Exploring the Unique Effects of α-Linolenic Acid and Docosahexaenoic Acid on Mononuclear Immune Cell Gene Expression and Function: Implications for Obesity and Atherosclerosis

By

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Abstract

Background: Monocytes play a central role in chronic inflammatory conditions such as obesity and atherosclerosis. Marine-derived omega-3 fatty acids (n-3 PUFAs) such as docosahexaenoic acid (DHA) have been shown to beneficially alter immune function and attenuate chronic inflammation in part by altering immune cell gene expression. Comparisons to the plant-derived n-3 PUFA, α-linolenic acid (ALA), on immune cell gene expression and function are limited.

Methods: Whole transcriptome analysis using a Clariom D microarray was performed on total mRNA isolated from a human THP-1 monocyte cell line treated with ALA, DHA or vehicle equivalent for 48 hr. Candidate genes (those responding to n-3 PUFA treatment) were identified via fold change and Ingenuity Pathway Analysis, and transcripts were subsequently validated by RT-qPCR. Assays to measure total cholesterol content and migration in response to chemokine (chemotaxis) were then performed on THP-1 monocytes treated with ALA or DHA to evaluate the outcomes of transcriptomic predictions. Candidate mRNA transcripts were examined via RT-qPCR in mononuclear immune cells isolated from a subgroup of clinical trial participants, where women with obesity received 4 g/day DHA or ALA for 4 weeks each.

Results & Conclusion: In THP-1 monocytes, transcriptome analysis identified ALA and DHA treatment differentially altered gene expression associated with cholesterol metabolism and chemotaxis. Based on these data, cholesterol content was predicted to be reduced by both fatty acids, while ALA would uniquely increase chemotaxis and DHA would have no effect. Functional assays revealed that ALA and DHA decreased cholesterol content to a similar extent. In contrast to our predictions, DHA significantly decreased chemotaxis, while ALA had no effect. Mononuclear immune cells obtained from a clinical n-3 PUFA supplementation trial displayed similar expression patterns to candidate transcripts identified in fatty acid treated THP-1 monocytes. Taken together, these results demonstrate that ALA and DHA differentially alter gene expression and cellular functions associated with chemotaxis and cholesterol metabolism in mononuclear immune cells. Thus, they may uniquely affect related disease processes contributing to obesity and atherosclerosis.

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List of Abbreviations

ALA, α-linolenic acid; ALT, alanine transaminase; AST, aspartate transaminase; ARP- WASP, actin related protein- Wiskott Aldrich Syndrome protein; ATP, adenosine triphosphate; BMI, body mass index; BP, blood pressure; CCR2, C-C chemokine receptor type 2; CMFDA, chloromethyl fluorescein diacetate; CRP, C-reactive protein; CVD, cardiovascular disease; CYP450, cytochrome P450; DHA, docosahexaenoic acid; ECAR, extracellular acidification rate; EFA, essential fatty acid; ELISA, enzyme-linked immunosorbent assay; EPA, eicosapentaenoic acid; FFA, free fatty acid; FFAR4, free fatty acid receptor 4; FMDV, foot-and-mouth disease virus; GPCR, g-protein coupled receptor; HEPE, hydroxy-docosahexaenoic acid; HDoHE, hydroxy docosahexaenoic acid; HMGCR, 3-hydroxy-3-methylglutaryl-CoA reductase; HIV, human immunodeficiency virus; ICAM-1, intercellular adhesion molecule-1; IL, interleukin; INF, interferon; IPA, Ingenuity Pathway Analysis; LA, linoleic acid; LDL, low-density lipoproteins; LOX, lipoxygenase; LSM, least squares mean; MetS, metabolic syndrome; MCP-1, monocyte chemoattractant protein-1; MUFA, monounsaturated fatty acid; n-3, omega-3; NAFLD, non-alcoholic fatty liver disease; NF, nuclear factor; OCR, oxygen consumption rate; oxLDL, oxidized low-density lipoprotein; PBMC, peripheral blood mononuclear cells; PL, phospholipid; PPARγ, peroxisome proliferator-activated receptor-γ; PUFA, polyunsaturated fatty acid; PWV, pulse wave velocity; RCT, randomized controlled trial; RT-qPCR, real time-quantitative polymerase chain reaction; SCD, stearoyl-CoA desaturase; T2D, type 2 diabetes; TAG, triacylglyceride; TLR, toll-like receptors; TNF-α, tumour necrosis

factor α; ULN, upper limit of normal; VCAM-1, vascular cell adhesion molecule-1; VEGF, vascular endothelial growth factor; VLDL, very low-density lipoprotein; WC, waist circumference; WHO, world health organization; PLS-DA, partial least squares discriminant analysis; VIP, variable interdependent parameters

Contribution Statement

The results presented in this thesis are arranged in manuscript format (Chapters 2, 3 and Chapter 5/Appendix 1). The contributions of each author for the respective manuscripts are described below.

Chapter 2

Contribution of Co-Authors to Chapter 2: Lisa Rodway: Wrote Chapter 2 and created Tables 1,3 and Figure 1. Samantha Pauls: Developed study design, created Figure 3, editing. Harold Aukema: Developed study design, editing, funding acquisition. Peter Zahradka: Developed study design, editing, funding acquisition. Carla Taylor: Submitted for ethics approval, editing, developed study design, funding acquisition.

Chapter 3

Contribution of Co-Authors to Chapter 3: **Lisa Rodway**: Wrote Chapter 3. Performed cell culture treatments, harvesting, RNA extraction and preparation of samples sent to Genome Quebec. Analyzed data using TAC and IPA and created Tables 2, 3 & 4. Performed all chemotaxis and cholesterol experiments and analysis. Extracted RNA from PBMC. Performed qPCR on THP-1 and PBMC RNA and analyzed data. Created Figures 3, 4, 5B and 5C. Samantha Pauls: Assisted with writing and with all analyses, specifically analyzed infection data and created Figure 3A, isolated PBMC from OXBIO trial participants. Christopher D Pascoe: Analyzed gene expression data using VIP and PLS-DA, created Figure 1. Harold Aukema: Developed study design, editing, funding acquisition. Carla Taylor: Submitted for ethics approval, editing, developed study design, funding acquisition. Peter Zahradka: Developed study design, editing, funding acquisition.

Chapter 5 (Appendix 1)

Contribution of Co-Authors to Chapter 5: Samantha Pauls: Designed research, performed experiments using plasma and immune cells, analyzed data, performed statistical analyses, and wrote the paper. **Lisa Rodway:** Recruited and screened participants, conducted all study visits (recruitment, consent procedures, data and personal health information management, measured blood pressure and performed pulse wave analysis, processed blood samples for use in experiments), entered food record data, constructed Table 1 and Figure 1. Karanbir Sidhu: Processed plasma for oxylipin analysis. Nikhil Sidhu: Processed plasma samples for fatty acid analyses. Tanja Winter: Maintained and ran samples on HPLC-MS/MS, calculated detector response factors for all oxylipins, calculated amount of internal standard to be added to each sample, assisted with questions and education regarding HPLC-MS/MS. Harold Aukema: Developed study design, editing, funding acquisition. Peter Zahradka: Developed study design, randomized samples for distribution, editing, funding acquisition. Carla Taylor: Obtained ethics approval, developed study design, editing, performed statistical analyses, funding acquisition.

Chapter 1: Introduction

1.1 General Introduction

The increasing prevalence of obesity and associated metabolic diseases has become an international concern. The number of persons with obesity has nearly doubled since the 1980s, and today approximately 39% of the world's adult population are overweight (BMI 25-30 kg/m^2) and more than 13% are obese (BMI>30-kg/m^2) [1]. Further, obesity is characterized by chronic adipose tissue inflammation [2]. The inflammatory mediators in adipose tissue recruit and activate immune cells creating systemic inflammation contributing to comorbidities such as type 2 diabetes (T2D), non-alcoholic fatty liver disease (NAFLD), and atherosclerosis [2].

Moreover, monocytes are a vital component of the innate immune system that respond to infection and act as important regulators of inflammation in disorders such as obesity [3]. Circulating monocytes mobilize to where they are needed and perform numerous functions including fighting infection and initiating tissue repair. This is beneficial where it is necessary to protect the body from harmful agents, or repair damage to the organism. However, if these processes occur in an uncontrolled manner, pathologic responses including chronic inflammation can ensue. Further, when exposed to chemotactic stimuli, monocytes in the circulation adhere to endothelial cells and cross by extravasation into the arterial wall, contributing to atherosclerotic lesion development [4]. This raises the possibility that treatments modulating monocyte function may be therapeutic in inflammatory disorders including obesity and comorbidities such as atherosclerosis.

Omega-3 (n-3) polyunsaturated fatty acids (PUFAs) have emerged as possible therapeutic agents, as marine-derived eicosapentaenoic acid (EPA; 20:5 n-3) and docosahexaenoic acid (DHA; 22:6 n-3) have previously been shown to reduce inflammatory markers and ameliorate symptoms of autoimmune and inflammatory disorders such as rheumatoid arthritis, obesity, and cardiovascular disease, both in vitro and in vivo [5-7]. A major mechanism by which n-3 PUFAs are thought to act in these conditions is by altering immune cell function, such as reducing production of immune cell-derived pro-inflammatory mediators [6]. Moreover, DHA is thought to possess superior immunomodulatory properties and reduce immune cell-derived pro-

inflammatory mediator secretion to a greater extent than EPA [8, 9]. The plant-derived omega-3 fatty acid α-linolenic acid (ALA, 18:3 n-3) has also demonstrated anti-inflammatory and immunomodulatory properties [10-12], however, far fewer studies have assessed its potential. Moreover, until very recently, most direct comparisons between plant and marine-derived n-3 PUFAs had been performed only in non-obese populations, where a dampening of inflammation and modulation of immune activity may not actually be relevant.

Recently, dietary fatty acids have been found to alter the transcriptome of immune cells in the blood, thus affecting their function in different disease states including obesity and other metabolic and immune disorders [7, 13-16]. Indeed, regulation of gene expression by n-3 PUFAs can occur through direct or indirect activation of transcription factors or other response factors, ultimately influencing mRNA synthesis [17]. DHA and its bioactive metabolites have been studied in this context and are thought to exert their beneficial effects in part by altering the expression of genes related to fatty acid metabolism and inflammation [18]. Similar studies with ALA have not been performed, as it is classically assumed that the biological effects of ALA are dependent on its conversion to longer chain fatty acids such as DHA. However, others, as well as our recent studies, indicate that ALA and its bioactive metabolites have immune modulatory effects distinct from other n-3 PUFAs [10-12]. In addition, most research to date has focused on studying the actions of n-3 PUFAs solely on production of inflammatory markers. Recently, many in vitro studies indicate n-3 PUFAs have effects in immune cells other than reducing the production of inflammatory compounds, including altering cell migration in response to chemokine (chemotaxis) [5, 8]. Overall, there is a need to characterize the biological effects of ALA and its metabolites both in function and in molecular mechanism, and to describe how their effects differ from other n-3 PUFAs such as DHA in a competitive or complementary manner. Further, comparing their effects in immune cells will guide and refine the use of n-3 PUFAs in different conditions, including, but not limited to, inflammatory disorders such as obesity.

1.2 Pathophysiology of Obesity

Obesity is broadly defined as an excess of body-fat mass and is recognized as a major public health concern. In 2016, the World Health Organization (WHO) estimated that at least 1.5 billion adults 20 years and older were overweight (body mass index (BMI) of 25-29 kg/m^2), and of

these, over 500 million were obese (BMI ≥ 30 kg/m^2) [19]. In addition, the numbers of overweight and obese individuals are estimated to increase in the coming years [20]. Moreover, obesity is characterized by chronic low-grade inflammation, a key factor in the development of obesity-related disorders, including T2D, non-alcoholic fatty liver disease (NAFLD) and cardiovascular disease (CVD) [2, 14, 21, 22]

The increase in adipose mass in obesity results from both increased adipocyte size (hypertrophy) and increased adipocyte numbers (hyperplasia) through adipogenesis [23]. Further, it has become clear that adipose tissue is an active endocrine organ with important roles in physiologic and pathophysiological processes [24]. In obesity, adipocytes take up excess circulating fatty acids due to positive energy balance, thus becoming hypertrophic, a state that produces increased amounts of pro-inflammatory adipokines (e.g. resistin), and cytokines (e.g. tumor necrosis factor-α) [25], as well as fewer beneficial adipokines (e.g. adiponectin) [26, 27]. This dysregulated secretory profile leads to recruitment of immune cells such as monocytes [24]. Moreover, resident and recruited immune cells within adipose tissue are activated by the local inflammatory milieu and secrete pro-inflammatory mediators of their own, further perpetuating adipose tissue inflammation and metabolic dysfunction, including local insulin resistance [28]. Indeed, there is an increase in the number of pro-inflammatory immune cells derived from monocytes (M1 macrophages) within adipose tissue [28]. Pro-inflammatory macrophages, referred to as M1, synthetize and release pro-inflammatory cytokines, such as tumor TNF-α and interleukin (IL)-6 [28]. When compared to lean individuals, adipose tissue in obese individuals shows higher amounts of pro-inflammatory mediators [27]. Pro-inflammatory cytokines and activated immune cells then 'spill' over from inflamed adipose tissue causing dysfunction in circulating immune cells, as well as other organ systems including the pancreas, liver, blood vessels and the heart [29]. This creates the chronic low-grade inflammatory state characteristic of obesity, which in turn causes numerous metabolic and physiologic malfunctions resulting in T2D, NAFLD and atherosclerosis [22, 27].

Foremost, inflammation is an acute, necessary protective response to infection or injury by harmful stimuli, such as bacteria, viruses, or toxic compounds [30] Generally, inflammation is an ordered sequence of events designed to preserve tissue and return the body to normal function [27]. This process includes the release of compounds and expression of receptors vital for

ensuring the safety of the organism in order to restore tissues to their original state [27]. In response to tissue injury or infection, a signaling cascade stimulates responses designed to deal with the infectious agent or heal the affected tissue [30]. These signals activate immune cell chemotaxis (directional movement) from the general circulation to sites of injury to fight infection and/or initiate tissue repair [30]. Monocytes play a central role in the acute inflammation response and are amongst the first immune cells to arrive at injured sites [31]. Infiltrated immune cells, such as monocytes, eliminate pathogens and initiate inflammatory cascades [21]. Further, macrophages can be generated from monocytes after tissue infiltration and activation to fight infection and/or initiate tissue repair [31].

Usually, acute inflammatory responses contribute to the restoration of tissue homeostasis, after which, the acute inflammation is resolved to prevent further tissue damage [30]. For example, cytokines in acute inflammation can act to increase blood vessel permeability, recruit other immune cells to fight infection, and initiate fibrosis and angiogenesis; these are all processes required for tissue repair [30]. As mentioned previously, under normal circumstances, inflammation is acute, and resolves after tissue repair is sufficient, or the threat of infection subsides. Resolution of inflammation involves the clearance of activated inflammatory cells (e.g. mononuclear cells), and the cessation of pro-inflammatory mediator release and signaling [30]. However, if inflammatory processes are uncontrolled or fail to resolve, these necessary processes contribute to a variety of chronic inflammatory diseases [21, 26, 30].

The chronic inflammatory response found in obesity is similar to the acute inflammatory response. It largely includes increases in circulating pro-inflammatory cytokines, and recruitment to and activation of immune cells to inflamed tissues. However, the chronic nature of obesity is unique and produces a longer-term low-grade activation of the immune system chronically affecting metabolic homeostasis over time [29]. Within obesity, the longer-term inflammatory state contributes to the disruption of metabolic homeostasis, including increases in circulating cytokines such as TNF-α and IL-6, decreases in protective factors (e.g. adiponectin), and disruption in cell signaling [21]. For example, inflammatory cytokines released from M1 macrophages impair insulin signaling and adipogenesis in adipocytes, leading to insulin resistance and T2D, and adipocyte hypertrophy, respectively [21]. In parallel, the long-term presence of pro-inflammatory cytokines, such as IL-6, dysregulates macrophage-hepatocyte

signaling, as well as lipoprotein receptor function, leading to NAFLD, impaired lipid handling (e.g. hyperlipidemia) and atherosclerotic plaque development [32]. Overall, the longer-term chronic inflammation in obesity affects the performance of many organ systems, tissues, and cell types, ultimately impairing their function [21, 25, 27, 28, 33]. As mentioned, lipid handling and the function of blood vessels, endothelial cells and circulating immune cells are pathologically altered in obesity, and all contribute to the development of atherosclerosis.

1.3 Pathophysiology of Atherosclerosis

Many of the metabolic functions impaired in obesity contribute to the development of atherosclerotic plaques within arteries [34]. Arteries are comprised of three layers. The intima, the innermost (luminal) layer that is responsible for mediating vascular tone, consists of a single layer of endothelial cells and subendothelial connective tissue [34]. The second (medial) layer is comprised of smooth muscle cells that respond to agents signaling contraction and dilation, thus determining vascular tone [34, 35]. The outermost layer (adventitia) is composed of connective tissue with fibroblasts, smooth muscle cells and progenitor cells [34, 35]. In health, the vascular endothelium maintains a semi-permeable barrier. In response to injury, this barrier is compromised, allowing the accumulation of apoB containing lipoproteins, such as low-density lipoprotein (LDL) [35]. Subendothelial lipoprotein accumulation is subject to oxidation by reactive oxygen species or enzymes (e.g. peroxidases, lipoxygenases), creating oxidized LDL (oxLDL) [35, 36]. The presence of oxLDL triggers an inflammatory response, both activating and disrupting the integrity and function of endothelial cells, resulting in secretion of chemoattractants that interact with receptors on circulating monocytes to promote their migration into the intima [35, 37]. In obesity, a large number of pro-inflammatory cytokines (e.g. TNF-α, IL-8) are released from adipose tissue. These pro-inflammatory cytokines contribute to endothelial dysfunction, a key process in atherosclerosis [25]. Dysfunctional endothelial cells in obesity are known to express higher levels of activated adhesion molecules (e.g. vascular cell adhesion molecule [VCAM-1] and intercellular adhesion molecule-1 [ICAM-1]) promoting the tethering/adhesion of monocytes to the endothelium of blood vessels [4, 25]. Subsequently, monocytes migrate into the subendothelial space, via a process termed intravasation, where they predominantly differentiate into pro-inflammatory M1 macrophages due to the local inflammatory environment [37]. These macrophages engulf oxidized lipoproteins, becoming

foam cells [35]. Within the local pro-inflammatory environment, foam cells continue to accumulate, thickening the arterial intima and resulting in a fatty streak [35]. The streak, or plaque, continues to develop, recruiting smooth muscle cells. Pro-inflammatory cytokines, oxLDL, and activated endothelial cells encourage the phenotypic transformation of smooth muscle cells resulting in their proliferation and migration into the atherosclerotic plaque, eventually forming a fibrous cap [38, 39]. Monocytes continue to be recruited, and their proliferation further accelerates plaque growth [40]. As the plaque progresses, it becomes more susceptible to rupture, releasing debris that triggers coagulation and thrombus formation; this increases the possibility of arterial occlusion resulting in myocardial infarction or stroke [35]. Therefore, finding treatments that counteract maladaptive functional alterations of key cell types, such as monocytes, within obesity is an important clinical objective.

1.4 Monocytes

Monocytes are a vital cell type responsible for regulating inflammation and fighting infection within the innate immune system [3]. They are a subset of white blood cells (leukocytes) originating from myeloid progenitors in the bone marrow [41]. Monocytes also reside in the spleen and lungs and can mobilize to other tissues [42]. When isolated from the blood for assessment (e.g. in clinical trials), monocytes comprise roughly 10-20% of the peripheral blood mononuclear cell (PBMC) population, with other mononuclear immune cells such as neutrophils and B-cells making up the rest [43]. Monocytes are recruited to tissues during acute infection and play an important role in the maintenance of tissue homeostasis, helping to remove pathogens and initiate tissue repair [3]. They are also phagocytic and remove infected and/or dying cells. Moreover, they play a role in adaptive immunity by directly activating T-cells or differentiating into macrophages and dendritic cells when exposed to the inflammatory milieu of the target tissue [3]. Within the context of obesity, migration of peripheral blood monocytes into adipose tissue and their subsequent differentiation to macrophages contributes to macrophage accumulation in adipose tissue [25, 44]. Moreover, monocyte infiltration contributes to the proliferation of resident macrophages, as more pro-inflammatory mediators are present initiating the differentiation of monocytes to M1 macrophages [44]. Monocyte chemoattractant protein-1 (MCP-1) and its receptor C-C chemokine receptor type 2 (CCR2) play a crucial role in the recruitment of monocytes into

adipose tissue, where they differentiate into M1 macrophages, contributing to inflammation [25, 28]. In healthy subjects, there is generally a balance of pro-and anti-inflammatory processes [45]. In obesity, the inflammatory milieu in inflamed adipose tissue disrupts this balance, promoting the polarization of monocytes to pro-inflammatory M1 (classically activated) macrophages as opposed to the alternatively activated, anti-inflammatory M2 type [24, 45]. Further, the local inflammatory environment in inflamed adipose tissue promotes the proliferation of monocytes, leading to increased numbers of mononuclear immune cells in the peripheral blood of obese individuals [46, 47]. Moreover, monocytes isolated from obese humans display altered function, including a higher chemotactic activity, increasing their ability to adhere and migrate through the endothelium, and therefore contribute to atherosclerosis [46, 47]. Thus, elucidating the complex roles of monocytes, and how they respond to therapeutic intervention will provide advantages both in preventing and controlling the progression of obesity and its associated comorbidities.

1.4.1 THP-1 Monocytes

THP-1 monocytes are an immortalized human monocyte cell line. They display a more mature monocytic phenotype than other immortalized human monocyte cell lines and have been shown to be more comparable to monocytes isolated from the blood versus RAW monocytes (mouse) or U937 monocytes (human) [48, 49] An article by Hu [50] illustrates they are an ideal monocyte proxy for basic and applied research, including their use in gene expression microarray analysis. Thus, THP-1 monocytes represent a valuable tool for investigating monocyte function and response to in vitro treatment with therapeutics.

1.5 Fatty Acids

The health effects of different types of dietary fatty acids have been a topic of interest for decades and have been studied in many cell types and disease pathologies. Moreover, they are an essential component of the human diet, comprising roughly 20-40% of total daily energy needs [51]. Fatty acids are hydrocarbons with a carboxylic acid group and methyl group at opposite ends; they vary in chain length and are hydrophobic [52]. They can be fully saturated, containing no double bonds, or unsaturated, containing one or more double bonds within their structure. Unsaturated fatty acids with one double bond are classified as monounsaturated fatty acids (MUFAs), while those with more than one double bond are termed polyunsaturated fatty acids

(PUFAs) [52]. Unsaturated fatty acids are classified based on the placement of their last double bond relative to the methyl group. For example, they can be omega-3 (n-3), omega-6 (n-6), or omega-9 (n-9) [53]. Among PUFAs, linoleic acid (n-6) is the most abundantly consumed fatty acid in the Western diet [53]. Both linoleic and α-linolenic acid (n-3) are considered essential fatty acids (EFAs), as they cannot be synthesized by the body, and are required for the complete nutrition of many species including humans [53].

Fatty acids have pluripotent roles within the human body and are obtained either through ingestion or conversion. The digestion of lipids, specifically triacylglycerides (TAGs) present in food, begins in the oral cavity through exposure to lingual lipases secreted by glands in the tongue [54]. Digestion continues in the stomach through the effects of both lingual and gastric enzymes [54]. Emulsification of fatty acids happens in the duodenum along with the actions of bile, which assists with the hydrolysis of TAGs by pancreatic lipase [54, 55]. TAGs are hydrolyzed resulting in the formation of 2-monoglycerides and FFAs, which then form into micelles in preparation for absorption across the cells of the intestinal wall (enterocytes) [54]. Once absorbed, longer chain (13+ carbons) hydrophobic fatty acids and 2-monoglycerides are re-esterified to TAGs and are transported in a lipoprotein complex (chylomicron) containing triglycerides, phospholipids and cholesteryl esters, first through the lymphatic system before entering the bloodstream at the thoracic duct. Short (<6 carbons), and medium chain (6 to 12 carbons) fatty acids are transported in the blood complexed to albumin (e.g. non-esterified fatty acids) [54].

In the tissues of the body, endothelial cells that line blood vessels contain the enzyme lipoprotein lipase (LPL) [54]. LPL cleaves the fatty acids from lipoprotein triglycerides so that the fatty acids can be taken up into tissues [54]. Fatty acids are then commonly integrated into several structures such as TAGs (e.g. in adipocytes), although they can also be maintained in their free form [52]. Dietary fatty acids are also incorporated into cell membrane phospholipids (PL), polar molecules composed of two fatty acids and a phosphate group attached to a glycerol backbone. Phospholipids make up most of the lipid bilayer in eukaryotic cells, with membrane proteins embedded in the bilayer selectively allowing the entry and exit of compounds [54, 56, 57]. Moreover, altering phospholipid composition (e.g. incorporation of different fatty acids, such as unsaturated) can alter membrane fluidity, and thus the function of proteins embedded in

the membrane. Most cellular fatty acids exist as part of a structure such as TAG or PLs, and are only released if needed for metabolic substrates, transport or signaling molecules [58]. For example, fatty acids can enter β-oxidation or undergo elongation and desaturation to other fatty acids, or when released from phospholipids, they may be altered enzymatically or non-enzymatically to act as signaling molecules, called oxylipins [58, 59]. Clearly, altering the type of fatty acids available within the body can have numerous effects on a variety of cellular functions, and can thus affect several health and disease states.

1.6 Omega-3 Polyunsaturated Fatty Acids

Generally, diets rich in PUFAs have many recognized benefits, including reduced all-cause mortality [60]. N-3 PUFAs in particular have emerged as dietary compounds capable of modulating immune, inflammatory and metabolic processes [6, 7, 58, 61-63]. Thus, they are thought to be beneficial in disease states such as obesity [15, 16, 64]. The three main n-3 PUFAs are plant-derived ALA and the marine-derived EPA and DHA. The health benefits of the marine-derived fatty acids were first observed in the low rates of CVD among the Inuit people, and subsequently the populations of Japan and Greenland, where diets are rich in marine life [65]. Many subsequent epidemiological and in vitro studies have shown the benefits of dietary consumption of n-3 PUFAs in dyslipidemia, atherosclerosis, hypertension, blood vessel function, T2D, metabolic syndrome, obesity and other inflammatory and immune disorders [65]. Although not completely understood, n-3 PUFAs are thought to convey their benefits through multiple mechanisms, including altering blood lipid profiles and membrane lipid composition, oxylipin synthesis, cell signaling, and gene expression [65]. However, both epidemiological and in vitro studies generally fail to account for the unique effects and actions of individual n-3 PUFAs. This highlights the need to separately characterize the biological effects of n-3 PUFAs and their metabolites both in function and in molecular mechanism, and to describe if their effects are competitive or complementary.

1.6.1 DHA + EPA

The most commonly studied omega-3s are the marine- derived n-3 PUFAs, EPA (20:5 n-3) and DHA (22:6 n-3). The recommended dietary intake of combined EPA + DHA is 0.16 g/d and 0.11 g/d for adult men and women, respectively [66, 67]. However, observational data show

that a majority of American adults are not meeting recommended levels [66]. The most common source of EPA + DHA is fatty fish, such as mackerel, containing approximately 3.2 g of n-3 PUFA per 100 g serving [68]. Other fish species containing an abundance of n-3 PUFA include salmon, sardines, and tuna [68]. EPA+ DHA can also be obtained via conversion of dietary ALA, which will be discussed in the next section. In 2015, a cross-sectional study found that the mean DHA plasma fatty acid levels of Canadian adults aged 20-29 (n=826) was 88.8 ± 36.8 μmol/L, with a range of 7.2 to 237.5 μmol/L [69]. In the OXBIO n-3 PUFA supplementation trial conducted by our group, baseline DHA plasma fatty acid levels were approximately 200 μmol/L for 22 participants. Interestingly, pre-menopausal females (18-30 years old) have higher baseline values of DHA compared to age-matched males (200 ± 6 versus 149 ± 8 μmol/L, p <0.0001) [70]. This could account for the difference between baseline values observed in the OXBIO trial (which included only pre-menopausal females), versus those in the cross-sectional Canadian study which included plasma fatty acid levels of both males and females. Further, many clinical supplementation trials report increases in DHA plasma concentrations, and their respective oxylipins in plasma after fish-oil supplementation depending on sex, study length, dose, and type of supplementation given [71, 72]. For example, the OXBIO trial found 4 g/day of DHA-rich fish oil supplementation increased DHA plasma levels to 588 μmol/L after 28 days [73, 74].

1.6.2 ALA

The actions of the plant-derived n-3 PUFA, ALA (18:3 n-3), have received far less attention than its marine counterparts, as it has commonly been assumed that its actions are due to its conversion to longer chain metabolites, such as DHA. ALA is considered an essential fatty acid as humans lack the desaturase enzymes required to synthesize ALA from other fatty acids [53]. EPA and DHA are not considered essential fatty acids as they can be synthesized from ALA via a series of alternating desaturation and elongation steps (Figure 1-1) [53]. ALA can be obtained through the diet from plant sources such as flaxseed, soybeans, canola oil, and walnuts [53]. ALA is also commonly consumed as ALA-rich flax oil supplements [53]. The Adequate Intake for ALA set by The Institute of Medicine is based on the average intakes of Americans: 1.1 g/day for females and 1.6 g/day for males [75]. Moreover, an analysis of the 2014 National Health and Nutrition Examination Survey discovered that approximately 41% of American

adults were consuming less than the Adequate Intake recommendations [76]. In 2015, one cross sectional study found that the average plasma concentrations of ALA in Canadian adults aged 20-29 (without supplementation) was approximately 50 μmol/L [69]. Plasma ALA levels have also been shown to reach 180 μmol/L (with flax oil supplementation), depending on sex, study length and dose given [77]. As mentioned previously, ALA is considered an essential fatty acid because of its ability to be converted into EPA and DHA (Figure 1-1) [73]. Though highly debated, conversion from ALA to DHA in humans is estimated to be limited. Dietary supplementation and isotope tracer studies in humans estimate ALA to EPA conversion to be approximately 5% [78]. However, biosynthesis of DHA from ALA is typically less efficient and is estimated to be approximately 0.5-8.0 % depending on biological sex, tissue or cell type examined [79]. For example, in persons supplemented with ALA in the form of flax-oil during the OXBIO trial, plasma DHA levels at baseline (218±125 μmol/L) displayed little difference to those on day 28 of supplementation (204±153 μmol/L). The main site of ALA desaturation and elongation is the liver [79, 80]. Desaturation and elongation occurs in other tissues to a lesser extent, such as the brain and heart [80]. A limitation of DHA synthesis estimates is that they are not usually based on measuring enzyme activity, or levels of DHA in the liver or other tissues. Rather, they are typically based on the measurement of labeled DHA in plasma following oral ingestion of stable-isotope ALA, thus, the efficiency of conversion in these studies is still debated [81-83]. However, within cells, the majority of the conversion pathway occurs in the endoplasmic reticulum [80]. Moreover, dietary LA (n-6 PUFA) and ALA compete for the same desaturase and elongase enzymes for long-chain PUFA biosynthesis [80]. As high levels of the dietary n-6 PUFA linoleic acid (LA, 18:2n-6) are consumed in the Western diet, LA out-competes ALA for Δ6-desaturation enzymes, providing little opportunity for the conversion of ALA to EPA and DHA [84, 85].

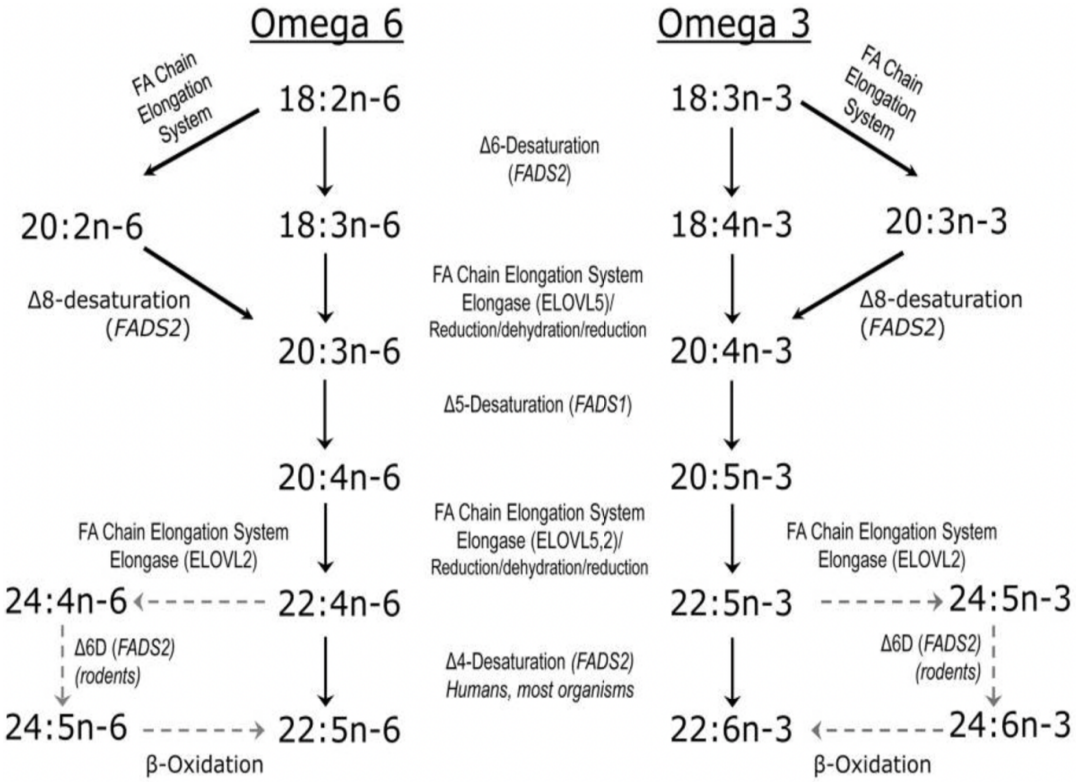

Figure 1-1 Omega-3 and omega-6 conversion pathway.

Linoleic acid (n-6 PUFA) and α-linolenic (n-3 PUFA) acid are converted to longer chain metabolites via several enzymatic steps. Figure obtained from Zhang et al. [86]. Copyright permission obtained.

1.7 Omega-3 Polyunsaturated Fatty Acids in Obesity

Since the hallmark studies in the Inuit people in the 1970s, n-3 PUFAs have continued to be of great research interest, with much focus on their actions in obesity, other metabolic disorders (e.g. T2D), and CVD. So far, within the context of obesity, most human trials have focused on fish oils rich in EPA and DHA and these have shown mixed results. However, generally, fish oils have demonstrated several beneficial effects on obesity-related changes in animals, such as reduced visceral fat accumulation, improved lipid profile, less insulin resistance and glucose intolerance, and decreased hepatic steatosis, as well as lowering inflammation in peripheral tissues [7, 15, 63-65, 87-90].

The most common mechanism by which n-3 PUFAs are thought to convey benefits in obesity are related to their capacity to reduce inflammation [18]. N-3 PUFAs are known to inhibit nuclear factor (NF)-κB activity and toll like receptor (TLR)-mediated inflammatory signaling and are thought to reduce adipose tissue inflammation by regulating the production of immune cell derived pro-inflammatory cytokines [8, 12, 65]. Fish oil supplementation has also been shown to promote the polarization of macrophages towards the anti-inflammatory (M2) phenotype [8]. Moreover, n-3 PUFAs also reduce the formation of n-6-derived pro-inflammatory lipid mediators via competition for desaturation and elongation enzymes, thereby limiting the amount pro-inflammatory mediators produced [8]. In addition, treatment with n-3 PUFA can increase the production of lipid mediators with pro-resolving properties (e.g. resolvins, protectins and maresins) promoting the reduction of adipose tissue and systemic inflammation [8]. However, to date, studies examining inflammatory markers in obese subjects have mostly used fish oil rich in both EPA and DHA and report mixed effects. For example, a study comparing DHA-rich oil to EPA-rich oil supplementation demonstrated unique anti-inflammatory effects of each n-3 PUFA after 10 weeks [91]. Further, some supplementation trials with ALA-rich oil report no effects [92] while others using higher dosages and longer durations or participants with more severe obesity [BMI $\geq$ 40 kg/m^2] reported reduced inflammation [93].

1.8 Omega-3 Polyunsaturated Fatty Acids in Cardiovascular Disease & Atherosclerosis

CVD is the leading cause of mortality in many economically developed nations, accounting for approximately 30% of all deaths [94, 95]. Current research aims to investigate and prevent the development of cardiovascular risk factors including atherosclerosis, hypertension, dyslipidemia, chronic inflammation, and insulin resistance. The cardioprotective actions of n-3 PUFAs are thought to be due to their anti-inflammatory, anti-atherogenic, and anti-arrhythmic properties [90, 96]. Moreover, many trials examining the effects of n-3 PUFAs have found improvements in endothelial function, and favorable lipid profile changes (e.g. TAG, lipoproteins [high-density lipoprotein (HDL), LDL]) [89, 97, 98]. Although mixed, there is a vast amount of evidence for the cardioprotective effects of fish oil-derived EPA and DHA in both epidemiologic studies and randomized controlled trials [5, 88, 90, 97]. For example, higher dietary intake of EPA+ DHA in Japan relative to that of North America is associated with considerably lower rates of myocardial infarction, ischemic heart disease and atherosclerosis despite only moderately lower blood cholesterol levels in the Japanese population [99]. Evidence regarding ALA is also mixed, however, most prospective cohort studies and controlled trials indicate that ALA may have cardioprotective actions similar to EPA and DHA. A recent meta-analysis indicated that countries with higher ALA intake have decreased rates of CVD mortality [100]. Moreover, in a randomized controlled trial supplementing with both plant (ALA) and marine oils (EPA+DHA), it was found that both had similar beneficial effects on CVD risk biomarkers (LDL, HDL, TGs, or inflammatory markers) compared with baseline [101]. Further, in a meta-analysis investigating the relation of ALA intake with fatal CVD in 5 prospective cohort studies, it was found that intakes of around 2 g/d of ALA were associated with a 21% lower risk of fatal CVD compared with ALA intakes of 0.8 g/d [102].

N-3 PUFAs have also been shown to have specific anti-atherogenic effects in epidemiological and experimental studies [60, 103-106]. For example, the Study on Prevention of Coronary Atherosclerosis by Intervention with Marine Omega-3 fatty acids demonstrated that a 1.65 g/day fish oil supplement resulted in slowed progression and reduced size of atherosclerotic plaques in patients with coronary artery disease [107]. N-3 PUFAs are thought to provide their anti-atherogenic effects through a combination of several mechanisms

including positively affecting endothelial and vascular smooth muscle cell function [108]. However, a critical factor in the development of atherosclerosis is the binding and intravasation of immune cells through the endothelial layer [4]. Despite this, when compared to endothelial cells, the actions of n-3 PUFAs on the function of immune cells in atherosclerosis is comparatively less examined. Further, the studies that have been conducted have mostly examined marine-derived oils.

As previously mentioned, actions and/or benefits are commonly attributed to n-3 PUFAs without specifying source or type. However, more recent studies indicate they have distinct physiological effects. For example, within marine oils, DHA supplementation led to a greater reduction of plasma IL-8 levels and a greater increase of adiponectin concentrations in healthy humans than EPA [91]. Other studies have also indicated that DHA may possess greater anti-inflammatory and immunomodulatory properties than EPA [9], making it an attractive therapeutic for disorders characterized by pathologically altered immune function and inflammation such as obesity. Moreover, to our knowledge, there is far less information comparing the actions and mechanisms of ALA with the marine-derived n-3 PUFAs.

1.9 Immunomodulatory Effects of Omega-3 Polyunsaturated Fatty Acids

N-3 PUFAs and their metabolites are thought to exert their bioactive effects in immune cells through multiple mechanisms, including altering cell membrane composition and surface receptor modulation, or through binding to G-protein coupled receptors and nuclear receptors [61]. For example, a recent report showed that DHA inhibits the production of pro-inflammatory cytokines (TNF-α, IL-6) in mouse monocytes and mouse primary macrophages by binding to a G-protein coupled receptor (GPCR) termed Free Fatty Acid Receptor 4 (FFAR4) [109]. Although the mechanisms are not yet completely understood, anti-inflammatory signaling via FFAR4 also has been shown to regulate glucose homeostasis, adiposity, and inhibit the inflammatory cascade [109]. EPA and DHA are also strong natural ligands for peroxisome proliferator-activated receptor gamma (PPARγ) type II nuclear receptors, which act as biosensors for fatty acids and play key roles in fatty acid metabolism and homeostasis [17, 110] PPARγ activation is thought to inhibit inflammatory responses in immune cells by blocking NF-κB activation, a pro-inflammatory transcription factor regulating multiple aspects of innate and

adaptive immune function [110]. Moreover, many of the biological effects of PUFAs have been attributed to the actions of their oxygenated free fatty acid metabolites called oxylipins [59]. For example, the DHA-derived metabolite, protectin D1, has been shown to be a potent ligand for both GPCRs such as FFAR4 and different nuclear receptors including PPARγ [109].

A major mechanism by which n-3 PUFAs are thought to exert their actions in immune cells is by altering gene expression [8]. Indeed, regulation of gene expression by n-3 PUFAs can occur through direct or indirect activation of transcription factors (e.g. PPARγ) or other response factors, ultimately influencing mRNA synthesis [17]. Previous investigations examining gene expression in PBMC during EPA + DHA supplementation demonstrated alterations in the expression of genes related to inflammation (decreased), cell cycle and apoptosis (increased), as well as fatty acid and cholesterol metabolism (decreased) [105, 111]. Studies have also reported DHA downregulates the expression of genes involved in atherogenesis and inflammation ,which play a role in several processes, including atherosclerotic plaque formation and the perpetuation of chronic inflammation in obesity and after infection [105, 111]. Both EPA and DHA supplementation were also found to decrease the expression of genes involved in cholesterol production, such as HMG-CoA reductase (*HMGCR*), and Stearoyl-CoA desaturase (*SCD*) [105, 111]. Similar studies examining the effects of ALA on the immune cell transcriptome have not been performed, although functional studies indicate ALA has biological actions both similar to and distinct from its marine counterparts both in cell lines and immune cells isolated from humans supplemented with n-3 PUFAs [10-12]. As well, transcriptomic studies examining n-3 PUFAs have so far been performed only in PBMC, a heterogenous mixture of immune cell types, making their effects even more difficult to differentiate.-Thus, there is a need to distinguish gene expression changes induced by ALA and DHA and the related functional consequences in monocytes.-This will help guide their therapeutic use in conditions with an immune component, such as obesity and atherosclerosis.

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1.11 Rationale

Monocytes have diverse roles in the body and are essential for regulating the inflammatory response, initiating tissue repair and fighting infection [1]. Their activity is known to be pathologically altered in chronic inflammatory disease states such as obesity, contributing to co-morbidities such as atherosclerosis [1-5]. N-3 PUFAs such as DHA have demonstrated immunomodulatory properties and hold promise as a dietary strategy to attenuate maladaptive immune function in obesity [6, 7]. ALA, the plant-derived n-3 PUFA, has also shown promise, however, far fewer studies have assessed its potential to affect immune function and chronic inflammation. Moreover, direct comparisons between DHA and ALA are limited, and have been mostly performed in non-obese populations [8, 9], where altered immune function and a dampening of inflammation may not be visible or relevant. In addition, it has been found that dietary fatty acids alter the transcriptome of immune cells in the blood [10-14], and thus affect their function. The n-3 PUFA DHA and its bioactive metabolites are thought to exert their effects in part by altering the expression of genes related to fatty acid metabolism and inflammation [8-12]. Similar studies with ALA have not been performed, as it is classically assumed that the biological effects of ALA are dependent on its conversion to longer chain fatty acids such as DHA. However, others, as well as our recent studies, indicate that ALA and its bioactive metabolites have direct immune modulatory effects of their own [15-20]. These findings highlight the need to characterize the biological effects of ALA and its metabolites both in function and in molecular mechanism, and to describe how their effects differ from those of DHA in a competitive or complementary manner.

1.12 Hypothesis

The overall hypothesis for this research project was:

ALA and DHA will have both distinct and similar effects on monocyte gene expression and function relating to inflammatory and atherogenic processes associated with obesity.

The overall hypothesis was tested by separating its various components into the following sub-hypotheses:

1. ALA and DHA treatment will have similar and divergent effects on the monocyte transcriptome related to inflammation and atherosclerosis.

2. Similar expression patterns of candidate genes (those responding to n-3 PUFA treatment) observed in ALA and DHA treated THP-1 monocytes will occur in mononuclear cells isolated from obese humans who are consuming ALA or DHA-rich oil supplements.

3. ALA and DHA treatment will have both distinct and similar effects on monocyte functions related to inflammation and atherosclerosis.

1.13 Objectives

1) To identify and compare candidate genes responding to acute ALA and DHA treatment, and to predict functions both commonly and distinctly affected by ALA and DHA in THP-1 human monocytes.

2) To examine whether the changes in gene expression observed in Objective 1 also occur in mononuclear cells isolated from humans who have consumed either ALA or DHA-rich oil supplements.

3) To evaluate the effects of ALA and DHA on monocyte functions predicted to be altered by treatment with ALA or DHA based on the gene expression profiles obtained in Objective 1.

Objective 1 utilized transcriptome profiling of THP-1 human monocytes to identify candidate genes responding to 48 hr ALA or DHA treatment (discussed in Chapter 3). Ingenuity Pathway Analysis (IPA) was then used to predict patterns and pathways affected by treatment, leading to functions that may be distinctly and similarly affected by ALA and DHA (Chapter 3). Objective 3 aimed to evaluate the validity of transcriptomic predictions and utilized functional experiments to examine candidate cellular functions in THP-1 monocytes related to

atherosclerosis, including cholesterol metabolism and migration towards chemokine (chemotaxis).

In parallel, Chapter 2 describes a randomized crossover clinical study I co-coordinated from 2018-2021, where 22 obese females consumed 4 g/day of ALA or DHA-rich oil supplements for 4 weeks each. I performed recruitment, screening, study visits and plasma processing for this trial. I also assisted with food record data entry, as well as table and figure generation for the manuscripts containing trial results (Appendix 1). This clinical trial (OXBIO) examined the impact of ALA and DHA-rich oil supplements on oxylipins and markers of metabolic health and immune function in obese humans. Objective 2 utilized mononuclear immune cells isolated from a subset of OXBIO participants to determine if expression patterns of candidate genes observed in ALA and DHA treated THP-1 monocytes (Objective 1) are similar to humans receiving n-3 PUFA supplementation (Chapter 3). Examining immune cells from an obese population allowed the cell culture findings to be interpreted in the context of a relevant disease state, since immune function would not be pathologically altered in immune cells from a healthy population.

Overall, this project compares the actions of ALA and DHA on mononuclear immune cell gene expression and function to aid in refining their specific, or differential use as therapeutics within obesity and related comorbidities, primarily atherosclerosis. The cell culture experiments illustrate the effects of shorter term (48 hr) treatment with DHA or ALA on mononuclear immune cell gene expression and function. The clinical samples provide information on longer term (28 day) in vivo supplementation in humans with obesity. Thus, this project provides a foundation for further investigation of the mechanisms underlying the beneficial properties of DHA and ALA, and in which circumstances they are each most useful. The knowledge of how, where and when these fatty acids are most beneficial will allow their use in targeted strategies to prevent and treat obesity and its associated comorbidities, including atherosclerosis.

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Chapter 2: Rationale and Design of a Randomized Controlled Trial Examining the Effects of Marine- and Plant-Sourced Omega-3 Fatty Acid Supplements on Octadecaniod Profiles and Inflammation in Females with Obesity (OXBIO trial)

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Contribution of Co-Authors to Chapter 2: Lisa Rodway: Wrote Chapter 2 and created Tables 1,3 and Figure 1. Samantha Pauls: Developed study design, created Figure 3, editing. Harold Aukema: Developed study design, editing, funding acquisition. Peter Zahradka: Developed study design, editing, funding acquisition. Carla Taylor: Submitted for ethics approval, editing, developed study design, funding acquisition.

Abstract

Introduction: Consumption of omega-3 polyunsaturated fatty acids (n-3 PUFAs) has been reported to provide health benefits, but it remains unknown whether the fatty acids themselves or their oxygenated metabolites, oxylipins, are responsible for the beneficial effects.

Purpose: This paper describes the design and rationale of a randomized, double-blinded, cross-over study comparing the effects of α-linolenic acid (ALA)-rich flax oil and docosahexaenoic acid (DHA)-rich fish oil supplementation on circulating oxylipin profiles in females with obesity, in relation to obesity-induced inflammation.

Methods and analysis: Pre-menopausal females (n=24) aged 20-55 with a BMI ≥30, will consume capsules containing flaxseed oil (4 g ALA/day) or fish oil (4 g DHA + 0.8 g EPA/day) during 4-week supplementation phases, with a minimum 4-week washout. The primary outcome is alterations in plasma oxylipin profiles. Secondary outcomes include effects of supplementation on circulating markers of inflammation, adipokines, plasma fatty acid composition, blood lipid profile, anthropometrics, oxylipin and cytokine profiles of stimulated immune cells, monocyte glucose metabolism, blood pressure and pulse wave velocity.

Ethics and significance: This trial has been approved by the University of Manitoba Biomedical Research Ethics Board and the St. Boniface Hospital Research Review Committee. The study will provide information regarding the effects of ALA and DHA supplementation on oxylipin profiles in obese but otherwise healthy females. Additionally, it will improve our understanding of the response of circulating inflammatory mediators originating from immune cells, adipose tissue and the liver to n-3 PUFA supplementation in relation to the metabolic features of obesity.

Keywords: Omega-3 fatty acids, ALA, DHA, Oxylipins, Obesity, Inflammation

2.1 Introduction

Obesity, defined as a BMI of 30 or greater, is a rapidly growing worldwide epidemic that is recognized as a major public health concern. The number of persons with obesity has nearly doubled since the 1980s, and today approximately 39% of the world's adult population are overweight (BMI 25-30) and more than 13% are obese (BMI>30) [1]. Moreover, obesity is characterized by chronic inflammation, which is implicated in the development of T2D, CVD, cancer, liver and kidney disease, arthritis and depression [2, 3].

Omega-3 polyunsaturated fatty acids (n-3 PUFAs), such as the marine-derived, EPA, and DHA, have demonstrated beneficial effects in the context of obesity, for example they are associated with reduced inflammation and improved glucose metabolism and plasma lipid profiles [4]. Studies examining the more prevalent dietary n-3 PUFA, ALA, indicate that it also may have beneficial effects for reducing inflammation [5]. These effects are likely mediated in part by metabolites formed from PUFAs called oxylipins, which are biologically active lipid metabolites oxygenated by cyclooxygenases, lipoxygenases, and cytochrome P450 enzymes. While much remains to be learned regarding their functions, they are known to influence processes involved in inflammation, immunity, vascular tone and pain [6]. Moreover, certain oxylipins synthesized from EPA and DHA, maresins and resolvins, respectively, have demonstrated potent anti-inflammatory properties [7]. This is an exciting area of research that should apply to ALA as well.

The oxylipin profile is thought to be pathologically altered in obesity [8]. Recently, Heemskirk et al. [9] reported that obese women with T2D had elevated levels of pro-inflammatory arachidonic acid-derived oxylipins and higher expression of 5-lipooxygenase in subcutaneous adipose tissue. Examination of how oxylipin profiles are altered in obesity may explain their beneficial and detrimental effects and allow targeted therapies and interventions to be developed [10]. N-3 PUFAs have emerged as potential therapeutic agents, however, their effects on oxylipin profiles in the context of obesity have not been thoroughly examined. A direct comparison of DHA and ALA supplementation in the context of obesity with respect to their effects on plasma oxylipin profiles, obesity-associated inflammation and other clinical parameters is needed to address these questions.

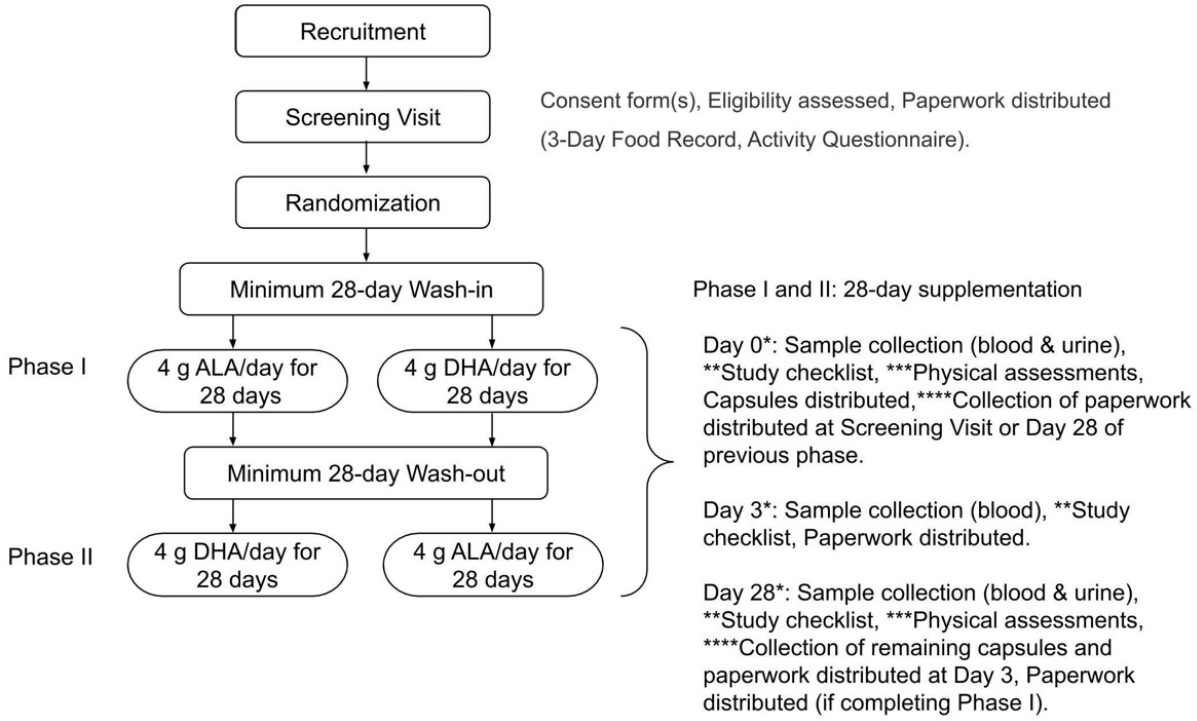
Our recently published clinical trial investigating the time course for oxylipin profile changes during supplementation with ALA or DHA in young healthy adults [11] found that the plasma oxylipin profiles of females are more responsive to n-3 PUFA supplementation compared to males. Thus, as an extension of our previous study, the Effects of Dietary Fatty Acids on Octadecanoid Production and Biological Actions in Obesity-Induced Inflammation (OXBIO) trial was designed as a randomized, double-blind, cross-over intervention trial to compare the effects of supplementation with ALA-rich flax oil and DHA-rich fish oil for four weeks on the plasma oxylipin profile and inflammation- and obesity-associated parameters of pre-menopausal cis-gendered females with obesity. Assessments before and after supplementation will include plasma oxylipin profile, fatty acid composition, adipokines, markers of inflammation, and markers of metabolic function (glycemia, lipid profile). In addition, the oxylipin profile and cytokines produced by PBMC in response to stimulation (TLR4, TLR7/8 ligands) and in relation to PBMC fatty acid composition will be investigated along with monocyte glucose metabolism and PBMC gene expression. This study design will provide new information on oxylipin profile changes in response to n-3 PUFA supplementation in a population of obese but otherwise healthy women and enable one of the first comparisons of ALA- and DHA-responsive oxylipins within the context of obesity. It will also inform our understanding of relationships among indices of inflammation originating from adipose tissue (adipokines), liver CRP and immune cells, with the plasma oxylipin profile and the metabolic health of the participants, in response to n-3 PUFA supplementation.

2.2 Patients and Methods

2.2.1 Study Design and Population

The OXBIO trial is a single site, double-blind, randomized, cross-over study conducted at the IH Asper Clinical Research Institute, St Boniface Hospital, Winnipeg, Canada. The objective of this study is to compare the effects of a 4-week supplementation with flax oil rich in ALA and fish oil rich in DHA in obese pre-menopausal females. The primary outcome is plasma oxylipin profile. Secondary outcomes include plasma fatty acid composition, adipokines, circulating markers of inflammation, metabolic function (glycemia, blood lipid profile), immune function, anthropometrics (BMI, waist circumference [WC], blood pressure [BP], and blood vessel health

(pulse wave velocity [PWV]) and augmentation index), and nutrient intake. A detailed list of outcomes and parameters to be measured are listed in Table 1. An overview of the study design and assessments is shown in Figure 2-1. The population for this study will be obese pre-menopausal females aged 20-55 years of age with an absence of chronic illness. Detailed inclusion and exclusion criteria are provided in Table 2-2.



*At each study visit changes in concomitant medication, medical condition, pregnancy status and adverse events are assessed.

**Study checklist to assess compliance (number of capsules missed, background omega-3 fatty acid intake), use of anti-inflammatory medication, and dates of menstrual cycle.

***Physical assessments include height, weight, waist circumference, blood pressure and pulse wave velocity.

****3-Day Food Record and Activity Questionnaire completed during each Wash-in/out Period and during week 3 of each Supplementation Phase.

Figure 2-1 Overview of study design and assessments.

Table 2-1 Study measurements and parameters.

Indication	Measured parameter	Biological sample
Oxylipin profile	· Oxylipin concentrations (168 individual oxylipins)	Plasma
Fatty acid profile	· Fatty acid composition	
Adipocyte (dys)function*	· Adipokines (adiponectin, resistin)	
Inflammatory markers	· CRP, interferon-γ, interleukin-1β, interleukin-6, interleukin-8, interleukin-10, tumour necrosis factor-α, vascular endothelial growth factor	
Metabolic function*	· Glucose · Glycated hemoglobin†‡ · Lipid profile† (total cholesterol, LDL-cholesterol, HDL-cholesterol, triglycerides)	
Liver function*	· Alanine aminotransferase† · Aspartate aminotransferase†	
Kidney function*	· Creatinine†	
Immune function*	· Oxylipin profile and cytokines (interferon-γ, interleukin-1β, interleukin-6, interleukin-8, interleukin-10, resistin, tumour necrosis factor-α) after stimulation of PBMC *via* TLR4 and TLR7/8; relationship to fatty acid composition of PBMC · PBMC gene expression · Glucose metabolism of monocytes	PBMC
Pregnancy status*	· Human Chorionic Gonadotropin test strip ¶	Urine
Physical assessments*	· Height§ · Weight§ · Waist circumference† · Blood pressure† · Pulse wave velocity and augmentation index	
Diet and lifestyle¥	· 3-Day Food Record · Activity Questionnaire	

†Measured at Screening Visit for Inclusion/Exclusion Criteria

‡Measured only at Screening Visit

§ Used to calculate Body Mass Index (BMI)

¶ Used on Day 0 and 28 of each Supplementation Phase to confirm not pregnant

* Measured on day 0 and 28 only

¥ Completed during each Wash-in/out Period and during week 3 of each Supplementation Phase

Table 2-2 Study inclusion and exclusion criteria

Inclusion Criteria	Exclusion Criteria
Non-pregnant, non-lactating pre-menopausal female, ≥20 and ≤55 years of age (must take adequate birth control measures through the trial and must immediately stop taking the study oil capsules and be withdrawn from the study if they become pregnant).	Has experienced a cardiovascular event (e.g. heart attack, stroke) or had a surgical procedure for cardiovascular disease (e.g. bypass, stent); presence of diabetes, chronic renal disease, liver disease (with exception of fatty liver), rheumatoid arthritis, immune disorder or disease (e.g. multiple sclerosis, leukemia), cancer in the previous 5 years, neurological disorders, gastrointestinal disorders or gastrointestinal surgery or bariatric surgery, or liposuction; or experiences migraines.
Fasting LDL-cholesterol ≤4.5 mmol/L and triglycerides ≤4.5 mmol/L (management with cholesterol-lowering and triglyceride-lowering medications is acceptable); plasma creatinine ≤265 µmol/L; AST <5× ULN where the normal range is 10 – 32 U/L; ALT <5× ULN where the normal range is <25 U/L; glycated hemoglobin <6.5%.	Taking medications for inflammation, pain or arthritis (e.g. COX inhibitors, steroids such as cortisone and prednisone), medications for blood glucose management, anti-coagulants/blood thinners, low dose acetylsalicylic acid within the last 3 months or while participating in the study.
Blood pressure <160/100 (management with anti-hypertensive medications is acceptable).	Regular use (> one day per week during two or more weeks) of acetylsalicylic acid (e.g. Aspirin), ibuprofen (e.g. Advil) or over-the-counter anti-inflammatory products such as naproxen (e.g. Aleve, Midol Extended Relief) or those containing steroids such as cortisone and prednisone, within the last 3 months or while participating in the study.
BMI ≥30 and waist circumference >80 cm for females of Asian ethnicity or ≥88 cm for females of non-Asian ethnicity.	Regular use (>three days per week during menstruation or >one day per week during two or more other weeks) of acetaminophen (e.g. Tylenol, Midol), within the last 3 months or while participating in the study.
Stable regime if taking vitamin and mineral/dietary/herbal supplements for the past 1 month and while participating in the study.	Allergy or sensitivity to any of the study product ingredients, such as flax oil or flaxseed, fish oil or its sources such as fish or shellfish.
Willing to maintain a stable level of activity while participating in the study.	Cigarette/cigar smoking or use of tobacco products within the past 12 months or during the study.
Willing to maintain dietary routine and to refrain from consuming omega-3 supplements or omega-3 rich foods (≥0.3 grams ALA/serving, or ≥0.1 grams EPA + DHA/serving), and to refrain from anti-inflammatory natural health products, from acceptance into the study until the final study visit.	Body weight has not been stable (±3 kg) over the past 6 months.
Must have normal menses.	Consumption of >15 alcoholic beverages per week (according to Canada's Low-Risk Alcohol Drinking Guidelines, 2012) within the last 3 months or while participating in the study.
Agrees to not donate blood or blood products (e.g. platelets) while participating in the study and for 2 months after participation in the study.	Current (within the past 30 days) bacterial, viral or fungal infection.
Willing to comply with the protocol requirements and procedures.	Unable to obtain blood sample at the screening, week 0 visit, or two consecutive study visits.
	Donated blood or blood products (e.g. platelets) or had blood collected in the 2 months prior to participation the study.

2.2.2 Ethics Approval

The protocol is approved by the University of Manitoba Biomedical Research Ethics Board and the St. Boniface Hospital Research Review Committee. The Natural and Non-Prescription Health Products Directorate at Health Canada issued a Notice of Authorization for the study protocol. The study is registered on Clinicaltrials.gov (NCT 03583281). The results of the study will be published in peer-reviewed journals.

2.2.3 Randomization and Blinding

The randomization sequence was generated by an unblinded statistician not affiliated with the research team. This ensures that participants are randomly assigned to the different oils in the first Supplementation Phase and then crossed over to the other oil during the subsequent Supplementation Phase. To reduce potential bias during data collection and analyses, the participants as well as individuals on the research team who interact directly with the participants and who are involved in analyses of the samples will be blinded to the intervention from the time of randomization and for the duration of the study. The capsules containing the oils will be dispensed by the Pharmacy Department at St. Boniface Hospital and provided in opaque packaging to reduce the risk of unblinding. Participants will be asked not to divulge any information about the characteristics of the oils during their interaction with the study coordinators.

2.2.4 Intervention

Daily doses of ~4 g of fatty acid (3.70 g of ALA or 3.88 g DHA) will be achieved by consumption of 6 flax oil capsules (NOW Foods, IL; NPN:80002313) (ALA group) or 7 DHA oil capsules (Super DHA, Carlson Laboratories, IL; NPN: 80012587) (DHA group) per day. The flax oil capsules (1000 mg) contain 617 mg of ALA, and the DHA oil capsules (1000 mg) contain 555 mg of DHA and 144 mg of EPA (Table 2-3), and this has been assessed by gas chromatography. The study capsules will be obtained from A1 nutrition (Winnipeg, MB) and distributed by St. Boniface Hospital Pharmacy (Winnipeg, MB) in packages (3 capsules/package for flax oil and 3 and 4 capsules/package for fish oil) to ensure consistent daily dosing. A 4-week supply of capsules will be provided in an opaque bag. Participants will be instructed to consume

the capsules in two portions (2 packs/day) in conjunction with a meal, preferably one portion at breakfast or lunch, and the other portion at supper, to promote digestion and absorption of the fatty acids [12].

The supplementation period and doses of fatty acids are based on a study showing that fish oil supplementation containing 3.6 g of n-3 PUFAs for 4 weeks resulted in increased EPA- and DHA-derived oxylipins [13]. Similarly, our group demonstrated changes in oxylipin profiles in healthy young males and females supplemented with 4 g of ALA or DHA over a 4-week time period, with most oxylipins reaching a plateau by 1 week [11]. There is also evidence that increased levels of EPA- and DHA-derived oxylipins can be detected as early as 3 days post-supplementation [11, 14]. As such oxylipin profiles and key cytokines will be measured at days 0, 3 and 28 to determine whether alterations occur early, and whether they are sustained throughout the supplementation period (see Table 2-1 for detailed list). Furthermore, supplementation with 4 g of fatty acid is within the range commonly used in studies investigating effects on inflammation [15-18]. Since oxylipin composition in response to ALA supplementation has not been fully investigated, a dose higher than what is usually obtained through the diet (1.0-1.6 g/day) was chosen [19]. Although adding 4 g of fatty acids is a greater change for fish-sourced than for plant-sourced n-3 PUFAs, we aim to examine how supplementation in equal amounts influences oxylipin and inflammatory profiles. Importantly, the European Food Safety Authority has determined that supplemental intakes of combined EPA + DHA up to 5 g per day do not present safety concerns for adults [20].

Table 2-3 Fatty acid composition of flaxseed (ALA) and fish oil (DHA) capsules for supplementation phases

	Flax oil Lot 1		Flax oil Lot 2		Fish oil Lot 1	
Fatty acid	**mg/capsule**	**mg/day***	**mg/capsule**	**mg/day***	**mg/capsule**	**mg/day¥**
C16:0	53.8	323	51.5	309	2.92	20.5
C16:1					2.9	20.3
C18:0	34.2	205	34.6	208	19	133
C18:1	185	1110	181	1090	50.9	356
C18:1n7c	5.4	32.2	6.4	38.6	5.56	38.9
C18:2	157	945	148	888	7.53	52.7
C18:3n3	**617**	**3700**	**597**	**3580**	3.87	27.1
C20:0	0.926	5.56	1.2	7.19	14.6	102
C20:1	0.954	5.72	1.21	7.24	17.6	123
C20:2					4.43	31
C20:4					10.3	71.9
C20:3n3					1.17	8.21
C20:5					144	1010
C22:0					3.26	22.8
C22:1					5.7	39.9
C22:4					2.05	14.3
C22:5n3					22.8	160
C22:6n3					**555**	**3880**
C24:1					16.5	115

Only FA present at > 0.9mg/capsule are shown

FA present at >100mg/capsule are underlined

ALA (C18:3n3) and DHA (C22:6n3) are indicated by bold text

* **ALA daily dose achieved** by consuming 6 capsules per day

¥ **DHA daily dose achieved** by consuming 7 capsules per day

2.2.5 Recruitment

Participants will be recruited through advertisement employing various media platforms, including bulletin board and email advertisements within the healthcare and academic communities and print ads distributed throughout the city of Winnipeg. After responding to an advertisement, participants will be provided a brief description of the study and they will be provided information about some key eligibility criteria over the phone. If a potential participant is still interested in the study and they may meet the eligibility criteria, they will be sent a copy of the informed consent for review and further consideration.

2.2.6 Screening Visit

Interested individuals will attend a screening visit where, prior to conducting any study-related procedure, consent forms will be reviewed and signed. The general consent form describes the study schedule and supplements to be provided, specific analyses planned as well as the handling of personal information, the storage of study samples and the potential risks associated with sampling of biological fluids. A separate consent form will provide the option of allowing collected samples to be used for genomic analyses. Participants will be asked questions regarding their demographic details (age, sex, ethnicity) and their medical history including clinically diagnosed diseases, usage of medications, vitamin and mineral supplements, dietary and herbal supplements, smoking and alcohol usage, body weight changes and body weight history, menstrual cycle regularity and length, and use of birth control. A physical assessment including blood pressure, height, weight and calculated BMI will also be conducted at the screening visit. A fasted (12 hr.) blood sample obtained by venipuncture will be used for analysis of blood lipid profile (total cholesterol, LDL-cholesterol, HDL-cholesterol and triglycerides), plasma creatinine, alanine transaminase (ALT), aspartate transaminase (AST) and glycated hemoglobin. At the Screening Visit, participants will be provided with information and forms for the Run-in Period in anticipation that they will meet the eligibility criteria.

2.2.7 Run-in and Wash-out Periods

During the Run-in Period and until the final study visit of the last Supplementation Phase, participants will be asked to adhere to a set of requirements to maintain a reasonably constant

background of factors which may affect oxylipin profiles. For the duration of the study participants will agree to forgo consumption of foods high in n-3 PUFAs (>0.3 g ALA/serving, or >0.1 g EPA + DHA/serving) as well as non-study n-3 PUFA supplements. Participants will be given a list of foods high in n-3 PUFAs to avoid during the study as well as suggestions for replacement items to assist with modifying their diet to reduce background n-3 PUFA intake. Participants will be asked to refrain from taking anti-inflammatory products, maintain a stable level of activity, refrain from consuming >15 alcoholic beverages per week (according to Canada's Low-Risk Alcohol Drinking Guidelines, 2012 [21]), and refrain from smoking and use of tobacco products.

The Run-in Period will be scheduled to start a minimum of 4 weeks before the Day 0 (baseline) study visit for the first Supplementation Phase. The minimum 4-week Wash-out Period before starting Supplementation Phase 2 will follow the same procedures as described for the Run-in Period. During the Run-in and Wash-out Periods, participants will complete a 3-day Food Record and Activity Questionnaire and return the completed documents at the Day 0 study visit for each phase.

2.2.8 Study Visits

Study visits will be scheduled at baseline (Day 0), Day 3 and Day 28 of each Supplementation Phase. The baseline (Day 0) visit will be scheduled 9±2 days after starting menses so that all participants are at a similar point in their menstrual cycle for sample collection days; this is to control as much as possible the effects of hormonal state on oxylipin and inflammatory status, similar to our previous study [11]. Participants will be asked to avoid strenuous exercise for 24 hours prior to all study visits, as well as all anti-inflammatory and over-the-counter pain medication such as acetaminophen and ibuprofen for 48 hours before a blood draw. During all study visits participants will be assessed for changes in medication profiles, health conditions and adverse events. At each study visit participants will complete a Study Checklist to monitor adherence to requirements regarding avoidance of n-3 PUFA rich foods and non-study n-3 PUFA supplements, to document alcohol consumption, activity levels, usage of pain medication or any anti-inflammatory products, to verify menstrual cycle dates, and to confirm fasting status for the blood draw.

An overview of the study visits is provided in Figure 2-1. At each study visit (Day 0, 3 and 28), fasting blood samples will be obtained by venipuncture and will be analyzed for oxylipin profiles using non-targeted high-performance liquid chromatography–tandem mass spectrometry. Oxylipin profile as well as other parameters measured are shown in Table 2-1. On Days 0 and 28, PBMC will be isolated from a portion of blood and analyzed for oxylipin profiles and immune cell parameters (Table 2-1). Urine samples collected on Days 0 and 28 of each Supplementation Phase will be used to test for pregnancy (Table 2-1). The other assessments conducted on Day 0 and 28 of each Supplementation Phase will include anthropometrics (height, weight, BMI, WC), BP, and non-invasive assessment of blood vessel health (PWV and augmentation index; Mobil-o-Graph, IEM, Stolberg, Germany) (Table 2-1).

On Day 0 of each Supplementation Phase, participants will return the 3-Day Food Record and Activity Questionnaire completed during the preceding Run-in or Wash-out Period, and they will receive another 3-Day Food Record and Activity Questionnaire to complete during week 3 of the Supplementation Phase and return on Day 28.

2.2.9 Compliance

Day 28 of each Supplementation Phase participants will be asked to return any remaining capsules. If more than 3 days of capsules are missed, or if capsules are not consumed during the 5 days preceding the study visit, the Supplementation Phase will be stopped, and the participant given the option of re-doing the Supplementation Phase after a minimum 4-week Wash-out phase. The 3-Day Food Records will be used to monitor compliance with the background diet (avoiding consumption of foods high in ALA, EPA and DHA as indicated on the handout). Plasma fatty acid composition will also be used to confirm consumption of the ALA or DHA capsules during the Supplementation Phases.

2.2.10 Sample Size Calculation

The sample size is based on the variance of the main outcome of the project, namely, the different oxylipins. The variance is not the same for all oxylipins, but power analysis for several of them using G*Power3 [22] indicates that n=20 will provide a power of at least 0.80 for the various oxylipins to be measured, specifically those derived from ALA and DHA. A sample size

of n=24 would provide further power for those oxylipins present at lower concentrations, and at the same time supply some allowance for potential dropouts given the length of this study (two Supplementation Phases with Wash-out Period in between) and if dropouts occur during the final Supplementation Phase. Based on a previous study by Zhao et al [5], a sample size of n=24 will provide sufficient power to detect a reduction in inflammation after n-3 PUFA supplementation with ALA and will support the identification of strong correlations ($r>0.5$) between oxylipin concentrations and levels of inflammatory markers.

2.2.11 Statistical Analyses

The effects of supplementation on plasma oxylipin concentrations (and other parameters) at day 28 as well as changes from baseline within a Supplementation Phase will be analyzed by PROC MIXED (Statistical Analysis Software, Cary, NC). Individuals will be treated as a random effect. Supplementation and time (day, phase) will be fixed effects; day and order of phase will be categorical variables. Adjusted p values (Tukey-Kramer correction) for differences of least squares means will be used to determine significant differences between ALA and DHA supplementation at a particular timepoint, and to determine significant differences from baseline during ALA or DHA supplementation. If necessary, values will be log transformed to achieve normal distribution within each group. To account for substantial instrument and handling errors but allow for true inter-individual variability, values will be excluded as outliers if they fall above or below the mean by 6× (oxylipins) or 5× (all other parameters) the standard deviation. Pearson's or Spearman's correlation analysis will be used to identify correlations between oxylipin concentrations and other parameters. The level of statistical significance will be $p<0.05$.

2.3 Discussion and Conclusions

The OXBIO study aims to compare the effects of short-term plant-based versus marine-based n-3 PUFA supplementation on oxylipin profiles and their relationship to clinically important metabolic parameters in females with obesity. Specifically, we will explore how these dietary fatty acids alter the plasma oxylipin profile and influence features of obesity-induced inflammation. Most research to date has focused on the investigation of fish oils in the context of inflammation and obesity [23, 24]. In contrast, knowledge about ALA in this context is limited, however, this is an important area of investigation given that ALA is an essential fatty acid and

an important plant-based n-3 PUFA in diets around the world. Whereas there is considerable information regarding the actions of the fatty acids present in fish oil [25-27], the same is not true for ALA even though certain studies have indicated this fatty acid has distinct biological actions [5, 28, 29]. Using the study design described here, supplementation with equal doses of ALA and DHA will be compared over 4 weeks in cis-gendered females with obesity. This study is unique as the impact on oxylipin profiles and inflammatory markers in plasma and secreted from stimulated immune cells will be examined side-by-side. It is predicted that ALA and DHA supplementation will uniquely alter the oxylipin profile and that oxylipin profiles will be different in participants with varying degrees of obesity. The oxylipin profile [8] as well as inflammatory marker production [1] are thought to be pathologically altered in obesity. We hypothesize that fatty acid supplementation with ALA or DHA may beneficially alter the oxylipin profile as well as inflammatory markers associated with obesity. Furthermore, certain oxylipins are predicted to be correlated with a reduction in inflammation and will be produced at higher levels in responders versus non-responders, as measured by reductions in key cytokines, such as interleukin IL-6, TNF-α, and CRP.

Clinical evidence supports the use of n-3 PUFAs to reduce risk factors for chronic diseases such as CVD and T2D [18, 30-32] although such findings are inconsistent. This evidence comes primarily from studies examining the fish oil-derived PUFAs, EPA and DHA, but studies examining ALA, the more prevalent dietary n-3 PUFA, indicate that it also may have beneficial effects for disease protection and management [5, 33]. These effects are likely mediated by metabolites of PUFAs called oxylipins. Oxylipins are biologically active lipid molecules formed through oxygenation reactions catalyzed by cyclooxygenases, lipoxygenases, and cytochrome P450 enzymes [34]. Although much remains to be learned with respect to their functions, oxylipins are known to influence inflammation, immunity, vascular tone and pain [6]. Examination of these oxylipins and how they are altered in disease states such as obesity may shed light on their beneficial and detrimental effects, allowing for the development of targeted treatments and interventions. The present study is designed to compare the impact of ALA and DHA supplementation on oxylipin profiles in humans with obesity, and to determine whether these alterations are associated with a reduction in inflammation and/or an improvement in clinical parameters.

Supplementing with 4 g/day of DHA represents a substantial increase in this fatty acid, given that the recommended dietary intake of combined EPA + DHA is 0.16 g/d and 0.11 g/d for men and women, respectively [19]. Similarly, increasing ALA intake by 4 g/day represents a considerable increase compared to typical dietary intakes of 1.2-1.6 g/d for men and 1.0-1.2 for women [19]. Although there is some debate, the conversion rate of ALA to DHA in humans is estimated to be 1-2% or lower [18]. Therefore, any increase in DHA as a result of ALA supplementation is expected to be far lower than the increase obtained through direct DHA supplementation. Although adding 4 g of fatty acids will be a more substantial change for fish-sourced n-3 PUFAs than for plant-sourced n-3 PUFAs, we aim to examine how supplementation in equal amounts influences oxylipin profiles. The dose of fatty acid selected is similar to other studies investigating effects of fish oil (EPA+DHA) provided in capsules [13, 14, 16] including a previous study by our group [11]. The 4 week supplementation period is based on previous studies [13], including our own, that found 4 grams of ALA or DHA altered oxylipin profiles within one week, with most changes occurring by 3 weeks [11]. Assessing oxylipin profiles and key cytokines at day 3 and day 28 will allow us to determine whether alterations are present early, and whether they are sustained throughout supplementation. A minimum 28-day Wash-in Period is selected to limit the background effect of n-3 PUFAs obtained through diet. There will be a minimum 4-week Wash-out between Supplementation Phases, as plasma and PBMC n-3 PUFA levels are expected to return to baseline levels within this time frame [35].

A major objective of the present study is to determine the effect that n-3 PUFAs have on inflammatory markers, which will be assessed using a Meso Scale Discovery platform. Obesity is commonly associated with elevated systemic inflammation, usually determined by liver-derived CRP levels [1]. However, cytokines such as IL-6 and TNF α are also indicative of systemic inflammation [1]. The best characterized anti-inflammatory cytokine, IL-10, is produced by a variety of immune cell types. IL-10 is positively associated with insulin sensitivity in healthy subjects and is reduced in those who are obese and have diabetes [36]. Additionally, certain adipose tissue-derived mediators (adipokines) are associated with metabolic function, either favorably (adiponectin) or unfavorably (resistin) [37]. Adiponectin is produced almost exclusively by adipocytes while resistin is also produced by immune cells in adipose tissue and in the circulation [38]. Adipocytes become hypertrophic in response to nutrient excess, resulting in a dysregulated secretory profile. This leads to infiltration of immune cells such as pro-

inflammatory macrophages, which perpetuate further dysfunction, including local insulin resistance [39]. Adipokines and cytokines can also impair the function of other organ systems including blood vessels and the heart. n-3 PUFAs originating from both plant [33] and fish oil [40] sources are reported to reduce both immune cell and adipocyte derived inflammatory markers in participants with obesity. Here we investigate this further with a head-to-head comparison of ALA and DHA in equal doses. To this end, we will measure cytokines (IL-6, IL-I-β, IL-8, interferon (INF)-γ, IL-10, TNF-α) and adipokines (adiponectin and resistin) in addition to factors related to adiposity (BMI, WC) and blood vessel function (vascular endothelial growth factor [VEGF], BP, PWV) at day 0 and 28 time points. We will also measure standard clinical markers related to obesity-associated co-morbidities including CRP, HDL-cholesterol and LDL-cholesterol, triglycerides, glucose, ALT, AST and creatinine. We hypothesize that 28-day n-3 PUFA supplementation will be sufficient to observe changes in cytokine and oxylipin profiles; however, this timeframe may be insufficient to observe the resulting effects on adipose and cardiovascular tissues. Regardless, we hope that characterizing early changes in oxylipins and cytokines in response to n-3 PUFA supplementation will inform future studies examining functional outcomes in larger, longer-term trials.

In addition to altering the plasma fatty acid and oxylipin profiles, n-3 PUFA supplementation is expected to alter the fatty acid profile of PBMC [41], which impacts the balance of fatty acid precursors available for oxylipin production. Thus, we will examine the effect of supplementation on PBMC fatty acid and oxylipin profiles, and how this relates to cytokine production (IL-6, TNF-α, IL-I-β IL-8, INF- γ, IL-10) at rest and in response to challenge with bacterial (TLR4 ligand) and viral (TLR 7/8 ligand) mimics. This will contribute to our understanding of how ALA and DHA supplementation influence immune cell responsiveness in the context of oxylipins and obesity, facets that have not received much attention.

Mitochondrial dysfunction is another immunological feature that has previously been linked to obesity and its complications [42, 43]. The release of reactive oxygen species by dysfunctional mitochondria is functionally linked to increased production of pro-inflammatory cytokines [44]. LPS stimulation of innate immune cells leads to a metabolic shift similar to the Warburg effect described in cancer cells. Here, oxidative phosphorylation of glucose (measured

by OCR) is reduced in favour of glycolysis (measured by ECAR) [45]. Shirai et al. demonstrated that these two processes are also out of balance in monocytes taken from coronary artery disease patients, although they observed increases in both ECAR and OCR [46] relative to monocytes from healthy controls. An increase in resting PBMC OCR had been reported in individuals with T2D [47] while others have reported a decrease in platelet OCR in T2D [48] and in PBMC OCR in systemic lupus erythematosus [49] relative to healthy controls. Thus, it is not entirely clear what direction of change would be indicative of better or worse immunometabolic state. Fish oil supplementation has been demonstrated to alter mitochondrial structure and function in skeletal and cardiac muscle cells [50]. Thus, there is sufficient rationale to predict that the bioenergetic profile of leukocytes may be correlated to markers of inflammation and/or metabolic health and that n-3 PUFA supplementation will alter the ECAR/OCR balance at rest and/or with LPS challenge. We will measure these parameters at Day 0 and Day 28 of supplementation using the Agilent Seahorse XF mitochondrial stress test. Since different leukocytes display different resting bioenergetic profiles [51] and supplementation may alter their relative numbers, a pure population of classical (CD14+CD16-) monocytes will be isolated for experimentation.

The present study has numerous strengths. One such strength is obtaining high-quality lipidomic information using high-performance liquid chromatography–tandem mass spectrometry. This method has a high degree of sensitivity for the quantitative assessment of oxylipins and can detect 168 molecular species of oxylipins in an unbiased manner [11]. Moreover, while some oxylipin profiles have been obtained for non-obese humans supplemented with DHA or ALA [11, 52], to our knowledge this will be one of the first trials examining the effect of ALA supplementation on oxylipins produced in humans with obesity. A relatively long term (12 weeks) and high dose (10 g/day) ALA supplementation study very recently reported alterations to the plasma oxylipin profile in participants with impaired fasting glucose [53]. The current study will reveal if the same changes can be observed with a more moderate dose, shorter term intervention. Excitingly, this may also be one of the first reports to examine the effect of ALA and DHA supplementation on oxylipin production by human PBMC upon stimulation.

Another strength of this study relates to the selection of our study population. Women have been traditionally underrepresented in clinical trials [54]. In our previous study [11], alterations in oxylipins were found to be occur more rapidly in females than males in response to

n-3 PUFA supplementation. Since we hypothesize that an increase in n-3 oxylipins will be associated with a decrease in inflammatory markers, we chose an all-female cohort in order to maximize our ability to detect these changes given the sample size and time period. Furthermore, it is known that people with abdominal obesity often have high circulating levels of inflammatory markers [55]. Thus, in selecting an obese population based on BMI and waist circumference, we hope to characterize alterations to oxylipins and cytokines that may not be observed in a healthy population where a dampening of inflammation may not be measurable or relevant.

Our study does have some limitations. As participants are required to begin supplementation 9 ± 2 days after the beginning of menses, we are only able to enroll women with verifiably regular menstrual cycles. Therefore, women using contraceptive methods that prevent visible menstruation, such as the intrauterine device Mirena [56], would be excluded from our study, and currently this includes a sizeable segment of the adult female population. Since sex hormones may directly influence lipid metabolism [52], omitting persons on a popular form of hormonal birth control may limit the applicability of our results to the broader population. Another limitation is the reliance on self-reporting for compliance during supplementation and adherence to a restricted n-3 PUFA diet. To document compliance, participants are required to return unused capsules, report missed doses during each Supplementation Phase, complete a questionnaire about consumption of n-3 PUFA rich foods at each study visit, and provide 3-Day Food Records for nutrient analysis. To further monitor compliance, plasma fatty acid composition of day 0 and 28 samples will be quantified by gas chromatography for each Supplementation Phase.

In our previous trial, significant changes in oxylipin and fatty acid profiles were observed at 3 weeks [11]. Here, with 4-week supplementation, changes in parameters such as weight, waist circumference, and blood vessel function (measured by PWV and BP) are not likely to be observed. However, we do expect that alterations in oxylipin and cytokine profiles would precede such changes and may serve as important biomarkers. For example, changes in 12-LOX oxylipins may provide the mechanistic basis for how obesity promotes the onset of CVD [10]. As such, the results we obtain may inform future studies examining functional outcomes in longer-term trials.

In summary, we expect the OXBIO study to enhance our understanding of the biological changes resulting from short-term supplementation with plant-based versus marine-based n-3 PUFA. The findings from this study will provide new information on how these important dietary fatty acids alter oxylipin profiles and how these, in turn, relate to markers of inflammation (such as pro-inflammatory cytokines) and metabolic function. Thus, this study may explain how changes in oxylipin levels influence the pathogenesis of obesity-induced inflammation in pre-menopausal females. Furthermore, examining the oxylipins and cytokines produced by immune cells challenged with pattern recognition receptor ligands, and the bioenergetic profiles of monocytes, will further contribute to our understanding of how ALA and DHA supplementation influence immune cell responsiveness. Taken together, this information will provide the basis for future studies elucidating the biological roles of these fatty acid metabolites. This may then assist in the development of more targeted strategies to treat and prevent obesity and its associated comorbidities.

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Transition Statement 1

Chapter 2 in this dissertation described the rationale and design for the randomized crossover clinical trial (OXBIO) that I co-coordinated from 2018-2020. The OXBIO trial compared the effects of equal doses of ALA and DHA-rich oil supplements (4 g/day) on plasma oxylipins and markers of metabolic health in women with obesity, along with plasma fatty acids, inflammatory markers, and monocyte glucose metabolism as secondary outcomes. This clinical study found that ALA-rich flaxseed oil and DHA-rich fish oil each had unique effects on metabolic parameters in women with obesity, including differentially altering adipokines and immune cell phenotype (Appendix 1). As discussed in earlier chapters, key studies have shown that adipose tissue of obese individuals is characterized by local inflammation [1-6], which in turn recruits and activates immune cells, promoting low grade systemic inflammation and an altered immune phenotype (e.g. monocytes display increased migration towards chemokines, secrete more pro-inflammatory cytokines and are more likely to polarize to pro-inflammatory M1 macrophages) [7, 8]. This contributes to numerous comorbidities including atherosclerosis. Moreover, the type of dietary fat consumed is known to alter immune cell gene expression, ultimately influencing their function [9-11]. Indeed, regulation of gene expression by PUFAs is thought to be one of the main mechanisms by which they convey their beneficial effects. However, to the authors knowledge, no research to date has compared the actions of ALA and DHA on immune cell gene expression or function, as it is commonly assumed that the biological effects of ALA are dependent on its conversion to longer chain fatty acids such as DHA. On the other hand, several studies, including our own, indicate that ALA and its bioactive metabolites have direct immune modulatory actions [12-14]. Thus, in parallel to the OXBIO trial, experiments using n-3 PUFA treated THP-1 human monocytes were conducted to aid in determining the various effects of ALA and DHA on immune cell gene expression and the resulting impacts on function. The next chapter utilizes (1) transcriptome analysis of THP-1 human monocytes to identify and compare genes responding to acute ALA and DHA treatment (i.e. candidate genes) , and to predict functions both commonly and distinctly affected by ALA and DHA, (2) functional assays to assess the validity of functions predicted to be altered by n-3 PUFA treatment, and (3) RT-qPCR of key transcripts in PBMC isolated from OXBIO trial participants to compare transcript levels observed in n-3 PUFA treated THP-1 monocytes with

those in mononuclear immune cells from humans consuming ALA or DHA-rich oil supplements. This will also help situate cell culture results in a disease state where immune function is known to be pathologically altered.

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Chapter 3: α-Linolenic Acid and Docosahexaenoic Acid Distinctly Affect Gene Expression and Functions Related to Chemotaxis, Response to Infection, and Cholesterol Metabolism in THP-1 Monocytes and Mononuclear Cells of Humans with Obesity

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Contribution of Co-Authors to Chapter 3: **Lisa Rodway**: Wrote Chapter 3. Performed cell culture treatments, harvesting, RNA extraction and preparation of samples sent to Genome Quebec. Analyzed data using TAC and IPA and created Tables 2, 3 & 4. Performed all chemotaxis and cholesterol experiments. Extracted RNA from PBMC. Performed qPCR on THP-1 and PBMC RNA and analyzed data. Created Figures 3, 4, 5B and 5C. Samantha Pauls: Assisted with writing and with all analyses, specifically analyzed infection data and created Figure 2 and 3A. Isolated PBMC from OXBIO trial participants. Christopher D Pascoe: Analyzed gene expression data using VIP and PLS-DA, created Figure 1. Harold Aukema: Developed study design, editing, funding acquisition. Carla Taylor: Submitted for ethics approval, editing, developed study design, funding acquisition. Peter Zahradka: Developed study design, editing, funding acquisition.

Abstract

Background: Monocytes play a large role in chronic inflammatory conditions such as obesity, atherosclerosis and infection. Marine-derived omega-3 fatty acids such as docosahexaenoic acid (DHA) beneficially alter immune function and attenuate chronic inflammation in part by altering gene expression. Comparisons with plant-derived omega-3 α-linolenic acid (ALA) on immune cell gene expression and function are limited.

Methods: Transcriptome analysis using a clariom D microarray was performed on THP-1 human monocytes treated with ALA, DHA or vehicle for 48 hr using fold change analysis, Partial Least Squares-Discriminant Analysis (PLS-DA), Variable Importance Analysis (VIP), Ingenuity Pathway Analysis (IPA). Candidate genes were validated by qPCR. Functional assays evaluating transcriptomic predictions were performed. Expression of candidate transcripts identified in THP-1 cells were examined in PBMC from clinical trial (OXBIO) participants consuming ALA- or DHA-rich oil supplements.

Findings: ALA and DHA presented distinct transcriptomic profiles as per VIP and PLS-DA. Based on fold change and IPA analyses, both fatty acids were predicted to reduce cellular cholesterol content, while ALA would uniquely increase the response to infection and chemotactic signals. Functional assays revealed ALA and DHA decreased cholesterol content. DHA significantly decreased the response to infection and chemotaxis, but ALA had no effect. Candidate transcripts had similar mRNA levels in OXBIO PBMC as n-3 PUFA treated THP-1 monocytes.

Conclusion: ALA and DHA differentially alter the transcription profiles and functions associated with the response to infection, chemotaxis, and cholesterol metabolism in mononuclear immune cells. Thus, they may uniquely affect related disease processes contributing to obesity, atherosclerosis, and the response to infection.

Keywords: THP-1 monocytes, Gene expression, Alpha-linolenic acid, Docosahexaenoic acid, Obesity

3.1 Introduction

Monocytes are a vital component of the innate immune system that respond to infection and act as important regulators of inflammation [1]. They are characterized by their ability to recognize pathogens and tissue damage via pattern recognition receptors, and can phagocytose, present antigens, secrete cytokines and chemokines, and proliferate in response to infection and injury [1, 2]. When activated, they adhere to endothelial cells, and cross by extravasation into inflamed or infected tissues where they are capable of differentiating into macrophages and dendritic cells [1]. The abilities of monocytes are beneficial in the case of acute infection or tissue repair. However, if these processes occur in an uncontrolled manner, pathologic responses including chronic inflammation can ensue. This raises the possibility that treatments modulating monocyte function may be therapeutic in inflammatory disorders including obesity, atherosclerosis, autoimmune disorders, and unresolved inflammation following infection [4, 5].

Omega-3 polyunsaturated fatty acids (n-3 PUFAs) have emerged as possible therapeutic agents, as marine-derived eicosapentaenoic acid (EPA; 20:5 n-3) and docosahexaenoic acid (DHA; 22:6 n-3) have previously been shown to reduce pro-inflammatory markers and ameliorate symptoms of autoimmune and inflammatory disorders, including rheumatoid arthritis, obesity, and cardiovascular disease, both in vitro and in vivo [6, 7]. A major mechanism by which n-3 PUFAs are thought to act in these conditions is by reducing production of immune cell pro-inflammatory mediators [8, 9]. For example, in THP-1 macrophages, Ndoul et al. [10] demonstrated that DHA reduced the production of pro-inflammatory cytokine IL-6 to a greater degree than EPA. Allaire et al. [11] also found that DHA supplementation led to a greater reduction of plasma IL-8 levels and greater increase of adiponectin concentrations in healthy individuals than EPA. Moreover, while research has focused on studying the actions of n-3 PUFAs in an inflammatory context, many in vitro studies indicate that they have effects other than reducing inflammation. Chao et al. [12] found DHA reduced adhesion of immune cells to the endothelium in an atherosclerosis model. DHA treatment has also been found to maintain an immature or inactivated phenotype in dendritic cells stimulated with lipopolysaccharide, a bacterial infection mimic [13].

In contrast, the actions of the plant-derived n-3 PUFA, ALA, have received far less attention, as it is usually assumed that the biological effects of ALA are dependent on its conversion to longer chain fatty acids such as DHA [14]. However, recent studies indicate that ALA and its bioactive metabolites not only have direct anti-inflammatory actions but also exhibit unique functional effects of their own[Monk, 2016 #277] [8, 9, 15]. A study completed in a rat model of metabolic syndrome (MetS) found equal doses of ALA, EPA and DHA produced different physiological responses that decreased risk factors for MetS; DHA reduced pro-inflammatory cytokines and reduced total body fat while ALA redistributed adipose tissue away from the abdominal area and improved insulin sensitivity [16]. Thus, a better understanding of the distinct effects of individual plant and marine-derived n-3 PUFAs in immune cells will be an important step towards understanding their potential therapeutic applications in different conditions, including, but not limited to inflammatory disorders.

N-3 PUFAs are thought to exert their bioactive effects in immune cells through multiple mechanisms, including altering cell membrane composition and surface receptor modulation, or through binding to G-protein coupled receptors and nuclear receptors [18]. It has also been found that the type of dietary fat consumed alters gene expression of immune cells in the blood, ultimately influencing their function [18]. Indeed, regulation of gene expression by PUFAs can occur through direct or indirect activation of transcription factors or other response factors, ultimately influencing mRNA synthesis [19]. Previous investigations examining gene expression in PBMC during fish oil supplementation demonstrate alterations in the expression of genes related to inflammation, cell cycle and apoptosis, as well as fatty acid and cholesterol metabolism [20, 21]. DHA also downregulates atherogenic and inflammatory genes, which play a role in several processes, including atherosclerotic plaque formation and the perpetuation of chronic inflammation in obesity and after infection [20, 21]. Both EPA and DHA supplementation were found to decrease the expression of genes involved in cholesterol production, such as HMG-CoA reductase (*HMGCR*), and Stearoyl-CoA desaturase (*SCD*) [20, 21]. Similar studies examining the effects of ALA on the immune cell transcriptome have not been performed, although functional studies indicate ALA has biological actions both similar and distinct from its marine counterparts in both cell lines and immune cells isolated from humans supplemented with n-3 PUFAs [8, 9, 22]. As well, to our knowledge, transcriptomic studies examining n-3 PUFAs have been performed only in PBMC, a heterogenous mixture of

immune cell types, making their effects even more difficult to differentiate. Distinguishing gene expression changes induced by marine and plant n-3 PUFA specifically in monocytes, and the associated functional changes, is expected to provide insight into their distinct effects on disease pathologies involving monocytes and possible targets for clinical intervention.

The objective of our study was to explore the impact of ALA and DHA exposure on gene expression and function relative to untreated human monocytes. Global transcriptome profiling of THP-1 human monocytes was used to identify candidate genes responding to the 48 hr ALA and DHA treatments, followed by Ingenuity Pathway Analysis (IPA) to predict common patterns and pathways affected by the fatty acids. The effects of n-3 PUFA treatment on candidate genes were subsequently validated in both THP-1 monocytes and in human PBMC isolated from participants of a clinical trial of ALA and DHA supplementation. Specific emphasis was placed on candidate cellular functions identified in THP-1 monocytes by fold change analysis and IPA, including those related to chemotaxis, cholesterol metabolism and infection. Overall, our results suggest that ALA and DHA have unique effects on the transcriptome of immune cells, leading to distinct functional consequences.

3.2 Methods

3.2.1 Cell culture and n-3 PUFA treatment

Prior to all experiments, THP-1 human monocytes (TIB-202, ATCC, VA, USA) were maintained at a density of 2.5×10^5 to 1×10^6 cells/mL in RPMI-1640 medium (Thermo Fisher Scientific, MA, USA) supplemented with 10% FBS (Wisent, QC, Canada) in a humidified atmosphere with 5% CO_2 at 37°C.

For fatty acid treatments, stock solutions of ALA and DHA (Cayman Chemicals, MI, USA) were diluted to working concentration in phosphate-buffered saline (PBS; Thermo Fisher Scientific, pH 7.45, catalogue no.18912014) containing 1% fatty acid-free bovine serum albumin (Roche, MH, Germany) and added to cell cultures at a final concentration of 50 μM.

3.2.2 PBMC isolation

Twenty-two females with obesity were recruited for a randomized crossover clinical study (described in Pauls 2021; NCT 03583281; approved by the University of Manitoba Biomedical Research Ethics Board and the St. Boniface Hospital Research Review Committee) [40] where they were supplemented with 4 g/day ALA or DHA for 4 weeks. However, due to the limited amount of blood collected for certain participants, PBMC were not available from all 22 participants, thus limiting the number of samples that could be used to test the actions of DHA and ALA on candidate gene expression in vivo (Figure 3-4). PBMC were isolated from anticoagulant-treated blood samples (10 mL EDTA tubes) collected before (day 0) and directly after (day 28) supplementation with ALA-rich flaxseed oil or DHA-rich fish oil using SepMate Tubes and Lymphoprep density gradient medium (Stem Cell Technologies, BC, Canada) according to the manufacturer's instructions, then stored at −80°C in cryogenic freezing medium.

3.2.3 RNA extraction

RNA was isolated from THP-1 cells directly after harvesting using the miRNeasy kit with DNase treatment (Qiagen, HD, Germany), then reverse transcribed to cDNA using the iScript advanced cDNA synthesis kit (Bio-Rad). PBMC were thawed and washed twice with 5 mL Phenol-Red Free RPMI-1640 medium (Gibco ON, Canada) prior to RNA isolation using the NEB RNA iso kit (New England Biolabs, MA, USA) with DNase treatment. RNA was then reverse transcribed using the iScript cDNA synthesis kit. The quantity of extracted RNA in both cases was checked with a spectrophotometer (NanoDrop, Thermo Fisher Scientific), and checked for quality using a 2100 Bioanalyzer (Agilent Technologies Canada Inc., Ontario, Canada). All samples used possessed a RIN of >9.0.

3.2.4 Transcriptomics analyses

Human Clariom D microarray (Agilent) analysis was performed by Genome Québec (Montreal, Québec) in duplicate using RNA extracted from each THP-1 treatment group. Transcriptomics data were analyzed using Transcriptome Analysis Console software (TAC, ThermoFisher). Additional filters were applied manually to exclude non-coding and non-annotated genes.

Partial Least Squares-Discriminant Analysis (PLS-DA) and Variable Importance Analysis (VIP) were performed using the mixOmics package for R. IPA software (Qiagen, software version 42012434) was used to perform Disease and Functions analysis and Canonical Pathway analysis using the software default settings. Input for each treatment included genes altered by >|2|-fold relative to Vehicle control with $p < 0.05$.

3.2.5 RT-qPCR

Real-time quantitative polymerase chain reaction (RT-qPCR) was performed with the StepOnePlus Real-Time PCR System (ThermoFisher Scientific, MA, USA) using the iTaq Universal SYBR Green Supermix (Bio-Rad, CA, USA) with 25 ng cDNA and 0.5 mM of each primer. Standard cycling conditions were used, with 35 and 40 cycles for THP-1 cells and PBMC, respectively. Relative gene expression (i.e., relative to chosen reference gene) was quantified by the 2^{-ddCt} method with β-2-microglobulin (*B2M*) as the reference gene for both THP-1 cells and PBMC. Primers were created using NCBI blast software or obtained via the Harvard primer bank (https://pga.mgh.harvard.edu/primerbank/). The specificities of the primers (sequences for forward and reverse primers are provided in Table 3-1) were assessed by BLAST (http://www.ncbi.nlm.nih.gov), BLAT (htttp://www.genome.ucsd.edu/), and oligoanalyzer (http://www.idtdna.com) analyses. Amplicon size and primer specificity were also verified after qPCR via agarose gel electrophoresis. Due to the low amount of RNA isolated from PBMC we measured the expression of a limited number of transcripts.

Table 3-1 RT-qPCR primers

Gene	Primer	Sequence	Source	Amplicon size (base pairs)
EGR1	Forward	GGTCAGTGGCCTAGTGAGC	Harvard PrimerBank	149
	Reverse	GTGCCGCTGAGTAAATGGGA	ID: 31317226c1	
PDK2	Forward	ATGAAAGAGATCAACCTGCTTCC	Harvard PrimerBank	82
	Reverse	GGCTCTGGACATACCAGCTC	ID: 315630350c1	
ITG1B	Forward	CCTACTTCTGCACGATGTGATG	Harvard PrimerBank	128
	Reverse	CCTTTGCTACGGTTGGTTACATT	ID: 182507160c1	
TOE1	Forward	TTGCTGAACCAGTGCATTGAG	Harvard PrimerBank	76
	Reverse	CCAGGGAAAGGATAGAACGGG	ID: 156564397c2	
IER2	Forward	TGG TGA AAC TGG GCC AAT CT	NCBI Primer Design Tool	91
	Reverse	AAG AAT CCA CCG CAC GAA AG		
CPT1A	Forward	ATCAATCGGACTCTGGAAACGG	Harvard PrimerBank	121
	Reverse	TCAGGGAGTAGCGCATGGT	ID: 188595713c2	
SCD	Forward	TCTAGCTCCTATACCACCACCA	Harvard PrimerBank	82
	Reverse	TCGTCTCCAACTTATCTCCTCC	ID: 53759150c1	
LDLR	Forward	TCTGCAACATGGCTAGAGACT	Harvard PrimerBank	76
	Reverse	TCCAAGCATTCGTTGGTCCC	ID: 307775415c1	
FADS2	Forward	GACCACGGCAAGAACTCAAAG	Harvard PrimerBank	183
	Reverse	GAGGGTAGGAATCCAGCCATT	ID: 14141180c2	
SREBF1	Forward	CGGAACCATCTTGGCAACAGT	Harvard PrimerBank	141
	Reverse	CGCTTCTCAATGGCGTTGT	ID: 256665250c3	
DFNA5	Forward	CCCAGGATGGACCATTAAGTGT	Harvard PrimerBank	157
	Reverse	GGTTCCAGGACCATGAGTAGTT	ID: 188536091c3	
CD99L2	Forward	GGGTAAAGGTGATGGCCGGT	NCBI Primer Design Tool	178
	Reverse	TGCGTTGAGACCCTGCTGAA		
PLK2	Forward	GCTGATGTCTGGCTGTTCATCAG	NCBI Primer Design Tool	139
	Reverse	CTTCCCTGTAGATCTCACAGTG		
B2M	Forward	GCTCGCGCTACTCTCTCTTT	NCBI Primer Design Tool	121
	Reverse	CCCAGACACATAGCAATTCAGG		
CXCL8	Forward	ATGACTTCCAAGCTGGCCGTGGCT	NCBI Primer Design Tool	292
	Reverse	TCTCAGCCCTCTTCAAAAACTTCT		
HMGCR	Forward	GCCCTCAGTTCCAACTCACA	NCBI Primer Design Tool	142
	Reverse	CAAGCTGACGTACCCCTGAC		

3.2.6 Chemotaxis

THP-1 monocytes were starved in serum-free (SF), phenol red (PR) free RPMI-1640 for 1.5 hr and then stained with 5 μM 5-chloromethylfluorescein diacetate (CMFDA)(Agilent) fluorescent dye for 45 min. Cells were then spun at 300g for 10 min with PBS to remove the dye. 10^6 cells were then resuspended in 200 μL SF PR free RPMI and loaded into the upper the upper insert (3 μm pore) of 24 well transwells (Corning, NY, USA). Inserts were lowered into wells containing 600 μL PR Free RPMI with 25 nM MCP-1 (R&D, MN, USA), where applicable, and allowed to migrate 1.5 hr as per the manufacturer's recommendations and as previously described [23]. Cells that migrated across the membrane to the lower chamber were imaged on a fluorescent microscope (Carl Zeiss, JA, Germany) and counted using ImageJ software [24].

3.2.7 Infection

THP-1 monocytes were stimulated with 10 μM TLR7 agonist CL-075 for 24 hr, then supernatants were harvested and frozen at -80°C until analyzed. IFN-β was then quantified by electrochemiluminescence using small-spot streptavadin plates (Meso Scale Discovery, MD, USA), according to the manufacturer's instructions, and read on a SECTOR imager (Meso Scale Discovery). Note that IFN-β levels were below detectable limits for unstimulated cells, thus only TLR7 stimulated cells were included in the analysis.

3.2.8 Cholesterol

As our microarray data predicted multiple mechanisms by which cellular cholesterol content was decreased (i.e., reduction in both uptake and synthesis), total cellular cholesterol content was determined using the Amplex Red Cholesterol Assay Kit (Life Technologies, CA, USA) using 10^5 THP-1 cells per well as per an established protocol [25]. Cells pretreated with a statin (10 μM Atorvastatin, Sigma-Aldrich, MO, USA, lot # 79530) at 48 hr and again at 24 hr prior to the experiments served as a control. In addition to 48 hr treatment with 50 μM ALA and DHA, 50 μM linoleic acid (LA: Cayman Chemicals, MI, USA) was used as a non-omega-3 PUFA control. Treated cells were rinsed twice in PBS. Cells were then suspended in 1× cholesterol reaction buffer, sonicated 3× for 15 s and centrifuged at 500x g for 5 min to remove cell debris. Fifty μL of cell lysate was then placed in the wells of a Fluroblock™ 96-well plate

(Corning, NY, USA). The Synergy2 plate reader (Biotek, VT, USA) was used to measure fluorescence intensities at 530 nm (excitation)/590 nm (emission) after 45 min. of incubation at 37°C. The cholesterol content was determined from a standard curve of 0.5–20 μg/mL cholesterol.

3.2.9 Statistical Analyses

All analyses were performed using GraphPad Prism software (version 9.12). Results for functional experiments are reported as means ± SD from at least three independent experiments with each performed in triplicate. Shapiro-Wilk test was used to assess normality for all experiments. To assess cholesterol content, IFN-β production and individual gene expression (RT-qPCR) between groups, a one-way ANOVA with Tukey's post-hoc test was used for normal data or a Kruskal-Wallis with Dunn's post-hoc test was used for non-normal data. Chemotaxis data were analyzed using a two-way ANOVA for fatty acid, MCP-1, or interaction effects, followed by Tukey's post-hoc test. For PBMC gene expression, comparisons between day 0 and 28 were done using Students *t*-test for normally distributed data or Wilcoxon matched-pairs signed rank test for non-parametric data. The level of significance was defined as $p < 0.05$ for all experiments. The ROUT coefficient Q was set to 5% to identify outliers prior to all analyses.

3.3 Results

3.3.1 ALA and DHA treatment differentially affect gene expression in THP-1 monocytes

To understand global transcriptome changes induced by ALA or DHA treatment in monocytes, total RNA from THP-1 human monocytes treated with vehicle, ALA or DHA for 48 hr was analyzed using an Affymetrix Clariom D microarray. Non-coding and non-annotated genes were filtered out. We found that ALA and DHA presented distinct profiles as demonstrated by the separate clusters identified by PLS-DA, each representing one of the treatments (Fig 1A). According to VIP analysis, there were no individual genes driving large degrees of separation in each group; rather, many genes had minor contributions. The 20 genes with the highest VIP scores for each component are shown in Figure 3-2. For illustration, a total of 7927 and 9217 genes had VIP scores >1 for components 1 and 2, respectively.

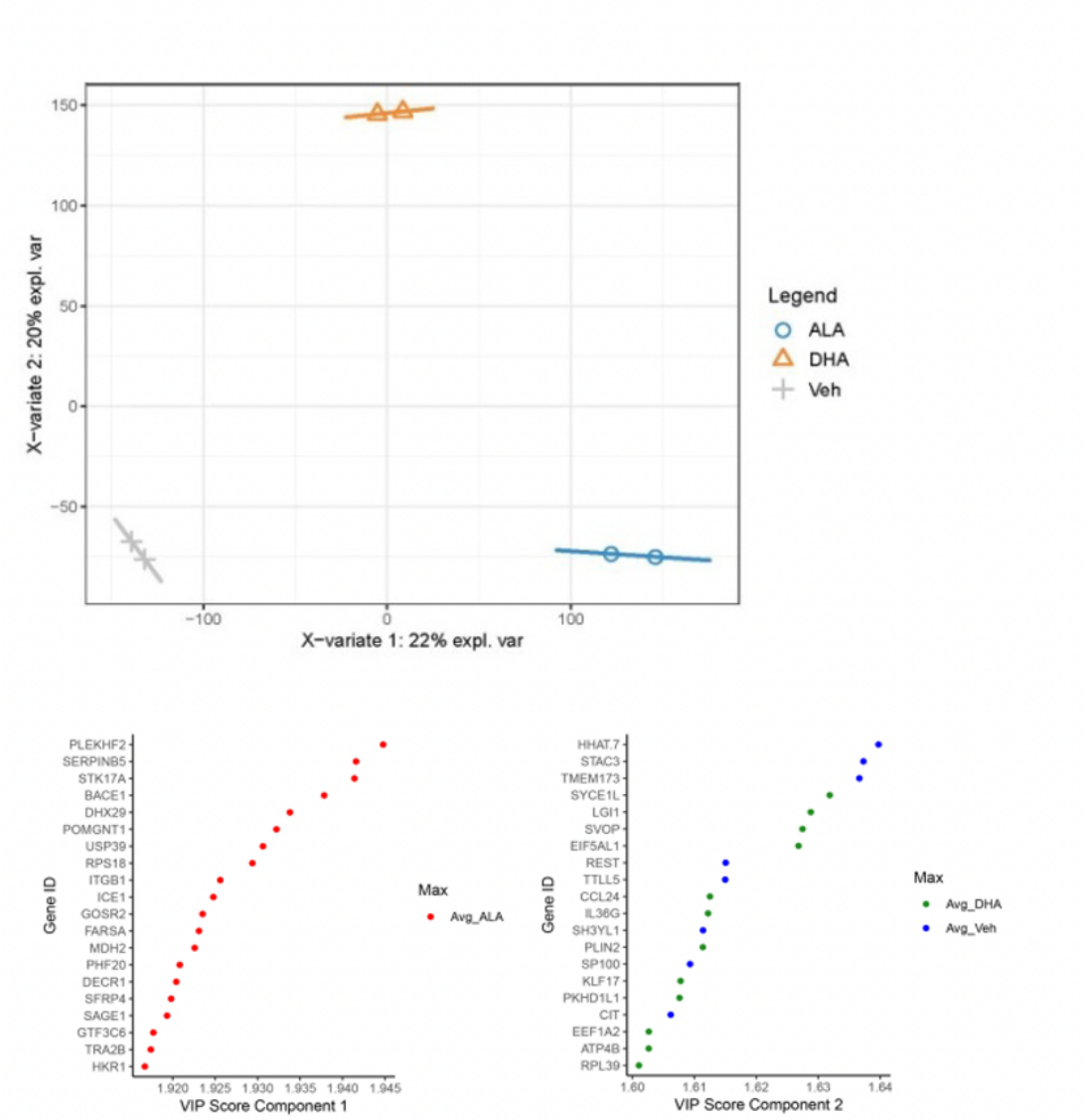


Figure 3-1 Distinct transcriptional signatures of vehicle, ALA and DHA treated monocytes.

(A) PLS-DA of microarray data reveals separation between treatment groups. (B) VIP scores identify the top 20 genes contributing to separation in component 1 [left, highest in ALA (red)] and component 2 [right, highest in DHA (green) or vehicle (blue)]. A total of 7927 and 9217 genes had VIP score >1 for component 1 and 2, respectively.

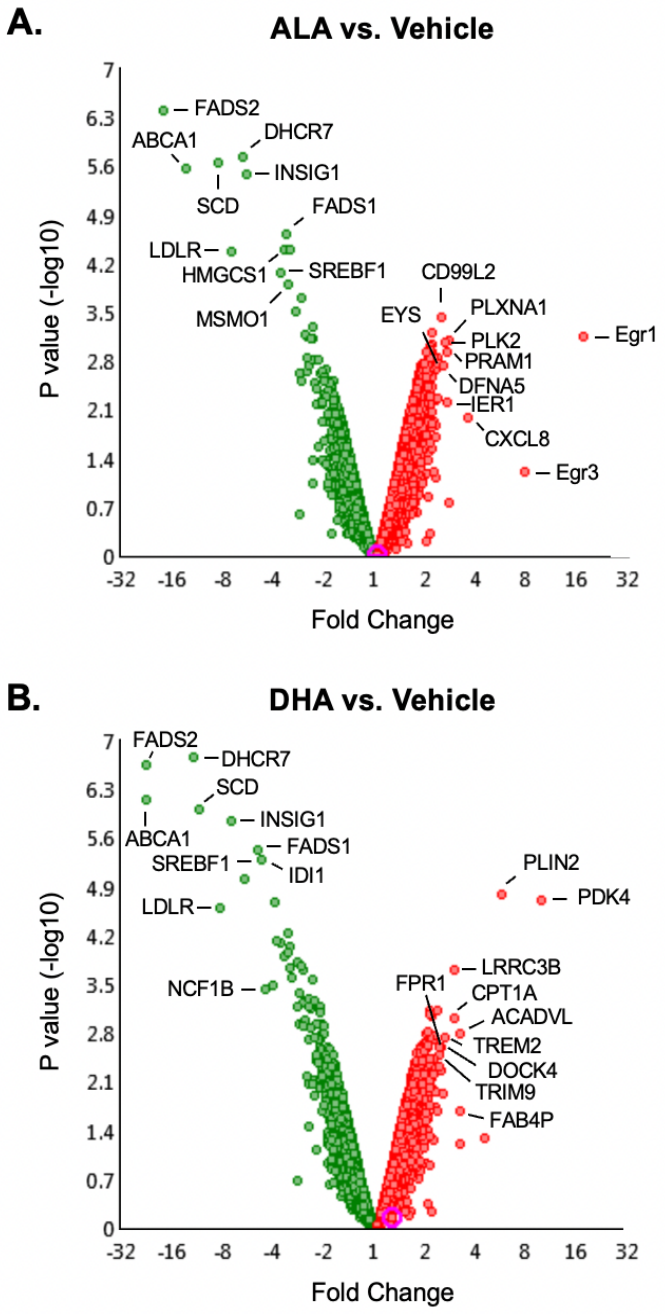


Figure 3-2 Individual genes regulated by n-3 PUFA treatment.

THP-1 monocytes were treated with 50 μM ALA, 50 μM DHA or vehicle equivalent for 48 hr before RNA extraction and microarray analysis, in duplicate. Volcano plots are shown for ALA vs. vehicle (A) and DHA vs. vehicle (B) gene expression analysis. The top 10 genes up- and down-regulated ($p < 0.05$) are listed on each plot.

3.3.2 ALA and DHA treatment differentially affect genes related to chemotaxis and infection response and similarly affect genes associated with cholesterol metabolism

Using Transcriptome Analysis Console (Affymetrix, CA, USA) software, a fold change and intensity-based filtering approach (>2-fold change and ANOVA $p < 0.05$) was applied to the transcriptomics data. Note that a false discovery rate adjustment was not made due to the small sample size (n=2) for each treatment condition, therefore, individual p values should be interpreted with caution. The screen was intended to identify global changes in gene expression and to identify candidate genes and pathways for downstream validation.

The top 10 putative coding genes upregulated by ALA relative to vehicle control included EGR1 (17.4-fold), an immediate early gene involved in multiple cellular processes, including response to infection [26], as well as *CD99L2, DFNA5* and *IER2*, genes all associated with pathways controlling migratory response towards chemokine (chemotaxis), cell motility and extravasation (Figure 3-2) [27-31]. ALA treatment also putatively upregulated expression of *CXCL8*, a chemokine [32] (Figure 3-2). The genes listed here related to response to infection and chemotaxis were also upregulated by ALA relative to DHA treatment ($p < 0.05$). In the top 10 coding genes upregulated by DHA treatment, there were no particular pathways affected, and only 2 genes were increased by more than 3.5-fold (Figure 3-2). There was no overlap in the genes upregulated the most by the ALA or DHA treatments. In contrast, the top 10 coding genes downregulated by ALA and DHA were similar and were related to cholesterol metabolism (e.g. *SREBF1, HMGCR, SCD, DHCR7*) [33, 34], and fatty acid metabolism (e.g. *FADS2, FADS1*) [33]. Descriptions of the top 10 up- and down-regulated genes by ALA and DHA can be found in supplementary tables(s) S3-4 & S3-5. For feasibility reasons, only the top 10 genes up or downregulated by each fatty acid are examined in this thesis.

3.3.3 ALA and DHA differentially regulate functions related to infection and chemotaxis while cholesterol metabolism is regulated similarly

The aim of the present study was to compare the effects of ALA and DHA in monocytes, with respect to both gene expression and function. IPA Core Analysis was used to cluster responsive genes according to the main biological functions of the protein they encode. Diseases and Functions analysis revealed ALA treatment was predicted to upregulate pathways related to

infection (e.g. viral infection, human immunodeficiency virus (HIV) infection) (Table 3-2). ALA treatment also putatively upregulated pathways controlling responses associated with migratory responses to chemokines (chemotaxis) (Table 3-2). In addition, Canonical Pathway analysis revealed that ALA treatment was predicted to upregulate the actin related protein Wiskott–Aldrich syndrome protein (ARP-WASP) pathway (Table 3-3), which promotes actin cytoskeleton rearrangements important for immune cell signaling and migration in response to chemokines [35]. Notably, the most highly downregulated Diseases and Functions and Canonical Pathways by both fatty acids were associated with cholesterol and fatty acid metabolism. Downregulated Diseases and Functions included metabolism of TAG, as well as synthesis and metabolism of cholesterol (Table 3-2). Downregulated Canonical pathways included cholesterol biosynthesis I and II and mevalonate synthesis (Table 3-3). The Z-score presented in our IPA analysis provides predictions about processes and considers the directional effect of genes in a process. For example, a positive Z score indicates an "activated" or increased state for a given pathway or biological function.

Table 3-2 Ingenuity Pathway Analysis (IPA): Diseases/Functions affected by PUFA treatment

ALA vs Vehicle Diseases & Function Annotation

Diseases or Functions Annotation	Predicted Activation State	Activation z-score	p-value
Infection by RNA virus	Increased	7.82	1.47E-02
Infection of cells	Increased	7.56	1.58E-03
Infection by HIV-1	Increased	7.34	3.31E-03
HIV infection	Increased	7.34	1.07E-02
Viral Infection	Increased	6.88	9.37E-04
Adhesion of connective tissue cells	Increased	2.93	1.80E-02
Adhesion of embryonic cells	Increased	2.78	1.70E-02
Adhesion of fibroblast cell lines	Increased	2.21	1.91E-02
Invasion of bladder cancer cell lines	Increased	2.19	1.21E-02
Invasion of bladder cancer cell lines	Increased	2.19	1.05E-02
NK cell migration	Increased	2.19	1.10E-03
Binding of osteoblasts	Increased	2.18	1.93E-02
Steroid metabolism	Decreased	-2.00	2.28E-16
Concentration of sterol	Decreased	-2.11	1.70E-07
Metabolism of triacylglycerol	Decreased	-2.20	7.91E-04
Synthesis of triacylglycerol	Decreased	-2.20	1.48E-03
Concentration of cholesterol ester	Decreased	-2.21	3.38E-05
Synthesis of steroid	Decreased	-2.31	4.60E-15
Synthesis of sterol	Decreased	-2.33	7.07E-23
Synthesis of cholesterol	Decreased	-2.40	1.35E-23
Metabolism of sterol	Decreased	-2.91	1.62E-21
Metabolism of cholesterol	Decreased	-2.91	1.76E-21

DHA vs Vehicle Diseases & Function Annotation

Diseases or Functions Annotation	Predicted Activation State	Activation z-score	p-value
Dermatitis	Increased	2.12	6.66E-03
Proliferation of neuronal cells	Increased	2.07	1.63E-03
Concentration of colfosceril palmitate	Decreased	-2.00	1.38E-04
Synthesis of cholesterol ester	Decreased	-2.00	4.00E-03
Metabolism of acylglycerol	Decreased	-2.03	3.54E-05
Synthesis of acylglycerol	Decreased	-2.03	9.40E-04
Metabolism of triacylglycerol	Decreased	-2.06	4.65E-05
Synthesis of triacylglycerol	Decreased	-2.06	2.92E-03
Synthesis of lipid	Decreased	-2.14	2.22E-08
Fatty acid metabolism	Decreased	-2.15	3.03E-07
Esterification of cholesterol	Decreased	-2.18	7.20E-03
Metabolism of terpenoid	Decreased	-2.30	8.04E-09
Synthesis of fatty acid	Decreased	-2.32	5.62E-03
Synthesis of steroid	Decreased	-2.47	1.34E-08
Synthesis of sterol	Decreased	-2.51	3.08E-14
Synthesis of cholesterol	Decreased	-2.57	1.97E-15
Synthesis of terpenoid	Decreased	-2.66	7.04E-08
Metabolism of sterol	Decreased	-2.70	1.19E-16
Conversion of fatty acid	Decreased	-2.80	1.40E-03
Metabolism of cholesterol	Decreased	-3.06	1.59E-15

Table 3-3 Ingenuity Pathway Analysis (IPA): Canonical Pathways affected by PUFA treatment

ALA vs Vehicle Canonical Pathways

Canonical Pathway	Z-score	p-value
NRF2-mediated Oxidative Stress Response	2.89	4.90E-02
Superpathway of Methionine Degradation	2.65	1.05E-02
UVC-Induced MAPK Signaling	2.65	3.09E-02
Cysteine Biosynthesis III (mammalia)	2.45	3.16E-03
TCA Cycle II (Eukaryotic)	2.45	6.61E-03
The Visual Cycle	2.24	7.94E-03
Methionine Degradation I (to Homocysteine)	2.24	1.02E-02
Retinol Biosynthesis	2.12	3.89E-03
Actin Nucleation by ARP-WASP Complex	2.11	1.26E-02
Superpathway of Cholesterol Biosynthesis	-3.90	3.16E-16
Cholesterol Biosynthesis I	-3.32	2.00E-11
Cholesterol Biosynthesis II (via 24,25-dihydrolanosterol)	-3.32	2.00E-11
Cholesterol Biosynthesis III (via Desmosterol)	-3.32	2.00E-11
Superpathway of Geranylgeranyldiphosphate Biosynthesis I (via Mevalonate)	-2.12	6.17E-06
Zymosterol Biosynthesis	-2.00	3.80E-04

DHA vs Vehicle Canonical Pathways

Canonical Pathway	Z-score	p-value
Oxidative Phosphorylation	0.10	1.62E-02
Superpathway of Cholesterol Biosynthesis	-4.12	6.31E-18
Cholesterol Biosynthesis I	-3.16	1.00E-11
Cholesterol Biosynthesis II (via 24,25-dihydrolanosterol)	-3.16	1.00E-11
Cholesterol Biosynthesis III (via Desmosterol)	-3.16	1.00E-11
Superpathway of Geranylgeranyldiphosphate Biosynthesis I (via Mevalonate)	-2.65	3.16E-07
Mevalonate Pathway I	-2.45	5.62E-07

Abbreviations: NRF2, nuclear factor-erythroid factor 2-related factor 2, UVC, ultraviolet-c, MAPK, mitogen-activated protein kinase, TCA, tricarboxylic acid, ARP-WASP, actin-related proteins-Wiskott–Aldrich syndrome protein

3.3.4 Validation of candidate genes up- or down-regulated by ALA and DHA

We chose a selection of transcripts identified as putative targets of ALA and/or DHA in the microarray screen that have known effects on infection response, chemotaxis, and lipid and cholesterol metabolism to validate alterations in transcript levels via RT-qPCR. The same fatty acid treatment and RNA extraction procedure was used to prepare THP-1 monocytes for RT-qPCR. Overall, most transcripts (11/15) examined via RT-qPCR displayed similar expression patterns as those obtained via microarray. Microarray data predicted transcripts related to infection response (*EGR1, TOE1, CXCL8, PDK2)* to be uniquely upregulated by ALA treatment, with EGR1 expression being the most highly upregulated (17.1 fold). Our RT-qPCR analyses confirmed that ALA upregulated *EGR1* expression, a gene which participates in the infection response and other cellular processes, by 6.7-fold, while DHA treatment had no effect. Moreover, 2/3 of the other transcripts related to infection response tested were uniquely upregulated by ALA (*CXCL8, PDK2*), with DHA having no effect. Based on microarray data, the expression of genes related to chemotaxis (*EGR1, CD99L2, IER2, PLK2, PDK2, ITG1B, DFNA5, CXCL8*) were also predicted to be uniquely upregulated by ALA treatment. Indeed, 5/8 transcripts related to chemotaxis and examined via RT-qPCR were uniquely upregulated by ALA, and unaffected by DHA treatment (Figure 3-3). Further, both ALA and DHA were predicted to reduce the expression of genes related to lipid and cholesterol metabolism (*FADS2, HMGCR, SCD, LDLR, SREBF1, CPT1A*) to similar degrees. Comparable to array data, we find both ALA and DHA decreased the expression of 6/6 genes related to cholesterol and fatty acid metabolism to similar degrees (Figure 3-3)

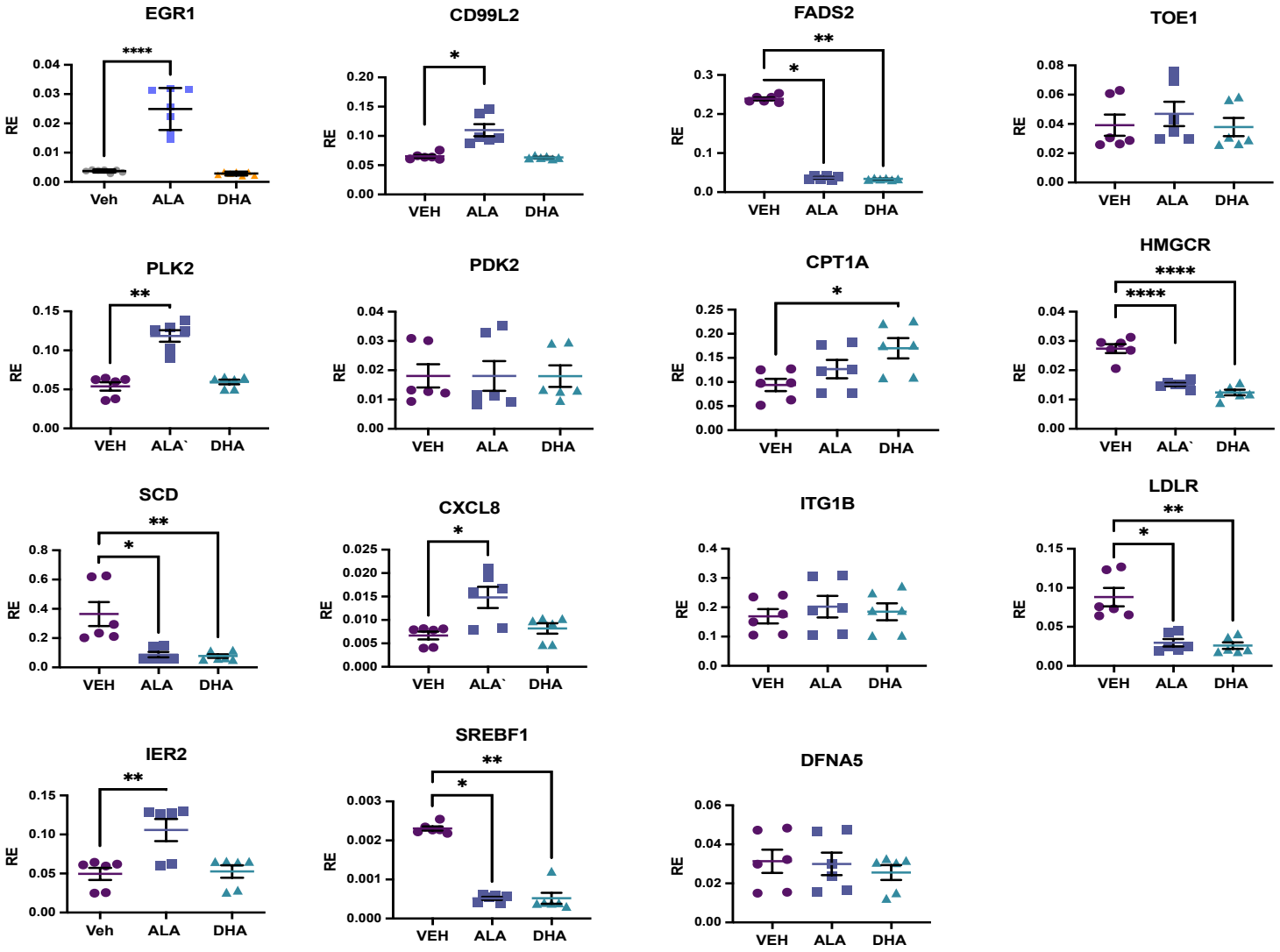

EGR1
****
RE
0.04
0.03
0.02
0.01
0.00
Veh
ALA
DHA
CD99L2
*
0.20
0.15
0.10
0.05
0.00
VEH
ALA
DHA
FADS2
**
*
0.3
0.2
0.1
0.0
VEH
ALA
DHA
TOE1
0.08
0.06
0.04
0.02
0.00
VEH
ALA
DHA
PLK2
**
0.15
0.10
0.05
0.00
VEH
ALA`
DHA
PDK2
0.04
0.03
0.02
0.01
0.00
VEH
ALA
DHA
CPT1A
*
0.25
0.20
0.15
0.10
0.05
0.00
VEH
ALA
DHA
HMGCR
****
****
0.04
0.03
0.02
0.01
0.00
VEH
ALA`
DHA
SCD
**
*
0.8
0.6
0.4
0.2
0.0
VEH
ALA
DHA
CXCL8
*
0.025
0.020
0.015
0.010
0.005
0.000
VEH
ALA`
DHA
ITG1B
0.4
0.3
0.2
0.1
0.0
VEH
ALA
DHA
LDLR
**
*
0.15
0.10
0.05
0.00
VEH
ALA
DHA
IER2
**
0.15
0.10
0.05
0.00
Veh
ALA
DHA
SREBF1
**
*
0.003
0.002
0.001
0.000
VEH
ALA
DHA
DFNA5
0.06
0.04
0.02
0.00
VEH
ALA
DHA

Figure 3-3 Effects of ALA and DHA treatment on expression of candidate genes related to infection, chemotaxis, and cholesterol metabolism in THP-1 monocytes.

THP-1 monocytes were treated with 50 μM ALA, 50 μM DHA or vehicle equivalent for 48 h before RNA extraction. Relative expression (RE) of Transcript levels were assessed by RT-qPCR. Relative quantification was used for statistical analysis by one-way ANOVA with Tukey's post-hoc test for normal data or a Kruskal-Wallis with Dunn's post-hoc test for non-normal data. Data are expressed as means ± SD of three separate experiments in duplicate. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

3.3.5 In vivo validation of target genes in PBMC from a clinical supplementation study

To determine if ALA and DHA treatment had a similar effect on the expression of candidate genes in humans, we performed qPCR on the RNA of PBMC collected from the OXBIO clinical supplementation trial [36] (Chapter 2 & Appendix 1). This randomized controlled crossover trial provided females with obesity (BMI >30) supplements consisting either of ALA-rich flaxseed oil or DHA-rich fish oil for 28 days, with PBMC collected at day 0 and 28. This study design allowed a comparison of both treatment conditions relative to their respective baseline. Consistent with the results obtained with THP-1 monocytes treated in vitro, supplementation in vivo with ALA-rich flaxseed oil upregulated *EGR1* both significantly (p=0.0069) and consistently (10/11 participants tested) (Figure 3-4). Flaxseed oil supplementation also upregulated two of the genes related to chemotaxis (*CD99L2, IER2*) in 9/11 and 7/8 participants, respectively. Both ALA- and DHA-rich oil supplementation downregulated *HMGCR,* which codes for the rate limiting enzyme of the cholesterol synthesis pathway, in 10/12 participants (Figure 3-4). Unlike direct treatment with ALA or DHA in vitro, neither oil directionally affected the transcript for *SREBF,* a major transcription factor controlling lipid metabolism and cholesterol metabolism (Figure 3-4).

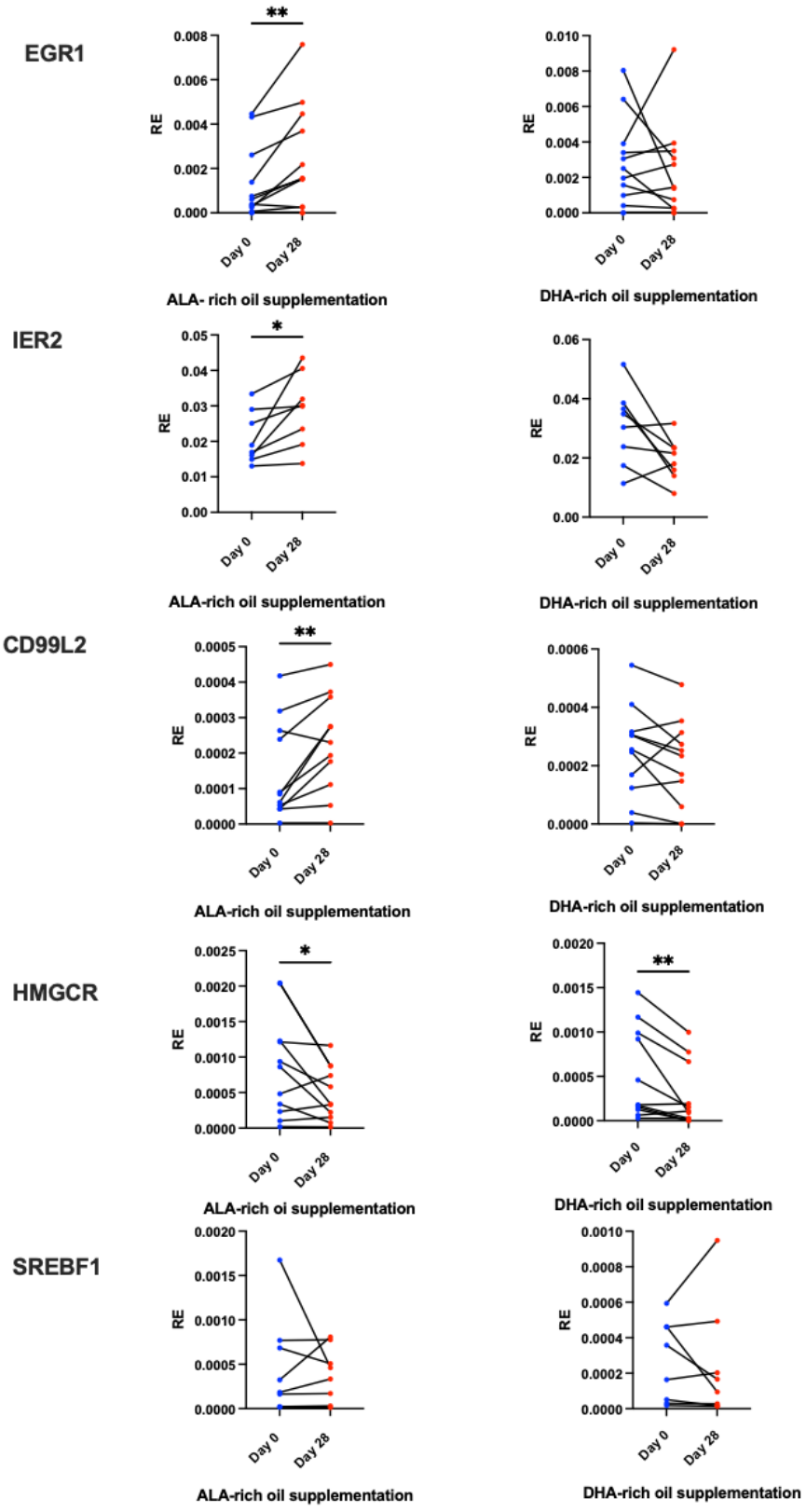

EGR1
**
RE
0.008
0.006
0.004
0.002
0.000
Day 0
Day 28
ALA- rich oil supplementation
0.010
0.008
0.006
0.004
0.002
0.000
DHA-rich oil supplementation
IER2
*
0.05
0.04
0.03
0.02
0.01
0.00
ALA-rich oil supplementation
0.06
0.04
0.02
0.00
DHA-rich oil supplementation
CD99L2
**
0.0005
0.0004
0.0003
0.0002
0.0001
0.0000
ALA-rich oil supplementation
0.0006
0.0004
0.0002
0.0000
DHA-rich oil supplementation
HMGCR
*
0.0025
0.0020
0.0015
0.0010
0.0005
0.0000
ALA-rich oi supplementation
**
0.0020
0.0015
0.0010
0.0005
0.0000
DHA-rich oil supplementation
SREBF1
0.0020
0.0015
0.0010
0.0005
0.0000
ALA-rich oil supplementation
0.0010
0.0008
0.0006
0.0004
0.0002
0.0000
DHA-rich oil supplementation

Figure 3-3 Effects of ALA-rich and DHA-rich oil supplementation on expression of candidate genes in human PBMC from the OXBIO clinical trial.

RNA was extracted from PBMC obtained from the OXBIO clinical trial. Relative expression (RE) of transcript levels were assessed by RT-qPCR. Data show differences between day 0 and 28 of supplementation phases with 2 replicates each and n=12 participants for H*MGCR*, n=10 participants for *EGR1*, n=11 participants for *CD99L2*, and n=8 participants for *SREBF1* and *IER2* (due to limited amount of RNA). Shapiro-Wilk was used to assess normality. The ROUT coefficient Q was set to 5% to identify outliers prior to analyses. A paired students t-test (normal) and Wilcoxon matched pairs rank (non-normal) were used to assess differences (significance) between day 0 and 28 of supplementation phases. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

3.3.6 DHA treated monocytes secrete less IFN-β in response to TLR-7 ligation

Although ALA treated cells were predicted to exhibit a more robust response to infection, we found no difference in IFN-β secretion between vehicle and ALA treated THP-1 cells that had been stimulated with CL-075, a TLR-7 agonist (Figure 3-5A). In contrast, IFN-β production induced by CL-075 was reduced in DHA treated cells relative to both vehicle and ALA (Figure 3-5A).

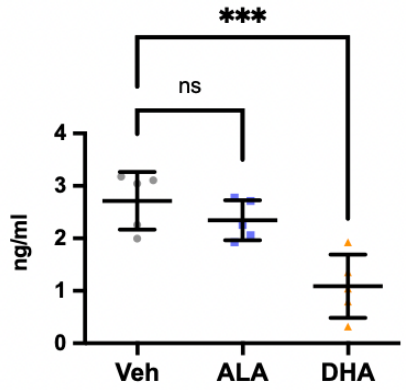
3.3.7 DHA treated monocytes migrate less robustly toward MCP-1

Based on our transcriptomics and pathway prediction analyses, we performed experiments examining functions predicted to be altered by fatty acid treatment. Transcriptomics and qPCR data indicated ALA uniquely upregulated genes and processes related to the migratory response towards chemokines (chemotaxis). As such, we hypothesized that ALA treated cells may have an increased propensity to migrate in response to in response to chemotactic signals. We examined the chemotactic response of fatty acid treated THP-1 monocytes and found that, contrary to our predictions, ALA did not stimulate cell migration towards MCP-1. On the other hand, DHA treated cells exhibited decreased (40%) migration towards MCP-1 compared to both vehicle and ALA (Figure 3-5B), consistent with previous literature [11, 37, 38].

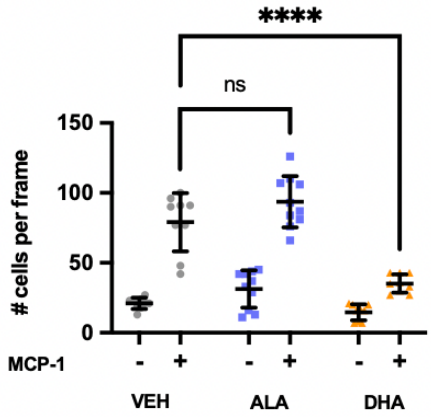
3.3.8 ALA and DHA treatment reduce cholesterol content in THP-1 human monocytes

ALA and DHA treatment downregulated multiple target genes related to cholesterol metabolism according to our gene expression analyses, and according to IPA these fatty acids were predicted to also reduce cholesterol synthesis. Consequently, the cholesterol content of THP-1 monocytes was measured after ALA and DHA treatment relative to untreated cells. Cellular cholesterol content is affected by both uptake and synthesis; therefore, we used an n-6 fatty acid (LA) and an HMGCR inhibitor (atorvastatin) as additional controls and measured total cholesterol content 48 h after fatty acid treatment. Consistent with the predictions, both ALA and DHA decreased the total cholesterol content of the monocytes to a similar degree. Moreover, both ALA and DHA decreased total cholesterol content to a similar degree as atorvastatin, while LA had no effect (Figure 3-5C).

A. TLR-7 stimulated INF-β production



B. MCP-1 induced migration



C. Cholesterol content

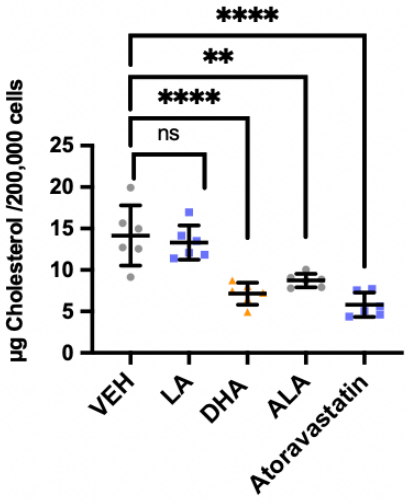


Figure 3-4 DHA treated monocytes secrete less IFN-β in response to TLR-7 ligation and display reduced MCP-1 induced chemotaxis, while both ALA and DHA treatment decrease monocyte cholesterol content.

THP-1 monocytes were treated with 50 μM ALA, 50 μM DHA or vehicle control for 48 hr before all experiments. (A) THP-1 monocytes were stimulated with 10 μM TLR7 agonist CL-075 for 24 h. IFN-β was then quantified by electrochemiluminescence streptavidin plates. Data are representative of two independent experiments with 3 replicates. Significance was determined using one-way ANOVA with Tukey's post hoc test. (B) Monocyte chemotaxis. Monocytes were treated with CMFDA fluorescent dye, washed, and placed in the upper insert of a transwell apparatus with 25 nM MCP-1 in the bottom well and allowed to migrate for 1.5 hr. Cells that migrated to the bottom chamber were counted via epifluorescence microscopy after capturing 3 pictures per well. Data are representative of three separate experiments with 2 replicates. Chemotaxis data were analyzed using a two-way ANOVA for fatty acid, MCP-1, or interaction effects, followed by Tukey's post-hoc test. (C) Cholesterol content of THP-1 monocytes. Medium was removed from fatty acid treated cells, and cells were lysed by sonication in amplex assay buffer. Cholesterol levels were then determined with the amplex cholesterol assay. Data are given as mean ± SD of 3 experiments. Significance was determined using one-way ANOVA with Tukey's post hoc test ** $P < 0.01$; *** $P < 0.001$, **** $P < 0.0001$

3.4 Discussion

It has previously been assumed that because the plant-derived n-3 PUFA, ALA, can be converted by elongation and desaturation to the marine-derived n-3 PUFA, DHA, their biological effects would be similar. However, evidence indicates that individual n-3 PUFAs, including ALA, have distinct effects [14-16]. Here, the actions of ALA and DHA on gene expression and function were compared in THP-1 monocytes. It was revealed that ALA and DHA treatment differentially affect gene expression in THP-1 monocytes, leading to distinct functional consequences. Specifically, ALA upregulated several mRNA transcripts related to proteins involved in infection and chemotaxis, however, neither IFN-β production in response to TLR7 ligation nor MCP1-induced chemotaxis was altered by ALA treatment. In contrast, DHA treatment decreased chemotactic propensity towards MCP-1 and IFN-β production. Both ALA and DHA downregulated multiple transcripts involved in cholesterol synthesis and consistently decreased cellular cholesterol content in THP-1 monocytes. Changes in key transcripts related to infection, chemotaxis and cholesterol metabolism in PBMC from humans receiving ALA- and DHA-rich oil supplementation were comparable to THP-1 monocytes, suggesting a majority of cells in PBMC may be similarly affected by n-3 PUFAs. To our knowledge, this study is the first directly comparing the acute effects of ALA and DHA on immune cell gene expression and function in cell culture and after long-term supplementation of humans with obesity. Based on the distinct transcriptional signatures and functional effects observed in ALA and DHA treated immune cells, it can be concluded that these important dietary n-3 PUFAs exert unique effects through different mechanisms and are not biologically interchangeable despite their structural similarities and biochemical linkages.

A marked effect of both ALA and DHA treatment was decreased expression of genes related to cholesterol metabolism and reduced cellular cholesterol, which alters membrane fluidity and cytoskeleton dynamics, thus affecting important functions such as cell signaling and chemotaxis [1, 31]. Contrasting the differences observed in chemotaxis and IFN-β production, we found that ALA and DHA had similar effects on cholesterol metabolism in THP-1 monocytes. Similar to our findings, studies previously published using PBMC obtained from humans receiving fish oil supplements (mixtures of EPA and DHA) reported that transcripts associated with cholesterol production (e.g. *MMSO1, HMGCR, SREBF1*) were downregulated

[21]. In addition, we observed that ALA and DHA treatment reduced total cholesterol content in THP-1 cells, an observation comparable to studies examining EPA and DHA in human carcinoma cell lines. In these cases, reductions in total cellular cholesterol levels as well as transcript levels of *SREBF1* and *HMGCR* were found [39]. The decrease in cholesterol is presumed to have occurred via a reduction in sterol regulatory element-binding protein 2 protein levels (protein product of *SREBF*), a major transcription factor which regulates the expression of genes involved in cholesterol synthesis. However, given that ALA and DHA downregulated the expression of genes related to both cholesterol uptake (e.g. *LDLR*) and synthesis (e.g. *HMGCR*), the reduction in cellular cholesterol observed in our functional experiments likely involves both mechanisms. It is also important to note that the objective of our study was to explore the impact of ALA and DHA exposure in comparison with normal (baseline) gene expression and function in monocytes. Thus, ALA and DHA were each compared with a vehicle equivalent. However, future experiments could use another fatty acid as an extra control (e.g. oleic acid) to compare the effects of ALA and DHA to an equal amount of non n-3 PUFA.

It should also be noted that in our study we used a fixed dose of 50 μM and a 48 hr treatment time. In humans, plasma ALA has been found to increase to 180 μM with flax oil supplementation [40]. Similarly, plasma DHA levels can also increase to 500 μM with fish oil supplementation. [40,41]. Thus, the concentration of ALA and DHA used in the experiments here is a level detected in human plasma and demonstrates that 50 μM of ALA and DHA is sufficient to induce changes in gene expression and function in THP-1 monocytes. Moreover, other in vitro studies with THP-1 monocytes demonstrate 50 μM of ALA or DHA elicits anti-inflammatory actions [42]. Additionally, the 48 hr treatment time was chosen based on our previous work, which found ALA and DHA to have functional effects at that time point in THP-1 monocytes with 50 μM of fatty acid treatment (unpublished). However, in future experiments, it would be beneficial to examine the timeline (e.g. acute vs longer term) and functional effects of different concentrations of PUFAs on cholesterol content and gene expression. Moreover, it would be useful to measure both cholesterol uptake and synthesis with n-3 PUFA treatment and establish which fraction of the cell cholesterol measured is derived from various cellular compartments (e.g. membrane or cytosol) as well as form (e.g. esterified) to further clarify the mechanistic actions of ALA and DHA. Cellular cholesterol is known to alter a variety of immune cell functions through modulation of cell stiffness, membrane fluidity, as well as the distribution

and activity of receptors [43]. Cholesterol intermediates have also been shown to play a role in prenylation of small G proteins (e.g. RhoA, Rac1), which are essential for coordinating immune cell responses including chemotaxis and response to infection [44]. Thus, understanding how different n-3 PUFAs affect immune cell gene expression and function in the context of cholesterol handling will be useful for better understanding the underlying pathogenesis, which in turn will assist in generating novel therapeutic approaches for disorders with altered immune function, such as atherosclerosis and HIV infection.

One of the most striking effects of ALA treatment was an increase in the expression of genes related to chemotaxis (migration towards chemokine), a critical process related to atherosclerosis as well as the response to infection [2, 26]. One of the earliest events in atherosclerotic plaque formation is the migration and extravasation of circulating blood monocytes through damaged endothelial cells lining the blood vessels [5]. There is currently little known regarding the effects of ALA on immune cell chemotaxis, however, DHA has been shown to inhibit this process in other immune cells [45], thus preventing monocytes from crossing through the endothelial cell layer where they differentiate to foam cells and contribute to plaque formation**.** Others have also reported that several marine-derived n-3 PUFA derived metabolites inhibit the chemotaxis of neutrophils both in vitro and in vivo. For example, the DHA-derived metabolite, resolvin D1, reduces actin polymerization leading to decreased chemotaxis of human leukocytes [38]. In line with these findings, we demonstrate that DHA treated monocytes have a reduced propensity for migration (chemotaxis) towards MCP-1. Contrasting this, we predicted an increase in chemotaxis following ALA treatment as ALA treatment upregulated pathways and functions (IPA) (Table 3-2 & 3-3) and individual genes, *CD99L2, DFNA5, PDK2* and *IER2*, related to monocyte migration and extravasation (Figure 3-2) However, our functional assays demonstrated ALA exposure had no effect on the migratory response to MCP-1. Intriguingly, there have been several reports of membrane cholesterol content influencing chemotaxis in immune cells. In a study using THP-1 monocytes, a 50% decrease in lipid raft cholesterol resulted in substantial decrease in chemotaxis towards MCP-1 [23]. In $CD4^+$ cells, EPA and DHA treatment reduced motility by interfering with cytoskeletal rearrangements required for cell motility and migration in response to chemokine. DHA also altered cell membrane fluidity and therefore may have subsequently interfered with receptor-mediated activation of signaling pathways also contributing to reduced chemotaxis [46].

Furthermore, intermediates in the cholesterol synthesis pathway serve as precursors for post-translational modification of proteins via prenylation [44]. The activity of Rho family small GTPases, many of which are central signaling proteins for chemotaxis and adhesion, is inhibited by statin treatment, resulting in inhibition of migration towards chemokine [44, 47]. Therefore, a reduction in active cholesterol synthesis by n-3 PUFAs might similarly reduce migratory responses by reducing small GTPase prenylation.

Given the reduced expression of *HMGCR* and reduced cholesterol content in ALA treated cells, this could compensate for an increased propensity for chemotaxis that may otherwise have resulted from upregulation of *CD99L2, DFNA5, PDK2 and IER2.* Future experiments where cholesterol content is kept constant with PUFA treatment, as well as examining actin polymerization would be able to clearly determine the effects of ALA on cell chemotaxis. It is also possible that ALA will interfere with chemotaxis stimulated by a different chemokine (e.g. IL-8). Moreover, it is conceivable that ALA and DHA may confer other benefits by decreasing cellular cholesterol content, ultimately influencing cellular processes such as those related to pro-inflammatory cytokine signaling. At this point, the specificity of the cellular effects of ALA and DHA have not been examined. Nonetheless, our results indicate ALA and DHA exert their actions on monocyte chemotaxis through different molecular mechanisms and are not equivalent in their effects on gene expression or function.

Given the IPA prediction that ALA would enhance the response to infection and given that the top gene upregulated by ALA, *EGR1*, is an immediate early gene important for anti-viral immunity [26], we tested the effect of ALA and DHA treatment on IFN- β secretion in response to a viral mimic. Contrary to our predictions, we found DHA decreased IFN- β release after TLR-7 ligation while ALA had no effect. Egr11 has been shown to be protective against Foot-and-Mouth Disease Virus (FMDV) by enhancing Type I IFN responses [26]. Although FMDV is a single stranded RNA virus, it does not infect human hosts and the importance of TLR7 relative to other innate sensors is not yet clear. Egr1 also plays a protective role against infection by another single stranded RNA virus, HIV [48]. Although TLR7 is important in immune recognition of HIV infection [49], the protective effect of Egr1 was shown to be mediated by its target gene called Target of Egr1 (TOE, also upregulated by ALA according to our transcriptomics screen) rather than through enhancement of IFN responses [49]. Therefore IFN-β

production may not be the most suitable functional read-out in the context of HIV infection. Notably, we attempted to also measure the effect of ALA and DHA treatment on IFN- β produced at rest and in response to TLR3 ligation with Poly I:C, however, levels were below the limit of detection under these conditions. Thus, although we determined that ALA treatment has no measurable effect on IFN-β produced by monocytes in response to TLR7 ligation, it is quite possible and even promising that ALA, through upregulation of Egr1, may enhance other types of anti-viral responses and ultimately confer protection.

The mechanisms by which n-3 PUFAs such as ALA and DHA exert their distinct effects on immune cells and disease pathologies is yet to be completely understood. Overall, our results demonstrate that ALA and DHA differentially alter gene expression and function associated with response to infection, chemotaxis, and cholesterol metabolism in THP-1 monocytes and PBMC, and thus may uniquely affect related disease processes. Discrepancies between the functional changes predicted based on pathway analysis versus those verified experimentally highlight the limitations of predictive software, particularly where up- and down-regulated pathways are inter-related. The findings of this study add to the current knowledge behind the unique actions of both plant and marine-derived n-3 PUFAs, offering putative targets for the development of novel therapeutics and/or interventions. Ultimately, continued study of each fatty acid and their metabolites in different disease states and cell types will aid in determining in which circumstances they are each most useful.

3.5 Literature Cited

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Supplementary Material

Table S3-4 ALA vs vehicle top 10 coding transcripts upregulated and downregulated

	Gene	Fold Change	p-value	Description
1	EGR1*	17.4	0.0007	Early growth response 1: Transcription factor; regulates response to growth factors, DNA damage, ischemia and hypoxia; role in cell survival/proliferation, differentiation; dampens response to cytokines, increases response to infection
2	CXCL8*	3.55	0.0099	Interleukin-8: chemotactic factor for lymphocytes, neutrophils, endothelial cells; pro-angiogenic
3	PLXNA1	2.72	0.0008	Plexin A1: Necessary for signaling by class 3 semaphorins and subsequent remodeling of the cytoskeleton. Plays a role in axon guidance, invasive growth and cell migration.
4	PRAM1	2.65	0.0012	PML-RARA regulated adaptor molecule 1: Adaptor protein involved in T cell receptor mediated signaling. This gene is expressed and regulated during normal myelopoiesis. Involved in integrin signaling
5	IER2*	2.65	0.0059	Immediate early response 2: DNA-binding protein that seems to act as a transcription factor Involved in the regulation of neuronal differentiation, acts upon JNK-signaling pathway activation and plays a role in neurite outgrowth in hippocampal cells (By similarity). Promotes cell motility in response to chemokine, seems to stimulate tumor metastasis
6	PLK2*	2.58	0.0009	Polo-like kinase 2: Tumor suppressor serine/threonine-protein kinase involved in synaptic plasticity, centriole duplication and G1/S phase transition. Polo-like kinases act by binding and phosphorylating proteins are that already phosphorylated on a specific motif recognized by the POLO box domains. Regulates Ras and Rap protein signaling
7	DFNA5*	2.53	0.0017	Deafness, autosomal dominant 5: Suppresses tumors by mediating granzyme-mediated pyroptosis in target cells of natural killer (NK) cells: cleavage by granzyme B (GZMB), delivered to target cells from NK-cells, triggers pyroptosis of tumor cells and tumor suppression. May play a role in the p53/TP53-regulated cellular response to DNA damage.
8	CD99L2	2.46	0.0004	CD99 molecule-like 2: Plays a role in leukocyte extravasation and migration helping cells to overcome the endothelial basement membrane. Homophilic adhesion molecule.
9	EYS*	2.36	0.0017	Eyes shut homolog: Required to maintain the integrity of photoreceptor cells. Specifically required for normal morphology of the photoreceptor ciliary pocket, and might thus facilitate protein trafficking between the photoreceptor inner and outer segments via the transition zone
10	WISP3	2.36	0.0014	WNT1 inducible signaling pathway protein 3: Plays a role in mitochondrial electron transport and mitochondrial respiration. Through its regulation of the mitochondrial function may play a role in normal postnatal skeletal growth and cartilage homeostasis.

1	FADS2	-18.3	3.82E-07	Fatty acid desaturase 2: Involved in the biosynthesis of highly unsaturated fatty acids (HUFA) from the essential polyunsaturated fatty acids (PUFA) linoleic acid (LA) (18:2n-6) and alpha-linolenic acid (ALA) (18:3n-3) precursors, acting as a fatty acyl-coenzyme A (CoA) desaturase that introduces a cis double bond at carbon 6 of the fatty acyl chain.
2	ABCA1	-13.38	2.61E-06	ATP binding cassette subfamily A member 1: Catalyzes the translocation of phospholipids from the cytoplasmic to the extracellular/lumenal leaflet of membrane coupled to the hydrolysis of ATP. Role in the efflux of intracellular cholesterol to apoliproteins and the formation of nascent high density lipoproteins/HDLs
3	SCD	-8.57	2.21E-06	Stearoyl-CoA desaturase (Delta-9 desaturase): Catalyzes the insertion of a cis double bond at the delta-9 position into fatty acyl-CoA substrates including palmitoyl-CoA and stearoyl-CoA. Plays an important role in lipid biosynthesis. Plays an important role in regulating the expression of genes that are involved in lipogenesis and in regulating mitochondrial fatty acid oxidation. Contributes to the biosynthesis of membrane phospholipids, cholesterol esters and triglycerides.
4	LDLR	-7.11	4.20E-05	Low density lipoprotein receptor: Binds LDL, the major cholesterol-carrying lipoprotein of plasma, and transports it into cells by endocytosis.
5	DHCR7	-6.1	1.78E-06	7-dehydrocholesterol reductase: Membrane-bound enzyme that catalyzes the final step of cholesterol biosynthesis (the reduction of the C7-8 double bond in 7-dehydrocholesterol to form cholesterol)
6	INSIG1	-5.82	3.12E-06	Insulin induced gene 1: Binds the sterol-sensing domains of sterol regulatory element-binding protein (SREBP) and 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoA reductase). Essential for the sterol-mediated trafficking of these two proteins. Promotes degradation of HMG-CoA reductase.
7	SREBF1	-3.65	8.31E-05	Sterol regulatory element binding transcription factor 1: Transcription factor that binds to the sterol regulatory element-1 (SRE1), which is a motif that is found in the promoter of the low density lipoprotein receptor gene and other genes involved in sterol biosynthesis.
8	HMGCS1	-3.44	3.92E-05	3-hydroxy-3-methylglutaryl-CoA synthase 1 (soluble): Catalyzes the condensation of acetyl-CoA with acetoacetyl-CoA to form HMG-CoA, which is converted by HMG-CoA reductase (HMGCR) into mevalonate, a precursor for cholesterol synthesis.
9	FADS1	-3.42	2.32E-05	Fatty acid desaturase 1: Acts as a front-end fatty acyl-coenzyme A (CoA) desaturase that introduces a cis double bond at carbon 5 located between a preexisting double bond and the carboxyl end of the fatty acyl chain. Involved in biosynthesis of HUFA from the essential PUFAs LA and ALA precursors.
10	MSMO1	-3.33	0.0001	Methylsterol monooxygenase 1: Catalyzes the three-step monooxygenation required for the demethylation of 4,4-dimethyl and 4alpha-methylsterols, which can be subsequently metabolized to cholesterol.

* Uniquely regulated by ALA

Table S3-5 DHA vs vehicle top 10 coding transcripts upregulated and downregulated

	Gene	Fold Change	p-value	Description
1	PDK4	9.66	1.84E-05	Pyruvate dehydrogenase kinase, isozyme 4: Phosphorylates and inhibits of pyruvate dehydrogenase, decrease glucose utilization and increase fat metabolism in response to fasting/starvation.
2	PLIN2	5.59	1.61E-05	Perilipin 2: Associated with the lipid droplet formation, and maybe involved in development and maintenance of adipose tissue.
3	ACADVL	3.21	0.0015	Acyl-CoA dehydrogenase, very long chain: Catalyzes the first step of the mitochondrial fatty acid beta-oxidation pathway. This acyl-Coenzyme A dehydrogenase is specific to long-chain and very-long-chain fatty acids.
4	LRRC3B	2.99	0.0002	Leucine rich repeat containing 3B: Putative tumor suppressor
5	CPT1A*	2.95	0.0009	Carnitine palmitoyltransferase 1A: Catalyzes the transfer of the acyl group of long-chain fatty acid-CoA conjugates onto carnitine, an essential step for the mitochondrial uptake of long-chain fatty acids and their subsequent beta-oxidation in the mitochondrion
6	TREM2	2.62	0.0018	Triggering receptor expressed on myeloid cells 2: Functions in immune response and may be involved in chronic inflammation by triggering the production of constitutive inflammatory cytokines.
7	DOCK4	2.47	0.0025	Dedicator of cytokinesis 4: Functions as a guanine nucleotide exchange factor (GEF) that promotes the exchange of GDP to GTP, converting inactive GDP-bound small GTPases into their active GTP-bound form. Plays a role in cell adhesion and migration
8	FPR1*	2.43	0.0024	Formyl peptide receptor 1: Encodes a G protein-coupled receptor of mammalian phagocytic cells that is a member of the G-protein coupled receptor 1 family. The protein mediates the response of phagocytic cells to invasion of the host by microorganisms and is important in host defense and inflammation
9	TRIM9*	2.42	0.0032	Tripartite motif containing 9: Ubiquitin-protein ligase which ubiquitinates itself in cooperation with an E2 enzyme and serves as a targeting signal for proteasomal degradation. May play a role in regulation of neuronal functions and may also participate in the formation or breakdown of abnormal inclusions in neurodegenerative disorders.
10	BST2	2.41	0.0047	Bone marrow stromal cell antigen 2: IFN-induced antiviral host restriction factor which efficiently blocks the release of diverse mammalian enveloped viruses by directly tethering nascent virions to the membranes of infected cells.
1	FADS2	-23.00	2.18E-07	Fatty acid desaturase 2: Involved in the biosynthesis of highly unsaturated fatty acids (HUFA) from the essential polyunsaturated fatty acids (PUFA) linoleic acid (LA) (18:2n-6) and alpha-linolenic acid (ALA) (18:3n-3) precursors, acting as a fatty acyl-coenzyme A (CoA) desaturase that introduces a cis double bond at carbon 6 of the fatty acyl chain.
2	ABCA1	-22.79	6.66E-06	ATP binding cassette subfamily A member 1: Catalyzes the translocation of phospholipids from the cytoplasmic to the extracellular/lumenal leaflet of membrane coupled to the hydrolysis of ATP. Role in the efflux of intracellular cholesterol to apoliproteins and the formation of nascent high-density lipoproteins/HDLs

3	DHCR7	-12.02	1.70E-07	7-Dehydrocholesterol reductase: Membrane-bound enzyme that catalyzes the final step of cholesterol biosynthesis (the reduction of the C7-8 double bond in 7-dehydrocholesterol to form cholesterol)
4	SCD	-11.23	9.30E-07	Stearoyl-CoA desaturase (Delta-9 desaturase): Catalyzes the insertion of a cis double bond at the delta-9 position into fatty acyl-CoA substrates including palmitoyl-CoA and stearoyl-CoA. Gives rise to a mixture of 16:1 and 18:1 unsaturated fatty acids. Plays an important role in lipid biosynthesis. Plays an important role in regulating the expression of genes that are involved in lipogenesis and in regulating mitochondrial fatty acid oxidation. Contributes to the biosynthesis of membrane phospholipids, cholesterol esters and triglycerides.
5	LDLR	-8.28	2.50E-05	Low density lipoprotein receptor: Binds LDL, the major cholesterol-carrying lipoprotein of plasma, and transports it into cells by endocytosis.
6	INSIG1	-7.14	1.40E-06	Insulin induced gene 1: Binds the sterol-sensing domains of sterol regulatory element-binding protein (SREBP) and 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoA reductase). Essential for the sterol-mediated trafficking of these two proteins. Promotes degradation of HMG-CoA reductase.
7	SREBF1	-5.89	9.25E-06	Sterol regulatory element binding transcription factor 1: Transcription factor that binds to the sterol regulatory element-1 (SRE1), which is a motif that is found in the promoter of the low-density lipoprotein receptor gene and other genes involved in sterol biosynthesis. The encoded protein is synthesized as a precursor that is initially attached to the nuclear membrane and endoplasmic reticulum. Following cleavage, the mature protein translocates to the nucleus and activates transcription. This cleaveage is inhibited by sterols.
8	FADS1	-4.93	3.59E-06	Fatty acid desaturase 1: Acts as a front-end fatty acyl-coenzyme A (CoA) desaturase that introduces a cis double bond at carbon 5 located between a preexisting double bond and the carboxyl end of the fatty acyl chain. Involved in biosynthesis of HUFA from the essential PUFAs LA and ALA precursors.
9	IDI1	-4.70	4.93E-06	Isopentenyl-diphosphate delta isomerase 1: Peroxisomally-localized enzyme that catalyzes the interconversion of isopentenyl diphosphate (IPP) to its highly electrophilic isomer, dimethylallyl diphosphate (DMAPP), which are the substrates for the successive reaction that results in the synthesis of farnesyl diphosphate and, ultimately, cholesterol.
10	NCF1*	-4.03	0.0003	Neutrophil cytosolic factor 1: Peroxisomally-localized enzyme that catalyzes the interconversion of isopentenyl diphosphate (IPP) to its highly electrophilic isomer, dimethylallyl diphosphate (DMAPP), which are the substrates for the successive reaction that results in the synthesis of farnesyl diphosphate and, ultimately, cholesterol.

* Uniquely regulated by DHA

Descriptions of gene functions in each table were obtained from GeneCards (https://www.GeneCards.org) and the NCBI database https://www.ncbi.nlm.nih.gov/

Chapter 4: Discussion

Monocytes are an innate immune cell with pluripotent roles in the body and are essential for regulating the inflammatory response, initiating tissue repair and fighting infection [1]. Moreover, their activity is pathologically altered in inflammatory disease states such as obesity, contributing to both systemic and local adipose tissue inflammation as well as co-morbidities including atherosclerosis, [1-4].Therefore, treatments targeting monocyte function are of particular interest within the context of obesity. N-3 PUFAs are one potential treatment and are proposed to block the progression of chronic disease in part by altering immune function[5-8]. Most research to date has focused on the marine-derived n-3 PUFAs, EPA and DHA. Far fewer studies have assessed the actions of ALA, as it is classically assumed that its biological effects are dependent on its conversion to longer chain fatty acids such as DHA. However, others, as well as our recent studies [9-12], indicate that ALA and its bioactive metabolites have beneficial effects and direct immune-modulatory actions of their own. The data presented in this thesis compare the unique actions of the n-3 PUFAs ALA and DHA on gene expression and function in immune cells, which will guide their therapeutic use within the context of obesity and associated conditions. Moreover, the work in this thesis adds to the growing evidence that n-3 PUFAs have beneficial effects in immune cells other than their ability to reduce the production of pro-inflammatory mediators.

A key finding of this thesis is that ALA and DHA differentially alter gene expression associated with the physiological responses to infection, chemotaxis, and cholesterol metabolism in THP-1 human monocytes. Thus, ALA and DHA may uniquely affect related disease processes, including complications arising from obesity such as atherosclerosis. Using a transcriptomic approach, we found ALA uniquely upregulated several genes involved in chemotaxis; however, functional experiments revealed ALA treatment did not alter chemokine-induced migration, a process related to atherosclerotic plaque development. In contrast, even though in our microarray screen DHA treatment did not downregulate the expression of genes related to chemotaxis, our functional assays demonstrated DHA treatment reduced MCP-1 mediated chemotaxis, highlighting the limitations of predictive software such as IPA. However, both ALA and DHA treatment downregulated multiple transcripts in the cholesterol synthesis

pathways and consistently decreased cellular cholesterol content compared to LA, an n-6 PUFA, and to the vehicle control as measured by the Amplex™ Red Cholesterol Assay. Additionally, candidate transcripts were validated in PBMC from participants in our group's clinical supplementation trial (See Chapter 2 for study description and the Appendix 1 for the OXBIO trial results) where females with obesity consumed ALA- or DHA-rich oils for 4 weeks. The changes in key transcripts related to chemotaxis and cholesterol metabolism in PBMC from OXBIO participants (Chapter 2, study design rationale; Appendix 1, OXBIO trial results), were similar to THP-1 monocytes, suggesting a majority of cells in PBMC are similarly affected by n-3 PUFAs. To the author's knowledge, this study is the first comparing the effects of ALA and DHA on immune cell gene expression and function both acutely in cell culture and in a longer-term supplementation trial in humans with obesity.

The first paper in this dissertation (Chapter 2) describes the rationale and design for the randomized crossover clinical trial that I co-coordinated from 2018-2020. In parallel to experiments using n-3 PUFA treated THP-1 monocytes, the OXBIO trial compared the effects of equal doses of ALA and DHA-rich oil supplements (4 g/day) on plasma oxylipins and markers of metabolic health in women with obesity, along with plasma fatty acids, inflammatory markers, and monocyte glucose metabolism as secondary outcomes. Adipose tissue of obese individuals is characterized by local inflammation, which in turn recruits and activates immune cells that promote low grade systemic inflammation and an altered immune phenotype [13-15]. Moreover, obesity is a significant risk factor for atherosclerosis, with evidence indicating the altered function of immune cells, including monocytes, promotes their accumulation in atherosclerotic lesions [4, 16]. Further, one action of the adipokine, adiponectin, is to suppress atherogenesis by inhibiting the ability of monocytes to adhere to endothelial cells, as well as reducing their phagocytic activity, thereby suppressing the accumulation of modified lipoproteins in the vascular wall [17, 18]. Adiponectin levels have shown to be decreased in obese humans, putting them at further risk for atherosclerosis [17]. Interestingly, previous work finds DHA and its bioactive metabolites to be beneficial in both aspects, through decreasing the migratory capacity of immune cells [19-22], as well as increasing adiponectin levels [23-25]. Experiments with THP-1 monocytes and the results from our group's clinical trial largely support and extend these findings. We find DHA but not ALA treated monocytes exhibit reduced chemotaxis. Moreover, ALA was also predicted to increase IFN-β production in response to viral challenge. However, in

our cell culture experiments, DHA, but not ALA treated monocytes were found to secrete less IFN-β, a cytokine that plays a central role in inflammation, and may also upregulate the production of chemokines [26]. While the increased production of IFN-β may be beneficial for enhancing anti-viral responses, it may not be advantageous in atherosclerotic processes, given that monocytes in obesity display an increased response to chemokines and are thus more likely to accumulate in atherosclerotic lesions [4, 16]. Further, in our clinical trial, DHA-rich oil supplementation uniquely increased adiponectin, which has been shown to reduce monocyte adherence to vascular endothelial cells and decrease oxylipins associated with improved cardiovascular function, including 12-HEPE and 14-HDoHE [27], while ALA had no effect on either. Together, this evidence may support a beneficial role for DHA over and above ALA in the prevention and treatment of atherosclerosis arising from obesity.

In Chapter 3, THP-1 cells were treated with 50 μM fatty acid, which is within the range of physiological circulating levels of ALA and DHA in human plasma [28]. In the OXBIO trial, flaxseed-oil supplementation increased total plasma ALA by only 50% (from 119±64.2 to 179 ± 98.8 μmol/L). However, flaxseed-oil supplementation studies designed with higher doses and/or longer durations have reported more substantial increases in plasma ALA levels [29-31] and oxylipins up to 5-fold [30, 32]. To our knowledge, the effect of supplementation on tissue ALA levels (in free fatty acid form) has not been reported. However, total tissue levels of ALA have been demonstrated to increase more than 10-fold in rodents supplemented with flax oil [9]. Furthermore, similar to our clinical study, other n-3 PUFA supplementation trials report increases in DHA plasma concentrations, and their respective oxylipins in plasma after fish-oil supplementation [30, 33], with total DHA levels in the OXBIO trial after supplementation reaching ~500 μmol/L [34]. Therefore, the concentration of ALA and DHA used in the treatment of THP-1 monocytes in this thesis is a level detected in human plasma and indicates 50 μM of both ALA or DHA is sufficient to induce changes in gene expression and function in THP-1 monocytes. Moreover, other in vitro studies with THP-1 monocytes demonstrate 50 μM ALA or DHA elicits anti-inflammatory actions [11]. The 48 hr treatment time was chosen based on our previous work (unpublished), which found ALA and DHA to have functional effects at that time point in THP-1 monocytes.

Further, in Chapter 3 the effect of ALA and DHA treatment on cellular cholesterol levels was examined. Cellular cholesterol content is known to alter a variety of immune cell properties including membrane fluidity, lipid raft composition, cell signaling and biomechanics, thus affecting several critical processes and functions [35-37]. Therefore, examining the impact of therapeutic agents on cellular cholesterol is of key importance in guiding and refining their use within obesity, cardiovascular and metabolic disorders. The effects of both plant and marine-derived n-3 PUFAs on blood cholesterol levels have been examined, with studies showing mixed results for whether they reduce LDL-cholesterol and/or increase HDL-cholesterol [38-41]There is comparatively less information regarding the actions of n-3 PUFAs on cellular cholesterol content. In the experiments performed here, we find both ALA and DHA decrease mRNA levels of genes related to cholesterol metabolism and reduce the total cellular cholesterol content in immune cells. These results support and extend the findings of the limited number of studies examining DHA treated cells, including human PBMC and human colon cancer cells [42-45]. In several immune cell types (e.g. monocytes, neutrophils), reduced cholesterol levels have been shown to result in less robust migration towards chemokines (e.g. MCP-1, IL-8) [46], resembling our findings with DHA treatment. Decreased membrane cholesterol content is known to affect membrane fluidity, which is thought to alter receptor-mediated activation of signaling pathways contributing to reduced chemotactic motility [46-48]. Furthermore, intermediates in the cholesterol synthesis pathway serve as precursors for post-translational modification of proteins via prenylation. The activity of Rho family small GTPases, many of which are central signaling proteins for chemotaxis, are inhibited by statin treatment, resulting in inhibition of migration towards chemokine [36, 49, 50]. Therefore, a reduction in active cholesterol synthesis by n-3 PUFAs might similarly reduce migratory responses by reducing small GTPase prenylation similar to statin treatment. As mentioned previously, monocyte recruitment to an atherosclerotic lesion is a vital step in the progression of atherosclerosis. Thus, the ability of n-3 PUFAs to reduce cellular cholesterol content and monocyte recruitment to atherosclerotic lesions represent mechanisms by which they convey beneficial effects in CVD.

Additionally, within the context of obesity, hypercholesterolemia is generally present, leading to cholesterol accumulation in monocytes, macrophages, and other immune cells [37]. In addition to affecting motility, cholesterol accumulation in immune cells is known to promote the inflammatory response, including TLR signaling, inflammasome activation, and the proliferation

of inflammatory macrophages within adipose tissue and in atherosclerotic lesions [37]. This increased inflammatory response contributes to the already dysregulated secretory profile within obese adipose tissue, further perpetuating systemic chronic inflammation [15]. Although acute inflammation is beneficial in the response to infection, the systemic chronic inflammation present in obesity along with pathologically altered immune function promotes atherosclerosis and the dysfunction of multiple organ systems, tissues and cell types [15]. The ability of both ALA and DHA to reduce cellular cholesterol content as demonstrated in our experiments may therefore represent a common mechanism by which they convey beneficial effects in obesity and atherosclerosis.

As mentioned, other cell types within the context of obesity are affected by cholesterol content. Cellular cholesterol regulates endothelial cell biomechanical properties, including permeability and leukocyte adhesion in atherosclerosis [51]. Further, increased cholesterol in adipocytes is associated with adipose tissue remodeling, triggering hypertrophic adipocyte expansion and immune cell infiltration [52]. Enlarged adipocytes exhibit decreased insulin responsiveness, reduced glucose uptake, and increased secretion of pro-inflammatory adipokines [52, 53]. Therefore, examining the ability of n-3 PUFAs to modify cellular cholesterol content may be useful in combatting the maladaptive functional alterations in multiple cell types in obesity, as well as reducing systemic inflammation and the associated metabolic consequences.

Generally, n-3 PUFAs such as ALA and DHA are thought to be beneficial in obesity due to their ability to alleviate adipose tissue inflammation [7, 54]. Similarly, from the OXBIO trial it was found that ALA-rich oil supplementation promotes an anti-inflammatory monocyte phenotype. This agrees with the results of others, which show metabolites of ALA dampen the polarization of macrophages in tissues such as adipose tissue, away from the pro-inflammatory (M1) phenotype [55] Although the complete mechanisms have yet to be fully elucidated, altering cellular cholesterol content, as well as promoting an anti-inflammatory immune phenotype may represent new mechanisms by which ALA and DHA impede the progression of chronic disease associated with obesity. Overall, the work in this thesis adds to the growing body of knowledge that n-3 PUFAs have effects in immune cells other than reducing the production of inflammatory compounds, including altering cholesterol content, and influencing response to chemokines.

4.1 Limitations and future directions

While this study had several strengths, which are outlined below, there were a few limitations. We found it difficult to schedule participants for day 3 appointments and were unable to collect sufficient blood for comparison of shorter-term supplementation with the experiments conducted in THP-1 cells (48 hr treatment). We were also unable to collect enough blood to isolate pure monocytes for both the bioenergetic experiments and for comparison of target gene expression to n-3 PUFA-treated THP-1 cells via qPCR. However, we were able to assess a limited number of transcripts in PBMC from most participants. As gene expression was similar to n-3 PUFA treated THP-1 cells, these results suggest that a majority of cells in PBMC (e.g. T cells, monocytes) may respond similarly to ALA and DHA treatment. Future studies could collect 50 mL blood specifically for the isolation of target immune cells (e.g. monocytes, neutrophils) for use in a microarray or qPCR, similar to Runblad et al. [45] who examined the effects of fish and krill oil supplementation on gene expression in human immune cells. In addition, for optimal RNA recovery we recommend future studies examining gene expression either immediately freeze isolated cells in liquid nitrogen or in the cognate lysis buffer of the kit selected for RNA isolation.

Studying the impact of n-3 PUFAs on the physiochemical properties of various cell types is a useful tool to further understand how they affect cholesterol metabolism, cell function and related disease processes. Although we predicted reduced cellular cholesterol content via multiple mechanisms in THP-1 cells (e.g. uptake, synthesis), we did not examine the specific mechanisms by which this occurred. We also did not determine which cellular fraction(s) contained the cholesterol which was measured by the assay. As such, an assay for receptor (e.g. LDLR, scavenger receptors) or enzyme activity (e.g. HMGCR) would assist in determining the specific mechanism by which n-3 PUFAs decrease cellular cholesterol. Further, different types of fatty acids are known to influence membrane fluidity via lipid rafts, which are microdomains enriched in cholesterol that regulate processes such as signal transduction, membrane trafficking, and cytoskeletal organization [5, 35, 51, 56]. Through altering lipid raft composition, fatty acids may affect the activity of receptors and cell signaling cascades [46-48]. Moreover, membrane cholesterol is thought to affect function differently than cholesterol located in the cytoplasm [35, 46, 57-59]. Thus, staining for cholesterol, or isolating cellular fractions (e.g. plasma membrane,

cytosol), or examining lipid raft formation after treatment would aid in determining how n-3 PUFAs affect cholesterol distribution and cell functions such as chemotaxis. Further, modification of proteins by cholesterol intermediates, known as prenylation, controls the localization and activity of many proteins, including those affecting immune cell motility and responses to chemokines [60]. As such, measuring prenylation of proteins (e.g Rho, part of the Ras superfamily of small GTPases and implicated in regulating cell motility) in monocytes as described in Berndt [61] after treatment with fatty acids such as ALA and DHA would also help determine how these n-3 PUFAs affect immune cell chemotaxis as well as other functions.

To assess if ALA treatment increases the propensity of monocyte chemotaxis separate from reducing cholesterol (as predicted by our transcriptomics array), future studies could hold cholesterol content constant before performing experiments. This could be achieved by enriching cells with cholesterol, or depleting cholesterol using an agent such as methyl β-cyclodextrin [46]. Further, actin polymerization is critical for cell migration, and for the directional migratory response of immune cells towards chemokines such as MCP-1 induced chemotaxis [35, 62]. Therefore, staining for actin-cytoskeleton rearrangements after n-3 PUFA treatment during a chemotaxis experiment would help to determine the mechanisms by which ALA and DHA affect immune cell chemotaxis.

Although we evaluated several functions experimentally based on our transcriptomics predictions, determining the protein levels of genes altered by n-3 PUFA treatment via Western blotting or enzyme-linked immunosorbent assay (ELISA) would be beneficial. For example, measuring the protein levels of CCR2, one primary receptor for MCP-1 involved in monocyte chemotaxis, would allow us to determine if reduced chemotaxis is in part due to a decrease in receptor levels.

Determining protein levels of LDLR, the scavenger receptor, and HMCGR would further support our findings of reduced cholesterol content via multiple mechanisms (uptake, synthesis), given their transcript levels were decreased. Determining the effect of n-3 PUFAs on enzyme activity, receptor /and or protein levels is essential for fully understanding the methods by which they exert their effects, as these are vital steps which determine whether altered gene expression affects cell function.

To further develop the chemotaxis experiments conducted in this thesis, a co-culture 'scratch' assay could be performed where both THP-1 monocytes and endothelial cells are treated with n-3 PUFAs. Using the transwell apparatus in our experiments, the bottom well could contain endothelial cells that have been purposefully damaged to release their own chemotactic factors (as opposed to addition of exogenous MCP-1 in our experiments). The chemotactic factors released by scratched/damaged endothelial cells would attract and bind monocytes (THP-1 cells), thus better representing atherogenesis in vivo.

In parallel to dose response and time course experiments using ALA and DHA, experiments in which the cells are treated with their metabolites (oxylipins) may also be of benefit. This experimental design has been used before [63]. Adipocyte lipid storage and adipokine production are modulated by lipoxygenase-derived oxylipins generated from 18-carbon fatty acids, but it requires considerable effort since these compounds should be tested individually. Measuring oxylipin as well as fatty acid levels throughout the experiments along with functional assessments would allow for further understanding of how ALA and DHA are utilized under different conditions and the biological effects of their oxylipin metabolites. For example, measuring fatty acids and their metabolites (oxylipins) in parallel to conducting chemotactic experiments (e.g. collecting samples of both cells and supernatant before, during and after migration towards chemokine) would aid in determining how n-3 PUFAs are utilized during this process.

As mentioned previously, both ALA and DHA are found at a range of concentrations in plasma, as well as other tissues and cell types within humans. Thus, future cell culture experiments could examine a dose response in comparison to physiological levels (e.g. with and without supplementation) and a time course of n-3 PUFA treatment (acute, mid- and longer term) could aid in translating our findings from in vitro experiments to relevant clinical information, and in determining the effects of n-3 PUFAs on an array of cellular functions in different conditions. Further, this study found that ALA and DHA have their own unique mechanisms and beneficial effects. Therefore, experiments co-treating, or supplementing with varying amounts of both ALA and DHA can determine if their effects are synergistic, and what proportions are ideal for different health and disease states.

4.2 Strengths

Both the cell culture and clinical supplementation trial have several design-related strengths. This is the first study of its kind comparing the effects of ALA and DHA on the human monocyte transcriptome, allowing a more detailed picture of their effects to be captured and explored. The methods employed for our cell culture study allowed us to generate predictions of functions uniquely altered by ALA and DHA treatment using both the expression of individual genes and transcriptomics software as guides. Further, our design was reinforced by our ability to directly test those functions predicted to be altered by PUFA treatment. This enabled the actions of ALA and DHA on cell function as well as the competing actions of pathways and processes affected by treatment to be assessed. Moreover, we were able to compare our cell culture experiments with immune cells obtained from humans supplemented with ALA- and DHA-rich oils, thus helping to situate our results in a relevant disease state where immune function is pathologically altered. Another strength of this study relates to the selection of the study population for the OXBIO clinical trial. It is known that abdominal obesity results in expansion and remodeling of adipose tissue, leading to chronic inflammation, promoting atherosclerosis and other inflammation-driven comorbidities [15, 53, 64]. Thus, examining immune cells from an obese population allowed us to interpret our cell culture findings in the context of a relevant disease state, in comparison to a healthy population, where a dampening of inflammation may not be measurable or relevant.

Overall, fatty acids are known to affect many cell types. Thus, the flow of experiments conducted in this thesis (e.g. transcriptomics, qPCR, functional assays) would be useful in examining the effects of multiple fatty acids in other cell types such as adipocytes, endothelial cells, hepatocytes and pancreatic cells, which play major roles in obesity and its co-morbidities such as atherosclerosis, non-alcoholic fatty liver disease, and type 2 diabetes.

4.3 Implications & Conclusions

This study provides information for both fundamental science and clinical use, adding to the growing body of work investigating the unique effects of plant and marine n-3 PUFAs. The cell culture experiments illustrate the effects of acute treatment with DHA or ALA on immune

cell gene expression and function, while our group's clinical trial provides information on longer term supplementation in humans with obesity.

Gene expression analyses suggest that ALA and DHA influence monocyte chemotaxis, or migration in response to chemokine, and response to infection through distinct mechanisms, while cholesterol metabolism may be regulated similarly. Functional cell culture assays revealed that, in contrast to our transcriptomic predictions, DHA reduced chemotaxis, while, in agreement with our predictions, both ALA and DHA reduced cholesterol content in THP-1 monocytes. Discrepancies between the functional changes predicted based on pathway analysis versus those verified experimentally highlight the limitations of predictive software, particularly where up- and down-regulated pathways are inter-related. Similar to cell culture results, our group's clinical trial finds that ALA and DHA have favorable, but different actions in obese humans; DHA increased adiponectin, an adipocyte derived hormone beneficial for atherosclerosis, and ALA promoted an anti-inflammatory monocyte phenotype. The work in this thesis also indicates that modifying cellular cholesterol content is a potential mechanism by which n-3 PUFAs exert their effects in immune cells.

In conclusion, we effectively employed multiple techniques, including transcriptomics analyses, functional cell culture experiments and a clinical supplementation trial to improve the current understanding of the unique actions of n-3 PUFAs in mononuclear immune cells within the context of obesity. Overall, the findings in this thesis indicate that ALA and DHA differentially affect monocyte chemotaxis, cellular cholesterol metabolism, mononuclear immune cell bioenergetics, and adiponectin levels. Evidence in this thesis also indicates DHA may be superior to ALA in preventing and treating processes in atherosclerosis. However, it is likely that ALA and DHA differentially affect more cellular functions than tested here, leading to different beneficial effects in several health and disease processes. Thus, one strategy when developing obesity treatments involving n-3 PUFAs is co-treating with ALA and DHA, targeting multiple pathologies (e.g. pro-inflammatory cytokine secretion, immune phenotype, response to chemokine, adipocyte hormones) present in obesity. The work in this thesis also reveals that the dose of ALA or DHA required to effectively assist with the treatment of obesity and its comorbidities remains to be determined. For example, the OXBIO clinical trial found that neither ALA- nor DHA-rich oil supplementation was adequate to reduce levels of pro-inflammatory

cytokines that contribute to the perpetuation of obesity and comorbidities. Nonetheless, plasma ALA and DHA concentrations in OXBIO participants were above those used in cell culture experiments, indicating that immune cells in participants may experience functional changes similar to fatty acid-treated THP-1 monocytes (e.g. reduced chemotaxis with DHA treatment). Thus, the dose of DHA used in the OXBIO trial may help reduce the development and progression of atherosclerotic lesions by altering mononuclear cell chemotaxis. Meanwhile, both ALA and DHA supplementation potentially reduced mononuclear immune cell cholesterol content, resulting in other beneficial functional effects in OXBIO participants.

Currently, n-3 PUFAs are widely recommended for health promotion to the general population. The findings described in this thesis provide a strong foundation for future investigation of the distinct mechanisms of ALA and DHA. Further, the work in this thesis supports the growing evidence that n-3 PUFAs have effects in immune cells other than reducing the production of inflammatory compounds. Understanding the distinct mechanisms and actions of n-3 PUFAs will help guide and refine their use, and the use of their bioactive derivatives as therapeutics in obesity and its comorbidities, including atherosclerosis. Understanding the unique properties and mechanisms of n-3 PUFAs will support their use within personalized medicine. Overall, the results presented in this thesis suggest that despite the structural similarity of ALA and DHA, they have different properties, and thus distinct beneficial effects on disease processes within obesity and atherosclerosis. Therefore, further investigation of how, where and when these fatty acids are most useful is warranted and will improve their specific use within obesity and its associated comorbidities.

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Chapter 5: Appendix 1

5.1 Oils Rich in α-Linolenic Acid or Docosahexaenoic Acid Have Distinct Effects on Plasma Oxylipin and Adiponectin Concentrations and on Monocyte Bioenergetics in Women with Obesity

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Contribution of Co-Authors to Chapter 5: Samantha Pauls: Designed research, performed experiments using plasma and immune cells, analyzed data, performed statistical analyses, and wrote the paper. **Lisa Rodway**: Recruited and screened participants, conducted all study visits (recruitment, consent procedures, measured blood pressure and performed pulse wave analysis, processed blood samples for use in experiments), entered food record data, constructed Table 1 and Figure 1. Karanbir Sidhu: Processed plasma for oxylipin analysis. Nikhil Sidhu: Processed plasma samples for fatty acid analyses. Tania Winter: Maintained and ran samples on HPLC-MS/MS, calculated detector response factors for all oxylipins, calculated amount of internal standard to be added to each sample, assisted with questions and education regarding HPLC-MS/MS. Harold Aukema: Developed study design, editing, funding acquisition. Peter Zahradka: Developed study design, randomized samples for distribution, editing, funding acquisition. Carla Taylor: Obtained ethics approval, developed study design, editing, performed statistical analyses, funding acquisition.

Abstract

Background: Omega-3 fatty acids including docosahexaenoic acid (DHA) and α-linolenic acid (ALA) are proposed to improve metabolic health by reducing obesity-associated inflammation. Their effects are mediated in part by conversion to oxylipins. ALA is relatively understudied and direct comparisons to other omega-3 fatty acids are limited.

Objective: We compared the effects of equal doses of ALA and DHA on plasma oxylipins and markers of metabolic health in women with obesity.

Methods: We carried out a randomized, double-blind crossover clinical trial (NCT03583281) where women aged 20-51 with a BMI of 30-51 were supplemented with 4 g/day ALA or DHA for 4 weeks in the form of ALA-rich flaxseed oil or DHA-rich fish oil. The primary outcome, the plasma oxylipin profile, was assessed at Day 0 and 28 of each phase by HPLC-tandem mass spectrometry. Plasma fatty acids, inflammatory markers, and monocyte glucose metabolism were key secondary outcomes. Data were analyzed using a mixed model.

Results: Compared to baseline visit, 28 days fish oil supplementation resulted in higher plasma levels of nearly all oxylipins derived from DHA (3.8-fold overall, $p<0.001$) and eicosapentaenoic acid (~2.7-fold overall, $p<0.05$). while no changes to oxylipins were observed after flaxseed oil supplementation. Neither supplement altered plasma cytokines; however, adiponectin was increased (1.1-fold, $p<0.05$) at the end of the fish oil phase. Compared to baseline visit, 28 days flaxseed oil supplementation reduced ATP-linked oxygen consumption (0.75-fold, $p<0.05$) and increased spare respiratory capacity (1.4-fold, $p<0.05$) in monocytes, and countered the shift in oxygen consumption induced by lipopolysaccharide.

Conclusions: Flaxseed oil and fish oil each had unique effects on metabolic parameters in women with obesity. The supplementation regimens were insufficient to reduce inflammatory markers but adequate to elicit increases in omega-3 oxylipins and adiponectin in response to fish oil and alter monocyte bioenergetics in response to flaxseed oil.

Introduction

Diets rich in PUFA have many documented benefits, including reduced all-cause mortality (1). The protective effects of omega-3 fatty acids in particular are usually attributed to their lipid-lowering and anti-inflammatory actions (2). Animal studies have demonstrated that supplementation with fish oil or flaxseed oil rich in omega-3 fatty acids reduces obesity-associated inflammatory markers and the progression of chronic diseases such as type 2 diabetes mellitus and cardiovascular disease (3-6). Results of human trials have been mixed and have so far been more focused on fish oils rich in DHA and EPA. Systemic anti-inflammatory effects, generally a decrease in C-reactive protein (CRP) or in pro-inflammatory cytokines IL-1β and TNF-α, have been demonstrated in several trials investigating the effects of DHA/EPA (7-10) or α-linolenic (ALA) (11-13) supplementation in subjects with obesity or hypercholesterolemia. It is clear that not all omega-3 PUFA are equal; for example, disparate effects of DHA-rich versus EPA-rich oils have been documented (9). So far direct comparisons between plant-derived ALA and fish oil-derived DHA or EPA in subjects with obesity have been limited.

Many of the biological effects of PUFA have been attributed to the actions of their oxygenated free fatty acid metabolites, called oxylipins. Oxylipins are produced from precursor PUFA by groups of intracellular enzymes in the cyclooxygenase (COX), lipoxygenase (LOX) and cytochrome P450 (CYP450) families. Dietary fatty acid intake influences the availability of precursor PUFA for conversion to oxylipins; however, changes to oxylipin profiles do not necessarily reflect changes in PUFA composition (14, 15). Many omega-3 oxylipins have been reported to exhibit direct anti-inflammatory or pro-resolving effects, while both pro- and anti-inflammatory effects have been demonstrated for various omega-6 oxylipins (16).

Multiple chronic diseases are characterized by the presence of mitochondrial dysfunction in immune and other cells (17). Mitochondrial function is closely tied to the production of reactive oxygen species, which influences pro-inflammatory cytokine production (18-20) and *vice versa* (21). Innate immune cells isolated from patients with obesity-related chronic diseases including type 2 diabetes (22) and coronary artery disease (23) exhibit elevated mitochondrial respiration rates compared to those from healthy control populations. Early changes to leukocyte mitochondrial function have not yet been investigated in at-risk (obese) participants who have

not developed secondary disease. Fish oil supplementation has been demonstrated to alter mitochondrial structure and function in skeletal and cardiac muscle cells (24-26); however, so far no such investigation has been performed in immune cells.

Here, we report the results of a two-phase, randomized, double-blind, crossover clinical trial (NCT03583281) where females with abdominal obesity were supplemented with 4 g/day ALA or DHA for 4 weeks in the form of ALA-rich flaxseed oil or DHA-rich fish oil (4:1 DHA/EPA). The primary outcome was the plasma oxylipin profile. Secondary outcomes included clinical makers and anthropometrics, plasma fatty acids, plasma cytokines and adipokines, and monocyte glucose metabolism.

Methods

Trial Design

As outlined in **Figure 1**, this was a randomized, double-blind crossover trial with two 28-day (± 2 days) phases. Each phase was preceded by a minimum 28-day wash-in period and each phase was scheduled to start at 9±2 days from last menses. Participants were randomly assigned to start with either flaxseed oil or DHA-rich fish oil using a simple randomization code generated by a statistician. All participants and all team members except one (who had no contact with participants or samples) were blinded to the supplement order. Study visits took place on Day 0, 3 and 28 of each phase. At each visit, participants were asked to report compliance to study protocols and changes in their health status or concomitant medications. Height, weight and blood pressure were recorded and a fasting blood sample was drawn. A Mobil-O-Graph PWA (I.E.M. GmbH) was used to assess blood vessel elasticity on Days 0 and 28. Fewer than half the participants (37%) were able to provide samples on Day 3 due to scheduling difficulties; therefore, this time point was dropped from the analyses. Participants were instructed to avoid consumption of foods high in omega-3 fatty acids, defined as no more than 0.3 g ALA/serving or 0.1 g EPA+DHA/serving (as per examples on a handout). Three-day food recall and activity questionnaires were to be completed during the 3rd week of each wash-in and supplementation phase. This trial, registered at clinicaltrials.gov as NCT03583281, was approved by the University of Manitoba Research Ethics Board and the St. Boniface Hospital Research Review Committee. Details are provided in Rodway et al. 2021 (27) which further describes the study rationale and design, inclusion criteria and sample size calculations. Some secondary outcomes will be reported separately [Fatty acid composition, oxylipin production and cytokine production by peripheral blood mononuclear cells (PBMC)] while others will not be reported as originally planned due to low sample numbers (vaginal fluid oxylipin profiles and immune composition).

Participants

This trial recruited non-pregnant, non-lactating premenopausal females between the ages of 20-55 with BMI>30 and waist circumference >80 cm (Asian descent) or >88 cm (all other ethnicities). Print, electronic and social media advertising was used in the Winnipeg, Manitoba,

area. Participants made contact by phone or email and those meeting general eligibility criteria (age, estimated BMI, non-smoker, premenopausal with regular menses) were invited to a screening visit. Written informed consent was given before any medical information was recorded or study-related procedures were conducted. Inclusion/exclusion criteria are described in detail in reference (27). In brief, participants were required to be in relatively good health apart from elevated BMI, as evidenced by lack of clinically diagnosed disease and bloodwork showing liver enzymes, creatinine and lipids within specified ranges. Additional exclusion criteria included unstable body weight, cigarette smoking, excessive consumption of alcoholic beverages and regular use of non-steroidal anti-inflammatory drugs or omega-3 PUFA supplements apart from those provided during the study. Participants were required to have regular menses and agreed to maintain stable levels of activity and their usual regimen of vitamin/mineral/dietary supplements, if applicable, during the course of their participation. If infection or illness (including cold, flu, yeast infection, etc.) was reported within 7 days of a study visit, the sample was not used and the participant was asked to repeat the phase or withdraw. Thirty individuals were enrolled by LR into the study (with oversight from CGT), with 22 and 21 participants completing phase 1 and 2, respectively.

Interventions

Thirty daily doses of ~4 g of ALA or DHA were provided in opaque, de-identified packaging by the St. Boniface Hospital Pharmacy and distributed to participants at their Day 0 visits. 3.70 g (first lot, 16 participants) or 3.58 g (second lot, 5 participants) of ALA per day was achieved by providing seven flaxseed capsules (7 g total oil, NOW Foods, IL; NPN:80002313). 3.86 g DHA (plus 1.01 g EPA) was achieved by providing six DHA-rich fish oil capsules (6 g total oil, Super DHA gems, Carlson Laboratories, IL; NPN: 80012587), according to fatty acid analysis of the capsule contents (27). Participants were instructed to consume half their daily capsules with a morning meal and the other half with an afternoon or evening meal. Any missed capsules were to be returned at their day 28 visit and were counted by the one team member not blinded. Self-reported compliance of >85% overall and 100% for the 72 h preceding the study visit was required. Plasma fatty acid analysis was also used to confirm compliance.

Sample collection

For screening visits, fasting blood samples were collected by venipuncture into BD Vacutainer collection tubes (Becton Dickinson) by a phlebotomist and analyzed by Shared Health Services at St Boniface Hospital. Lithium heparin-containing tubes were used for lipid panel, creatinine and liver enzymes while K2-EDTA tubes were used for Hemoglobin A1c.

For study visits, fasting blood samples were collected into 10 mL K2-EDTA Vacutainer collection tubes (Becton Dickinson) and processed within 2 h. To isolate platelet-poor plasma, blood was centrifuged at 1100g for 10 min at room temperature, then the top ¾ of the plasma was transferred and centrifuged again at 1500g for 15 min. Antioxidant cocktail (0.2 mg/mL BHT, 0.2 mg/mL EDTA, 2 mg/mL triphenylphosphine, 2 mg/mL indomethacin in a solution of 2:1:1 methanol:ethanol:water) was added to plasma aliquots reserved for fatty acid and oxylipin extraction at 3.3% of sample volume. All plasma aliquots were kept frozen at -80°C until analyzed.

Oxylipin analysis

Following a previously established protocol (28) oxylipins were extracted from plasma samples (thawed on ice) using Strata-X-SPE columns (Phenomenex), analyzed by HPLC/MS/MS (QTRAP 6500; Sciex), and quantified by the stable isotope dilution method (29). Peaks were considered quantifiable if peak height exceeded 5 times Blank levels in multiple samples. Detailed descriptions of quantification methods, mass transitions and retention times for the 157 oxylipin panel can be found in previous publications (14, 30, 31).

Fatty acid analysis

Total lipids were extracted from 250 µL plasma using the Folch method and quantified by gas chromatography as previously described (28, 32-34).

Plasma cytokine analysis

Cytokines and adipokines were quantified by electrochemiluminescence detection according to manufacturer's instructions using a SECTOR imager (Meso Scale Discovery), with all antibodies, diluents and standards purchased from Meso Scale Discovery. Pre-coated multi-spot V-Plex plates were used to quantify IL-1β, TNF-α, IL-10, IL-6, IL-8, IL-17A and vascular

endothelial growth factor (VEGF) while single-spot R-Plex plates coated in-house were used to quantify adiponectin and resistin.

Analysis of clinical markers in plasma

Alanine transaminase (ALT), aspartate aminotransferase (AST), creatinine, glucose, lipid panel (total cholesterol, LDL-cholesterol, HDL-cholesterol, triglycerides) and CRP were measured for all study visit samples using a cobas c111 analyzer (Roche).

Monocyte isolation

PBMC were first isolated using SepMate tubes with Lymphoprep Density Gradient Medium (Stem Cell Technologies) according to the manufacturer's instructions. Briefly, blood was mixed 1:1 with PBS/2%FBS [PBS prepared from disks (Calbiochem), FBS (Multicell)], dispensed into SepMate tube inserts, then centrifuged at 1200g for 10 min at room temperature. Plasma was removed to ~2 cm above the buffy coat, then the remainder was poured into a fresh 50 mL tube, topped up with PBS/2%FBS and centrifuged at 300g for 8 min. Cells were washed once more and spun at 120g for 10 min to deplete platelets. The PBMC were assessed for viability and counted using a hemocytometer and trypan blue dye (Gibco) then re-suspended in PBS/2%FBS containing 1 mM EDTA. The EasySep Human Monocyte Isolation Kit (Stem Cell Technologies) was then used to purify $CD14^{+}CD16^{-}$ monocytes by immunomagnetic negative selection as per the manufacturer's instructions. Viability (target >95%) was assessed for every sample using trypan blue and purity (target > 90%) was assessed periodically by blocking with TruStain FcX (Biolegend), staining with Fluorescein isothiocyanate-conjugated anti-CD14 antibody (Stem Cell Technologies) and examining cells under a fluorescence microscope (Olympus).

Monocyte bioenergetics

Bioenergetics parameters were assessed by modifying previously published protocols (35). Seahorse XF24 culture plates were coated with 45 μg/mL CellTak (Corning) in 0.1 M sodium bicarbonate (pH 7.5) for 20-30 min then washed with sterile de-ionized water. Monocytes were plated at 4×10^5 cells per well in 200 μL serum-free RPMI. Plates were centrifuged at 300g for 5 min at room temperature; 200 μL 2× media (20% FBS) was added

along with media control or 10 ng/mL LPS (from *Escherichia coli* O111:B4, γ-irradiated, Sigma-Aldrich) with 5 wells per condition as the target. Some samples were limited to 3-4 wells per condition due to low monocyte numbers (because of difficulties in drawing blood or purifying cells). Cells were incubated for 2 h in a humidified cell culture incubator (37°C, 5% CO_2) after which pre-warmed Seahorse XF Assay medium [XF base medium (Agilent Technologies) with 0.5% FBS (Multicell), 2 mM L-glutamine (Hyclone), 1 mM sodium pyruvate (Gibco), 10 mM glucose (Agilent Technologies), pH 7.4] was used to wash twice and replace the culture medium. A mitochondrial stress test protocol was followed where 0.5 M oligomycin (Sigma-Aldrich), 1 µM carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP, Sigma-Aldrich) and 10 µM antimycin A (Sigma-Aldrich) plus 10 µM rotenone (Sigma-Aldrich) were injected sequentially as oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were read on a Seahorse XFe24 analyzer (Agilent Technologies).

Statistical analysis

SAS University Edition (SAS Institute Inc.) was used for statistical analyses. Primary outcomes (plasma oxylipins) and secondary outcomes (plasma fatty acids, plasma cytokines and adipokines, anthropometric data, blood lipids and other clinical parameters, nutrient intake data, activity data, and monocyte metabolic flux parameters) were all analyzed using a mixed model (28). Individuals were treated as random effects while supplement, day and phase order were fixed effects and entered as categorical values. Phase order had no effect on the majority of parameters (fixed effect $p \geq 0.05$), and so was dropped from the model unless otherwise stated, in which case the full data are reported in **Supplemental Table 1**. Type 3 fixed effects of Day (0 and 28), Supplement (flaxseed oil and fish oil) and Day*Supplement interaction were determined and difference of least square means with adjusted p values (Tukey-Kramer correction) are reported to compare D28 to D0 for each supplementation and to compare flaxseed oil to fish oil at each day. If necessary, values were log transformed to achieve a normal distribution within each group, as assessed by the distribution of Conditional Studentized Residuals. Values were excluded as outliers if they fell above or below the mean by 6× (oxylipins) or 5× (all other parameters) the standard deviation, unless its paired values for the same participant in the same phase was similarly elevated. Some data are missing due to, for example, insufficient sample volume, values outside assay detection range, or unexpected retention time. Missing values were

not imputed. Pearson's correlation was performed to identify associations between parameters. For all analyses, $p<0.05$ was considered significant.

Results

General Results

Recruitment took place between June 2018 and December 2019. A participant flow diagram can be found in **Figure 1**. Thirty participants were randomized, however, eight dropped out due to unrelated health issues or inability to comply with study protocols or schedule. One additional participant was unable to complete phase 2 due to cessation of operations in response to the COVID-19 pandemic in March 2020. No adverse events were reported. Self-declared ethnicities included Caucasian (19 of 22), Asian (2 of 22) and Hispanic (1 of 22). Ten participants received flaxseed oil for phase 1 and all ten went on to receive DHA-rich fish oil for phase 2. Twelve participants received DHA-rich oil for phase 1 and eleven of these went on to receive flaxseed oil for phase 2. One participant repeated their DHA phase after retroactively reporting a yeast infection that was present at their day 28 visit. Participant characteristics at the screening visit are described in **Table 1**. All participants had abdominal obesity according to BMI and waist circumference measurements, as specified in the inclusion criteria. Mean self-reported capsule consumption compliance was > 95% for both supplements. This was supported by plasma fatty acid analysis, which confirmed an overall increase in the supplemented PUFA for each phase (**Table 2**). One participant reported relatively low compliance (86%) in the flaxseed oil phase and another completed their flaxseed oil phase prematurely by 6 days due to availability. A substantial increase in plasma ALA was nonetheless observed in both of these participants (a rise of 34% and 124%, respectively). In total, 21 and 22 participants completed the flaxseed oil and fish oil phases, respectively, and were analysed for the primary outcome. Self-reported 3-day food records (**Supplemental Table 2**) and activity (**Supplemental Table 3**) indicated similar nutrient intakes and activity levels at week 3 of each wash-out and supplementation phase.

Oxylipin profiles

Plasma oxylipin concentrations were measured as the primary outcome of this study and are reported in **Table 3** with statistical details and effect sizes in **Supplemental Table 4**. Note that due to the large number of parameters being examined statistically, there is increased risk of

type 1 error for a given oxylipin. As such, we emphasize results pertaining to oxylipin groups, defined by precursor PUFA or enzymatic pathway, rather than individual lipids, whose p values should be interpreted with caution. Flaxseed oil had no effect on any plasma oxylipin (**Table 3**). Total ALA oxylipins were statistically similar on D0 and D28 of flaxseed oil supplementation, although the concentrations were higher on D28 of ALA compared to DHA-rich fish oil supplementation (**Figure 2A**, upper left panel). By contrast, DHA-rich fish oil supplementation increased the total concentration of EPA- and DHA-derived oxylipins (**Figure 2A**, middle and lower left panels) by 2.7-fold and 3.8-fold, respectively. Nearly all individual EPA and DHA oxylipins were significantly increased as well (**Table 3**). These were: the LOX products 8-, 11-, 12-, 15-, and 18-hydroxy-eicosapentaenoic acid (HEPE) and 7-, 10-, 11-, 13-, 14-, 16- and 17-hydroxy-docosahexaenoic acid (HDoHE), the CYP450 epoxygenase products 19,20-epoxy-docosapentaenoic acid (EpDoPE), 16,17- and 19,20-dihydroxy-docosapentaenoic acid (DiHDoPE) and the CYP450 hydroxylase product 20-HDoHE. Levels of three ARA oxylipins, all secondary metabolites of CYP450 epoxygenase, were reduced in response to DHA-rich fish oil supplementation (**Table 3).**

Fatty acid profiles

Plasma fatty acid concentrations are reported in **Table 2** and **Supplemental Table 5.** Only two plasma fatty acids were higher (by 1.5-fold each) after 28 days flaxseed oil supplementation: ALA itself and a low-abundance omega-3 PUFA, 20:3n3 (eicosatrienoic acid, **Table 2**). DHA-rich fish oil supplementation resulted in elevated plasma levels of EPA (4.6-fold) and DHA (3.1-fold, **Figure 2B**, middle and lower right panels). There were changes in several other plasma fatty acids (reported in **Table 2** and **Supplemental Table 5**) that were not accompanied by changes in their derived oxylipins. For example, DHA-rich fish oil supplementation resulted in lower levels of several omega-6 PUFA including γ-linolenic acid (GLA, 18:3n6) and dihomo-γ-linolenic acid (DGLA, 20:3n6, **Table 2**). Oxylipins derived from these PUFA were unchanged (**Table 3**). Also, DHA-rich fish oil supplementation had no measurable effect on plasma ARA (**Table 2**), despite reductions in three ARA oxylipins (**Table 3**). Overall, both plasma fatty acid and oxylipin profiles were altered substantially by DHA-rich fish oil supplementation but were relatively resistant to flaxseed oil supplementation.

Clinical parameters

Anthropometric, vascular and blood biochemistry measurements are reported in **Table 4** and **Supplemental Table 6**. None of these parameters were altered by flaxseed oil supplementation. One marker of liver injury, alanine aminotransferase (ALT) was slightly (1.18× fold) but significantly increased after 28 days of DHA-rich fish oil supplementation whereas aspartate aminotransferase (AST) was unchanged. HDL-cholesterol was increased while triglyceride levels were decreased after 28 days of DHA-rich fish oil supplementation, similar to previous clinical trials that employed longer intervention periods (9). Although supplementation with both ALA and DHA has been previously reported to reduce CRP levels (7, 8, 11, 12), we did not detect a significant effect of either fatty acid supplement on CRP in this study.

Cytokines and adipokines

Inflammatory mediator concentrations are reported in **Table 5** and **Supplemental Table 7**. Based on previous studies we expected to observe a reduction in plasma inflammatory markers with DHA (8, 9) and ALA (13) supplementation. Instead, no changes were detected in any of the measured pro-inflammatory cytokines (IFN-γ, IL-1β, IL-6, IL-8, IL-17A, TNF-α, VEGF), anti-inflammatory cytokine (IL-10) or resistin. However, we did observe a small but significant increase in plasma adiponectin after 28 days of DHA-rich fish oil supplementation, consistent with previous reports (36), which is indicative of improved adipocyte function in these participants.

Associations between BMI and monocyte bioenergetics

Several studies have reported altered glucose metabolism in circulating immune cells of patients with chronic disease, however, the direction of change (higher or lower) seems to depend on the cellular population and/or the specific disease condition (22, 23, 37, 38). Here, we measured live monocyte metabolism *ex vivo* using a Seahorse XF platform in the presence of glucose. We found that BMI is negatively associated with ECAR and positively associated with OCR and ATP-linked OCR (**Figure 3A**). Consistent with experiments performed in dendritic cells and macrophages (39, 40), we observed a Warburg effect induced by LPS stimulation of monocytes (**Supplemental Figure 1A-C**), that is, a slight decrease in OCR accompanied by a

large increase in ECAR. Furthermore, the LPS-induced decrease in OCR is amplified with increasing BMI (**Supplemental Figure 1D)**. Thus, in the most unhealthy state (highest BMI), resting OCR is increased but the dampening of OCR by LPS is enhanced.

Supplementation effects on monocyte bioenergetics

Complete data for monocyte bioenergetics parameters are reported in **Table 6** and **Supplemental Table 8**. Flaxseed oil supplementation for 28 days reduced basal respiration and ATP-linked OCR in monocytes, and increased spare respiratory capacity (**Table 6**, **Figure 3B).** Thus, flaxseed oil supplementation countered the increase in monocyte oxidative glucose metabolism observed with higher BMI. In contrast, DHA-rich fish oil supplementation had no effect on any bioenergetics parameters.

Furthermore, flaxseed oil supplementation countered the changes in oxidative phosphorylation parameters induced by LPS. Specifically, after supplementation, the LPS-induced reduction in basal respiration, ATP-linked OCR and OCR were lessened (**Table 6**). This can also be visualized in **Figure 3C** where the LPS-induced change in ECAR *versus* OCR is traced with an arrow. 2 h LPS stimulation increased ECAR and decreased OCR in cells harvested on Day 0 and on DHA Day 28. In contrast, cells harvested from flaxseed oil-supplemented participants, LPS stimulation increased both ECAR and OCR. Thus, flaxseed oil opposed the enhanced Warburg effect of LPS observed with higher BMI.

Discussion

Omega-3 PUFAs are a promising group of dietary bioactive compounds proposed to block the progression of chronic disease in part by modulating obesity-associated inflammation (2). However, before they can be utilized effectively, much remains to be learned in humans with respect to the differences between plant and marine PUFA and the types of inflammatory markers and processes that are affected. Here, we present the results of a randomized, double-blind, crossover clinical trial that compared the effects of supplementation with ALA-rich oil versus DHA-rich oil (4:1 DHA/EPA) for 4 weeks in women with obesity. We report that DHA-rich fish oil supplementation increased plasma levels of nearly all detectable oxylipins derived from DHA and EPA, including CYP450 products with anti-cancer activities (41) and LOX

products that are precursors to pro-resolving mediators (42). Plasma oxylipins were unaffected by flaxseed oil supplementation. There were also no measurable changes to inflammatory cytokines (IFN-γ, IL-1β, IL-6, IL-8, IL-17A, TNF-α, VEGF), an anti-inflammatory cytokine (IL-10) or resistin with either supplement; however, plasma levels of adiponectin and HDL-cholesterol were increased and triglycerides were decreased after 28 days of DHA-rich fish oil supplementation. We also investigated effects on monocyte glucose metabolism and found that ALA but not DHA reduced OCR and increased spare respiratory capacity. These appear to be favourable changes since higher BMI was associated with an increase in oxygen consumption and a decrease in spare respiratory capacity. Finally, flaxseed oil supplementation also countered the Warburg effect of LPS stimulation. Overall, monocytes in ALA-supplemented participants with obesity shift to a more quiescent phenotype with respect to glucose metabolism. Thus, we report that fish oil and flaxseed oil supplementation have distinct effects in this population: fish oil enhances pro-resolving lipid mediators and adipocyte function while flaxseed oil seems to promote immune-metabolic quiescence at the cellular level.

The effect of ALA- and DHA-rich oils on plasma oxylipin profiles described here largely agrees with earlier work. We previously reported that the same DHA-rich oil increased most DHA- and EPA-derived oxylipins in healthy weight men and women, while flaxseed oil had little effect on circulating oxylipins (28). At least three independent clinical trials similarly report increased DHA- and EPA-derived oxylipins in plasma after fish oil supplementation (43-45). Flaxseed oil supplementation studies designed with higher doses and/or longer durations have reported increases in ALA oxylipins (44, 46), unlike what we observed here using ~4 g/day for 4 weeks. The degree of precursor PUFA enrichment is likely an important factor contributing to oxylipin profile changes. Here, DHA-rich fish oil supplementation increased total plasma EPA and DHA by more than 200% each while flaxseed oil supplementation increased total plasma ALA by only 50%. We previously reported that better vascular health was associated with higher plasma levels of 12-LOX products including 12-HEPE and 14-HDoHE (47). Both of these products increased with DHA-rich fish oil supplementation. Other omega-3 oxylipins have known anti-inflammatory and pro-resolving effects (48, 49). Thus, omega-3 oxylipins may mediate some of the physiological benefits of short-term DHA-rich fish oil supplementation and long-term flaxseed oil supplementation.

In rats, supplementation with pure omega-3 PUFAs reduces circulating omega-6 PUFA oxylipins, including pro-inflammatory eicosanoids derived from ARA, with DHA having a stronger effect compared to ALA (15). On the other hand, human studies examining the impact of supplementation with ALA, DHA or a combination of EPA and DHA on ARA-derived oxylipins have reported either minimal effect (28, 46) or a reduction in a few discrete products only (43-45, 50). Similarly, here we report minimal effects of supplementation on omega-6 oxylipins, despite the observation that DHA- (but not ALA-) rich oil reduced total plasma levels of several omega-6 PUFA. Only three omega-6 oxylipins were reduced by DHA-rich fish oil supplementation here, all secondary CYP450 epoxygenase metabolites of ARA.

Despite potent anti-inflammatory effects reported in animal feeding studies (3-6), the outcomes of ALA-rich and DHA-rich oil supplementation in humans with obesity have been mixed. These trials commonly use circulating CRP, IL-6 and/or TNF-α as the markers of interest. Many (8, 10) but not all (51, 52) studies providing fish oil rich in both EPA and DHA to subjects with obesity for similar time frames have reported systemic anti-inflammatory effects. A study comparing DHA-rich to EPA-rich oil demonstrated some unique anti-inflammatory effects of each after 10 weeks (9). Some ALA-rich oil supplementation trials report no effect (7, 52, 53), while others that used higher doses [~17.5 g/day (12, 13)], longer durations [24 weeks (54)] or participants with more severe obesity [BMI ≥ 40 (10, 11)] do report reduced inflammation. Here, we used a moderate dose (~4 g/day), short time frame (4 weeks) and participants with BMI of 30-44 without secondary disease. Note that consensus healthy reference ranges are not available for these inflammatory mediators. According to values reported in other studies, levels of IFNγ, IL-6 and TNFα in our participants are not higher than expected in healthy female controls, but IL-10 levels are lower than expected (55-57). Other factors such as background diet and co-administered functional ingredients likely play an important role; for example, other FA present in the oil or fibre and protein components when milled flaxseed is used as the source of ALA. Gene diet interactions could also be at play (58). It is important to note that the current study reports only on circulating cytokine levels; other aspects of leukocyte function and tissue-level cytokine expression may be more sensitive to omega-3 PUFA intervention.

According to current disease models, abdominal obesity is harmful because adipocytes become hypertrophic and begin secreting chemokines and other factors that attract immune cells

that then perpetuate a local, and eventually systemic, inflammatory state (59, 60). A well-established marker of adipocyte dysfunction is reduced levels of adiponectin (61). Here we report that plasma adiponectin levels are slightly but significantly elevated after DHA-rich fish oil supplementation, in agreement with previously published studies (7, 36). Since adiponectin is primarily produced by adipocytes, this is evidence of improved adipocyte function. Although the change in adiponectin occurred in the absence of reduced adiposity defined by either body weight or BMI, it is possible that beneficial effects related to metabolic and/or cardiovascular function might still occur. Indeed, high plasma adiponectin is thought to reduce triglycerides and increase HDL-cholesterol (62), both of which were observed here with DHA-rich fish oil supplementation. Adiponectin can also bind to and inactivate oxidized LDL, reducing the atherogenic potential of LDL-cholesterol without altering its levels (63). Our group previously reported no change in triglyceride levels when the same DHA-rich oil was supplemented to healthy weight men and women for 4 weeks (28). That study population was younger and healthier, according to blood biochemistry, thus elevated triglycerides at baseline might be required to detect a significant reduction with supplementation.

The most novel finding of this study is that monocyte OCR and ATP-linked OCR are reduced and spare respiratory capacity is increased after 4 weeks of flaxseed oil supplementation. Evidence from our study population suggests that this is a beneficial effect since higher BMI is associated with higher OCR and ATP-linked OCR but lower spare respiratory capacity. A limitation to this result is the lack of healthy weight participants and a small sample size. Nevertheless, our results are in agreement with other findings. Hartmann et al. reported increased basal respiration in PBMC from patients with type 2 diabetes compared to cells from control patients (22). Shirai et al. demonstrated a similar OCR elevation in monocytes of coronary artery disease patients, although this was accompanied by a greater increase in ECAR and was measured in LPS/IFNγ-stimulated monocytes without an unstimulated reference (23). Here we found that, in parallel to a reduction in resting OCR, flaxseed oil supplementation also led to a dampened Warburg shift in response to 2 h LPS stimulation. In the presence of adequate glucose, the Warburg shift promotes a pro-inflammatory phenotype in innate immune cells (64, 65). Thus, we predict that the combined effect of decreasing oxidative glucose metabolism at rest and diminishing the LPS-induced Warburg shift will reduce mitochondrial ROS production in

monocytes and suppress a pro-inflammatory phenotype. Although the mechanism is as yet unclear, this may represent a new means by which ALA impedes the progression of chronic disease and is of interest for future investigations. Note that although we achieved cellular purity of >90% (tested sporadically), we cannot rule out that population shifts in the contaminating minority component may have contributed to the observed changes.

The role of glucose availability and the effects of obesity, ALA and other PUFA on oxidative phosphorylation of lipid substrates remain to be investigated. For example, it is possible that the decrease in OCR observed with flaxseed oil supplementation may be accompanied by an increase in fatty acid oxidation to support cellular functions. Furthermore, the relationship between immune cell metabolism and whole-body physiology is controversial and seemingly context-dependent. Clarity may be achieved by characterizing the effects of ALA on bioenergetics in other cells and tissues (37, 66), on the response to acute cellular stress (67) and in the context of autoimmune disease (38).

In summary, we report that ALA- and DHA-rich oils each have unique effects on plasma oxylipins and on markers of metabolic health in women with obesity. Four week supplementation with 4 g/day DHA (plus 1 g/day EPA) was sufficient to increase plasma levels of oxylipins derived from EPA and DHA, to improve clinical blood lipid profiles and to increase plasma adiponectin, but insufficient to alter systemic inflammatory markers. Four week supplementation with 4 g/day ALA was insufficient to alter oxylipin, blood lipid or inflammatory profiles in our study population, however, monocyte bioenergetics profiles were markedly changed. These disparate effects provide further evidence that omega-3 PUFA are not interchangeable and may provide metabolic benefits by entirely different, and possibly complementary, mechanisms. This raises the possibility that the full benefits of plant and marine omega-3 fatty acids may be optimally realized when present together in a given diet.

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Author Contributions

SP designed research, conducted research, analyzed data, performed statistical analyses and wrote the paper; LR conducted research, analyzed data and other (constructed 1 table and 1 figure); KS conducted research and analyzed data; TW and NS conducted research; HMA and PZ designed research, provided essential reagents/materials and other (edited the paper); CGT designed research, performed statistical analyses, provided essential reagents/material, had primary responsibility for final content and other (responsible for ethics approval, edited the paper.

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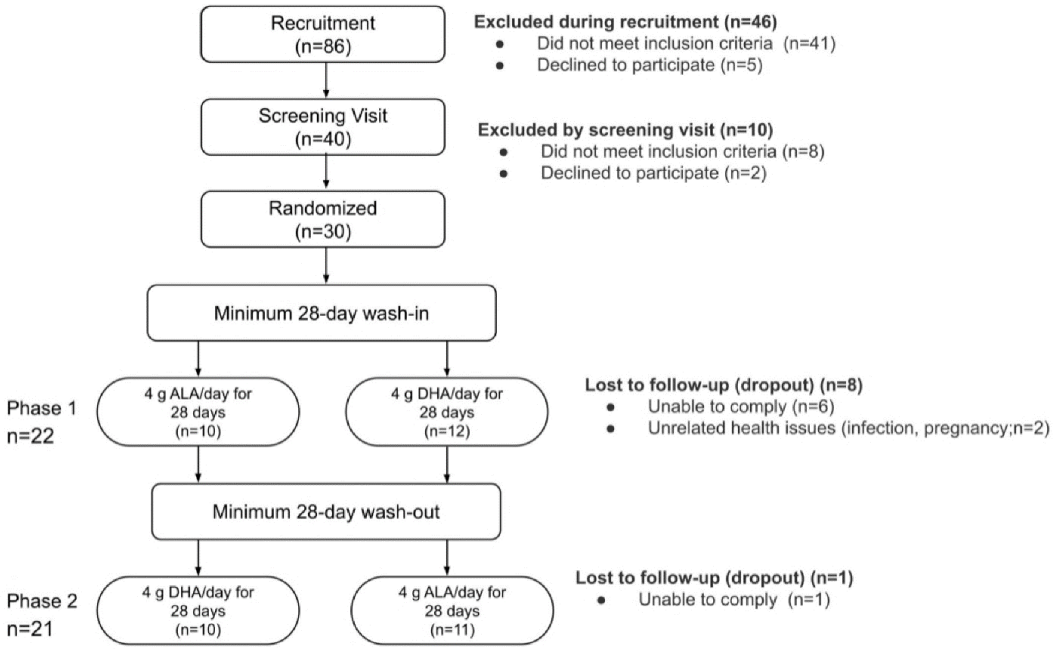


Figure 1. Consolidated Standards of Reporting Trials (CONSORT) chart of study subjects. After contacting 86 individuals and screening 40, eight individuals did not meet the inclusion criteria. Of 32 eligible individuals, 30 were randomized while two declined to participate. The completion rate varied between phases due to the randomized crossover nature of the study. Analysis of the fish oil arm of the study was based on $n = 22$ individuals, and flaxseed oil $n = 21$.

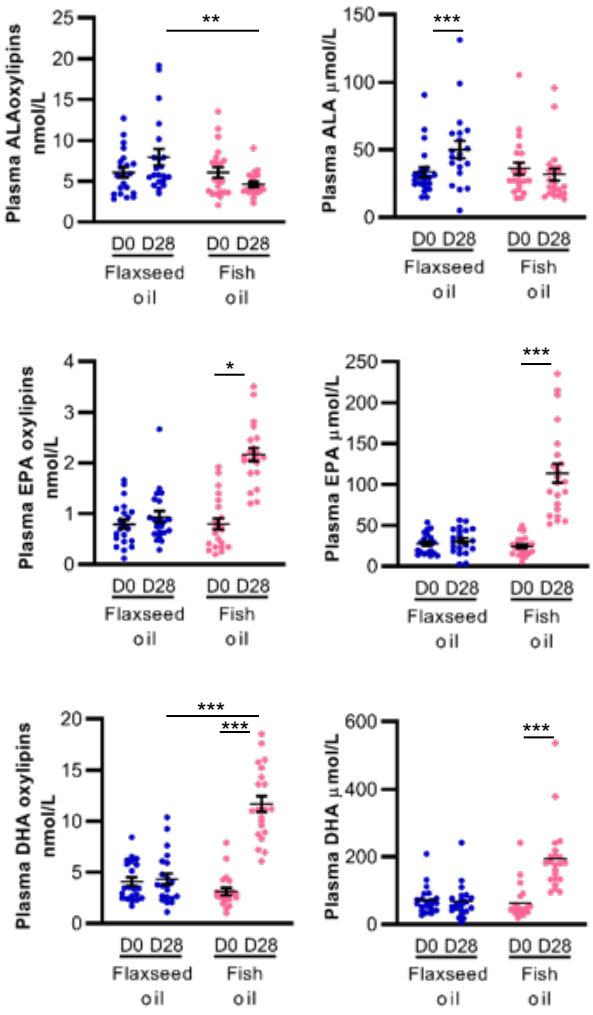


Figure 2. Effect of flaxseed oil and fish oil supplementation on plasma ALA, EPA and DHA oxylipins and their fatty acid precursors in women with obesity. Plasma oxylipins and total plasma fatty acids were measured in samples from day 0 and day 28 of each phase. Selected oxylipin groups (A, n=21) and fatty acids (B, n=21-22) are shown. Data were analyzed using a mixed model and pairwise comparisons were assessed by difference of least square means with adjusted p values (Tukey-Kramer correction) where *, ** and *** indicate $p < 0.05$, 0.01 and 0.01, respectively. Complete statistical analyses are provided **in Table 3 and Supplemental Table 4** for all oxylipins and in **Table 2** and **Supplemental Table 5** for all fatty acids.

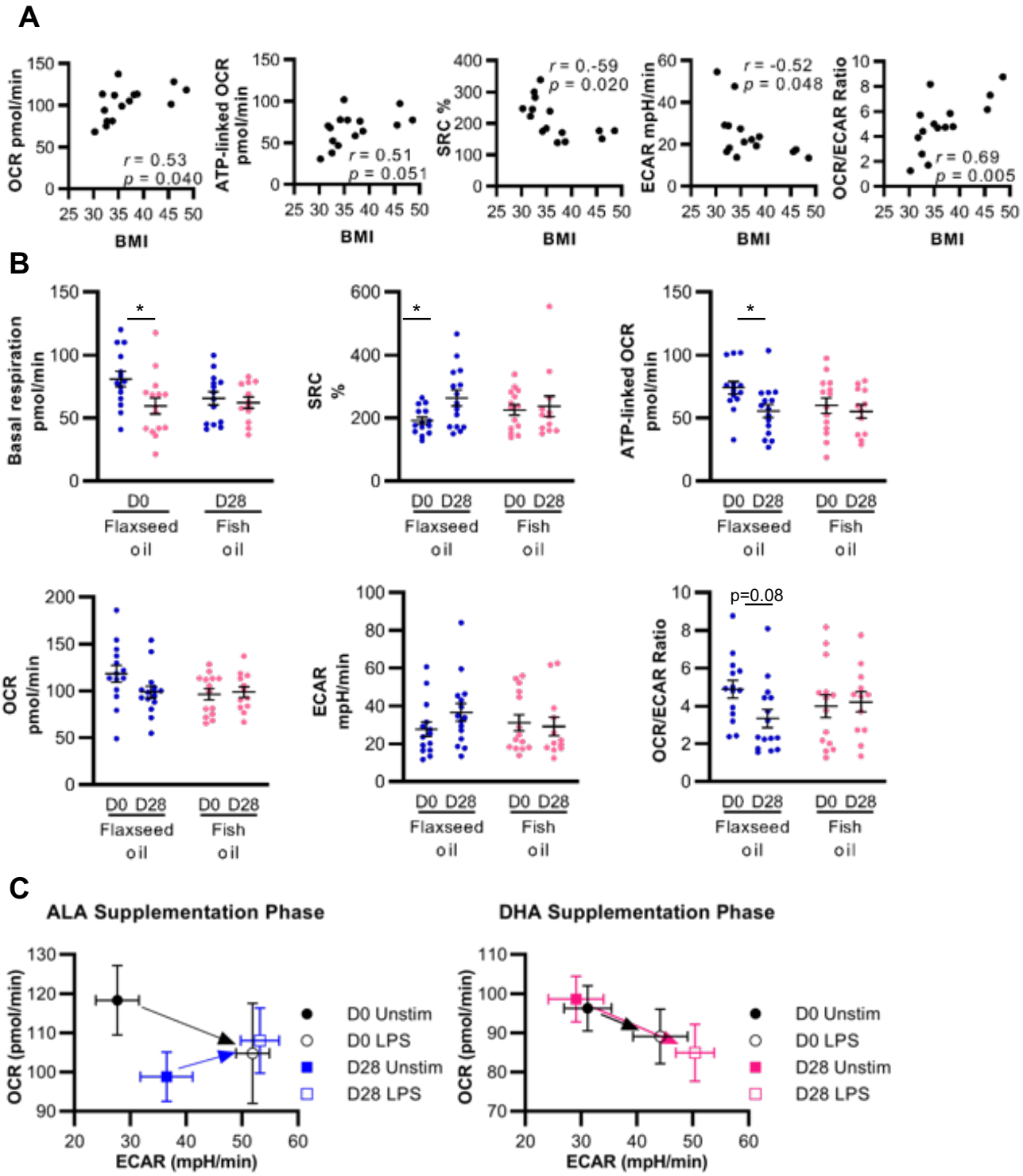

A
OCR pmol/min
r = 0.53
p = 0.040
BMI
ATP-linked OCR pmol/min
r = 0.51
p = 0.051
SRC %
r = 0.-59
p = 0.020
ECAR mpH/min
r = -0.52
p = 0.048
OCR/ECAR Ratio
r = 0.69
p = 0.005
B
Basal respiration pmol/min
SRC %
ATP-linked OCR pmol/min
OCR pmol/min
ECAR mpH/min
OCR/ECAR Ratio
p=0.08
D0 Flaxseed oil
D28 Fish oil
C
ALA Supplementation Phase
DHA Supplementation Phase
OCR (pmol/min)
ECAR (mpH/min)
D0 Unstim
D0 LPS
D28 Unstim
D28 LPS

Figure 3. Flaxseed oil supplementation alters monocyte glucose metabolism in women with obesity. Bioenergetics parameters of $CD14^{+}CD16^{-}$ monocytes purified from peripheral blood on day 0 and 28 of each phase were assessed using a Seahorse XF mitochondrial stress test in the presence of glucose. (A) Correlations between BMI and parameters at first study visit (n=15). Pearson coefficient (*r*) and *p* values are indicated. (B) Supplementation effects on selected bioenergetics parameters (n=12-15). Data were analyzed using a mixed model and pairwise comparisons were assessed by difference of least square means with adjusted p values (Tukey-Kramer correction) where * indicates $p < 0.05$. Complete statistical analyses of all bioenergetics parameters are in **Table 6** and **Supplemental Table 8.** (C) Visualization of cell energy phenotype of unstimulated and 2 h LPS-stimulated cells. OCR and ECAR measurements represent mean values from 10-15 participants. Abbreviations: OCR, oxygen consumption rate; SRC, spare respiratory capacity; ECAR, extracellular acidification rate

Table 1. Anthropometrics and plasma analyte concentrations of the women with obesity[1]

Parameters	
Age, *years*	38.0 ± 8.6 (21-51)
Weight, *kg*	99.8 ± 22.1 (70.0-146)
BMI, *kg/m²*	36.8 ± 6.10 (30.0-51.4)
Waist Circumference, *cm*	102 ± 12.7 (82.0-134)
BP Systolic, *mmHg*	128 ± 14.2 (98-155)
BP Diastolic, *mmHg*	82.5 ± 9.90 (63 -99)
Creatinine, *μmol/L*	68.0 ± 8.90 (48-89)
AST, *U/L*	17.1 ± 4.73 (11-29)
ALT, *U/L*	17.1 ± 7.10 (10-37)
Cholesterol, *mmol/L*	4.60 ± 0.70 (3.1-6.3)
HDL-cholesterol, *mmol/L*	1.50 ± 0.50 (1.0-3.1)
LDL-cholesterol, *mmol/L*	2.53 ± 0.80 (0.8-4.4)
TG, *mmol/L*	1.30 ± 0.41 (0.5-1.9)
CRP, *mmol/L*	7.60 ± 6.20 (1-26)
HbA1c, *%*	5.41 ± 0.30 (4.8-6.3)
Compliance[2] Flaxseed oil phase, %	96.7 ± 4.1 (86.1-100)
Compliance[2] Fish oil phase, %	97.8 ± 2.2 (93.2-100)

[1]Values are reported as mean ± SD (range) from n=21 (flaxseed oil phase) or 22 (fish oil phase) participants

[2] calculated from self-reported doses missed

Abbreviations: BP, blood pressure; CRP, C-reactive protein; HbA1c, hemoglobin A1c

Table 2. Effects of flaxseed oil and fish oil supplementation on plasma FA in women with obesity[1]

	Flaxseed oil		DHA-rich fish oil		Pr>F for type 3 fixed effect		
	D0	D28	D0	D28	Day	Suppl.	Day*Suppl
SFA, *µmol/L*							
12:0[2,5]	9.77±5.19	9.71±4.92	13.28±15.9	9.53±8.1	0.234	0.256	0.500
14:0[2]	155±97.0	139±60.5	164±121	115±67.1†	0.004	0.069	0.106
16:0[2]	3410±1530	3390±1720	3640±2260	3030±1540†	0.012	0.170	0.109
17:0[2]	33.5±9.92	34.4±14.6	36.8±19.9	33.6±13.9	0.402	0.816	0.585
18:0[2]	928±346	932±397	976±519	883±412	0.174	0.308	0.392
20:0[2]	35.2±12.8	34.7±13.1	35.9±17.4	36.3±14.7	0.918	0.689	0.346
22:0	83.9±29.2	82.7±31.2	86.4±39.2	83.0±36.2	0.361	0.516	0.667
24:0	61.7±21.5	61.8±26.8	64.0±29.2	59.9±27.4	0.303	0.835	0.291
MUFA, *µmol/L*							
14:1[2]	9.91±6.78	9.01±4.62	10.6±8.98	5.75±3.75†,‡	.0004	0.016	0.030
16:1[2,5]	336±213	291±169	338±246	223±146†	<.0001	0.100	0.234
16:1t[2]	62.2±30.2	58.7±33.0	67.8±47.3	55.1±32.4	0.008	0.835	0.530
18:1[2]	2780±1150	2630±1400	3030±1830	2300±1100†	0.003	0.742	0.463
18:1n-7[2,5]	228±89.8	218±111	243±141	188±77.8†	0.002	0.231	0.349
20:1[2]	20.6±13.3	20.0±14.1	22.5±16.8	16.2±9.75†,‡	0.001	0.182	0.019
22:1[2]	1.08±4.42	0.760±5.06	2.36±7.84	0.380±4.69†	0.024	0.642	0.045
24:1[2]	126±38.6	120±56.3	128±67.1	142±54.7	0.846	0.064	0.019
n-6 PUFA, *µmol/L*							
18:2[2]	3800±1180	3690±1730	3980±1840	3380±1400	0.018	0.939	0.946
18:3n-6	49.7±18.8	43.2±21.8	50.6±23.8	26.7±10.6†,‡	<.0001	0.009	0.002
20:2[2]	24.5±16.4	23.2±18.42	259±21.5	18.0±11.5†	0.001	0.474	0.389
20:3n-6	237±158	205±144	240±164	136±80.6†,‡	<.0001	0.020	0.005
20:4[2]	809±255	778±400	859±402	671±246	0.004	0.949	0.731
22:2[2]	3.40±1.94	3.80±3.02	4.00±2.83	3.02±2.24†	0.006	0.463	0.042

22:4[2]	20.9±9.99	18.9±9.65	23.3±14.6	10.4±6.29†,‡	<.0001	.0003	<.0001
22:5n6[2]	13.7±8.45	12.1±8.19	16.3±12.9	11.7±6.19†	0.001	0.151	0.555
n-3 PUFA, *µmol/L*							
18:3n-3	119±64.2	179±98.8†,‡	130±75.2	113.±73.8	0.015	0.002	<.0001
20:3n-3[2]	2.79±1.96	4.11±3.73†,‡	2.77±2.68	2.24±1.74	0.184	<.0001	0.003
20:5[2]	91.1±41.0	102±50.6	81.1±38.4	376±180†,‡	<.0001	<.0001	<.0001
22:5n-3[2,3]	49.0±20.2	53.9±29.2	52.7±26.7	44.6±25.3	0.142	0.733	0.419
22:6n-3[2,4]	218±125	204±153	191±154	588±301†,‡	<.0001	<.0001	<.0001
Totals, *µmol/L*							
SFA[2]	4720±2020	4683±2240	5020±2980	4250±2080†	0.020	0.195	0.148
MUFA[2,3]	3560±1480	3350±1750	38340±2320	2930±1370†	0.002	0.687	0.490
PUFA	5440±1770	5320±2560	5650±2680	5380±2220	0.317	0.466	0.713
n-3[2]	480±219	543±299	457±269	1120±547†,‡	<.0001	<.0001	<.0001
n-6[2,3]	4960±1560	4770±2270	5190±2420	4260±1690	0.007	0.844	0.798

[1] Concentrations are shown as Mean±SD; *n*=19-21. Differences of least square means and adjusted *p*-values are in **Supplemental Table 5**.

[2] Log transformed for statistics;

[3] Residuals distribution may limit validity of the model

[4] Phase order effect (refer to **Supplemental Table 1** for values separated by phase)

[5] One outlier removed

† indicates different ($p<0.05$) from Day 0 of the same phase

‡ indicates different ($p<0.05$) from other supplement at the same time point

Table 3. Effects of flaxseed oil and fish oil supplementation on plasma oxylipins in women with obesity[1]

	Flaxseed oil supplementation		DHA-rich fish oil supplementation		Pr>F for type 3 fixed effect		
	D0	D28	D0	D28	Day	Suppl.	Day*Suppl
LA oxylipins, *nmol/L*							
9-HODE[2]	24.6±9.78	28.7±19.2	23.3±10.5	18.9±9.20	0.422	0.069	0.077
9-oxoODE[2]	4.43±1.75	4.38±2.75	4.79±2.87	3.40±1.78	0.070	0.479	0.415
13-HODE[2]	11.1±4.19	12.8±8.19	11.0±5.18	8.91±4.26	0.438	0.119	0.101
13-oxoODE[2]	4.55±2.20	5.91±5.51	5.09±2.69	3.32±1.93	0.239	0.153	0.057
9,10,13-TriHOME	1.19±0.564	1.11±0.676	1.16±0.751	0.722±0.366	0.039	0.155	0.160
9,12,13-TriHOME	0.763±0.317	0.733±0.409	0.767±0.476	0.519±0.229	0.080	0.202	0.166
9,10-DiHOME[2,3,5]	0.212±0.063	0.200±0.101	0.202±0.072	0.195±0.136	0.111	0.506	0.948
12,13-DiHOME[2,3]	0.621±0.203	0.549±0.186	0.611±0.361	0.501±0.176	0.040	0.440	0.739
GLA oxylipins, *nmol/L*							
13-HOTrE-γ[2]	0.180±0.104	0.190±0.132	0.195±0.141	0.119±0.048	0.032	0.193	0.010
DGLA oxylipins, *nmol/L*							
15k PGE1	0.008±0.002	0.011±0.008	0.009±0.003	0.008±0.003	0.207	0.232	0.026
15-HETrE	0.191±0.092	0.180±0.086	0.167±0.096	0.155±0.063	0.471	0.467	0.688
ARA oxylipins, *nmol/L*							
PGK2	0.079±0.044	0.098±0.072	0.085±0.049	0.109±0.051		0.550	0.623
5-HETE	1.54±0.681	1.76±0.822	1.59±0.734	1.35±0.516	0.968	0.465	0.024
5-oxoETE	0.177±0.102	0.140±0.080	0.128±0.079	0.139±0.073	0.389	0.252	0.100
8-HETE[2]	0.560±0.193	0.557±0.176	0.570±0.281	0.514±0.165	0.795	0.790	0.606
9-HETE	0.344±0.128	0.305±0.145	0.347±0.166	0.267±0.112	0.016	0.693	0.422
11-HETE[2]	0.230±0.088	0.216±0.075	0.218±0.089	0.191±0.050	0.140	0.448	0.814
12-HETE	0.817±0.333	0.861±0.398	0.788±0.323	0.693±0.288	0.746	0.279	0.291
15-HETE	0.643±0.228	0.767±0.381	0.701±0.343	0.579±0.263	0.683	0.449	0.020

15-oxoETE	0.105±0.045	0.125±0.111	0.136±0.067	0.101±0.075	0.171	0.926	0.039
5,6-EpETrE	0.020±0.011	0.021±0.010	0.023±0.016	0.019±0.009	0.338	0.780	0.364
8,9-DiHETrE	0.119±0.054	0.112±0.049	0.110±0.037	0.091±0.028†	0.006	0.232	0.158
11,12 DiHETrE[2]	0.208±0.067	0.205±0.077	0.209±0.063	0.162±0.045†	0.002	0.204	0.005
14,15 DiHETrE[2]	0.190±0.062	0.186±0.071	0.182±0.050	0.145±0.033†	0.002	0.131	0.012
16-HETE	0.162±0.075	0.150±0.089	0.123±0.070	0.091±0.053	0.066	0.017	0.420
17-HETE[2]	0.068±0.030	0.064±0.032	0.068±0.040	0.064±0.026	0.660	0.925	0.966
18-HETE[2,3]	0.077±0.045	0.065±0.043	0.076±0.070	0.092±0.092	0.810	0.478	0.185
ALA oxylipins, *nmol/L*							
9-HOTrE[2]	4.07±2.02	5.78±3.955	3.93±1.89	3.10±1.11‡	0.498	0.012	0.016
9-oxoOTrE[2]	0.762±0.337	0.839±0.546	0.805±0.547	0.572±0.202	0.407	0.232	0.365
13-HOTrE[2]	1.24±0.587	1.38±0.63	1.30±0.771	0.940±0.241	0.489	0.105	0.034
12,13 EpODE[2]	0.012±0.007	0.013±0.007	0.012±0.006	0.008±0.005‡	0.285	0.095	0.011
EPA oxylipins, *nmol/L*							
8-HEPE[2]	0.322±0.131	0.338±0.331	0.354±0.216	0.697±0.208†,‡	0.005	0.001	0.001
11-HEPE	0.101±0.042	0.104±0.055	0.106±0.046	0.171±0.063†,‡	0.010	0.030	0.002
12-HEPE[2]	0.180±0.077	0.201±0.088	0.215±0.291	0.453±0.300†,‡	<.0001	0.041	<.0001
15-HEPE[2]	0.122±0.051	0.122±0.041	0.103±0.049	0.248±0.086†,‡	<.0001	0.020	<.0001
18-HEPE	0.225±0.119	0.265±0.120	0.202±0.097	0.592±0.183†,‡	<.0001	<.0001	<.0001
DHA oxylipins, *nmol/L*							
7-HDoHE	0.556±0.353	0.563±0.462	0.395±0.211	1.80±0.697†,‡	<.0001	<.0001	<.0001
10-HDoHE	0.193±0.105	0.205±0.144	0.142±0.089	0.629±0.208†,‡	<.0001	<.0001	<.0001
11-HDoHE	0.272±0.149	0.291±0.196	0.196±0.127	0.910±0.301†,‡	<.0001	<.0001	<.0001
13-HDoHE[2]	0.397±0.174	0.427±0.272	0.346±0.185	1.12±0.430†,‡	<.0001	0.001	<.0001
14-HDoHE[2]	0.275±0.130	0.313±0.196	0.201±0.108	0.861±0.346†,‡	<.0001	0.005	<.0001
16-HDoHE	0.227±0.115	0.256±0.168	0.171±0.095	0.652±0.223†,‡	<.0001	<.0001	<.0001
17-HDoHE	0.795±0.364	0.866±0.550	0.567±0.300	2.224±0.768†,‡	<.0001	<.0001	<.0001
RvD1	0.041±0.012	0.042±0.008	0.040±0.015	0.048±0.011		0.370	0.133

	Flaxseed oil supplementation		DHA-rich fish oil supplementation		Pr>F for type 3 fixed effect		
	D0	D28	D0	D28	Day	Suppl.	Day*Suppl
DHA oxylipins cont							
16,17-DiHDoPE	0.051±0.022	0.048±0.021	0.043±0.023	0.124±0.030†,‡	<.0001	<.0001	<.0001
19,20-EpDoPE[2]	0.044±0.030	0.046±0.030	0.031±0.021	0.132±0.061†,‡	<.0001	0.013	<.0001
19,20-DiHDoPE	0.743±0.449	0.709±0.332	0.594±0.336	1.649±0.402†,‡	<.0001	.0004	<.0001
20-HDoHE[2,4]	0.497±0.273	0.588±0.399	0.387±0.222	1.526±0.730†,‡	<.0001	0.006	<.0001
Free FA, *nmol/L*							
ALA	2350±873	2520±761	2270±838	1981±740	0.656	0.154	0.100
ARA	183±75.9	195±72.2	190±81.4	177±63.1	0.911	0.919	0.213
DHA	957±423	995±505	797.±398	2320±702†,‡	<.0001	.0001	<.0001
EPA[2]	123±70.8	136±59.9	109±61.5	324±135†,‡	<.0001	0.008	<.0001

[1] Concentrations are shown as Mean±SD; *n*=19-20 for all oxylipins except PGK2 (*n*=10-17), 8-HEPE (*n*=13-21), 11-HEPE (*n*=13-21) and 15-oxoETE (*n*=18-19). Two participant samples were excluded from analysis because >⅓ of oxylipins qualified as outlier values, suggesting a technical error. Differences of least square means and adjusted *p*-values are in **Supplemental Table 4.**

[2] Log transformed for statistics;

[3] Residuals distribution may limit validity of the model

[4] Phase order effect (refer to **Supplemental Table 1** for values separated by phase)

[5] One outlier removed

† indicates different ($p<0.05$) from Day 0 of the same phase

‡ indicates different ($p<0.05$) from other supplement at the same time point

Abbreviations: ARA, arachidonic acid; ALA, α-linolenic acid; DGLA, dihomo-gamma-linolenic acid; DiHDoPE, dihydroxy-docosapentaenoic acid; DiHETrE, dihydroxy-eicosatrienoic acid; DiHODE, dihydroxy-octadecadienoic acid; DiHOME, dihydroxy-octadecenoic acid; EpDPE, epoxydocosapentaenoic acid, EpETrE, epoxy-eicosatrienoic acid; EpODE, epoxy-octadecadienoic acid; EpOME, epoxy-octadecenoic acid; GLA, gamma-linolenic acid; HDoHE, hydroxy-docosahexaenoic acid; HEPE, hydroxy-eicosapentaenoic acid; HETE, hydroxy-eicosatetraenoic acid; HETrE, hydroxy-eicosatrienoic acid; HODE, hydroxy-octadecadienoic

acid; HOTrE, hydroxy-octadecatrienoic acid; LA, linoleic acid; oxoETE, oxo-eicosatetraenoic acid; oxoOTrE, oxo-octadecatrienoic acid; TriHOME, trihydroxy-octadecenoic acid

Table 4. Effects of flaxseed oil and fish oil supplementation on anthropometric and clinical parameters in women with obesity[1]

	Flaxseed oil supplementation		DHA-rich fish oil supplementation		Pr>F for type 3 fixed effect		
	D0	D28	D0	D28	Day	Suppl.	Day*Suppl
Anthropometrics							
Body weight, *kg*	100±21.4	100±23.0	100±23.3	100±24.0	0.963	0.998	0.513
Waist circumference, *cm*	102±13.5	102±12.9	102±13.7	103±13.3	0.712	0.932	0.770
BMI, *kg/m²*	36.9±6.12	36.7±6.67	36.9±6.59	37±6.84	0.915	0.962	0.479
Vascular							
SBP, *mmHg*	127±16.6	129±16.7	128±14.3	127±15.7	0.899	0.986	0.232
DBP, *mmHg*	80.5±11.3	80.8±10.8	80.4±10.1	81.4±9.4	0.803	0.836	0.952
AIx, *%*	16.3±9.99	17.4±11.6	20.2±9.46	20.5±16.4	0.822	0.268	0.890
PWV, *m/s*	6.22±0.806	5.97±0.903	6.15±1.19	5.86±0.995	0.467	0.947	0.340
Blood biochemistry							
ALT. *U/L*[2]	17.9±7.27	17.5±6.76	18.5±9.37	21.8±11.19[†]	0.039	0.420	0.003
AST, *U/L*	22.7±5.29	24.1±6.51	24.3±7	26.1±7.32	0.075	0.306	0.816
Creatinine, *µmol/L*[4]	67.3±8.35	68±9.51	68.2±7.84	68.3±8.06	0.604	0.810	0.759
CRP, *mg/L*[2,3]	9.28±11.92	8.04±7.74	8.44±8.69	7.97±8.23	0.083	0.813	0.838
Glucose, *mmol/L*	5.53±0.653	5.62±0.761	5.55±0.66	5.74±0.761	0.035	0.731	0.489
Chol, *mmol/L*	4.74±0.735	4.62±0.664	4.66±0.84	4.81±0.828	0.859	0.802	0.054
HDL-cholesterol, *mmol/L*	1.42±0.425	1.38±0.423	1.35±0.401	1.50±0.432[†]	.0004	0.847	<.0001
LDL-cholesterol, *mmol/L*[2]	2.80±0.805	2.67±0.654	2.72±0.835	2.79±0.89	0.858	0.968	0.230
TG, *mmol/L*	1.25±0.333	1.26±0.381	1.41±0.483	1.08±0.339[†]	0.001	0.910	.0005

[1] Concentrations are shown as Mean±SD; *n*=20-22 for all parameters except AIx and PWV (*n*=12-16).

[2] Log transformed for statistics;

[3] Residuals distribution may limit validity of the model

[4] Phase order effect (refer to **Supplemental Table 1** for values separated by phase)

† indicates different (p<0.05) from Day 0 of the same phase

‡ indicates different (p<0.05) from other supplement at the same time point

Abbreviations: AIx, augmentation index; ALT, Alanine transaminase (ALT); AST, aspartate aminotransferase; BMI, body mass index; circ., circumference; CRP, C-reactive protein; DBP, diastolic blood pressure; PWV, pulse wave velocity; SBP, systolic blood pressure.

Differences of least square means and adjusted p-values are in **Supplemental Table 6.**

Table 5. Effects of flaxseed oil and fish oil supplementation on plasma inflammatory markers in women with obesity[1]

	Flaxseed oil supplementation		DHA-rich fish oil supplementation		Pr>F for type 3 fixed effect		
	D0	D28	D0	D28	Day	Suppl.	Day*Suppl
IFN-γ, *pg/mL*[2,6]	4.34±2.56	4.52±2.80	3.79±1.71	3.51±1.58	0.425	0.460	0.781
IL-10, *pg/mL*[5]	0.235±0.083	0.227±0.081	0.231±0.089	0.246±0.079	0.843	0.694	0.408
IL-1β, *pg/mL*[2,4]	0.098±0.056	0.099±0.047	0.092±0.054	0.093±0.051	0.656	0.481	0.887
IL-6, *pg/mL*[3]	1.31±1.33	1.05±1.08	1.07±1.09	1.03±1.25	0.307	0.635	0.478
IL-8, *pg/mL*	2.72±1.17	3.26±2.13	2.62±1.42	2.63±1.37	0.278	0.393	0.250
TNF-α, *pg/mL*[2]	2.01±0.740	1.95±0.620	1.81±0.500	1.90±0.490	0.689	0.612	0.280
VEGF, *pg/mL*[2]	37.8±29.6	35.1±23.1	39.5±21.5	31.3±13.7	0.185	0.810	0.197
IL-17A, *pg/m*[2,5]	0.557±0.278	0.491±0.187	0.466±0.167	0.519±0.146	0.956	0.834	0.026
Resistin, *pg/m*[2]	2530±747	2630±712	2710±851	2560±672	0.786	0.919	0.263
Adiponectin, *μg/mL*	237±77.1	242±83.4	237±73.2	261±87.9†	0.010	0.903	0.079

[1] Concentrations are shown as Mean±SD; *n*=19-20 for all markers except VEGF (*n*=18-19) and IL-17A (*n*=16-19). Differences of least square means and adjusted *p*-values are in **Supplemental Table 7.**

[2] Log transformed for statistics;

[3] indicates residuals distribution may limit validity of the model

[4] Phase order effect (refer to **Supplemental Table 1** for values separated by phase)

[5] One outlier removed

[6] Two outliers removed

† indicates different ($p<0.05$) from Day 0 of the same phase

‡ indicates different ($p<0.05$) from other supplement at the same time point

Abbreviations: VEGF, vascular endothelial growth factor

Table 6. Effects of flaxseed oil and fish oil supplementation on monocyte bioenergetics parameters in women with obesity[1]

	Flaxseed oil supplementation		DHA-rich fish oil supplementation		Pr>F for type 3 fixed effect		
	D0	D28	D0	D28	Day	Suppl.	Day*Suppl
Unstimulated							
Basal Resp, *pmol/min*	80.8±22.8	59.7±24.5†	65.6±19.4	62.3±15.4	0.022	0.439	0.048
Proton Leak, *pmol/min*	8.89±12.2	5.47±8.3	5.78±6.2	6.98±7.3	0.238	0.830	0.080
Max Resp, *pmol/min*	152±44.4	144±31.8	147±45.1	125±22.8	0.129	0.244	0.471
SRC, *pmol/min*	71.0±33.4	83.8±30.1	80.8±37.5	62.4±20.2	0.698	0.514	0.058
Non Mito Ox, *pmol/min*	37.5±21.1	39±11.3	30.7±6.6	36.3±18	0.360	0.298	0.538
ATP-linked OCR, *pmol/min*	74.0±18.6	55.5±19.9†	60.0±22.9	55.3±18.3	0.018	0.422	0.057
Coupling Effic, *pmol/min*[2]	0.948±0.241	0.943±0.124	0.889±0.173	0.926±0.211	0.280	0.546	0.579
SRC, %[2,4]	1.91±0.42	2.63±0.975†	2.26±0.649	2.37±1.13	0.029	0.992	0.028
OCR, *pmol/min*[2]	118±33.2	98.8±24.3	96.3±21.6	98.6±20.3	0.180	0.180	0.067
ECAR, *mpH/min*	27.7±14.5	36.5±18.1	31.2±15.9	29.1±17.1	0.613	0.610	0.110
OCR/ECAR Ratio[2]	4.88±1.75	3.33±1.85	3.98±2.24	4.21±1.85	0.293	0.785	0.021
Change by LPS							
∆Basal, *pmol/min*[3]	-26.4±21.2	-2.08±23.5†	-8.68±17.0	-19.6±15.5	0.213	0.867	.0003
∆Proton Leak, *pmol/min*	1.96±5.09	7.07±5.88	5.65±6.12	3.31±5.66	0.395	0.984	0.030
∆Max Resp, *pmol/min*	-11.6±23.9	20.2±37.2†	9.44±25.1	5.05±16.1	0.077	0.713	0.026
∆SRC, *pmol/min*	14.8±30.9	22.3±25.4	19.2±30.1	24.7±22.8	0.409	0.662	0.899
∆Non Mito Ox, *pmol/min*	11.1±19.6	11.4±16.0	4.92±12.8	10.5±21.1	0.472	0.530	0.488
∆ATP-linked OCR, *pmol/min*	-27.9±18.9	-9.72±21.3†	-14.6±19.6	-23.0±16.5	0.459	0.796	0.001
∆Coupling efficiency, *pmol/min*[2]	-0.043±0.163	-0.125±0.150	-0.089±0.166	-0.176±0.229	0.082	0.347	0.942
∆SRC, %[2]	0.875±1.17	0.399±0.725	0.701±0.699	0.832±1.167	0.557	0.581	0.221
∆OCR, *pmol/min*[3]	-15.3±17.3	9.30±23.1†	-3.76±21.1	-9.15±21	0.071	0.525	0.003

ΔECAR, *mpH/min*	23.3±13.0	17.5±11.1	13.6±15.2	21.1±13.9	0.692	0.518	0.048
ΔOCR/ECAR Ratio	-2.86±1.96	-1.37±1.80	-1.44±1.87	-2.32±1.49	0.894	0.895	0.003

[1] Concentrations are shown as Mean±SD; *n*=12-15 for Unstim and *n*=10-14 for Change by LPS. Differences of least square means and adjusted *p*-values are in **Supplemental Table 8.**

[2] Log transformed for statistics;

[3] Phase order effect (refer to **Supplemental Table 1** for values separated by phase)

[4] One outlier removed

† indicates different ($p<0.05$) from Day 0 of the same phase

‡ indicates different ($p<0.05$) from other supplement at the same time point

Abbreviations: ECAR, extracellular acidification rate; effic, efficiency; mito, mitochondrial; OCR, oxygen consumption rate; Ox, oxidation; Resp, respiration; SCR, spare respiratory capacity

Supplemental Table 1. Effects of flaxseed oil and fish oil supplementation on parameters with a phase effect in women with obesity[1]

	Flaxseed oil Phase 1		Flaxseed oil Phase 2		Fish oil Phase 1		Fish oil Phase 2		Pr>F for type 3 fixed efects						
	D0	D28	D0	D28	D0	D28	D0	D28	Day	Suppl	Day* Suppl	Phase	Day* Phase	Suppl* Phase	Day* Suppl* Phase
IL-1β, *ng/mL*	0.103±0.081	0.111±0.056	0.091±0.026	0.088±0.035	0.119±0.063	0.100±0.06	0.068±0.023	0.084±0.038	0.615	0.595	0.988	0.235	0.627	0.448	0.064
20-HDoHE, *nmol/L*	0.385±0.269	0.321±0.194	0.599±0.244	0.830±0.386¥	0.404±0.194	1.64±0.807†	0.368±0.26‡	1.42±0.674†	<.0001	0.001	<.0001	0.029	0.134	0.001	0.064
22:6n3, *μmol/L*[2]	172±95.4	157±119	260±137	247±172	206±181	619±349†	176±127	557±258†	<.0001	0.001	<.0001	0.187	0.325	0.029	0.615
Creatinine, *μmol/L*	68.1±10.7	67.8±11.6	66.6±5.99	68.2±7.77	66.0±5.72	65.6±7.04	70.3±9.27	71.1±8.36	0.620	0.810	0.779	0.347	0.330	0.247	0.816
Monocyte OCR/ECAR Ratio	5.74±1.54	3.82±1.06	4.01±1.59	3.09±2.15	4.36±2.49	4.44±2.03	3.48±1.96	3.90±1.73	0.313	0.847	0.012	0.142	0.116	0.648	0.373
Monocyte ΔBasal Resp, *pmol/min*	-38.8±19.5	-14.9±23.2	-17.5±18.8	5.03±21.7	-12.3±17.5	-20.1±17.6	-1.51±15.4	-18.9±14.3	0.277	0.625	.0004	0.039	0.601	0.300	0.739
Monocyte ΔOCR, *pmol/min*	-28.3±12.7	0.929±12.3	-6.00±14.1	14.0±27.0	-11.3±20.6	-12.4±17.7	11.2±14.1	-4.3±27.4	0.097	0.934	0.002	0.016	0.191	0.872	0.841
EPA+DHA intake, *g/day*[3]	0.036±0.021	0.036±0.031	0.039±0.052	0.057±0.052	0.036±0.03	0.038±0.026	0.052±0.061	0.057±0.038	0.578	0.531	0.637	0.0266	0.4829	0.719	0.5963

[1]Values are shown as Mean±SD in the indicated units. Data were analyzed using a mixed model with individuals as a random effect, and phase, day and supplement as fixed effects. n=10-15 for monocyte bioenergetics parameters or n=20-21 for all other measurements.

[2] indicates log transformed for statistics;

[3] dietary intake data, Timepoints are Before and During Supplementation instead of Days 0 and 28

† indicates different (p<0.05) from Day 0 of the same phase

‡ indicates different (p<0.05) from other supplement at the same time point

¥ indicates different (p<0.05) from matched timepoint in phase 1

Shading:

Differences in least square means	Increased from D0
	Decreased from D0

The lack of statistical symbols and shading indicates no significant differences among groups

Abbreviations: ECAR, extracellular acidification rate; HDoHE, hydroxy-docosahexaenoic acid; OCR, oxygen consumption rate; Resp, respiration

Supplemental Table 2. Effects of flaxseed oil and fish oil supplementation on self-reported food intake in women with obesity[1]

	Flaxseed oil supplementation		Fish oil supplementation		Pr>F for type 3 fixed effect		
	Before	During	Before	During	Time	Suppl.	Day*Supp
Energy, *kcal/day*	2050±648	2060±798	2060±604	2080±871	0.900	0.881	0.905
Carbohydrate, *g/day*	252±88.1	238±94.4	239±73.6	241±113.3	0.828	0.793	0.586
Fibre, *g/day*	18.6±7.42	18.9±10.04	19.5±8.46	18.0±6.71	0.835	0.976	0.630
Protein, *g/day*[2]	80.2±26.9	88.6±36.0	88.3±35.1	82.9±39.1	0.925	0.956	0.469
Fat, *g/day*	79.5±29.3	82.0±43.7	82.7±37.0	86.8±35.7	0.682	0.470	0.901
Saturated Fat, *g/day*	29.8±14.7	28.7±17.5	25.9±12.3	31.1±14.7	0.555	0.852	0.264
Trans Fat, *g/day*[2]	1.10±0.784	1.07±0.894	1.21±0.866	1.49±1.30	0.690	0.047	0.384
MUFA, *g/day*[2]	26.5±9.04	28.3±15.2	30.5±18.2	31.2±12.7	0.619	0.344	0.536
PUFA, *g/day*	14.5±7.52	14.9±7.90	16.1±8.55	15.0±6.99	0.885	0.549	0.652
Cholesterol, *mg/day*	258±123	295±145	283±199	315±143	0.351	0.458	0.960
n-6, *g/day*[2]	11.2±5.64	11.7±6.75	11.1±5.31	11.7±6.08	0.621	0.978	0.946
LA, *g/day*[2]	11.3±5.74	11.8±7.03	11.3±5.50	11.8±5.99	0.625	0.927	0.977
n-3, *g/day*	1.91±0.880	1.83±1.03	1.92±1.13	2.01±1.26	0.964	0.651	0.681
ALA, *g/day*	1.24±0.510	1.24±0.602	1.25±0.574	1.38±0.778	0.653	0.556	0.601
EPA+DHA, *g/day*[3]	0.038±0.038	0.047±0.044	0.043±0.045	0.046±0.033	0.524	0.696	0.665
n-6/n-3 Ratio[2]	2.04±0.876	2.24±0.743	2.10±0.476	2.20±1.02	0.503	0.811	0.307
Sugars, *g/day*	92.3±45.6	83.2±50.6	78.7±38.9	84.6±36.53	0.933	0.450	0.324
Added Sugar, *g/day*[2]	48.4±38.7	43.8±31.4	37.6±19.1	42.9±27.9	0.946	0.631	0.633
Vit K, *µg/day*[2]	73.0±35.4	132±157	104±107	92.9±62.6	0.450	0.810	0.878
β-carotene, *µg/day*[2]	3960±2860	3030±3800	3860±4780	3810±4140	0.513	0.971	0.123
Lycopene, *µg/day*[2]	4880±6700	2970±5330	4500±6090	2410±3290	0.616	0.384	0.980
Magnesium, *mg/day*	256±83.8	271±130	250±95.7	254±103	0.661	0.590	0.853
Manganese, *mg/day*	3.34±1.96	3.49±2.25	3.04±1.87	3.16±1.85	0.743	0.405	0.954
Phosphorous, *mg/day*[2]	1170±453	1260±536	1200±471	1230±662	0.726	0.893	0.867
Selenium, *µg/day*[2]	97.3±45.6	99.6±31.7	101±36.0	88.0±42.8	0.529	0.237	0.113
Zing, *mg/day*	9.03±3.35	10.7±5.34	11.4±5.91	10.3±4.73	0.851	0.343	0.190
Vit A, *µg/day*	803±448	710±474	793±570	830±428	0.842	0.479	0.380
Vit D, *IU/day*[2]	102±86.6	107±71.7	133±99.6	124±112	0.837	0.024	0.708
Vit E, *mg/day*	6.91±3.57	8.41±4.06	9.18±5.82	9.21±4.23	0.455	0.057	0.412
Thiamin, *mg/day*[2]	1.51±0.794	1.50±0.437	1.46±0.450	1.42±0.647	0.960	0.634	0.473
Riboflavin, *mg/day*	1.81±0.861	1.98±0.741	1.87±0.795	1.74±0.602	0.880	0.491	0.351
Niacin *ne/day*	35.2±13.1	38.7±12.6	37.6±15.5	33.9±12.0	0.996	0.610	0.207
Vit B6, *mg/day*	1.42±0.570	1.61±0.720	1.63±0.620	1.53±0.680	0.754	0.632	0.303
Folate, *µg/day*[2]	392±210	368±151	403±145	380±189	0.410	0.660	0.802
Vit B12, *mg/day*[2]	2.95±1.37	3.50±2.07	3.95±2.57	3.60±2.21	0.958	0.347	0.923
Vit C, *mg/day*	62.7±37.4	70.4±52.4	59.1±50.8	63.6±38.0	0.528	0.472	0.888
Sodium, *mg/day*	2860±987	3120±1480	3290±1350	3200±1640	0.790	0.349	0.558
Potassium, *mg/day*[2]	2470±795	2520±1020	2480±981	2490±868	0.728	0.887	0.670
Calcium, *mg/day*	911±434	954±437	889±377	860±300	0.895	0.469	0.662
Iron, *mg/day*	11.4±4.44	12.9±4.36	13.9±4.59	12.8±5.64	0.854	0.268	0.237

[1]Values are shown as Mean±SD, average of 3-day Food Record*; n*=19-20; No significant pairwise comparisons were observed.

[2] indicates log transformed values used for statistics

[3] indicates phase order effect (refer to **Supplemental Table S1** for values separated by phase)

Supplemental Table 3. Effects of flaxseed oil and fish oil supplementation on self-reported activity in women with obesity[1]

	Flaxseed oil supplementation		Fish oil supplementation		Pr>F for type 3 fixed effect		
	Before	During	Before	During	Time	Suppl.	Day*Suppl
Vigorous activity, *days/week*	1.63±1.61	2±1.88	1.67±1.72	1.79±1.96	0.617	0.715	0.454
Vigorous activity, *avg min/day* [2]	7.89±8.02	12.2±12.5	12.5±18.3	11.7±15.7	0.848	0.423	0.153
Moderate activity, *days/week*	1.95±1.81	1.94±2.21	2.33±2.43	2.21±2.51	0.808	0.437	0.587
Moderate activity, *avg min/day* [2]	18.9±28.6	8.8±9.1	18.8±36.9	17.9±36.1	0.526	0.864	0.623
Walking, *days/week*	5.19±1.97	5.21±1.69	5.00±2.00	5.11±2.26	0.953	0.823	0.982
Walking, *avg min/day*	68.3±78	51.5±60.5	61.4±82.9	66.6±101	0.994	0.540	0.857
Sitting, *avg min/day*	366±154	423±152	381±177	428±212	0.684	0.531	0.370

[1]Values are shown as Mean±SD; *n*=14-19; No significant pairwise comparisons were observed.
[2] indicates log transformed values used for statistics

Supplemental Table 4. Effects of flaxseed oil and fish oil supplementation on plasma oxylipins in women with obesity

	Flaxseed oil D28 vs. D0	*p*	Fish oil D28 vs. D0	*p*	D0 flax vs. fish	*p*	D28 flax vs. fish	*p*
LA oxylipins, *nmol/L*								
9-HODE[2]	4.13±0.110	*0.892*	-4.04±0.111	*0.267*	0.066±0.136	*0.962*	0.350±0.136	*0.065*
9-oxoODE[2]	-0.053±0.173	*0.882*	-1.39±0.173	*0.245*	-0.009±0.178	*1.00*	0.193±0.178	*0.700*
13-HODE[2]	1.74±0.110	*0.920*	-1.90±0.111	*0.302*	0.037±0.131	*0.992*	0.299±0.131	*0.121*
13-oxoODE[2]	1.36±0.182	*0.948*	-1.77±0.182	*0.133*	-0.042±0.193	*0.996*	0.463±0.193	*0.095*
9,10,13-TriHOME	-0.084±0.168	*0.958*	-0.426±0.171	*0.076*	0.033±0.184	*0.998*	0.376±0.186	*0.198*
9,12,13-TriHOME	-0.030±0.109	*0.993*	-0.247±0.109	*0.123*	-0.004±0.111	*1.00*	0.213±0.111	*0.240*
9,10-DiHOME[2,3]	-0.012±0.078	*0.617*	-0.005±0.081	*0.697*	0.071±0.114	*0.925*	0.063±0.115	*0.946*
12,13-DiHOME[2,3]	-0.072±0.071	*0.312*	-0.095±0.072	*0.597*	0.103±0.121	*0.831*	0.069±0.121	*0.941*
GLA oxylipins, *nmol/L*								
13-HOTrE-γ[2]	0.010±0.106	*0.986*	-0.058±0.111	*0.008*	-0.038±0.149	*0.994*	0.377±0.149	*0.072*
DGLA oxylipins, *nmol/L*								
15k PGE1	0.004±0.001	*0.076*	-0.001±0.001	*0.875*	-0.001±0.001	*0.863*	0.004±0.001	*0.073*
15-HETrE	-0.011±0.013	*0.850*	-0.003±0.013	*0.996*	0.022±0.026	*0.840*	0.014±0.026	*0.948*
AA oxylipins, *nmol/L*								
PGK2	0.018±0.015	*0.670*	0.028±0.013	*0.202*	-0.005±0.019	*0.994*	-0.015±0.019	*0.865*
5-HETE	0.217±0.127	*0.333*	-0.210±0.129	*0.377*	-0.072±0.212	*0.987*	0.355±0.212	*0.352*
5-oxoETE	-0.039±0.021	*0.280*	0.012±0.021	*0.938*	0.050±0.026	*0.243*	-0.001±0.026	*1.00*
8-HETE[2]	-0.003±0.067	*0.998*	-0.039±0.068	*0.946*	0.002±0.111	*1.00*	0.052±0.111	*0.966*
9-HETE	-0.039±0.031	*0.613*	-0.075±0.032	*0.110*	-0.004±0.042	*1.00*	0.032±0.042	*0.865*
11-HETE[2]	-0.014±0.062	*0.803*	-0.021±0.063	*0.616*	0.058±0.099	*0.937*	0.079±0.099	*0.858*
12-HETE	0.044±0.083	*0.950*	-0.083±0.085	*0.766*	0.029±0.102	*0.992*	0.156±0.104	*0.449*
15-HETE	0.080±0.056	*0.492*	-0.113±0.056	*0.201*	-0.027±0.099	*0.993*	0.166±0.099	*0.348*
15-oxoETE	0.020±0.155	*0.949*	-0.035±0.158	*0.082*	-0.250±0.165	*0.444*	0.227±0.167	*0.538*
5,6-EpETrE	0.00±0.003	*1.00*	-0.004±0.003	*0.551*	-0.003±0.004	*0.883*	0.001±0.004	*0.994*

8,9-DiHETrE	-0.007±0.007	*0.733*	-0.022±0.007	*0.022*	0.007±0.013	*0.942*	0.022±0.013	*0.350*
11,12 DiHETrE[2]	-0.002±0.055	*0.995*	-0.049±0.056	*.0004*	-0.013±0.09	*0.999*	0.222±0.090	*0.081*
14,15 DiHETrE[2]	-0.004±0.051	*0.960*	-0.039±0.052	*0.001*	0.024±0.086	*0.992*	0.216±0.086	*0.074*
16-HETE	-0.012±0.016	*0.869*	-0.030±0.016	*0.243*	0.038±0.022	*0.317*	0.056±0.022	*0.066*
17-HETE[2]	-0.004±0.112	*0.983*	-0.006±0.125	*0.993*	-0.007±0.137	*1.00*	-0.014±0.142	*1.00*
18-HETE[2,3]	-0.011±0.156	*0.680*	0.016±0.154	*0.859*	0.063±0.164	*0.980*	-0.233±0.158	*0.465*
ALA oxylipins, *nmol/L*								
9-HOTrE[2]	1.71±0.135	*0.124*	-0.833±0.135	*0.571*	0.034±0.142	*0.995*	0.515±0.142	*0.004*
9-oxoOTrE[2]	0.078±0.165	*1.00*	-0.233±0.165	*0.605*	0.037±0.167	*0.996*	0.251±0.167	*0.442*
13-HOTrE[2]	0.140±0.113	*0.718*	-0.361±0.112	*0.185*	-0.023±0.120	*0.997*	0.327±0.122	*0.051*
12,13 EpODE[2]	0.001±0.115	*0.678*	-0.003±0.116	*0.055*	-0.006±0.148	*1.00*	0.427±0.148	*0.030*
EPA oxylipins, *nmol/L*								
8-HEPE[2]	0.016±0.165	*0.966*	0.343±0.164	*.0003*	0.005±0.179	*1.00*	-0.884±0.149	*<.0001*
11-HEPE	-0.006±0.015	*0.971*	0.063±0.014	*0.001*	-0.003±0.02	*0.999*	-0.072±0.018	*0.003*
12-HEPE[2]	0.025±0.127	*0.709*	0.238±0.128	*<.0001*	0.118±0.180	*0.912*	-0.766±0.176	*0.001*
15-HEPE[2]	0.00±0.092	*0.992*	0.148±0.091	*<.0001*	0.208±0.125	*0.359*	-0.727±0.124	*<.0001*
18-HEPE	0.039±0.032	*0.624*	0.395±0.032	*<.0001*	0.025±0.040	*0.921*	-0.330±0.041	*<.0001*
DHA oxylipins, *nmol/L*								
7-HDoHE	0.008±0.127	*1.00*	1.41±0.128	*<.0001*	0.161±0.14	*0.663*	-1.24±0.14	*<.0001*
10-HDoHE	0.011±0.037	*0.990*	0.490±0.037	*<.0001*	0.052±0.043	*0.624*	-0.426±0.043	*<.0001*
11-HDoHE	0.019±0.052	*0.983*	0.715±0.052	*<.0001*	0.076±0.061	*0.604*	-0.62±0.061	*<.0001*
13-HDoHE	0.031±0.109	*0.999*	0.785±0.110	*<.0001*	0.177±0.145	*0.617*	-1.09±0.145	*<.0001*
14-HDoHE	0.038±0.097	*0.930*	0.661±0.099	*<.0001*	0.323±0.152	*0.161*	-1.13±0.152	*<.0001*
16-HDoHE	0.030±0.039	*0.872*	0.484±0.039	*<.0001*	0.057±0.048	*0.636*	-0.397±0.048	*<.0001*
17-HDoHE	0.072±0.125	*0.939*	1.67±0.126	*<.0001*	0.238±0.159	*0.448*	-1.36±0.159	*<.0001*
RvD1	0.002±0.003	*0.953*	0.008±0.003	*0.053*	0.001±0.004	*0.995*	-0.006±0.004	*0.362*

	Flaxseed oil D28 vs. D0	*p*	Fish oil D28 vs. D0	*p*	D0 flax vs. fish	*p*	D28 flax vs. fish	*p*
DHA oxylipins cont								
16,17 DiHDoPE	-0.003±0.006	*0.960*	0.081±0.006	*<.0001*	0.008±0.007	*0.675*	-0.076±0.007	*<.0001*
19,20 EpDoPE	0.002±0.131	*0.990*	0.101±0.133	*<.0001*	0.334±0.188	*0.298*	-1.18±0.188	*<.0001*
19,20 DiHDoPE	-0.035±0.071	*0.962*	1.05±0.072	*<.0001*	0.141±0.115	*0.612*	-0.940±0.115	*<.0001*
20-HDoHE[4]	0.091±0.103	*0.816*	1.14±0.104	*<.0001*	0.238±0.164	*0.476*	-1.09±0.164	*<.0001*
Free FA, *nmol/L*								
ALA	169.6±193	*0.815*	-292±194	*0.445*	59.9±243	*0.995*	522±243	*0.155*
ARA	11.36±13.8	*0.844*	-13.6±14.0	*0.768*	-10.4±22.2	*0.966*	14.5±22.2	*0.914*
DHA	38.3±96.8	*0.979*	1500±98.2	*<.0001*	132±157	*0.833*	-1330±157	*<.0001*
EPA	12.4±0.086	*0.423*	214±0.087	*<.0001*	0.124±0.159	*0.864*	-0.941±0.159	*<.0001*

[1]Differences of least square means (estimate±SE) in nmol/L and adjusted *p* values (Tukey-Kramer correction); *n*=19-20 for all oxylipins except PGK2 (10-17), 8-HEPE (13-21), 11-HEPE (13-21) and 15-oxoETE (18-19). Data were analyzed using a mixed model with individuals as a random effect, and day and supplement as fixed effects; phase order was removed from the model.

[2] indicates log transformed for statistics;

[3] indicates residuals distribution may limit validity of the model;

[4] indicates phase order effect (refer to **Supplemental Table S1** for values separated by phase)

Significantly different comparisons ($p<0.05$) are shaded in gray

Abbreviations: ARA, arachidonic acid; ALA, α-linolenic acid; DGLA, dihomo-gamma-linolenic acid; DiHDoPE, dihydroxy-docosapentaenoic acid; DiHETrE, dihydroxy-eicosatrienoic acid; DiHODE, dihydroxy-octadecadienoic acid; DiHOME, dihydroxy-octadecenoic acid; EpDPE, epoxydocosapentaenoic acid, EpETrE, epoxy-eicosatrienoic acid; EpODE, epoxy-octadecadienoic acid; EpOME, epoxy-octadecenoic acid; GLA, gamma-linolenic acid; HDoHE, hydroxy-docosahexaenoic acid; HEPE, hydroxy-eicosapentaenoic acid; HETE, hydroxy-eicosatetraenoic acid; HETrE, hydroxy-eicosatrienoic acid; HODE, hydroxy-octadecadienoic acid; HOTrE, hydroxy-octadecatrienoic acid; LA, linoleic acid; oxoETE, oxo-eicosatetraenoic acid; oxoOTrE, oxo-octadecatrienoic acid; TriHOME, trihydroxy-octadecenoic acid

Supplemental Table 5. Effects of flaxseed oil and fish oil supplementation on plasma FA in women with obesity[1]

	Flaxseed oil D28 vs. D0	*p*	Fish oil D28 vs. D0	*p*	D0 flax vs. fish	*p*	D28 flax vs. fish	*p*
SFA, *µmol/L*								
12:0[2]	-0.851±2.36	*0.983*	-3.760±2.28	*0.531*	-1.98±2.35	*0.987*	0.923±2.31	*0.565*
14:0[2]	-15.3±15.3	*0.775*	-49.5±14.9	*0.008*	-5.24±15.12	*0.999*	28.9±15.2	*0.075*
16:0[2]	-25.3±190	*0.908*	-609±186	*0.019*	-222±189	*0.999*	361±189	*0.155*
17:0[2]	0.916±2.12	*0.997*	-3.18±2.07	*0.752*	-2.88±2.11	*0.996*	1.22±2.11	*0.946*
18:0[2]	4.44±46.4	*0.984*	-93.8±45.3	*0.386*	-46.3±46.1	*0.999*	51.9±46.1	*0.543*
20:0[2]	-0.566±1.70	*0.934*	0.393±1.66	*0.875*	-0.838±1.68	*0.981*	-1.80±1.68	*0.776*
22:0	-1.23±3.60	*0.986*	-3.41±3.52	*0.768*	-2.75±3.58	*0.868*	-0.573±3.58	*0.999*
24:0	0.052±2.77	*1.00*	-4.08±2.71	*0.440*	-2.47±2.75	*0.806*	1.66±2.75	*0.931*
MUFA, *µmol/L*								
14:1[2]	-1.09±1.39	*0.727*	-4.83±1.34	*.0004*	-0.382±1.36	*0.998*	3.36±1.38	*0.009*
16:1[2]	-44.9±28.0	*0.121*	-116±27.4	*0.001*	-5.07±27.9	*0.987*	65.6±27.9	*0.182*
16:1t[22]	-3.53±4.53	*0.453*	-12.7±4.42	*0.083*	-6.04±4.50	*0.991*	3.13±4.50	*0.934*
18:1[2]	-145±194	*0.354*	-723±190	*0.038*	-236±193	*0.992*	342±193	*0.874*
18:1n-7[2]	-10.3±15.4	*0.365*	-54.2±15.0	*0.020*	-13.9±15.3	*0.997*	30.1±15.3	*0.423*
20:1[2]	-1.07±1.83	*0.830*	-6.31±1.76	*.0003*	-1.82±1.79	*0.877*	3.42±1.81	*0.050*
22:1[2]	-0.325±1.08	*0.997*	-1.98±1.06	*0.015*	-1.76±1.08	*0.698*	-0.109±1.08	*0.267*
24:1[2]	-5.63±7.45	*0.275*	14.1±7.28	*0.396*	-3.52±7.41	*0.985*	-23.2±7.41	*0.018*
n-6 PUFA, *µmol/L*								
18:2[2]	-114±195	*0.360*	-594±190	*0.290*	-168±193	*1.00*	312±193	*1.00*
18:3n-6	-6.42±3.88	*0.357*	-23.8±3.79	*<.0001*	-1.32±3.85	*0.986*	16.1±3.85	*0.001*
20:2[2]	-1.26±1.89	*0.304*	-7.87±1.85	*0.018*	-1.53±1.88	*1.00*	5.09±1.88	*0.677*
20:3n-6	-31.8±17.5	*0.274*	-104±17.1	*<.0001*	-6.49±17.3	*0.982*	65.4±17.3	*0.002*
20:4[2]	-31.2±51.2	*0.250*	-188±50.0	*0.085*	-54.3±50.9	*0.997*	102±50.9	*0.992*
22:2[2]	0.224±0.348	*0.944*	-0.983±0.329	*0.004*	-0.627±0.335	*0.778*	0.579±0.346	*0.218*

22:4[2]	-1.92±1.62	*0.231*	-13.0±1.58	*<.0001*	-2.66±1.61	*0.818*	8.39±1.61	*<.0001*
22:5n-6[2]	-1.53±1.29	*0.199*	-4.60±1.26	*0.026*	-2.73±1.28	*0.473*	0.333±1.28	*0.927*
n-3 PUFA, *µmol/L*								
18:3n3	60.3±12.5	*<.0001*	-16.4±12.2	*0.537*	-10.2±12.4	*0.843*	66.5±12.4	*<.0001*
20:3n-3[2,4]	1.24±0.394	*0.018*	-0.538±0.378	*0.610*	0.042±0.385	*0.600*	1.82±0.391	*<.0001*
20:5[2]	11.1±24.1	*1.00*	295±23.5	*<.0001*	10.2±23.9	*0.573*	-274±23.9	*<.0001*
22:5n-3[2,3]	4.88±3.81	*0.965*	-8.16±3.72	*0.361*	-4.15±3.79	*0.988*	8.88±3.79	*0.846*
22:6n-3[2]	-13.9±31.9	*0.205*	397±31.2	*<.0001*	26.3±31.7	*0.160*	-385±31.7	*<.0001*
Totals, *µmol/L*								
SFA[2]	-36.6±252	0.919	-766±246	0.038	-285±251	1.000	444±250	0.214
MUFA[2,3]	-213±243	0.298	-905±237	0.033	-268±241	0.997	424±241	0.865
PUFA	-124±278	0.970	-268±272	0.757	-216±276	0.862	-72.3±276	0.994
n-3[2]	63.5±56.3	0.989	667±55.0	<.0001	22.2±55.9	0.682	-581±55.9	<.0001
n-6[2,3]	-188±256	0.300	-935±251	0.142	-238±255	1.000	509±255	0.989

[1]Differences of least square means (estimate±SE) and adjusted *p* values (Tukey-Kramer correction); *n*=19-21. Data were analyzed using a mixed model with individuals as a random effect, and day and supplement as fixed effects; phase order was removed from the model.

[2] indicates log transformed for statistics; [3] indicates residuals distribution may limit validity of the model;

[4] indicates phase order effect (refer to **Supplemental Table S1** for values separated by phase)
Significantly different comparisons ($p<0.05$) are shaded in gray

Supplementary Table 6. Effects of flaxseed oil and fish oil supplementation on anthropometric and clinical parameters in women with obesity[1]

	Flaxseed oil D28 vs. D0	*p*	Fish oil D28 vs. D0	*p*	D0 flax vs. fish	*p*	D28 flax vs. fish	*p*
Anthropometrics								
Body weight, *kg*	-0.471±0.955	*0.960*	0.409±0.933	*0.971*	0.460±6.84	*1.000*	-0.420±6.84	*1.000*
Waist circumference, *cm*	0.050±0.932	*1.000*	0.434±0.911	*0.964*	-0.143±3.974	*1.000*	-0.527±3.97	*0.999*
BMI, *kg/m²*	-0.197±0.343	*0.939*	0.146±0.335	*0.972*	0.079±1.955	*1.000*	-0.263±1.955	*0.999*
Vascular								
SBP, *mmHg*	2.05±2.19	*0.785*	-1.66±2.13	*0.864*	-1.93±4.74	*0.977*	1.77±4.74	*0.982*
DBP, *mmHg*	0.206±1.55	*0.999*	0.339±1.52	*0.996*	-0.547±3.13	*0.998*	-0.679±3.13	*0.996*
AIx, *%*	1.12±4.10	*0.993*	0.268±4.49	*1.000*	-3.92±4.20	*0.787*	-3.07±4.44	*0.899*
PWV, *m/s*	0.099±0.078	*0.589*	-0.014±0.085	*0.999*	-0.079±0.333	*0.995*	0.034±0.335	*1.000*
Blood biochemistry								
ALT. *U/L*[2]	0.597±2.64	*0.904*	3.37±0.902	*0.003*	-0.597±2.64	*1.000*	-4.289±2.64	*0.433*
AST, *U/L*	1.44±1.30	*0.685*	1.87±1.27	*0.463*	-1.59±1.96	*0.849*	-2.02±1.96	*0.735*
Creatinine, *µmol/L*[4]	0.671±1.16	*0.937*	0.173±1.128	*0.999*	-0.826±2.52	*0.988*	-0.327±2.52	*0.999*
CRP, *mg/L*[2,3]	-1.24±1.42	*0.692*	-0.468±1.39	*0.495*	0.847±2.762	*0.997*	0.071±2.762	*0.993*
Glucose, *mmol/L*	0.097±0.094	*0.729*	0.189±0.092	*0.183*	-0.024±0.212	*1.000*	-0.115±0.212	*0.947*
Cholesterol, *mmol/L*	-0.122±0.096	*0.590*	0.146±0.094	*0.418*	0.078±0.23	*0.986*	-0.189±0.23	*0.843*
HDL-cholesterp;, *mmol/L*	-0.035±0.021	*0.365*	0.151±0.021	*<.0001*	0.069±0.125	*0.946*	-0.117±0.125	*0.786*
LDL-cholesterol, *mmol/L*[2]	-0.132±0.090	*0.763*	0.069±0.088	*0.879*	0.083±0.239	*0.986*	-0.117±0.239	*0.994*
Trig, *mmol/L*	0.011±0.063	*0.998*	-0.326±0.062	*<.0001*	-0.156±0.116	*0.539*	0.181±0.116	*0.413*

[1]Differences of least square means (estimate±SE) and adjusted *p* values (Tukey-Kramer correction); *n*=20-22 for all parameters except AIx and PWV (*n*=12-16). Data were analyzed using a mixed model with individuals as a random effect, and day and supplement as fixed effects; phase order was removed from the model.

[2] indicates log transformed for statistics;

[3] indicates residuals distribution may limit validity of the model; [4] indicates phase order effect (refer to **Supplemental Table S1** for values separated by phase)

Significantly different comparisons ($p<0.05$) are shaded in gray

Abbreviations: AIx, augmentation index; BMI, body mass index; chol, cholesterol; circ, circumference; CRP, C-reactive protein; DBP, diastolic blood pressure; PWV, pulse wave velocity; SBP, systolic blood pressure.

Supplemental Table 7. Effects of flaxseed oil and fish oil supplementation on plasma inflammatory markers in women with obesity[1]

	Flaxseed oil D28 vs. D0	*p*	Fish oil D28 vs. D0	*p*	D0 flax vs. fish	*p*	D28 flax vs. fish	*p*
IFN-γ, *pg/mL*[3]	-0.269±0.416	*0.982*	-0.27±0.393	*0.867*	0.922±0.72	*0.935*	0.925±0.725	*0.856*
IL-10, *pg/mL*	-0.003±0.02	*0.985*	0.01±0.019	*0.908*	0.008±0.026	*1.000*	-0.003±0.026	*0.898*
IL-1β, *pg/mL*[2,4]	-0.002±0.012	*0.996*	-0.001±0.011	*0.975*	0.004±0.016	*0.898*	0.005±0.016	*0.941*
IL-6, *pg/mL*[3]	-0.254±0.21	*0.627*	-0.046±0.199	*0.996*	0.264±0.365	*0.888*	0.056±0.363	*0.999*
IL-8, *pg/mL*	0.545±0.341	*0.392*	-0.016±0.339	*1.000*	0.075±0.48	*0.999*	0.637±0.475	*0.544*
TNF-α, *pg/mL*[2]	0±0.074	*0.961*	0.015±0.071	*0.715*	0.155±0.181	*0.835*	0.14±0.181	*0.999*
VEGF, *pg/m*[2]	-3.18±4.116	*1.000*	-8.06±3.99	*0.244*	-0.697±7.157	*0.867*	4.18±7.204	*0.988*
IL-17A, *pg/mL*[2]	-0.078±0.042	*0.386*	0.049±0.042	*0.346*	0.109±0.065	*0.677*	-0.018±0.066	*0.887*
Resistin, *pg/mL*[2]	87.2±108	*0.759*	-102±105	*0.926*	-95.8±230	*0.958*	93.5±230	*0.989*
Adiponectin, *μg/mL*	5.15±7.95	*0.916*	25.8±8.14	*0.016*	7.33±24.9	*0.991*	-13.3±25.0	*0.951*

[1]Differences of least square means (estimate±SE) and adjusted *p* values (Tukey-Kramer correction); units are in pg/mL except adiponectin is in μg/mL; *n*=19-20 for all markers except VEGF (18-19) and IL-17A (16-19). Data were analyzed using a mixed model with individuals as a random effect, and day and supplement as fixed effects; phase order was removed from the model.

[2] indicates log transformed for statistics;

[3] indicates residuals distribution may limit validity of the model; [4] indicates phase order effect (refer to **Supplemental Table S1** for values separated by phase)

Significantly different comparisons ($p<0.05$) are shaded in gray

Abbreviations: VEGF, vascular endothelial growth factor

Supplemental Table 8. Effects of flaxseed oil and fish oil supplementation on monocyte bioenergetics parameters in women with obesity[1]

	Flaxseed oil D28 vs. D0	*p*	Fish oil D28 vs. D0	*p*	D0 flax vs. fish	*p*	D28 flax vs. fish	*p*
Unstimulated								
Basal Resp, *pmol/min*	-21.1±6.43	*0.018*	-1.68±6.61	*0.994*	14.56±7.62	*0.254*	-4.82±7.79	*0.925*
Proton Leak, *pmol/min*	-3.96±1.84	*0.171*	0.81±1.82	*0.970*	2.99±3.06	*0.763*	-1.78±3.10	*0.939*
Max Resp, *pmol/min*	-8.29±13.44	*0.926*	-22.7±14.2	*0.404*	4.56±13.67	*0.987*	18.92±14	*0.542*
SRC, *pmol/min*	12.3±10.54	*0.654*	-18.3±11.0	*0.369*	-9.56±11.38	*0.835*	21.02±11.66	*0.300*
Non Mito Ox, *pmol/min*	1.09±4.91	*0.996*	5.53±5.11	*0.703*	6.78±5.49	*0.612*	2.34±5.62	*0.975*
ATP-linked OCR, *pmol/min*	-18.2±5.59	*0.018*	-2.15±5.68	*0.981*	13.05±7.23	*0.299*	-3.03±7.38	*0.976*
Coupling Effic, *pmol/min*[2]	-0.004±0.048	*0.980*	0.045±0.048	*0.641*	0.057±0.07	*0.852*	0.009±0.068	*0.990*
SRC, %[2,4]	0.68±0.229	*0.016*	0.086±0.232	*1.000*	-0.31±0.297	*0.675*	0.285±0.303	*0.675*
OCR, *pmol/min*[2]	-19.9±8.33	*0.109*	3.27±8.66	*0.981*	21.58±9.26	*0.123*	-1.62±9.48	*0.998*
ECAR, *mpH/min*	6.37±4.12	*0.429*	-3.37±4.13	*0.846*	-2.22±5.86	*0.981*	7.53±5.96	*0.595*
OCR/ECAR Ratio[2]	-1.28±0.50	*0.084*	0.505±0.507	*0.754*	0.723±0.696	*0.729*	-1.06±0.71	*0.459*
Change by LPS								
∆Basal, *pmol/min*[3]	23.6±5.59	*0.002*	-13.0±5.99	*0.166*	-17.2±7.67	*0.148*	19.4±7.76	*0.092*
∆Proton Leak, *pmol/min*	5.11±2.15	*0.117*	-2.34±2.34	*0.752*	-3.69±2.23	*0.375*	3.76±2.26	*0.372*
∆Max Resp, *pmol/min*	31.39±9.94	*0.024*	-3.99±10.78	*0.982*	-20.68±10.78	*0.254*	14.7±10.93	*0.548*
∆SRC, *pmol/min*	7.47±10.38	*0.888*	5.50±11.3	*0.961*	-4.4±10.79	*0.976*	-2.43±10.94	*0.996*
∆Non Mito Ox, *pmol/min*	0.111±5.701	*1.000*	6.04±6.15	*0.761*	6.41±6.77	*0.781*	0.476±6.86	*1.000*
∆ATP-linked OCR, *pmol/min*	16.9±5.1	*0.017*	-11.3±5.44	*0.197*	-12.4±7.47	*0.370*	15.8±7.54	*0.190*
∆Coupling efficiency, *pmol/min*[2]	-0.084±0.065	*0.583*	-0.091±0.069	*0.564*	0.046±0.07	*0.912*	0.053±0.07	*0.870*
∆SRC, %[2]	-0.445±0.329	*0.543*	0.160±0.345	*0.966*	0.14±0.38	*0.981*	0.388±0.372	*0.607*
∆OCR, *pmol/min*[3]	23.7±6.16	*0.005*	-6.47±6.61	*0.763*	-10.8±8.02	*0.550*	19.4±8.12	*0.113*
∆ECAR, *mpH/min*	-5.61±4.46	*0.599*	8.25±4.81	*0.344*	9.60±5.18	*0.280*	-4.26±5.25	*0.848*

ΔOCR/ECAR Ratio	1.26±0.48	*0.071*	-1.16±0.509	*0.137*	-1.291±0.702	*0.286*	1.13±0.709	*0.407*

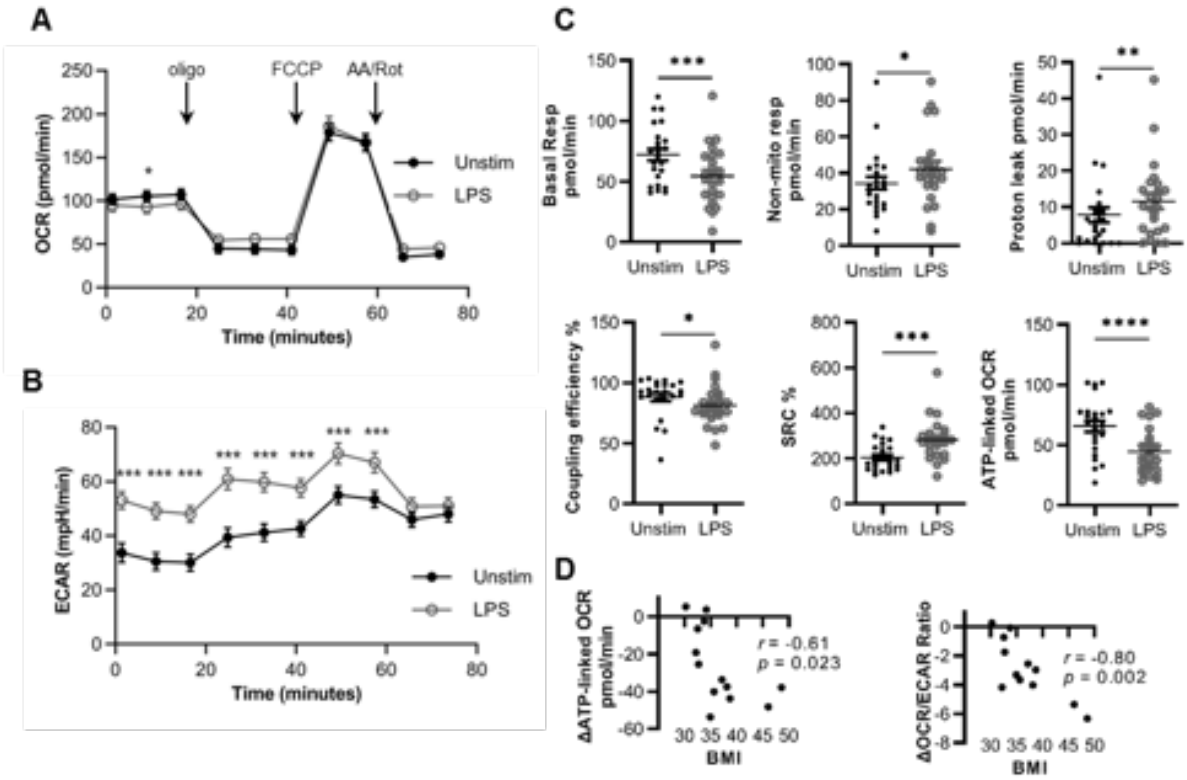
[1]Differences of least square means (estimate±SE) and adjusted *p* values (Tukey-Kramer correction); *n*= 12-15 (unstim), *n*=10-14 (Change by LPS). Data were analyzed using a mixed model with individuals as a random effect, and day and supplement as fixed effects; phase order was removed from the model.

[2] indicates log transformed for statistics;

[3] indicates phase order effect (refer to **Supplemental Table S1** for values separated by phase)

Significantly different comparisons ($p<0.05$) are shaded in gray

Abbreviations: ECAR, extracellular acidification rate; effic, efficiency; mito, mitochondrial; OCR, oxygen consumption rate; Ox, oxidation; Resp, respiration; SCR, spare respiratory capacity



Supplemental Figure 1. LPS triggers a Warburg shift in human monocytes. $CD14^{+}CD16^{-}$ monocytes were isolated, transferred to CellTak-coated XF24 plates, treated with complete cell culture medium alone (unstim) or with 10 ng/mL LPS for 2 h, and assessed for *ex vivo* bioenergetic parameters using the Seahorse XF mitochondrial stress test in the presence of glucose. (A) Oxygen consumption rate and (B) extracellular acidification rate were averaged from n=24 day 0 measurements. Differences between unstimulated and LPS-stimulated cells at each time point were assessed by repeated measures 2-way ANOVA followed by Sidak's test. (C) Bioenergetics parameters compared between unstimulated and LPS-stimulated monocytes. Paired t-test (or Wilcoxin test for non-parametric data) was used to assess differences. *, ** and *** indicate $p < 0.05$, 0.01 and 0.001, respectively. (D) Pearson's correlation between BMI and LPS-induced change (Δ) in the indicated parameter (value in LPS-stimulated cells *minus* value in unstimulated cells). Abbreviations: AA, antimycin A; ECAR, extracellular acidification rate; FCCP, carbonyl cyanide-4-(trifluoromethoxy)-phenylhydrazon; Non-mito resp, non-mitochondrial respiration; OCR, oxygen consumption rate; oligo, oligomycin; Resp, respiration; SRC, spare respiratory capacity.