fatty acid (mead acid) significantly declined during pregnancy. However, there was no evidence of any changes in n-3 fatty acids. Concentrations of three AA-derived oxylipins—12-HETE, 15-HETE and TXB2—also significantly declined between weeks 14-24 of pregnancy, however no changes were observed for DHA-derivatives or other oxylipins.

Table 2: Changes in unesterified precursor fatty acids and oxylipins between weeks 14 and 24 in pregnancy without DHA supplementation (n=21)

	Week 14	Week 24	p-value*
	Median (IQR)	Median (IQR)	
Omega-6 fatty acids and oxylipins			
LA**	7.6 (4.8, 11)	7.3 (4.1, 9.9)	.24
9-HODE	4.2 (1.4, 11)	3.4 (1.3, 7.9)	.42
13-HODE	11 (3.4, 23)	7.6 (5, 21)	.54
9,10-DiHOME	2.4 (1.1, 5.1)	1.6 (1.1, 4.3)	.56
13H-9,10E-LA	.25 (.16, .48)	.22 (.16, .36)	.73
9H-12,13E-LA	.16 (.067, .38)	.16 (.12, .24)	.93
9,12,13-TriHOME	.12 (.088, .19)	.14 (.098, .17)	.95
GLA	.086 (.044, .13)	.049 (.036, .074)	.030
DGLA	.12 (.086, .15)	.094 (.077, .11)	.059
AA	.18 (.14, .25)	.14 (.12, .17)	.011
5-HETE	.17 (.14, .23)	.16 (.12, .17)	.08
12-HETE	.13 (.1, .24)	.088 (.08, .12)	.039
15-HETE	.29 (.26, .5)	.21 (.17, .22)	.001
TxB2	.11 (.066, .22)	.053 (.039, .11)	.048
DTA	.045 (.034, .067)	.035 (.028, .048)	.033
DPA n-6	.042 (.033, .067)	.044 (.036, .05)	.45
Omega-3 fatty acids and oxylipins			
ALA	.44 (.23, .79)	.26 (.18, .51)	.08
SDA (18:4 n-3)	.012 (.0061, .016)	.0058 (.0045, .0093)	.11
ETA (20:4 n-3)	.014 (.011, .018)	.012 (.0089, .014)	.15
EPA	.093 (.086, .1)	.083 (.056, .099)	.12
DPA n-3	.024 (.015, .038)	.023 (.016, .032)	.91
DHA	.65 (.43, .75)	.54 (.4, .78)	.97
4-HDHA	.066 (.042, .09)	.05 (.03, .076)	.57
10-HDHA	.026 (.021, .035)	.019 (.014, .038)	.11
14-HDHA	.12 (.085, .22)	.078 (.059, .12)	.06
17-HDHA	.17 (.13, .23)	.12 (.1, .19)	.55
19,20-EpDPA	.032 (.018, .047)	.034 (.019, .063)	.31
Other			
Mead	.016 (.011, .02)	.011 (.0073, .013)	.003

* Based on the Wilcoxon matched-pairs signed-ranks test

** 9,10-EpOME & 9,10,13-TriHOME had >50% values below LOQ.

Concentrations: fatty acids (ug/mL); oxylipins (ng/mL)

Concentrations of unesterified fatty acids and oxylipins in the DHA supplementation group in pregnancy weeks 14 and 24 are shown in **Table 3**. With DHA supplementation, within-group analysis suggested that unesterified concentrations of three n-3 fatty acids (EPA, DPAn-3, and DHA) and one n-6 fatty acid (DPAn-6) increased, while LA (18:2n-6), GLA (18:3n-6) and SDA (18:4n-3) decreased. Unesterified AA did not change. Two DHA-derived oxylipins (4-HDHA and 19,20-EpDPA) increased and one AA-derivative (15-HETE) decreased. Importantly, these within-group changes in this DHA cohort reflect a combination of effects of DHA supplementation and normal trajectory of change, and as such should be interpreted cautiously.

In order to determine the effects of DHA supplementation on plasma oxylipins and precursor fatty acids, we compared levels of each variable at week 24 in the DHA group versus the control group in the combined term and preterm pregnancies, with each variable adjusted for baseline values. We observed that DHA supplementation significantly increased concentrations of unesterified n-3 DHA, DPA(n-3), EPA, as well as n-6 AA and DPAn-6, but did not alter linoleic acid, alpha-linolenic acid, or other fatty acids (**Table 4**). DHA supplementation also significantly increased three DHA-derived oxylipins (4-HDHA, 10-HDHA and 19,20-EpDPA), and one AA-derived oxylipin (12-HETE).

Table 3: Changes in unesterified precursor fatty acids and oxylipins between weeks 14 and 24 in pregnancy with DHA supplementation (n=27)

	Week 14	Week 24	p-value*
	Median (IQR)	Median (IQR)	
Omega-6 fatty acids and oxylipins			
LA**	8.5 (6.4, 13)	7.6 (4.8, 11)	.038
9-HODE	4.4 (2.7, 6.8)	2.6 (1.1, 6.2)	.26
13-HODE	8.4 (5.6, 15)	5.6 (2.6, 13)	.15
9,10-DiHOME	2 (1.1, 2.6)	1.3 (.64, 4.1)	.61
13H-9,10E-LA	.25 (.21, .37)	.19 (.12, .31)	.21
9H-12,13E-LA	.19 (.13, .24)	.1 (.075, .28)	.85
9,12,13-TriHOME	.13 (.09, .22)	.11 (.086, .17)	.26
GLA	.083 (.05, .13)	.05 (.028, .075)	<0.001
DGLA	.096 (.081, .13)	.098 (.082, .12)	.71
AA	.15 (.12, .18)	.18 (.13, .21)	.20
5-HETE	.19 (.14, .28)	.14 (.12, .19)	.012
12-HETE	.21 (.1, .28)	.17 (.1, .23)	.36
15-HETE	.33 (.25, .6)	.19 (.12, .32)	<0.001
TxB2	.098 (.06, .22)	.057 (.036, .14)	.16
DTA	.04 (.03, .055)	.035 (.028, .048)	.54
DPA n-6	.04 (.03, .055)	.054 (.042, .085)	<0.001
Omega-3 fatty acids and oxylipins			
ALA	.59 (.3, .82)	.29 (.18, .51)	<0.001
SDA (18:4 n-3)	.011 (.007, .02)	.008 (.004, .014)	.008
ETA (20:4 n-3)	.012 (.009, .02)	.014 (.011, .019)	.28
EPA	.075 (.049, .1)	.14 (.11, .19)	<0.001
DPA n-3	.02 (.014, .03)	.031 (.022, .04)	.023
DHA	.62 (.47, .8)	1.1 (.84, 1.6)	<0.001
4-HDHA	.07 (.04, .12)	.12 (.069, .17)	.004
10-HDHA	.03 (.024, .041)	.034 (.021, .072)	.058
14-HDHA	.19 (.15, .31)	.2 (.12, .31)	.71
17-HDHA	.19 (.12, .3)	.21 (.15, .28)	.60
19,20-EpDPA	.03 (.02, .05)	.077 (.057, .11)	<0.001
Other			
Mead	.01 (.008, .018)	.009 (.006, .016)	.041

* Based on the Wilcoxon matched-pairs signed-ranks test

** 9,10-EpOME & 9,10,13-TriHOME had >50% values below LOQ.

Concentrations: fatty acids (ug/mL); oxylipins (ng/mL)

Table 4: DHA supplementation vs Control at week 24

	Control (n=21)	DHA (n=27)	Nonparametric ANCOVA*	
	Median (IQR)	Median (IQR)	Difference (95% CI)	p-value
Omega-6 fatty acids and oxylipins				
LA**	7.3 (4.1, 9.9)	7.6 (4.8, 11)	.64 (-1.1, 2.6)	.50
9-HODE	3.4 (1.3, 7.9)	2.6 (1.1, 6.2)	-.58 (-4.7, 4.4)	.78
13-HODE	7.6 (5, 21)	5.6 (2.6, 13)	-1.3 (-11, 8.3)	.77
9,10-DiHOME	1.6 (1.1, 4.3)	1.3 (.64, 4.1)	.39 (-2, 2.9)	.74
13H-9,10E-LA	.22 (.16, .36)	.19 (.12, .31)	-.029 (-.22, .18)	.77
9H-12,13E-LA	.16 (.12, .24)	.1 (.075, .28)	.028 (-.19, .26)	.80
9,12,13-TriHOME	.14 (.098, .17)	.11 (.086, .17)	-.021 (-.089, .041)	.53
GLA	.049 (.036, .074)	.05 (.028, .075)	-.000018 (-.018, .016)	.99
DGLA	.094 (.077, .11)	.098 (.082, .12)	.016 (-.00089, .031)	.052
AA	.14 (.12, .17)	.18 (.13, .21)	.037 (.015, .062)	.002
5-HETE	.16 (.12, .17)	.14 (.12, .19)	.02 (-.052, .098)	.58
12-HETE	.088 (.08, .12)	.17 (.1, .23)	.071 (.0082, .13)	.036
15-HETE	.21 (.17, .22)	.19 (.12, .32)	.023 (-.055, .1)	.55
TxB2	.053 (.039, .11)	.057 (.036, .14)	.011 (-.051, .069)	.73
DTA	.035 (.028, .048)	.035 (.028, .048)	.0051 (-.0038, .013)	.25
DPA n-6	.044 (.036, .05)	.054 (.042, .085)	.024 (.013, .037)	<0.001
Omega-3 fatty acids and oxylipins				
ALA	.26 (.18, .51)	.29 (.18, .51)	.021 (-.1, .13)	.71
SDA (18:4 n-3)	.0058 (.0045, .0093)	.0079 (.004, .014)	.0013 (-.0019, .004)	.40
ETA (20:4 n-3)	.012 (.0089, .014)	.014 (.011, .019)	.0025 (-.0013, .0055)	.16
EPA	.083 (.056, .099)	.14 (.11, .19)	.072 (.049, .099)	<0.001
DPA n-3	.023 (.016, .032)	.031 (.022, .04)	.008 (-.00029, .015)	.035
DHA	.54 (.4, .78)	1.1 (.84, 1.6)	.56 (.36, .78)	<0.001
4-HDHA	.05 (.03, .076)	.12 (.069, .17)	.099 (.011, .21)	.048
10-HDHA	.019 (.014, .038)	.034 (.021, .072)	.019 (.0037, .036)	.010
14-HDHA	.078 (.059, .12)	.2 (.12, .31)	.12 (-.0085, .25)	.06
17-HDHA	.12 (.1, .19)	.21 (.15, .28)	.019 (-.15, .16)	.81
19,20-EpDPA	.034 (.019, .063)	.077 (.057, .11)	.033 (.01, .055)	.002
Other				
Mead	.011 (.007, .013)	.009 (.006, .016)	.0028 (-.0005, .006)	.09

* Difference is estimated using a nonparametric ANCOVA controlling for the mediator at Week 14.
A negative difference means the DHA group had a lower concentration than the control group.

** 9,10-EpOME & 9,10,13-TriHOME had >50% values below LOQ.

Concentrations: fatty acids (ug/mL); oxylipins (ng/mL)

To explore whether differences in precursor fatty acids at week 14 (initial visit) or 24 (shortly before the gestational limit for viable pregnancy) hold promise as biomarkers for predicting risk of preterm birth, we compared the proportion of cases (spontaneous preterm births, n=12) vs controls (spontaneous matched term births, n=36), independent of DHA supplementation, that were above the median level at 14 and 24 weeks using logistic regression adjusting for age and parity. This analysis showed that participants with concentrations of unesterified AA above the median at 24 weeks had higher risk of spontaneous preterm birth (Odds ratio (OR) 5.1; p=0.038) (**Table 5, Figure 1 and 2**). None of the other fatty acids were predictive of spontaneous preterm birth at week 14 or 24.

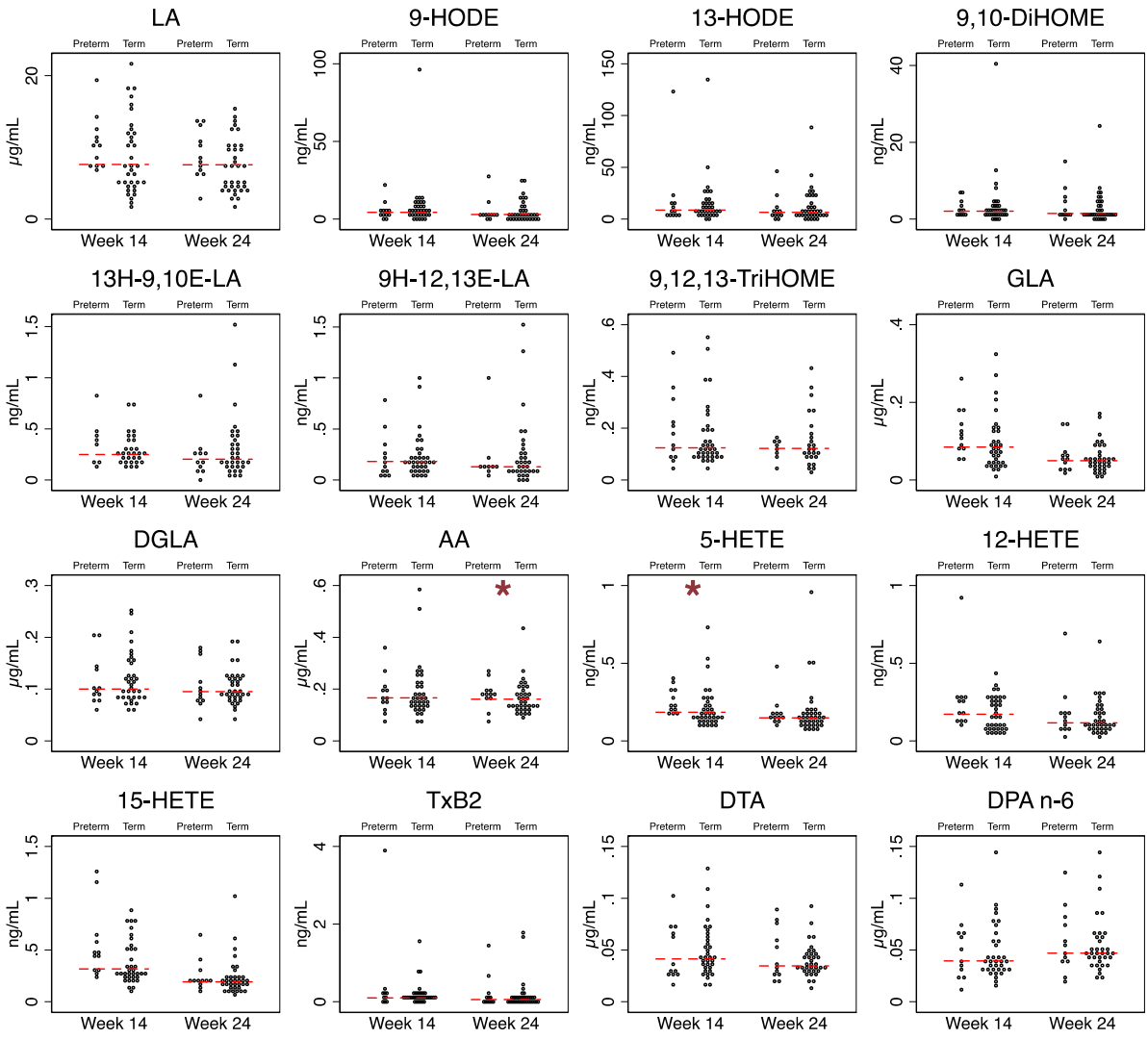
Table 5: Risk of spontaneous preterm birth according to concentrations of oxylipins & precursor fatty acids (n=48 including 12 cases of spontaneous preterm birth (25 %))

	Week 14 (n=48)				Week 24 (n=48)			
	Below Median	Above Median	Odds Ratio	p-value	Below Median	Above Median	Odds Ratio	p-value
Omega-6 fatty acids and oxylipins								
LA**	17%	35%	2.8	.14	21%	30%	1.7	.43
9-HODE	39%	14%	0.2	.05	22%	26%	1.2	.75
13-HODE	26%	26%	1.0	.98	29%	21%	0.6	.49
9,10-DiHOME	25%	25%	1.0	.99	24%	26%	1.1	.87
13H-9,10E-LA	16%	28%	2.0	.41	27%	23%	0.8	.70
9H-12,13E-LA	25%	26%	1.1	.92	24%	19%	0.7	.62
9,12,13-TriHOME	17%	33%	2.5	.18	24%	25%	1.1	.93
GLA	17%	35%	2.8	.14	21%	30%	1.7	.44
DGLA	33%	17%	0.4	.20	29%	22%	0.7	.64
AA	24%	27%	1.2	.79	12%	39%	5.1	.038
5-HETE	8%	42%	8.2	.014	21%	29%	1.6	.49
12-HETE	21%	29%	1.6	.50	21%	29%	1.6	.50
15-HETE	12%	38%	4.4	.050	17%	33%	2.7	.16
TxB2	13%	32%	3.7	.10	25%	25%	1.0	.97
DTA	29%	22%	0.7	.53	29%	22%	0.7	.58
DPA n-6	25%	26%	1.1	.94	25%	26%	1.1	.91
Omega-3 fatty acids and oxylipins								
ALA	17%	35%	2.9	.13	17%	35%	2.8	.15
SDA (18:4 n-3)	15%	38%	3.6	.07	25%	26%	1.1	.93
ETA (20:4 n-3)	29%	22%	0.6	.51	25%	26%	1.0	.94
EPA	21%	30%	1.7	.46	25%	26%	1.1	.93
DPA n-3	25%	26%	1.0	.99	21%	30%	1.7	.45
DHA	25%	26%	1.1	.91	21%	30%	1.7	.42
4-HDHA	8%	42%	8.0	.015	25%	26%	1.1	.91
10-HDHA	17%	33%	2.6	.17	22%	30%	1.6	.48
14-HDHA	17%	33%	2.8	.15	25%	25%	1.0	.98
17-HDHA	18%	36%	2.8	.15	18%	33%	2.3	.24
19,20-EpDPA	12%	39%	4.8	.041	17%	35%	2.6	.16
Other								
Mead	21%	30%	1.7	.45	25%	26%	1.1	.87

* Each compound was dichotomized according to median split at each timepoint. Odds ratios and p-values were from logistic regressions adjusted for age and parity. Due to the selection process for inclusion in this substudy, the overall risk of preterm birth is 25%.

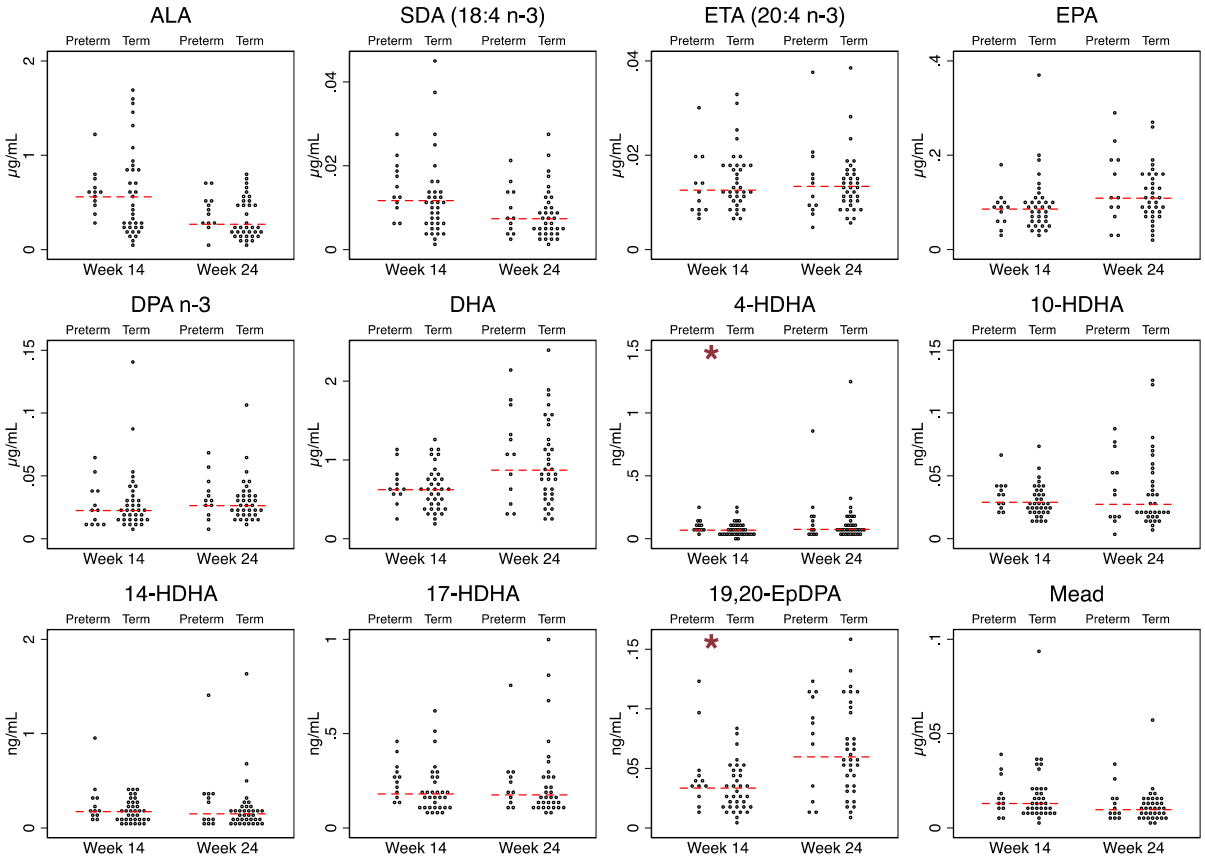
** 9,10-EpOME & 9,10,13-TriHOME had >50% values below LOQ.

Figure 1. Concentrations of n-6 fatty acids and oxylipin derivatives in the ORIP plasma substudy (n=48)



*corresponds to p<0.05 Table 5.

Figure 2. Concentrations of n-3 fatty acids and oxylipin derivatives in the ORIP plasma substudy (n=48)



*corresponds to p<0.05 in Table 5.

None of the oxylipins were predictive of spontaneous preterm birth at pregnancy week 24. However, at 14 weeks of gestation, participants with concentrations of 5-lipoxygenase derivatives of two different precursor fatty acids—5-HETE (OR 8.2; p=0.014) from AA and 4-HDHA (OR 8.0; p=0.015) from DHA—above the median had higher risk of spontaneous preterm birth. Participants with concentrations of 15-HETE and 19,20-EpDPA above the median, or 9-HODE below the median, also tended to have higher risk of spontaneous preterm birth (**Table 5, Figure 1 and 2**).

5.6 Discussion

Preterm birth is a leading cause of infant death and long-term neurological disabilities in children (37, 38). A better understanding of the molecular mechanisms underlying early parturition and the identification of non-invasive mechanism-based biomarkers predicting preterm birth are urgently needed. Supplementation with n-3 DHA during pregnancy has been shown to decrease the risk of preterm birth in singleton pregnancies (19), which may be due, at least in part, to consequent increases in DHA-derived oxylipins with anti-inflammatory and pro-resolving properties, reductions in AA-derived oxylipins that are implicated in immune activation (e.g. leukotrienes, HETEs), or cervical ripening and uterine contractility (e.g. prostanoids), or both. In the present study, DHA supplementation did produce the anticipated increases in unesterified DHA and several DHA-derived oxylipins in plasma. Unexpectedly, however, DHA supplementation did not decrease measured AA-derived oxylipins but rather appeared to increase unesterified AA and one of its oxylipin derivatives (12-HETE). This increase in unesterified AA stands in contrast to the finding that DHA supplementation in the full ORIP cohort decreased total AA in whole blood (23). Given the small sample size these unexpected findings could be a random error. Alternatively, they may reflect different effects of DHA supplementation on the abundance of AA and AA-derived oxylipins in unesterified versus total lipid pools. Since AA and oxylipins in the unesterified pool are bioactive and could potentially affect early parturition, the effect of supplemental DHA on the unesterified pool of AA and AA-derived oxylipins is a topic that is worthy of investigation in future, larger studies. Concentrations of prostanoids were below the limit of quantitation in all plasma samples, thus the effects of DHA supplementation on plasma prostaglandins could not be tested.

Unesterified fatty acids as potential biomarkers of spontaneous preterm birth

Unesterified PUFAs and their oxylipin derivatives are attractive candidate biomarkers for predicting risk of spontaneous preterm birth because they may reflect underlying biological mechanisms and are likely to be readily modifiable via diet or supplementation. In the present study, women with AA above the median at 24 weeks of gestation had a higher risk of spontaneous preterm birth than women with AA below the median. Since AA is the precursor to 2-series prostaglandins that promote initiation and progression of labor (9, 39) and 5-lipoxygenase derivatives (e.g. leukotriene B4) that are also involved in parturition (3, 16, 17) in pregnancy, there are plausible biological mechanisms that could account for this finding. However, given the small size of the ORIP plasma subgroup, larger, more adequately powered studies are needed to test the hypothesis that unesterified AA could serve as a mechanism-based biomarker for spontaneous preterm birth.

Unesterified oxylipins as potential biomarkers of preterm birth

At 14 weeks of gestation, participants with concentrations of 5-lipoxygenase derivatives of AA (5-HETE) and DHA (4-HDHA) above the median had 8-fold higher odds of spontaneous preterm birth compared to participants with concentrations below the median. The 5-lipoxygenase metabolite of AA, leukotriene B4, has previously been shown to be elevated in amniotic fluid during normal parturition (17), and to be elevated in amniotic fluid of mothers with premature rupture of membranes in the presence of infection, labor, or both (3). In 2016, Maddipati at al (40) reported that two 5-lipoxygenase derivatives of AA (5-HETE and leukotriene B4) were elevated in amniotic fluid in pregnant women who had intra-amniotic infection, and suggested that these 5-lipoxygenase derivatives could serve as potential biomarkers of microbial invasion of the amniotic cavity, a well-established cause of preterm

birth (41). The above authors did not measure unesterified AA or 5-lipoxygenase derivatives of DHA such as 4-HDHA that was reported in the present study. However, these collective findings in amniotic fluid, together with our findings of higher risk of preterm birth in mothers with higher concentration of 5-HETE and 4-HDHA at 14-weeks, and higher AA concentrations at 24-weeks of gestation, provide an intriguing clue that 5-lipoxygenase derivatives of AA and/or DHA could potentially help identify mothers at risk for spontaneous preterm birth and/or chorioamniotic infection. These preliminary findings should be followed-up in adequately powered studies.

Oxidized linoleic acid metabolites (OXLAMs) in pregnancy

In the present study LA and unesterified LA-derived oxylipins did not change during pregnancy, were not altered by DHA supplementation, and did not appear to have any relationship with preterm birth. Given the small sample size of this study, these findings do not rule out an effect of OXLAMs or LA in spontaneous preterm birth. Nevertheless, the findings here suggest that follow-up studies emphasizing the relationships between plasma AA, DHA and their respective oxylipin derivatives and spontaneous preterm birth are likely to be more productive. Since the majority of OXLAMs in oxidized lipoproteins are esterified in phospholipids and cholesteryl-esters pools, future studies in this domain should consider quantifying total or esterified OXLAMs rather than the unesterified pool.

Limitations

This study had several important limitations. First, although participants were chosen from a randomized trial of DHA supplementation, treatment group was not considered in the selection procedure for this study. Treatment group comparisons may be subject to confounding

and the small sample size precludes adequate controls for confounding. Results of comparisons between treatment groups should thus be interpreted with some caution. Additional limitations include the lack of dietary fatty acid data, incomplete understanding of bioavailability and metabolic fate of unesterified fatty acids and small sample sizes. Findings from this substudy are not necessarily generalizable to a more general population of pregnant women. A substantial number of samples had oxylipin values that were below the LOQ. Blood was kept in EDTA tubes at room temperature for up to 2-hours prior to centrifugation and therefore changes in 12-lipoxygenase (12-HETE, 14-HDHA) and platelet derived (TXB2) should be interpreted with some caution in this setting.

Summary and Conclusion

Here we take an initial step toward understanding the role of oxylipins and their precursor unesterified fatty acids during pregnancy using plasma samples collected from a subset of pregnant Australian women enrolled in the ORIP trial. We showed that DHA supplementation produced marked alterations in unesterified fatty acids and several of their oxylipin derivatives and suggest that unesterified AA and/or 5-lipoxygenase derivatives of AA and DHA hold promise as candidate biomarkers for predicting spontaneous preterm birth.

Acknowledgements

Supported by the intramural programs of the National Institute on Alcohol Abuse and Alcoholism and National Institute on Aging, National Institutes of Health, the University of Adelaide, the South Australian Health and Medical Research Institute, and USDA National Institute of Food and Agriculture, Hatch/Taha (project #1008787). The original study was supported by grants from the Australian National Health and Medical Research Council (NHMRC) (1050468) and the Thyne Reid Foundation, and fellowships awarded to M Makrides (1061704), RA Gibson (1046207) and LN Yelland (1052388) from the Australian NHMRC. Croda UK provided the oils and Wassen UK the encapsulation. Collection of the extra samples used in this study were supported by the NHMRC Centre of Research Excellence in Foods for Future Australians (1035530). We would like to acknowledge the ORIP Steering Committee for their contributions to the main trial and Beverly Muhlhausler for assistance with location and transport of samples. We would also like to acknowledge the ORIP study participants.

5.7 References (Chapter 5)

1. Yuan ZX, Majchrzak-Hong S, Keyes GS, Iadarola MJ, Mannes AJ, Ramsden CE. Lipidomic profiling of targeted oxylipins with ultra-performance liquid chromatography-tandem mass spectrometry. Anal Bioanal Chem. 2018;410(23):6009-29.

2. Romero R, Emamian M, Wan M, Quintero R, Hobbins JC, Mitchell MD. Prostaglandin concentrations in amniotic fluid of women with intra-amniotic infection and preterm labor. Am J Obstet Gynecol. 1987;157(6):1461-7.

3. Romero R, Quintero R, Emamian M, Wan M, Grzyboski C, Hobbins JC, et al. Arachidonate lipoxygenase metabolites in amniotic fluid of women with intra-amniotic infection and preterm labor. Am J Obstet Gynecol. 1987;157(6):1454-60.

4. Serhan CN. Pro-resolving lipid mediators are leads for resolution physiology. Nature. 2014;510(7503):92-101.

5. Nordgren TM, Anderson Berry A, Van Ormer M, Zoucha S, Elliott E, Johnson R, et al. Omega-3 Fatty Acid Supplementation, Pro-Resolving Mediators, and Clinical Outcomes in Maternal-Infant Pairs. Nutrients. 2019;11(1).

6. Shearer GC, Newman JW. Impact of circulating esterified eicosanoids and other oxylipins on endothelial function. Curr Atheroscler Rep. 2009;11(6):403-10.

7. Adili R, Hawley M, Holinstat M. Regulation of platelet function and thrombosis by omega-3 and omega-6 polyunsaturated fatty acids. Prostaglandins Other Lipid Mediat. 2018;139:10-8.

8. Gouveia-Figueira S, Martens DS, Nawrot TS, Nording ML. Cord blood eicosanoid signatures and newborn gestational age. Prostaglandins Other Lipid Mediat. 2017;133:123-7.

9. Olson DM, Ammann C. Role of the prostaglandins in labour and prostaglandin receptor inhibitors in the prevention of preterm labour. Front Biosci. 2007;12:1329-43.

10. Thomas J, Fairclough A, Kavanagh J, Kelly AJ. Vaginal prostaglandin (PGE2 and PGF2a) for induction of labour at term. Cochrane Database Syst Rev. 2014(6):CD003101.

11. Boyle AK, Rinaldi SF, Norman JE, Stock SJ. Preterm birth: Inflammation, fetal injury and treatment strategies. J Reprod Immunol. 2017;119:62-6.

12. Christiaens I, Zaragoza DB, Guilbert L, Robertson SA, Mitchell BF, Olson DM. Inflammatory processes in preterm and term parturition. J Reprod Immunol. 2008;79(1):50-7.

13. Reece MS, McGregor JA, Allen KG, Harris MA. Maternal and perinatal long-chain fatty acids: possible roles in preterm birth. Am J Obstet Gynecol. 1997;176(4):907-14.

14. Martens DS, Gouveia S, Madhloum N, Janssen BG, Plusquin M, Vanpoucke C, et al. Neonatal Cord Blood Oxylipins and Exposure to Particulate Matter in the Early-Life Environment: An ENVIR. Environ Health Perspect. 2017;125(4):691-8.

15. Bernard JY, Pan H, Aris IM, Moreno-Betancur M, Soh SE, Yap F, et al. Long-chain polyunsaturated fatty acids, gestation duration, and birth size: a Mendelian randomization study using fatty acid desaturase variants. Am J Clin Nutr. 2018;108(1):92-100.

16. van der Elst CW, Lòpez Bernal A, Sinclair-Smith CC. The role of chorioamnionitis and prostaglandins in preterm labor. Obstet Gynecol. 1991;77(5):672-6.

17. Romero R, Emamian M, Wan M, Grzyboski C, Hobbins JC, Mitchell MD. Increased concentrations of arachidonic acid lipoxygenase metabolites in amniotic fluid during parturition. Obstet Gynecol. 1987;70(6):849-51.

18. Kuipers RS, Luxwolda MF, Sango WS, Kwesigabo G, Dijck-Brouwer DA, Muskiet FA. Maternal DHA equilibrium during pregnancy and lactation is reached at an erythrocyte DHA content of 8 g/100 g fatty acids. J Nutr. 2011;141(3):418-27.

19. Makrides M, Gibson RA, McPhee AJ, Yelland L, Quinlivan J, Ryan P, et al. Effect of DHA supplementation during pregnancy on maternal depression and neurodevelopment of young children: a randomized controlled trial. JAMA. 2010;304(15):1675-83.

20. Makrides M. DHA supplementation during the perinatal period and neurodevelopment: Do some babies benefit more than others? Prostaglandins Leukot Essent Fatty Acids. 2013;88(1):87-90.

21. Middleton P, Gomersall JC, Gould JF, Shepherd E, Olsen SF, Makrides M. Omega-3 fatty acid addition during pregnancy. Cochrane Database Syst Rev. 2018;11:CD003402.

22. Zhou SJ, Best K, Gibson R, McPhee A, Yelland L, Quinlivan J, et al. Study protocol for a randomised controlled trial evaluating the effect of prenatal omega-3 LCPUFA supplementation to reduce the incidence of preterm birth: the ORIP trial. BMJ Open. 2017;7(9):e018360.

23. Makrides M, Best K, Yelland L, McPhee A, Zhou S, Quinlivan J, et al. A Randomized Trial of Prenatal n-3 Fatty Acid Supplementation and Preterm Delivery. N Engl J Med. 2019;381(11):1035-45.

24. Foster BA, Escaname E, Powell TL, Larsen B, Siddiqui SK, Menchaca J, et al. Randomized Controlled Trial of DHA Supplementation during Pregnancy: Child Adiposity Outcomes. Nutrients. 2017;9(6).

25. Olsen SF. Is supplementation with marine omega-3 fatty acids during pregnancy a useful tool in the prevention of preterm birth? Clin Obstet Gynecol. 2004;47(4):768-74; discussion 881-2.

26. Roman AS, Schreher J, Mackenzie AP, Nathanielsz PW. Omega-3 fatty acids and decidual cell prostaglandin production in response to the inflammatory cytokine IL-1beta. Am J Obstet Gynecol. 2006;195(6):1693-9.

27. Keelan JA, Mas E, D'Vaz N, Dunstan JA, Li S, Barden AE, et al. Effects of maternal n-3 fatty acid supplementation on placental cytokines, pro-resolving lipid mediators and their precursors. Reproduction. 2015;149(2):171-8.

28. Ramsden CE, Zamora D, Majchrzak-Hong S, Faurot KR, Broste SK, Frantz RP, et al. Re-evaluation of the traditional diet-heart hypothesis: analysis of recovered data from Minnesota Coronary Experiment (1968-73). BMJ. 2016;353:i1246.

29. Dye JF, Jablenska R, Donnelly JL, Lawrence L, Leach L, Clark P, et al. Phenotype of the endothelium in the human term placenta. Placenta. 2001;22(1):32-43.

30. Fournier T, Handschuh K, Tsatsaris V, Evain-Brion D. Involvement of PPARgamma in human trophoblast invasion. Placenta. 2007;28 Suppl A:S76-81.

31. Palani S, Maksimow M, Miiluniemi M, Auvinen K, Jalkanen S, Salmi M. Stabilin-1/CLEVER-1, a type 2 macrophage marker, is an adhesion and scavenging molecule on human placental macrophages. Eur J Immunol. 2011;41(7):2052-63.

32. Dubé E, Gravel A, Martin C, Desparois G, Moussa I, Ethier-Chiasson M, et al. Modulation of fatty acid transport and metabolism by maternal obesity in the human full-term placenta. Biol Reprod. 2012;87(1):14, 1-1.

33. Choi SH, Sviridov D, Miller YI. Oxidized cholesteryl esters and inflammation. Biochim Biophys Acta Mol Cell Biol Lipids. 2017;1862(4):393-7.

34. Indart A, Viana M, Grootveld MC, Silwood CJ, Sanchez-Vera I, Bonet B. Teratogenic actions of thermally-stressed culinary oils in rats. Free Radic Res. 2002;36(10):1051-8.

35. Ramsden CE, Hennebelle M, Schuster S, Keyes GS, Johnson CD, Kirpich IA, et al. Effects of diets enriched in linoleic acid and its peroxidation products on brain fatty acids, oxylipins, and aldehydes in mice. Biochim Biophys Acta Mol Cell Biol Lipids. 2018;1863(10):1206-13.

36. Stuart EA. Matching methods for causal inference: A review and a look forward. Stat Sci. 2010;25(1):1-21.

37. Keelan JA, Newnham JP. Recent advances in the prevention of preterm birth. F1000Res. 2017;6.

38. Govindaswami B, Jegatheesan P, Nudelman M, Narasimhan SR. Prevention of Prematurity: Advances and Opportunities. Clin Perinatol. 2018;45(3):579-95.

39. Hsu CD, Meaddough E, Aversa K, Hong SF, Lee IS, Bahodo-Singh RO, et al. Dual roles of amniotic fluid nitric oxide and prostaglandin E2 in preterm labor with intra-amniotic infection. Am J Perinatol. 1998;15(12):683-7.

40. Maddipati KR, Romero R, Chaiworapongsa T, Chaemsaithong P, Zhou SL, Xu Z, et al. Lipidomic analysis of patients with microbial invasion of the amniotic cavity reveals up-regulation of leukotriene B4. FASEB J. 2016;30(10):3296-307.

41. Peng CC, Chang JH, Lin HY, Cheng PJ, Su BH. Intrauterine inflammation, infection, or both (Triple I): A new concept for chorioamnionitis. Pediatr Neonatol. 2018;59(3):231-7.

5.8 Appendices

Table S1: Oxylipins and unesterified fatty acids included in LC-MS/MS assays

Table S2: Changes in unesterified precursor fatty acids and oxylipins between weeks 14 and 24 in pregnancy without DHA supplementation (n=21), missing values imputed

Table S3: Changes in unesterified precursor fatty acids and oxylipins between weeks 14 and 24 in pregnancy with DHA supplementation (n=27)

Table S4: DHA supplementation vs Control at week 24, missing values imputed

Table S5: Risk of spontaneous preterm birth according to concentrations of oxylipins & precursor fatty acids, missing values imputed

Table S1: Oxylipins and unesterified fatty acids included in LC-MS/MS assays

Oxylipins	LOQ (ng/mL)
9-HODE	0.2
13-HODE	0.2
9,10-EpOME**	0.5
9,10-DiHOME	0.1
9,10,13-TriHOME	0.1
9,12,13-TriHOME	0.1
PGE2*	N/A
PGF2a*	N/A
8-isoPGF2a*	N/A
TxB2	0.02
5-HETE	0.02
12-HETE	0.1
15-HETE	0.05
4-HDHA	0.02
10-HDHA	0.02
14-HDHA	0.05
17-HDHA	0.1
19,20-EpDPA	0.02
9,10,13-TriHOME**	0.05
9,12,13-TriHOME	0.1
Fatty acids	**LOQ (ug/mL)**
ALA	0.02
GLA	0.01
EPA	0.02
DHA	0.04
LA	0.40
DGLA	0.01
AA	0.01
ETA (20:4n-3)	0.004
DTA	0.01
Mead	0.002
DPAn-3	0.004
DPAn-6	0.004
SDA (18:4 n-3)	0.002

* indicates below the limit of detection

** indicates that 9,10-EpOME & 9,10,13-TriHOME had >50% values below limit of quantitation (LOQ).

Table S2: Changes in unesterified precursor fatty acids and oxylipins between weeks 14 and 24 in pregnancy without DHA supplementation (n=21), missing values imputed

	Week 14	Week 24	p-value*
	Median (IQR)	Median (IQR)	
Omega-6 fatty acids and oxylipins			
LA**	7.6 (4.8, 11)	7.1 (4.1, 9.6)	.17
9-HODE	4.1 (1.1, 11)	3.1 (1.2, 7.4)	.50
13-HODE	8.1 (2.7, 19)	7.6 (5, 21)	.63
9,10-DiHOME	2.4 (1.1, 5.1)	1.6 (1.1, 4.3)	.56
13H-9,10E-LA	.17 (.05, .29)	.21 (.15, .34)	.57
9H-12,13E-LA	.14 (.066, .36)	.14 (.1, .22)	.75
9,12,13-TriHOME	.12 (.088, .19)	.1 (.05, .15)	.27
GLA	.086 (.044, .13)	.048 (.033, .063)	.023
DGLA	.12 (.086, .15)	.092 (.077, .11)	.034
AA	.18 (.14, .25)	.14 (.12, .17)	.006
5-HETE	.17 (.14, .23)	.16 (.12, .17)	.08
12-HETE	.13 (.1, .24)	.088 (.08, .12)	.039
15-HETE	.29 (.26, .5)	.21 (.17, .22)	.001
TxB2	.096 (.057, .21)	.053 (.039, .11)	.054
DTA	.045 (.034, .067)	.034 (.027, .047)	.019
DPA n-6	.042 (.033, .067)	.042 (.036, .049)	.28
Omega-3 fatty acids and oxylipins			
ALA	.44 (.23, .79)	.26 (.17, .48)	.06
SDA (18:4 n-3)	.012 (.0061, .016)	.006 (.0045, .0093)	.11
ETA (20:4 n-3)	.014 (.011, .018)	.012 (.0089, .014)	.15
EPA	.093 (.086, .1)	.082 (.054, .095)	.07
DPA n-3	.024 (.015, .038)	.023 (.015, .031)	.66
DHA	.65 (.43, .75)	.52 (.4, .73)	.71
4-HDHA	.066 (.042, .09)	.05 (.03, .076)	.57
10-HDHA	.026 (.021, .035)	.018 (.013, .037)	.056
14-HDHA	.12 (.085, .22)	.078 (.059, .12)	.06
17-HDHA	.16 (.1, .19)	.11 (.094, .19)	.34
19,20-EpDPA	.028 (.018, .044)	.034 (.018, .056)	.30
Other			
Mead	.016 (.011, .02)	.011 (.0071, .013)	.002

* Based on the Wilcoxon matched-pairs signed-ranks test

** 9,10-EpOME & 9,10,13-TriHOME had >50% values below LOQ.

Concentrations: fatty acids (ug/mL); oxylipins (ng/mL)

Table S3: Changes in unesterified precursor fatty acids and oxylipins between weeks 14 and 24 in pregnancy with DHA supplementation (n=27)

	Week 14	Week 24	p-value*
	Median (IQR)	Median (IQR)	
Omega-6 fatty acids and oxylipins			
LA**	8.3 (5.3, 13)	7.6 (4.8, 11)	.07
9-HODE	4.4 (2.7, 6.8)	2.4 (1, 6.2)	.21
13-HODE	8.4 (5.6, 15)	5.6 (2.6, 13)	.15
9,10-DiHOME	2 (1.1, 2.6)	1.3 (.64, 4.1)	.61
13H-9,10E-LA	.24 (.16, .37)	.17 (.077, .28)	.46
9H-12,13E-LA	.19 (.13, .24)	.1 (.025, .23)	.41
9,12,13-TriHOME	.13 (.09, .22)	.086 (.05, .12)	.007
GLA	.081 (.047, .13)	.05 (.028, .075)	<0.001
DGLA	.096 (.08, .13)	.098 (.082, .12)	.96
AA	.15 (.12, .18)	.18 (.13, .21)	.14
5-HETE	.19 (.14, .28)	.14 (.12, .19)	.012
12-HETE	.21 (.1, .28)	.17 (.1, .23)	.36
15-HETE	.33 (.25, .6)	.19 (.12, .32)	<0.001
TxB2	.095 (.056, .22)	.057 (.036, .14)	.23
DTA	.036 (.026, .055)	.035 (.028, .048)	.76
DPA n-6	.037 (.03, .055)	.054 (.042, .085)	<0.001
Omega-3 fatty acids and oxylipins			
ALA	.58 (.3, .82)	.29 (.18, .51)	<0.001
SDA (18:4 n-3)	.011 (.0069, .015)	.008 (.004, .014)	.008
ETA (20:4 n-3)	.012 (.0092, .018)	.014 (.011, .019)	.28
EPA	.067 (.047, .1)	.14 (.11, .19)	<0.001
DPA n-3	.021 (.014, .03)	.031 (.022, .04)	.015
DHA	.62 (.43, .8)	1.1 (.84, 1.6)	<0.001
4-HDHA	.074 (.041, .12)	.12 (.066, .17)	.004
10-HDHA	.03 (.024, .041)	.034 (.021, .072)	.058
14-HDHA	.19 (.15, .31)	.2 (.12, .31)	.71
17-HDHA	.18 (.12, .3)	.2 (.14, .28)	.59
19,20-EpDPA	.033 (.018, .049)	.077 (.057, .11)	<0.001
Other			
Mead	.01 (.0071, .018)	.009 (.006, .016)	.08

* Based on the Wilcoxon matched-pairs signed-ranks test

** 9,10-EpOME & 9,10,13-TriHOME had >50% values below LOQ.

Concentrations: fatty acids (ug/mL); oxylipins (ng/mL)

Table S4: DHA supplementation vs Control at week 24, missing values imputed

	Control (n=21)	DHA (n=27)	Nonparametric ANCOVA*	
	Median (IQR)	Median (IQR)	Difference (95% CI)	p-value
Omega-6 fatty acids and oxylipins				
LA**	7.1 (4.1, 9.6)	7.6 (4.8, 11)	.75 (-.99, 2.5)	.43
9-HODE	3.1 (1.2, 7.4)	2.4 (1, 6.2)	.039 (-4.5, 3.9)	.98
13-HODE	7.6 (5, 21)	5.6 (2.6, 13)	-.79 (-8.5, 8)	.86
9,10-DiHOME	1.6 (1.1, 4.3)	1.3 (.64, 4.1)	.39 (-2, 2.9)	.74
13H-9,10E-LA	.21 (.15, .34)	.17 (.077, .28)	.0037 (-.18, .19)	.96
9H-12,13E-LA	.14 (.1, .22)	.1 (.025, .23)	.013 (-.2, .23)	.89
9,12,13-TriHOME	.1 (.05, .15)	.086 (.05, .12)	-.019 (-.066, .033)	.44
GLA	.048 (.033, .063)	.05 (.028, .075)	.0014 (-.019, .019)	.88
DGLA	.092 (.077, .11)	.098 (.082, .12)	.02 (.0023, .034)	.019
AA	.14 (.12, .17)	.18 (.13, .21)	.044 (.016, .072)	.002
5-HETE	.16 (.12, .17)	.14 (.12, .19)	.02 (-.052, .098)	.58
12-HETE	.088 (.08, .12)	.17 (.1, .23)	.071 (.0082, .13)	.036
15-HETE	.21 (.17, .22)	.19 (.12, .32)	.023 (-.055, .1)	.55
TxB2	.053 (.039, .11)	.057 (.036, .14)	.01 (-.038, .064)	.69
DTA	.034 (.027, .047)	.035 (.028, .048)	.007 (-.0015, .015)	.09
DPA n-6	.042 (.036, .049)	.054 (.042, .085)	.027 (.012, .04)	<0.001
Omega-3 fatty acids and oxylipins				
ALA	.26 (.17, .48)	.29 (.18, .51)	.03 (-.1, .13)	.60
SDA (18:4 n-3)	.006 (.0045, .009)	.008 (.004, .014)	.0013 (-.0019, .004)	.40
ETA (20:4 n-3)	.012 (.0089, .014)	.014 (.011, .019)	.0025 (-.001, .0055)	.16
EPA	.082 (.054, .095)	.14 (.11, .19)	.078 (.05, .1)	<0.001
DPA n-3	.023 (.015, .031)	.031 (.022, .04)	.009 (-.00005, .016)	.022
DHA	.52 (.4, .73)	1.1 (.84, 1.6)	.63 (.4, .86)	<0.001
4-HDHA	.05 (.03, .076)	.12 (.066, .17)	.095 (.008, .2)	.055
10-HDHA	.018 (.013, .037)	.034 (.021, .072)	.02 (.0044, .036)	.008
14-HDHA	.078 (.059, .12)	.2 (.12, .31)	.12 (-.0085, .25)	.06
17-HDHA	.11 (.094, .19)	.2 (.14, .28)	.052 (-.095, .16)	.40
19,20-EpDPA	.034 (.018, .056)	.077 (.057, .11)	.035 (.018, .054)	<0.001
Other				
Mead	.011 (.0071, .013)	.009 (.006, .016)	.003 (.0001, .007)	.049

* Difference is estimated using a nonparametric ANCOVA controlling for the mediator at Week 14. A negative difference means the DHA group had a lower concentration than the control group.

** 9,10-EpOME & 9,10,13-TriHOME had >50% values below LOQ.

Concentrations: fatty acids (ug/mL); oxylipins (ng/mL)

Table S5: Risk of spontaneous preterm birth according to concentrations of oxylipins & precursor fatty acids, missing values imputed*

	Week 14 (n=48)				Week 24 (n=48)			
	Below Median	Above Median	Odds Ratio	p-value	Below Median	Above Median	Odds Ratio	p-value
Omega-6 fatty acids and oxylipins								
LA**	17%	33%	2.7	.16	21%	29%	1.6	.48
9-HODE	32%	17%	0.4	.22	25%	25%	1.0	.98
13-HODE	25%	25%	1.0	.99	29%	21%	0.6	.49
9,10-DiHOME	25%	25%	1.0	.99	24%	26%	1.1	.87
13H-9,10E-LA	29%	21%	0.6	.52	25%	25%	1.0	.99
9H-12,13E-LA	25%	25%	1.0	.98	25%	25%	1.0	.99
9,12,13-TriHOME	17%	33%	2.5	.18	21%	29%	1.6	.52
GLA	17%	33%	2.7	.16	21%	29%	1.6	.46
DGLA	32%	17%	0.4	.23	25%	25%	1.1	.90
AA	25%	25%	1.0	.99	12%	38%	4.4	.050
5-HETE	8%	42%	8.2	.014	21%	29%	1.6	.49
12-HETE	21%	29%	1.6	.50	21%	29%	1.6	.50
15-HETE	12%	38%	4.4	.050	17%	33%	2.7	.16
TxB2	21%	29%	1.6	.49	25%	25%	1.0	.97
DTA	29%	21%	0.6	.50	25%	25%	1.1	.93
DPA n-6	25%	25%	1.0	.98	25%	25%	1.0	.95
Omega-3 fatty acids and oxylipins								
ALA	17%	33%	2.7	.16	12%	38%	4.3	.053
SDA (18:4 n-3)	15%	38%	3.6	.07	25%	26%	1.1	.93
ETA (20:4 n-3)	29%	22%	0.6	.51	25%	26%	1.0	.94
EPA	21%	29%	1.6	.51	21%	29%	1.6	.48
DPA n-3	25%	25%	1.0	.94	20%	30%	1.8	.39
DHA	24%	26%	1.1	.85	21%	29%	1.6	.49
4-HDHA	8%	42%	8.0	.015	21%	29%	1.6	.49
10-HDHA	17%	33%	2.6	.17	21%	29%	1.6	.47
14-HDHA	17%	33%	2.8	.15	25%	25%	1.0	.98
17-HDHA	12%	38%	4.5	.049	17%	33%	2.5	.18
19,20-EpDPA	12%	38%	4.4	.050	17%	33%	2.5	.19
Other								
Mead	17%	33%	2.5	.18	25%	25%	1.0	.97

* Each compound was dichotomized according to median split at each timepoint. Odds ratios and p-values were from logistic regressions adjusted for age and parity. Due to the selection process for inclusion in this substudy, the overall risk of preterm birth is 25%.

** 9,10-EpOME & 9,10,13-TriHOME had >50% values below LOQ.

CHAPTER 6:

GENERAL DISCUSSION

6.1 Significance/contributions to the discipline and future directions

6.1.1 Perspective: Oxylipins in pregnancy and neurodevelopment as a nascent field

The ultimate motivation that led to this Thesis was the prospect of determining roles that specific oxylipins play in pregnancy and neurodevelopment. However, at the time this Thesis was initiated, little was known about oxylipin exposures or actions in pregnancy or development, and many of the tools needed to address these gaps were not widely available. Thus, much of the work for this Thesis was directed toward filling research gaps to enable, and stimulate, future research to definitively determine whether and how specific oxylipins affect pregnancy, parturition, and neurodevelopment. The collective work has advanced the field by distilling new questions and hypotheses, and by generating findings and scientific leads that can inform the design of future, definitive studies. This future work could ultimately have important implications for human populations by informing strategies for manipulating oxylipins as in a manner to decrease the risk preterm birth and/or to favorably impact neurodevelopmental trajectory. Accomplishments and future studies that grew out of these Thesis projects are summarized below. The most direct accomplishment of this Thesis is the publication of two manuscripts in peer-reviewed scientific journals, and a third manuscript that is currently being reviewed for publication. Key findings from these three Thesis projects and future studies that grew out of each project are summarized below.

6.1.2 Thesis Project 1: Effects of diets enriched in linoleic acid and its peroxidation products on brain fatty acids, oxylipins, and aldehydes in mice

A literature review identified manuscripts from the 1960s-1980s that demonstrated in chicks that consumption of heated oils rich in LA without adequate vitamin E or intravenous administration of HPODEs (1-4) produces cerebellar necrosis and ataxia, suggesting that dietary or endogenously produced OXLAMs could potentially have adverse effects in CNS tissues. However, it was not yet known whether eating non-oxidized or heated vegetable oils has biochemical consequences in mammalian brain. To help fill this gap, in Project 1 we tested biochemical consequences of feeding of either non-oxidized high LA oil or heated high LA corn oil to mice. The central finding of Project 1 was that increasing dietary non-oxidized LA significantly increased n-6 fatty acids, decreased n-3 fatty acids, and increased OXLAMs in brain. Dietary OXLAMs had minimal effect on oxidized lipids but did decrease both n-6 and n-3 fatty acids. Together, findings indicate that brain fatty acids are modulated by both dietary LA and OXLAMs, while brain OXLAMs are regulated by endogenous synthesis from LA, rather than incorporation of preformed OXLAMs. These findings represent an important first step by demonstrating that there are biochemical consequences in the mammalian brain from consuming high LA diets. Two projects in rats (Section 6.3.1) have grown out of these findings, further investigating the brain-related biochemical, pathological and behavioral effects of exposure to high amounts of either dietary LA or amounts of dietary OXLAMs that are consistent with industrialized diets containing heated high-LA oils, respectively.

6.1.3 Thesis Project 2: Temperature and time-dependent effects of blood processing on oxylipin concentrations in human plasma

The second and third Thesis projects are closely related and share the overarching goal of investigating whether oxylipins measured in plasma can serve as mechanism-based, pharmacodynamic biomarkers to help predict the risk of preterm birth and/or developmental endpoints in humans. The term pharmacodynamic here signifies that these compounds can likely to altered with targeted provision of dietary interventions or supplements. These studies required the design, development and validation of a targeted lipid mediator profiling assay that prioritizes the most relevant oxylipins and unesterified fatty acids. I planned to use these assays to profile oxylipins and unesterified fatty acids on the highly unique plasma samples collected in pregnant Australian women at weeks 14 and 24 of gestation as part of the ORIP study. Like most clinical studies, however, whole blood collected in ORIP was not immediately processed to plasma, which this could potentially impact results and interpretation. Thus, in Project 2, I systematically tested effects of delays in processing, and of temperature (cold versus room temperature) prior to processing, on oxylipin concentrations. Using the ORIP procedures and using plasma EDTA tubes, I observed that many plasma oxylipins can be measured with reasonable accuracy despite delayed processing for up to two hours, even at room temperature. 12-lipoxygenase- and platelet-derived oxylipins were observed to be sensitive to post-collection artifact with delayed processing at room temperature, however these were stable when whole blood is stored on ice prior to centrifugation. These observations could have important implications for design, implementation, and interpretation of clinical studies measuring oxylipins in plasma. For the current thesis, these findings indicated that our team could proceed with the ORIP plasma analyses with confidence, with the caveat that any findings related to 12-

HETE, 14-HDHA and TXB2 should be interpreted with some caution. In Manuscript 2, I also emphasized several additional pre-analytical variables—including venipuncture location, needle gauge, blood collection tube type, duration and temperature of centrifugation, and post-processing storage conditions—that have not been adequately assessed and are worthy of investigation. While these investigations are beyond the scope of our current work, it is anticipated that the unexpected results presented here will spur other investigators to systematically investigate the impacts of each of these pre-analytical variables on measured oxylipin concentrations in relevant tissues.

6.1.4 Thesis Project 3: Plasma oxylipins and unesterified precursor fatty acids are altered by DHA supplementation in pregnancy: Can they help predict risk of preterm birth?

Plausible mechanisms exist whereby oxylipins derived from DHA, AA and LA could have favorable or unfavorable effects on the placental function and brain development. However, there is currently a limited understanding of whether and how oxylipins influence biochemical and clinical endpoints in pregnancy and development. In Thesis project 3, I sought to help fill these gaps by profiling oxylipins and their precursor fatty acids using plasma samples collected from pregnant Australian women enrolled in the ORIP (Omega-3 fats to Reduce the Incidence of Prematurity) study. ORIP collected plasma from a subgroup of participants at study entry (pregnancy week 14) and again at ≈pregnancy week 24, providing a unique opportunity to: (1) describe the levels and trajectory of changes in plasma unesterified oxylipins and precursor fatty acids in early pregnancy and when nearing the time of preterm or term birth; (2) determine the impact of DHA supplementation on oxylipins and precursor fatty acids; and (3) to explore whether differences in oxylipins and precursor fatty acids at pregnancy weeks 14 and 24 hold promise as biomarkers for predicting risk of preterm birth. Our team therefore used liquid chromatography-tandem mass spectrometry to profile precursor fatty acids and oxylipins in plasma collected at study entry (≈pregnancy week 14) and again at ≈week 24, in a subgroup of 48 ORIP participants—12 cases with spontaneous preterm (<37 weeks) birth and 36 matched controls with spontaneous term (>40 weeks) birth. I observed that: (1) unesterified AA and AA-derived oxylipins 12-HETE, 15-HETE and TXB2 declined between weeks 14-24 of pregnancy without DHA supplementation; (2) compared to placebo, DHA supplementation increased unesterified DHA, EPA, and AA, DHA-derived 4-HDHA, 10-HDHA and 19,20-EpDPA, and AA-derived 12-HETE; (3) participants with concentrations above the median for 5-lipoxygenase

derivatives of AA (5-HETE) or DHA (4-HDHA) at 14 weeks or unesterified AA at 24 weeks had higher risk of preterm birth. These results provide an intriguing clue that 5-lipoxygenase-derived oxylipins and/or unesterified AA, which are indicative of one arm of immune activation, could potentially serve as mechanism-based biomarkers for predicting preterm birth. One important limitation of the present studies is that PGE2 was below the limit of quantitation in most of our plasma samples. Thus, we were not able to adequately test whether plasma PGE2 is a useful biomarker for predicting preterm birth. Since PGE2 and other 2-series prostaglandins are known to play a central role in cervical ripening and uterine contractility, future studies are needed to determine whether circulating levels of PGE2 are predictive. This may require collecting another tissue (i.e. serum, urine) or using a different methodology (i.e. enzyme linked immunosorbent assay, or a different mass spectrometry method). Together with previous work by others, the findings of this ORIP plasma substudy, suggest candidate mechanism-based biomarkers that hold promise for predicting preterm birth (**Fig. 1**).

My hypothesis is that 5-lipoxygenase-derived oxylipins, unesterified AA, and/or 2-series prostaglandins should be evaluated in larger, adequately powered studies. Given the small size of this plasma cohort I examined, the latter exploration is anticipated to inform sample size calculations for larger, more adequately powered studies testing and associations between these variables and preterm birth. I anticipate that these data will be used to perform sample size calculations and to inform the design of such studies. Such studies could ultimately determine whether specific oxylipins (or combinations as part of a risk metric) measured in plasma can serve as mechanism-based, pharmacodynamic biomarkers to help predict the risk of preterm birth and/or neurodevelopmental endpoints (see planned future study in Section 6.4).

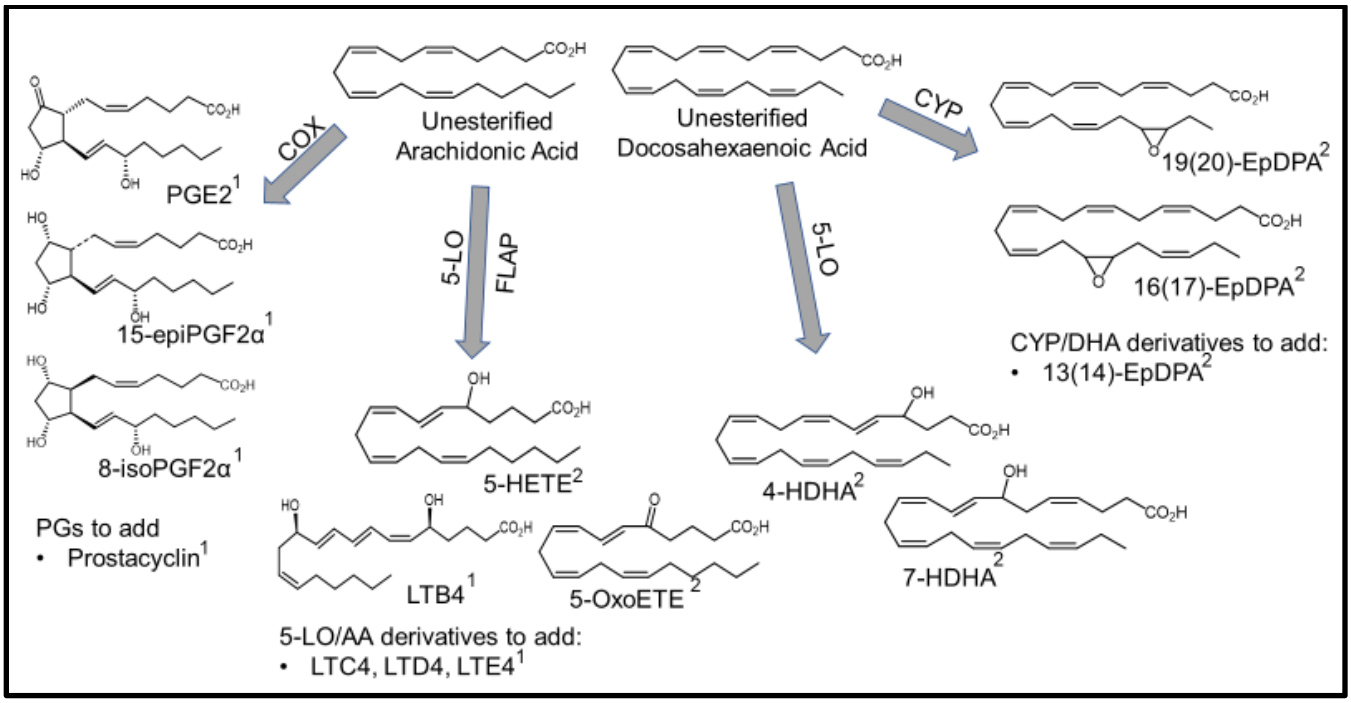


Figure 1. Candidate mechanism-based biomarkers for predicting preterm birth. *Preliminary evidence suggests that 5-lipoxygenase (5-LO) derivatives of AA and DHA, cytochrome P450 (CYP) derivatives of DHA and 2-series prostaglandins could potentially serve as prognostic markers for birth.* [1]*indicates that alternative tissues or methods may be required to measure these compounds;* [2]*indicates that these oxylipins are present in both free and esterified lipid pools. Future studies should assess both pools as candidate biomarkers.*

6.2. Research limitations

Central limitations of each of these projects are included in the respective Discussion sections of each published manuscript and will be briefly summarized here. An important limitation of Project 1 was the use of chow rather than gavage to control the nutrient compositions of the diet interventions. Like chow, gavage allows for precise control of the nutrient composition of provided foods. However, because gavage also controls the *amount* of food provided, gavage allows for a tighter control over nutrient intakes. To address this limitation of Project 1, gavage is used in follow-up Study 1 (described in Section 6.3.1). Project 2 and 3 limitations included the small sample sizes and the substantial number of samples with oxylipin values that were below the limits of quantitation. Some oxylipins have potent actions at low concentrations, and it is therefore possible that certain labile oxylipins were already degraded before quantitation or during sample preparation. Projects 2 and 3 used standard clinical "purple top" potassium EDTA tubes for blood collection and therefore findings are not necessarily generalizable to blood collected using other tubes or for serum or other blood fractions. As with all clinical biomarker studies, an inherent limitation of these two studies is that measured compounds could potentially be altered by numerous pre-analytical variables and are therefore do not exactly reflect *in vivo* conditions. The potential for preanalytical oxidation or degradation of oxylipins and/or precursor fatty acids is a limitation shared by all research in this field that should be considered when interpreting findings. We analyzed oxylipins as free acids only and therefore did not account for the oxylipins present in the phospholipids, triglycerides, or cholesteryl esters that are abundant in circulating lipoproteins. Due to the observational nature of Project 3, findings should not be interpreted as causal. Findings form Project 3 also require replication and expansion, as described in the Future Directions section 6.3 below.

6.3 Future directions

The findings presented in this Thesis have provided a foundation for, and helped to stimulate, ongoing research into the roles of oxylipins and their manipulation in pregnancy and neurodevelopment. Here I summarize three projects that have grown out of the Thesis work.

6.3.1 New Research resulting from Thesis Project 1

Follow-up project 1: Role of excess maternal LA intake on infant neurodevelopment (A. Taha, R21-HD095391-01A1)

Since LA itself (rather than dietary OXLAMs) appears to be the primary source of brain OXLAMs in vivo (Thesis Project 1), understanding how this process is regulated during development is an important next step. Ameer Taha, PhD, and his UC-Davis team submitted a successful NIH R21 grant to test the overall hypothesis that early maternal exposure to excess (non-oxidized) dietary LA will impair neurodevelopment by *in vivo* generation of excessive levels of OXLAMs in the developing brain. The specific aims are to: (1) Determine the accumulation of specific OXLAMs in the developing brain in response to varying levels of maternal dietary LA during pregnancy, lactation and both periods to identify potential windows of maternal LA vulnerability; (2) Assess neurodevelopmental endpoints— including neuroinflammation, neurogenesis, dendritic arborization, and apoptosis in the neocortex and hippocampus—in offspring of dams fed varying levels of LA during pregnancy, lactation or both periods; and (3) Test specific OXLAMs identified *in vivo* in primary neuron-glia co-cultures derived from the developing rat neocortex and hippocampus to investigate links between OXLAMs and neuronal viability and morphogenesis in brain regions critical to development.

6.3.2 Follow-up project 2: Effects of Heated Oil and Hydroperoxy Fatty Acid Consumption on Rodent Behavior and Neuropathology (NIH animal protocol# ASP-1409).

My Unit in the intramural program of NIA/NIAAA is conducting a tightly controlled feeding study that is designed to isolate the effects of heated high-LA oils, or a specific component of heated oils (hydroperoxy-octadecadienoic acids), on biochemical, histopathological, and cognitive endpoints in male and female rats (led by A. Domenichiello and J. Jensen in my Unit).

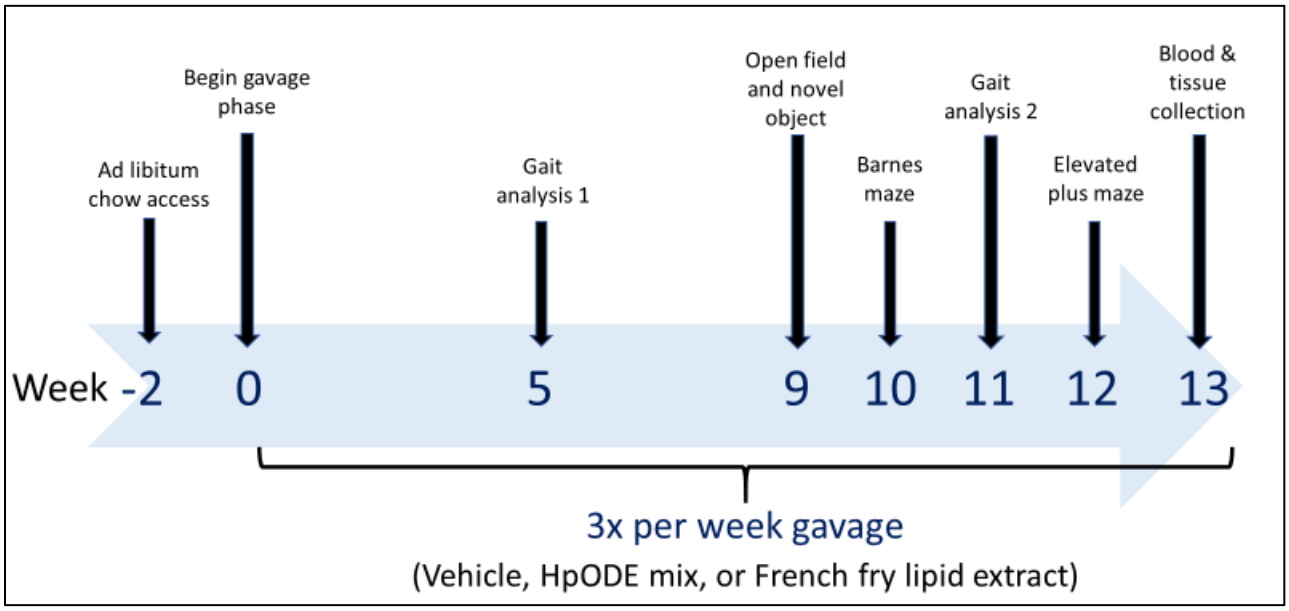


Figure 2. Timeline of rat HPODE and french fry lipid extract feeding study

The goal is to investigate the overall hypothesis that heated high-LA oil, or HPODEs that enriched in such oils, could affect vascular and central nervous system (CNS) biochemistry and contribute to CNS pathologies. Specific aims are to determine the effects of heated oil lipid extract and HPODEs, versus control gavage on: (1) markers of CNS pathologies including endothelial activation, blood brain barrier dysfunction, and neuroinflammation; (2) levels of specific oxylipins and related peroxidation products in blood, liver and brain tissues; and (3) to

explore effects on cognition and behavior. In Project 1 of the Thesis, we observed that mice consuming chow made with heated corn oil gained less weight than those consuming normal chow. It was not clear whether this was due to biochemical effects of the components of the heated oil or an aversion to the chow containing it, with lower dietary consumption. To address this gap, and to ensure tightly controlled alterations in dietary variables, we are currently delivering the interventions via gavage with otherwise identical background diets. The timeline for gavage and study endpoints are shown in **Fig 2**. Notably, commercially available rodent chows are generally supplemented with 10-50-times higher amounts of vitamin E than is relevant for humans consuming modern diets. Since tocopherols can protect against lipid peroxidation (5, 6), these unnaturally high amounts of Vitamin E may mask any effects of eating high amounts of LA or OXLAMs. We therefore designed the background diets and gavage components to ensure that levels of tocopherols are adequate but consistent with normal human diets. Together, these two follow-up studies that grew out of Thesis Project 1 will fill key evidence gaps and advance our understanding of the effects of exposures to high amounts of dietary LA and heated high-LA oils on central nervous system biochemistry, pathology, and cognitive endpoints.

6.3.3 Follow-up study 3: Oxylipins and the molecular pathways involved in their biosynthesis, transport and inactivation in human placenta

Omega-6 and omega-3 fatty acids and their oxylipin derivatives are proposed to play important roles in pregnancy, parturition, and neurodevelopment (reviewed in Thesis section 1.3). However, our understanding of the specific oxylipins and molecular pathways involved in their biosynthesis, transport and inactivation in human placenta is incomplete. In follow-up project 3, we are applying a systems approach to characterize a broad range of oxylipins and their precursor fatty acids in human placental tissues. We also extracted open data allowing for

quantitative characterization of the expression of genes coding for proteins involved in oxylipin biosynthesis, transport, signaling and inactivation from placental specimens, collected from the maternofetal interface. The observed oxylipins, precursor fatty acids, and gene expression signatures are intended to provide clues for molecular pathways and specific oxylipins that are active in placenta and modulated by parturition. For this project, we are focusing on pathways involved in the synthesis and signaling of several families of oxylipins with plausible links to parturition including prostaglandins, 5-lipoxygenase derivatives of AA (leukotrienes, 5-HETE), monohydroxy- and epoxy- and specialized pro-resolving mediators derived from DHA.

These pathways and mediators could potentially play a role in preterm and normal term parturition, as well as the synthesis and maternofetal transport of lipids during this period of rapid brain growth. Therefore, improved understanding of the pathways investigated in this project could have implications for pregnancy, parturition, and/or neurodevelopment. These studies are intended to determine whether any oxylipins derived from DHA, AA, or LA, including prostaglandins, leukotrienes, n-3 epoxides and monohydroxy-fatty acids and oxidized linoleic acid metabolites, are abundant in placenta following normal spontaneous delivery, or following uncomplicated C-section (no labor). Further, in combination with gene expression profiles and precursor fatty acids, placental oxylipin concentrations will provide insight into the molecular pathways involved in biosynthesis, transport and inactivation of oxylipins. Collective findings are intended to help refine our hypotheses and to serve as a basis for future, more targeted studies characterizing the molecular pathways regulating oxylipin biosynthesis, transport and inactivation in human placenta. This project will further expand and refine the target list of oxylipins that should be assessed as mechanism-based biomarkers in future, larger, prospective observational cohorts.

6.3.4 Follow-up study 4: Can oxylipins serve as mechanism-based biomarkers for risk of preterm birth?

The intriguing results of Thesis project 3 (ORIP Plasma Substudy (see Chapter 5)) suggest that 5-lipoxygenase-derived oxylipins and/or unesterified AA (or combinations as part of a risk metric) measured in plasma could potentially serve as mechanism-based, pharmacodynamic biomarker to help predict the risk of preterm birth and/or neurodevelopmental endpoints. Drs. Taha, Gibson, Makrides and I are currently using the results of Thesis Project 3 as the basis for sample size calculations to inform the design of a much larger observational study to definitively test this hypothesis.

6.4 Summary and conclusion

The series of projects undertaken for this Thesis has helped open-up a new line of inquiry with the long-term goal of determining whether and how oxylipins derived from AA, DHA and/or LA and their unesterified precursor fatty acids affect pregnancy, parturition, and development. Important findings include the demonstration that increasing dietary LA alters brain biochemistry in a mammalian model, effects of delayed processing on clinical plasma samples, and identification of candidate oxylipins as mechanism-based biomarkers in plasma that may help predict preterm birth in humans. Findings suggest the need to move past the traditional measure of esterified fatty acid levels in blood and tissues and to measure unesterified fatty acids their oxylipin derivatives as likely mediators of change in humans. The work described herein has revealed important insights and has stimulated ongoing and future research into the roles of oxylipins in pregnancy and development. Such work is likely to have important implications for human populations by informing strategies for manipulating oxylipins as in a manner to decrease the risk preterm birth and/or favorably impact developmental trajectory.

6.5 References (Chapter 6)

1. Budowski P, Bartov I, Dror Y, Frankel EN. Lipid oxidation products and chick nutritional encephalopathy. Lipids. 1979;14(9):768-72.

2. TSUCHIYAMA H, NISHIDA T, KUMMEROW FA. Pathology of chick encephalomalacia. Peroxides or vitamin E deficiency as causes. Arch Pathol. 1962;74:208-13.

3. CENTURY B, HORWITT MK, BAILEY P. Lipid factors in the production of encephalomalacia in the chick. AMA Arch Gen Psychiatry. 1959;1:420-4.

4. Cheville NF. The pathology of vitamin E deficiency in the chick. Pathol Vet. 1966;3(3):208-25.

5. Fukuzawa K. Dynamics of lipid peroxidation and antioxidion of alpha-tocopherol in membranes. J Nutr Sci Vitaminol (Tokyo). 2008;54(4):273-85.

6. Atkinson J, Epand RF, Epand RM. Tocopherols and tocotrienols in membranes: a critical review. Free Radic Biol Med. 2008;44(5):739-64.

APPENDICES

Appendix 1. Methods used for oxylipin analyses in Projects 2 and 3 (published methods paper) Yuan ZX, Majchrzak-Hong S, Keyes GS, Iadarola MJ, Mannes AJ, Ramsden CE. Lipidomic profiling of targeted oxylipins with ultra-performance liquid chromatography-tandem mass spectrometry. *Anal Bioanal Chem*. 2018;410(23):6009-29. doi: 10.1007/s00216-018-1222-4. (PMID: 30074088)

Appendix 2. De-identification agreement for sample analysis and approved exemption for analysis of de-identified plasma specimens

Appendix 3 (A3.1-A3.3). Manuscripts preceded by author attestations

A3.1. Published manuscript 1 for Chapter 3: C.E. Ramsden, M. Hennebelle, S. Schuster, G.S. Keyes, C. Johnson, I. Kirpich, J. E. Dahlen, M.S. Horowitz, D. Zamora, A.E. Feldstein, C. McClain, B. Muhlhausler, M. Makrides, R.A. Gibson, A.Y. Taha. Effects of diets enriched in linoleic acid and its peroxidation products on brain fatty acids, oxylipins, and aldehydes in mice. *Biochim Biphys Acta (BBA) - Molecular and Cell Biology of Lipids*. 2018 Oct; 1863(10):1206-1213). doi:10.1016/j.bbalip. 2018.07.007.

A3.2. Published manuscript 2 for Chapter 4: C.E. Ramsden, Z. Yuan, M.S. Horowitz, D. Zamora, S. Majchrzak-Hong, B. Muhlhausler, A.Y. Taha, M. Makrides, R.A. Gibson. Temperature and time-dependent effects of delayed blood processing on oxylipin concentrations in human plasma. *Prostaglandins, Leukotrienes and Essential Fatty Acids*. 2019; 150; 31-37; Sept 4 https://doi.org/10.1016/j.plefa.2019.09.001

A3.3 Published manuscript 3 for Chapter 5: C.E. Ramsden, M. Makrides, Z. Yuan, M.S. Horowitz, D. Zamora, L.N. Yelland, K. Best, J. Jensen, A.Y. Taha, R.A. Gibson. Plasma oxylipins and unesterified precursor fatty acids are altered by DHA supplementation in pregnancy: Can they help predict risk of preterm birth? Prostaglandins, Leukotrienes and Essential Fatty Acids. 2020; 153; 102041 https://doi: 10.1016/j.plefa.2019.102041 (published online 2019 Dec 13, 2019).

Appendix 2

Dr. Ramsden requested to add an additional collaborator (Maria Makrides, PhD, University of Adelaide and SAHMRI Institute (Adelaide, Australia)) as a collaborator via an amendment to ongoing project 17-NIA-00007-1 (03/20/2018) entitled “Lipid measurements in coded human specimens”. In this project we use our liquid chromatography tandem mass spectrometry assays to quantify lipid metabolites and their precursor fatty acids in blood and other specimens collected from multiple human studies. This ongoing project was deemed exempt by the National Institutes of Health Human Research Protection Program so long as the specified oxylipin analyses are performed on coded samples provided from an IRB-approved protocol on the condition that a de-identification agreement is documented between Professor Makrides and Dr.

Institute (Adelaide, Australia)).

Statement of Authorship

Title of Paper	Effects of diets enriched in linoleic acid and its peroxidation products on brain fatty acids, oxylipins, and aldehydes in mice.
Publication Status	☑ Published ☐ Accepted for Publication ☐ Submitted for Publication ☐ Unpublished and Unsubmitted w ork w ritten in manuscript style
Publication Details	Published in 2018 in Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids (2018 Oct;1863(10) 1206-1213). doi: 10.1016/j.bbalip.2018.07.007; Epub 2018 Jul 25; PMID: 30053599.

Principal Author

Name of Principal Author (Candidate)	Christopher E. Ramsden
Contribution to the Paper	Drafted the manuscript, compiled revisions from co-authors, submitted and revised manuscript for publication. Led development of the brain-related hypotheses. Led design of the study diets. Designed and developed the 'OXCORN' (thermally stressed corn oil product enriched in HPODEs). Contributed to experimental design, data and statistical analyses, and table and figure preparation.
Overall percentage (%)	65%
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
Signature	Date 12/6/2019

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Hennebelle M		
Contribution to the paper	Performed the oxylipin analyses and quantitation. Critically revised the manuscript.		
Signature		Date	

Name of Co-Author	Schuster S		
Contribution to the Paper	Performed and led parallel mouse liver experiments including feeding, tissue harvest, and western blot analysis. This project provided the brain specimens. Critically revised this manuscript.		
Signature		Date	

Name of Co-Author	Keyes GS		
Contribution to the Paper	Developed and synthesized the OXCORN (thermally stressed high linoleic acid oil) product in collaboration with C. Ramsden. Critically revised the manuscript.		
Signature		Date	12/6/2019

Name of Co-Author	Johnson CD		
Contribution to the Paper	Intellectual input into analyses. Critically revised the manuscript.		
Signature		Date	

Name of Co-Author	Kirpich IA		
Contribution to the Paper	Intellectual input into study design. Critically revised the manuscript.		
Signature		Date	

Name of Co-Author	Dahlen JE		
Contribution to the Paper	Performed the mouse tissue harvest and critically revised the manuscript.		
Signature		Date	

Name of Co-Author	Horowitz MS		
Contribution to the Paper	Made key contributions to data analysis, table and figure preparation in collaboration with C. Ramsden. Critically revised the manuscript.		
Signature		Date	

Name of Co-Author	Zamora D		
Contribution to the Paper	Led the statistical analyses in collaboration with C. Ramsden. Critically revised the manuscript.		
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Name of Co-Author	Feldstein AE		
Contribution to the Paper	Intellectual input into study design. Critically revised the manuscript.		
Signature		Date	

Name of Co-Author	McClain CJ		
Contribution to the Paper	Intellectual input into study design. Critically revised the manuscript.		
Signature		Date	

Name of Co-Author	Muhlhausler BS		
Contribution to the Paper	Intellectual input into study design. Critically revised the manuscript.		
Signature		Date	

Name of Co-Author	Makrides M		
Contribution to the Paper	Provided intellectual input into hypothesis development, study design and analyses. Critically revised the manuscript.		
Signature		Date	19/11/19

Name of Co-Author	Gibson RA		
Contribution to the Paper	Provided intellectual input into hypothesis development, study design and analyses. Critically revised the manuscript..		
Signature		Date	19/11/19

Name of Co-Author	Taha AY
Contribution to the Paper	Provided intellectual input into hypothesis development, study design and analyses. Oversaw the oxylipin and fatty acid analyses. Critically revised the manuscript.

Statement of Authorship

Title of Paper	Effects of diets enriched in linoleic acid and its peroxidation products on brain fatty acids, oxylipins, and aldehydes in mice.
Publication Status	☑ Published ☐ Accepted for Publication ☐ Submitted for Publication ☐ Unpublished and Unsubmitted w ork w ritten in manuscript style
Publication Details	Published in 2018 in Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids (2018 Oct;1863(10):1206-1213). doi: 10.1016/j.bbalip.2018.07.007; Epub 2018 Jul 25; PMID: 30053599.

Principal Author

Name of Principal Author (Candidate)	Christopher E. Ramsden		
Contribution to the Paper	Drafted the manuscript, compiled revisions from co-authors, submitted and revised manuscript for publication. Led development of the brain-related hypotheses. Led design of the study diets. Designed and developed the 'OXCORN' (thermally stressed corn oil product enriched in HPODEs). Contributed to experimental design, data and statistical analyses, and table and figure preparation.		
Overall percentage (%)	65%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Hennebelle M		
Contribution to the paper	Performed the oxylipin analyses and quantitation. Critically revised the manuscript.		
Signature		Date	18th November 2019

Name of Co-Author	Schuster S
Contribution to the Paper	Performed and led parallel mouse liver experiments including feeding, tissue harvest, and western blot analysis. This project provided the brain specimens. Critically revised this manuscript.

Name of Co-Author	Feldstein AE		
Contribution to the Paper	Intellectual input into study design. Critically revised the manuscript.		
Signature		Date	

Name of Co-Author	McClain CJ		
Contribution to the Paper	Intellectual input into study design. Critically revised the manuscript.		
Signature		Date	

Name of Co-Author	Muhlhausler BS		
Contribution to the Paper	Intellectual input into study design. Critically revised the manuscript.		
Signature		Date	

Name of Co-Author	Makrides M		
Contribution to the Paper	Provided intellectual input into hypothesis development, study design and analyses. Critically revised the manuscript.		
Signature		Date	

Name of Co-Author	Gibson RA		
Contribution to the Paper	Provided intellectual input into hypothesis development, study design and analyses. Critically revised the manuscript..		
Signature		Date	

Name of Co-Author	Taha AY		
Contribution to the Paper	Provided intellectual input into hypothesis development, study design and analyses. Oversaw the oxylipin and fatty acid analyses. Critically revised the manuscript.		
Signature		Date	Nov 11th 2019

Statement of Authorship

Title of Paper	Effects of diets enriched in linoleic acid and its peroxidation products on brain fatty acids, oxylipins, and aldehydes in mice.
Publication Status	☑ Published ☐ Accepted for Publication ☐ Submitted for Publication ☐ Unpublished and Unsubmitted w ork w ritten in manuscript style
Publication Details	Published in 2018 in Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids (2018 Oct;1863(10):1206-1213). doi: 10.1016/j.bbalip.2018.07.007; Epub 2018 Jul 25; PMID: 30053599.

Principal Author

Name of Principal Author (Candidate)	Christopher E. Ramsden		
Contribution to the Paper	Drafted the manuscript, compiled revisions from co-authors, submitted and revised manuscript for publication. Led development of the brain-related hypotheses. Led design of the study diets. Designed and developed the 'OXCORN' (thermally stressed corn oil product enriched in HPODEs). Contributed to experimental design, data and statistical analyses, and table and figure preparation.		
Overall percentage (%)	65%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Contribution to the Paper	Performed and led parallel mouse liver experiments including feeding, tissue harvest, and western blot analysis. This project provided the brain specimens. Critically revised this manuscript.		
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Contribution to the Paper	Developed and synthesized the OXCORN (thermally stressed high linoleic acid oil) product in collaboration with C. Ramsden. Critically revised the manuscript.		
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Contribution to the Paper	Intellectual input into analyses. Critically revised the manuscript.		
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Contribution to the Paper	Made key contributions to data analysis, table and figure preparation in collaboration with C. Ramsden. Critically revised the manuscript.		
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Statement of Authorship

Title of Paper	Temperature and time-dependent effects of delayed blood processing on oxylipin concentrations in human plasma.
Publication Status	☑ Published ☐ Accepted for Publication ☐ Submitted for Publication ☐ Unpublished and Unsubmitted w ork w ritten in manuscript style
Publication Details	Published in 2019 in Prostaglandins Leukotrienes and Essential Fatty Acids. (2019 Sep 5;150:31-37; PMID: 31568925 DOI: 10.1016/j.plefa.2019.09.001).

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Name of Principal Author (Candidate)	Christopher E. Ramsden
Contribution to the Paper	Drafted the manuscript, compiled revisions from co-authors, submitted and revised manuscript for publication. Led development of the hypotheses. Led design and implementation of the blood collection and processing. Oversaw the oxylipin analyses. Led interpretation of findings. Contributed to data and statistical analyses, and table and figure preparation.
Overall percentage (%)	80%
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
Signature	Date 12/6/2019

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Name of Co-Author	Taha AY		
Contribution to the Paper	Input into study design and interpretation. Critically revised the manuscript.		
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Statement of Authorship

Title of Paper	Temperature and time-dependent effects of delayed blood processing on oxylipin concentrations in human plasma.
Publication Status	☑ Published ☐ Accepted for Publication ☐ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style
Publication Details	Published in 2019 in Prostaglandins Leukotrienes and Essential Fatty Acids. (2019 Sep 5;150:31-37, PMID: 31568925 DOI: 10.1016/j.plefa.2019.09.001).

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Overall percentage (%)	80%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
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Name of Co-Author	Yuan ZX		
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Statement of Authorship

Title of Paper	Plasma oxylipins and unesterified precursor fatty acids are altered by DHA supplementation in pregnancy: Can they help predict risk of preterm birth?
Publication Status	☐ Published ☐ Accepted for Publication ☑ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style
Publication Details	Submitted to Prostaglandins, Leukotrienes and Essential Fatty Acids

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Contribution to the Paper	Drafted the manuscript, compiled revisions from co-authors, submitted and revised manuscript for publication. Led development of the hypotheses under guidance of Drs Gibson, Makrides and Taha. Led the design and wrote application requesting samples for case-control study. Oversaw the oxylipin analyses. Worked with statistical team on interpretation and presentation of findings. Contributed to table and figure preparation.
Overall percentage (%)	75%
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
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Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

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ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Contribution to the Paper	Input into study design and interpretation. Critically revised the manuscript.		
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