Eicosapentaenoic Acid and Docosahexaenoic Acid Differentially Modulate Monocytes in Chronic Inflammation: A Randomized Clinical Trial

A dissertation presented to the faculty of the Gerald J. and Dorothy R. Friedman School of Nutrition Science and Policy

By

Jisun So, MS

A dissertation submitted in partial fulfillment of the requirements for the degree of **Doctor of Philosophy**

Tufts University
August 2020

Thesis Committee:

Stefania Lamon-Fava, M.D., Ph.D. (Chair)

Alice H. Lichtenstein, D.Sc.

Dayong Wu, M.D., Ph.D.

Albert K. Tai, Ph.D.

Acknowledgements

I would like to acknowledge the guidance and support of the many individuals who have helped me complete this dissertation. First and foremost, my advisor Dr. Stefania Lamon-Fava, for her wholehearted support, wisdom, patience, and hearty encouragement. She has shaped me as a scientist who has not only a critical thinking but also a positive outlook. The members of my thesis committee Dr. Alice H. Lichtenstein, Dr. Dayong Wu, and Dr Albert K. Tai were instrumental in helping me move forward and complete this dissertation. I would like to recognize Dr. Nirupa R. Matthan, Dr. Bela Asztalos, Katalin V. Horvath, Dr. Weimin Guo, and Lijun Li for their time and dedication to this project. I would like to thank Drs. Joan and Peter Cohn for the award that helped to expand and support this research. I would like to express thanks to all of the members of the Cardiovascular Nutrition Laboratory, especially my officemates Simon and Julie, and Boo Boo, Jelena, Maria, Esra, Mengxi, Ji Ye, Kenny, Jen and other BMN colleagues. Last but not least, special thanks to my family and Byungsu for their endless support.

To God Be the Glory

Abstract

Background and objective: The anti-inflammatory effects of the very-long-chain n-3 polyunsaturated fatty acids (n-3 LC-PUFA) eicosapentaenoic acid (EPA, 20:5 n-3) and docosapentaenoic acid (DHA, 22:6 n-3) have been extensively studied *in vitro*, especially in monocytes and macrophages, the key cells involved in the pathogenesis of atherosclerosis. However, the results of clinical studies examining combinations of these two fatty acids in varying doses and ratios have been mixed. The objective of this thesis was to characterize the individual effects of EPA and DHA in modulating systemic inflammation and monocyte inflammatory profile, and identify the mechanisms involved, in individuals with chronic inflammation.

Methods: In a randomized, double-blind, crossover study, individuals with chronic inflammation received 3 g/day purified EPA and DHA for 10 weeks, with a 10-week washout phase in between. Systemic inflammation and monocyte inflammatory response were determined by assessing the pro-inflammatory cytokines tumor necrosis factor (TNF)-a, interleukin (IL)-6, and monocyte chemoattractant protein 1 (MCP-1) and the anti-inflammatory cytokine IL-10 in serum and lipo-polysaccharide (LPS)-stimulated monocytes, respectively, at baseline and the end of each supplementation. The effects of EPA and DHA on the plasma PUFA-derived lipidome, including specialized pro-resolving lipid mediators (SPM), and on the transcriptome of unstimulated blood monocytes were examined using liquid chromatography-mass spectrometry and RNA sequencing, respectively.

Results: Relative to baseline, serum concentrations of inflammatory markers were not significantly affected by EPA or DHA. However, EPA and DHA significantly, but differentially, modulated the monocyte inflammatory response: relative to baseline, DHA reduced the LPS-stimulated expression of all pro-inflammatory and anti-inflammatory cytokines, while EPA improved the balances between pro- and anti-inflammatory cytokines. Supplementation with EPA and DHA significantly enhanced the lipidome derived from the respective fatty acids, with DHA showing a more pronounced effect by increasing not only DHA-derived lipid mediators but also lipid mediators derived from EPA and DPA and lowering those from arachidonic acid (20:4 n-6). An exploratory mediation analysis showed that changes in distinct PUFA lipidome signatures following EPA and DHA supplementation mediated, at least in part, the effect of each supplementation on the LPS-stimulated monocyte cytokine expression. Monocyte transcriptomic analysis revealed a clear sex dimorphism at baseline, and therefore, the effect of EPA and DHA on monocyte gene expression was examined separately in men and women. Pathway analysis of the genes differentially expressed by EPA and DHA, relative to baseline, indicated that EPA but not DHA modulated gene expression differently in men versus women. EPA had a unique effect in men by upregulating pathways involved in cell proliferation and differentiation and specifically macrophage phenotype with anti-inflammatory/pro-resolving properties. However, in women, EPA led to the downregulation of mitochondrial oxidative phosphorylation (OXPHOS), suggesting a reduced metabolic activation. The OXPHOS downregulation was observed after DHA supplementation as well in both men and women. DHA also modulated the biological pathways related to cellular reactive oxygen species (ROS) control in both men and women, by upregulating antioxidant pathways and downregulating protein translation.

Conclusions: Taken together, these findings indicate that EPA and DHA have distinct immunomodulatory effects on monocytes. EPA is effective in balancing the pro- and anti-inflammatory responses of monocytes, with sex-dependent modulations of genes involved in cell differentiation and phenotype in men and energy metabolism in women. DHA has a more pronounced effect in attenuating the pro-inflammatory status of monocytes, by reducing the activated metabolic state and enhancing protection against ROS. Results from this thesis provide strong evidence of fatty acid- and sex-specific effects of n-3 LC-PUFA.

Abbreviations

AA: arachidonic acid
AHR: aryl hydrocarbon receptor
Akt (PKB): protein kinase B
AMPK: 5' adenosine monophosphate-activated protein kinase
AP-1: activator protein 1
APC: antigen-presenting cells
ATP: adenosine triphosphate
BMDM: bone marrow-derived macrophages
cAMP: cyclic adenosine monophosphate
COX: cyclooxygenase
CVD: cardiovascular disease
DHA: docosahexaenoic acid
DPA: docosapentaenoic acid
ECL: electrochemiluminescence
eIF2: eukaryotic initiation factor 2
EPA: eicosapentaenoic acid
ER: endoplasmic reticulum
ETC: electron transfer chain
$FADH_2$: flavin adenine dinucleotide
FDR: false discovery rate
fMLP: N-formylmethionine-leucyl-phenylalanine
GAPDH: glyceraldehyde 3-phosphate dehydrogenase
GPR: G protein-coupled receptor
HDHA: hydroxy-docosahexaenoic acid
HEPE: hydroxy-eicosapentaenoic acid
HETE: hydroxy-eicosatetraenoic acid
HHTrE: hydroxy-heptadecatrienoic acid
Hs-CRP: high sensitivity C-reactive protein
ICAM: intercellular adhesion molecule
IFN: interferon
IL: interleukin
ImmGen: the Immunological Genome project
IPA: Ingenuity Pathway Analysis
IRF: interferon regulatory factors
ISG: interferon signature genes
JELIS: the Japan EPA Lipid Intervention Study
LC-MS: liquid chromatography-mass spectrometry
LC-PUFA: very-long-chain polyunsaturated fatty acids
LDL-C: low-density lipoprotein cholesterol
LOX: lipoxygenase
LPS: lipopolysaccharide
LT: leukotriene
LX: lipoxin

LXR/RXR: liver X receptor/retinoid X receptor
MaR: maresin
MCP-1: monocyte chemoattractant protein 1
MF-ISG: macrophage-specific interferon signature genes
MHC: major histocompatibility complex
mol %: molar percentage
MyD88: myeloid differentiation primary response gene 88
NADH: nicotinamide adenine dinucleotide
NF-κB: nuclear factor-kappa B
NFE2L2 (NRF2): nuclear factor erythroid 2-related factor 2
NSAID: non-steroidal anti-inflammatory drugs
OXPHOS: oxidative phosphorylation
PBMC: peripheral blood mononuclear cells
PC(A): principal component (analysis)
PD-1: programmed cell death protein 1
PD-L1: programmed cell death-ligand 1
PD: protectin
PG: prostaglandin
PKA: protein kinase A
PLA_2: phospholipase A2
PPAR: peroxisome proliferator activated receptor
PUFA: polyunsaturated fatty acids
RCT: randomized controlled trials
REDUCE-IT: the Reduction of Cardiovascular Events with Icosapent Ethyl-Intervention Trial
ROS: reactive oxygen species
RvD/E: D/E-series resolvin
SLE: systemic lupus erythematosus
SPM: specialized pro-resolving lipid mediator
TAX1: mitogen-activated protein kinase kinase kinase 7
TG: triglycerides
Th1: T helper type 1
TLR: toll-like receptor
TNF: tumor necrosis factor
TRAF: tumor necrosis factor receptor-associated factor
TX: thromboxane
VCAM: vascular adhesion molecule

Table of Contents

List of Tables

List of Figures

Chapter 1 Introduction

1.1 Introduction

Chronic low-grade inflammation plays a key role in some of the most challenging diseases in our time, including diabetes, cardiovascular disease (CVD), rheumatoid arthritis, and neurodegenerative disorders (1–3). Inflammation is a defense mechanism, part of the innate immune response to maintain homeostasis by attacking exogenous pathogens (4). However, unchecked inflammation that persists even after the initial threat has been eliminated, activating immune cells in excess to attack normal tissues and organs, leading to pathological conditions (5). For example, chronic inflammation is one of the major mechanisms leading to atherosclerosis by activating monocyte/macrophages to infiltrate the arterial intima (6,7) and promoting the accumulation of cholesterol (8). Pro-inflammatory mediators that are secreted by these activated monocyte/macrophages and other immune cells further promote the development of atherogenic lesions (6).

The very-long-chain n-3 polyunsaturated fatty acids (n-3 LC-PUFA), especially eicosapentaenoic acid (EPA, 20:5 n-3) and docosahexaenoic acid (DHA, 22:6 n-3), have long been accepted to be protective against CVD primarily through their anti-inflammatory effects (9–11). Based on initial epidemiological evidence for the role of n-3 LC-PUFA in CVD prevention (12), remarkable progress has been made to elucidate the underlying mechanisms using *in vitro* and animal models. N-3 LC-PUFA are known to act as signaling molecules *per se* by activating several membrane-associated and/or intracellular receptors, modulating the expression of target genes involved in inflammation (13). In addition, n-3 LC-PUFA counterbalance the pro-inflammatory lipid mediators that are derived from n-6 arachidonic acid (AA, 20:4 n-6), the major constituent of membrane phospholipids, by replacing AA in the membrane and then

producing less inflammatory lipid mediators via enzymatic conversions (13). More recently, the concept of active process of inflammation resolution has risen, where n-3 LC-PUFA act as precursors of a novel family of lipid mediators called specialized pro-resolving mediators (SPM) that promote resolution (14).

However, in contrast to early large-scale randomized controlled trials (RCT) of n-3 LC-PUFA supplementation (9,15) that reported promising clinical benefits, later RCT and meta-analyses have shown conflicting results (16–19). Considerable heterogeneity among the trials concerning methodologies (e.g., composition, dose, and duration) may have confounded the true effect of n-3 LC-PUFA on inflammation and CVD risk. Particularly, fish oil supplements were usually prepared in varying ratios of EPA and DHA based on the assumption that EPA and DHA have similar biological effects. It has now been recognized that benefits from lower doses of 1-2 g/day of n-3 LC-PUFA are very limited (20) and higher doses may be needed to achieve CVD risk reduction (18,21). However, we still lack a comprehensive understanding of the common and differential effects of EPA and DHA on inflammation in humans that may contribute to the prevention of CVD.

In order to study the effects of n-3 LC-PUFA on inflammation and CVD risk factors, an appropriate research model is required in humans. We addressed this gap using a crossover design with pure EPA and DHA at the dose of 3 g/day each. Compared to a parallel-arm design, a crossover design can minimize substantial interindividual variations due to genetic and environmental differences among participants. We previously completed a randomized, placebo-controlled, parallel-arm study investigating individual effects of 1.8 g/day EPA and DHA supplementation on peripheral blood

mononuclear cells (PBMC) in healthy normolipidemic individuals, and found that each supplementation affected different signaling pathways (22). Based on these results, we used a crossover design to examine and compare the effects of EPA and DHA at a higher dose (i.e., 3 g/day), relative to high-oleic sunflower oil, on systemic inflammation and inflammatory responses of peripheral blood monocytes in individuals with chronic inflammation. We characterized the effects of EPA and DHA supplementation on the plasma profile of n-3 and n-6 PUFA-derived lipid mediators and gene expression of peripheral blood monocytes. Collectively, this work provides novel insights into the differential mechanisms of actions of EPA and DHA on inflammation in individuals with chronic inflammation.

1.1.2 References

1. Libby P, Ridker PM, Hansson GK. Inflammation in Atherosclerosis. From Pathophysiology to Practice. J Am Coll Cardiol. 2009;54:2129–38.
2. Demoruelle MK, Deane KD, Holers VM. When and where does inflammation begin in rheumatoid arthritis? Curr Opin Rheumatol. 2014;26:64–71.
3. Amor S, Peferoen LAN, Vogel DYS, Breur M, van der Valk P, Baker D, Van Noort JM. Inflammation in neurodegenerative diseases - an update. Immunology. 2014;142:151–66.
4. Kotas ME, Medzhitov R. Homeostasis, Inflammation, and Disease Susceptibility. Cell. 2015;160:816–27.
5. Cotran RS, Kumar V, Collins T, Robbins SL. Robbins Pathologic Basis of Disease. 6th ed. Saunders. Philadelphia, PA; 1999.
6. Hansson GK, Libby P, Schönbeck U, Yan ZQ. Innate and adaptive immunity in the pathogenesis of atherosclerosis. Circ Res. 2002;91:281–91.
7. Tabas I, García-Cardeña G, Owens GK. Recent insights into the cellular biology of atherosclerosis. J Cell Biol. 2015;209:13–22.
8. Pirillo A, Norata GD, Catapano AL. LOX-1, OxLDL, and Atherosclerosis. Mediators Inflamm. 2013;2013:12.
9. Burr ML, Gilbert JF, Holliday RM, Elwood PC, Fehily AM, Rogers S, Sweetnam PM, Deadman NM. Effects of changes in fat, fish, and fibre intakes on death and myocardial reinfarction: Diet and Reinfarction Trial (DART). Lancet. 1989;334:757–61.
10. Simopoulos AP. Omega-3 fatty acids in inflammation and autoimmune diseases. J Am Coll Nutr. 2002;21:495–505.
11. Mori TA, Beilin LJ. Omega-3 fatty acids and inflammation. Curr Atheroscler Rep. 2004;6:461–7.
12. Bang HO, Dyerberg J, Nielsen AB o. ndu. Plasma lipid and lipoprotein pattern in Greenlandic West-coast Eskimos. Lancet. 1971;297:1143–6.
13. Calder PC. Marine omega-3 fatty acids and inflammatory processes: Effects, mechanisms and clinical relevance. Biochim Biophys Acta - Mol Cell Biol Lipids. 2015;1851:469–84.
14. Serhan CN. Pro-resolving lipid mediators are leads for resolution physiology. Nature. 2014;510:92–101.
15. Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto miocardico. Dietary supplementation with n-3 polyunsaturated fatty acids and vitamin E after myocardial infarction: results of the GISSI-Prevenzione trial. Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto miocardico. Lancet. 1999;354:447–55.
16. Bowman L, Mafham M, Wallendszus K, Stevens W, Buck G, Barton J, Murphy K, Aung T, Haynes R, Cox J, et al. Effects of n-3 fatty acid supplements in diabetes

mellitus. N Engl J Med. 2018;379:1540–50.

17. Manson JE, Cook NR, Lee I-M, Christen W, Bassuk SS, Mora S, Gibson H, Albert CM, Gordon D, Copeland T, et al. Marine n−3 Fatty Acids and Prevention of Cardiovascular Disease and Cancer. N Engl J Med. 2019;380:23–32.

18. Bhatt DL, Steg PG, Miller M, Brinton EA, Jacobson TA, Ketchum SB, Doyle RT, Juliano RA, Jiao L, Granowitz C, et al. Cardiovascular risk reduction with icosapent ethyl for hypertriglyceridemia. N Engl J Med. 2019;380:11–22.

19. Aung T, Halsey J, Kromhout D, Gerstein HC, Marchioli R, Tavazzi L, Geleijnse JM, Rauch B, Ness A, Galan P, et al. Associations of omega-3 fatty acid supplement use with cardiovascular disease risks meta-analysis of 10 trials involving 77 917 individuals. JAMA Cardiol. American Medical Association; 2018;3:225–34.

20. Siscovick DS, Barringer TA, Fretts AM, Wu JHY, Lichtenstein AH, Costello RB, Kris-Etherton PM, Jacobson TA, Engler MB, Alger HM, et al. Omega-3 Polyunsaturated Fatty Acid (Fish Oil) Supplementation and the Prevention of Clinical Cardiovascular Disease: A Science Advisory from the American Heart Association. Circulation. 2017;135:e867–84.

21. Yokoyama M, Origasa H, Matsuzaki M, Matsuzawa Y, Saito Y, Ishikawa Y, Oikawa S, Sasaki J, Hishida H, Itakura H, et al. Effects of eicosapentaenoic acid on major coronary events in hypercholesterolaemic patients (JELIS): a randomised open-label, blinded endpoint analysis. Lancet. 2007;369:1090–8.

22. Tsunoda F, Lamon-Fava S, Asztalos BF, Iyer LK, Richardson K, Schaefer EJ. Effects of oral eicosapentaenoic acid versus docosahexaenoic acid on human peripheral blood mononuclear cell gene expression. Atherosclerosis. 2015;241:400–8.

1.2 Statement of Hypothesis

The overall objective of this thesis was to determine, in individuals with chronic inflammation: a) the effects of EPA and DHA on markers of systemic inflammation and lipopolysaccharide (LPS)-induced inflammatory responses of peripheral blood monocytes; b) the effects of EPA and DHA on the profile of PUFA-derived lipid mediators, including SPM, and its association with inflammation; and c) the effects of EPA and DHA on gene expression in peripheral blood monocytes.

The objective of this study was pursued using blood samples obtained from a parent study designed to assess the effects of EPA and DHA on systemic inflammation and lipoprotein metabolism. Men and postmenopausal women aged 50-75 years with chronically elevated inflammation were recruited based on the following inclusion criteria: i) plasma high-sensitivity C-reactive protein (hs-CRP) ≥ 2 μg/mL, ii) fasting plasma triglyceride (TG) between 90 and 500 mg/dL, and iii) at least of one of the following: waist circumference > 40 inches in men and > 34 inches in women, blood pressure > 135/80 mmHg or anti-hypertensive drug use, and fasting plasma glucose ≥ 100 mg/dL. Individuals using anti-inflammatory medications were excluded from the study. After completing a four-week lead-in phase with high oleic acid sunflower oil, participants were randomized to a 2x2 crossover design with two 10-week supplementation phases using 3 g/day pure EPA and DHA (ethyl ester form, purity > 97%) and a 10-week washout phase using high oleic acid sunflower oil. Blood samples were collected at the end of the lead-in phase (considered as baseline hereafter) and the two supplementation phases.

Specific Aim 1: Identify the effects of EPA and DHA supplementation on systemic inflammation and inflammatory response of peripheral blood monocytes.

Hypothesis 1: EPA and DHA will differentially modulate systemic inflammation-related markers and LPS-induced inflammatory response of peripheral blood monocytes.

1a. Determine serum concentrations of markers of inflammation (hs-CRP, tumor necrosis factor [TNF]-α, interleukin [IL]-6, monocyte chemoattractant protein [MCP-1], and IL-10).

1b. Determine LPS-stimulated gene and protein expression of cytokines (TNF-α, IL-6, MCP-1, and IL-10) in peripheral blood monocytes.

Specific Aim 2: Identify the effects of EPA and DHA on plasma profiles of n-3 PUFA downstream lipid mediators, including SPM, and the associations between PUFA lipidome signatures and inflammation.

Hypothesis 2: EPA and DHA will differently change plasma profiles of n-3 PUFA downstream lipid mediators, including SPM, and the resulting lipid mediator signatures will be associated with their differential effects on inflammation.

2a. Characterize plasma concentrations of EPA-, DHA-, and AA-derived lipid mediators, including their respective downstream SPM, using targeted lipidomic analysis.

2b. Identify plasma PUFA lipidome signatures and determine their associations with the measures of inflammation.

Specific Aim 3: Identify the effects of EPA and DHA on gene expression and the biological processes in peripheral blood monocytes.

Hypothesis 3: EPA and DHA will have differential immunomodulatory effects on peripheral blood monocytes by affecting unique gene expression signatures that are associated with immune and inflammatory signaling pathways.

3a. Characterize monocyte gene expression attributable to EPA and DHA supplementation using transcriptome analysis.

1.3 Literature Review

1.3.1 Inflammation

Inflammation, a component of the normal defense mechanisms, provides protection from infection and other tissue insults. In the process of pathogen killing and tissue repair for the restoration of homeostasis, inflammation involves multiple cell types and chemical mediators (1). The inflammatory response consists of increased blood supply to the infected or damaged site, increased vascular permeability, influx of leukocytes through the vascular wall, and release of inflammatory chemical mediators by the migrated leukocytes (1,2). The chemical mediators include lipid-derived prostaglandins (PG), thromboxanes (TX) and leukotrienes (LT), peptide-based chemokines and cytokines, reactive oxygen species (e.g. superoxide), and biogenic amines (e.g. histamine). Since the inflammatory cells and mediators can be damaging not only to pathogens but to host tissues, inflammation is normally a well-regulated and self-limiting process with negative feedback mechanisms: the inhibition of pro-inflammatory signaling cascades, the removal of inflammatory proteins, the production of anti-inflammatory mediators, and the activation of regulatory immune cells (2,3). Loss of these regulatory responses can result in inappropriate and excessive inflammation which contributes to a range of diseases (4–6).

In contrast to acute inflammation, which is often initiated by exogenous factors and usually resolves within hours or days, chronic inflammation can be induced by endogenous materials from tissue damage and persist over long periods of time (7). Excessive inflammatory mediators produced by the inflammatory cells are released into the bloodstream, affecting blood vessels and other organs. Chronic inflammation leads to

prolonged destructive processes, contributing to the pathology of many conditions, such as rheumatoid arthritis (5), inflammatory bowel disease (8), and atherosclerosis (4). These conditions are characterized by extensive infiltration of inflammatory cells at the site of affected tissue and elevated concentrations of inflammatory mediators both at this site and released into the systemic circulation (9) .

1.3.2 N-6 and N-3 Polyunsaturated Fatty Acids

N-6 and n-3 PUFA are two major PUFA families. Linoleic acid (18:2 n-6) and α-linolenic acid (18:3 n-3), found in seeds, nuts, and seed oils, are synthesized in plants but not in animals, and therefore, must be obtained from diet (10,11). Once ingested, both may be converted into their respective downstream n-6 and n-3 PUFA via a series of desaturation and elongation reactions (12–14) (Figure 1). These reactions occur in a competitive manner due to competition between n-6 and n-3 PUFA, for example, α-linolenic acid to EPA and linoleic acid to AA (14). In addition, the conversion of EPA to DHA via docosapentaenoic acid (DPA; 22:5 n-3) involves additional steps of translocation from the endoplasmic reticulum to peroxisomes where limited β-oxidation occurs (15). Studies have demonstrated that the conversion of α-linolenic acid to EPA, DPA, and DHA is generally low in humans (12). These very-long-chain n-3 PUFA, which are collectively referred to as marine n-3 PUFA (hereafter, n-3 LC-PUFA), are found in fish and seafood products, especially in fatty fish like salmon, tuna, and mackerel (11,16). A single fatty fish meal (e.g. 3.5 ounces of salmon) provides approximately 1.0 to 2.0 g of these FA (16). However, in most adults eating Western diets, intake of EPA and DHA is typically low (< 0.2 g/day) (16).

1.3.3 Long-chain N-3 Polyunsaturated Fatty Acids and Inflammation

The early observations of a lower CVD rate in Greenland Inuits (17) and Japanese (18) have generated several studies to explore the cardioprotective effects of n-3 LC-PUFA and the mechanisms involved. With increasing recognition of inflammation as a key player in the pathology of cardiometabolic diseases, the anti-inflammatory actions of n-3 LC-PUFA have been widely studied and reported (2). More recent studies are further suggesting that they may also be involved in the active processes during the resolution of inflammation (19).

Long-chain N-3 Polyunsaturated Fatty Acids and Eicosanoid Production

N-6 and n-3 PUFA are critical constituents of membrane phospholipids, affecting cell membrane properties such as fluidity, that can alter the activity of membrane-embedded enzymes and receptors (20,21). Membrane phospholipids preferentially incorporate PUFA into the sn-2 position, and AA is the most prevalent type PUFA due to its high content in Western diets. For example, the mean relative contents of AA, EPA, and DHA were about 20, 0.5, and 2.5% of total fatty acids in PBMC from healthy adults consuming a Western diet (22).

The metabolism of AA, which is released from the membrane phospholipids by phospholipase A2 (PLA_2), is important regulator of inflammation. It serves as a substrate for the enzymes cyclooxygenase (COX), lipoxygenase (LOX) and cytochrome P450. The products are eicosanoid mediators with pro-inflammatory properties (Figure 2). Eicosanoids include PG/TX and LT and act as regulators of various immune and inflammatory responses (23–25). Upon inflammatory stimuli, the production of eicosanoids is elevated due to activation of the enzymes and the upregulated expression

of the genes encoding the enzymes. Many anti-inflammatory drugs such as COX inhibitors and non-steroidal anti-inflammatory drugs (NSAID) target the metabolism of AA-derived eicosanoids.

Increased intake of EPA and DHA from food sources and/or supplements increases their contents in the cell membrane phospholipids in a time- and dose-dependent manner, at the expense of n-6 PUFA, primarily AA (26,27). This results in not only decreases the substrate availability of AA for PLA_2, but also inhibits the expression of COX-1/2 genes, thus leading to decreased production of AA-derived pro-inflammatory eicosanoids (28–30). In addition, EPA itself acts as a substrate for the eicosanoid-producing enzymes, resulting in the production of less potent pro-inflammatory eicosanoids (29). For example, the EPA-derived LTB_5 exhibits 10- to 100-fold less chemotactic potency for human neutrophils than AA-derived LTB_4 (31). In summary, increased n-3 LC-PUFA intake, particularly EPA, results in decreased production of potent AA-derived eicosanoids and increased production of less potent eicosanoids.

Long-chain N-3 Polyunsaturated Fatty Acids and Inflammatory Signaling

In addition to modulating the profiles of eicosanoids involved in inflammatory processes, n-3 LC-PUFA also act as signaling molecules *per se* and affect the production of inflammatory proteins such as cytokines and adhesion molecules (31). N-3 LC-PUFA or fish oil supplementation have been shown to decrease the production of pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6 in endotoxin-stimulated human endothelial cells and macrophages *in vitro* (32–34) and in macrophages from mice fed fish oil (35,36). Some but not all studies in human have reported decreased expression and secretion of these cytokines in LPS-stimulated PBMC or monocytes following n-3

LC-PUFA supplementation only at doses greater than 1-2 g/day (37,38). N-3 LC-PUFA have also been shown to reduce the expression of adhesion molecules such as intercellular adhesion molecule (ICAM)-1 on endothelial cells or leukocytes in culture (39,40) and plasma concentrations of soluble vascular cell adhesion molecule (VCAM)-1 (41). This effect could result in inhibition of leukocyte migration from blood into the site of inflammation.

N-3 LC-PUFA affect plasma concentrations of cytokines and other inflammation-related proteins through regulation of the expression of genes encoding these proteins. N-3 LC-PUFA function as ligands for some membrane-associated and nuclear receptors, thereby activating transcription factors involved in immune and inflammatory responses. Much work has focused on nuclear factor kappa B (NF-κB) inhibition (34–36), a transcription factor engaged in pro-inflammatory signaling pathways. Upon binding of LPS to extracellular Toll-like receptor 4 (TLR4), phosphorylation results in dissociation of an inhibitory subunit called inhibitory subunit of NF-κB (IκB) and translocation of the remaining NF-kB dimer to the nucleus (42). N-3 LC-PUFA may interfere with the TLR4-mediated NF-κB signaling cascade, possibly by disrupting the assembly of signaling proteins such as TLR4 and myeloid differentiation primary response gene 88 (MyD88) into lipid rafts within the membrane of inflammatory cells exposed to LPS (43). Other mechanisms by which n-3 LC-PUFA may influence NF-κB signaling involve peroxisome proliferator-activated receptor (PPAR)-γ (44) and G protein-coupled receptor (GPR) 120 (45), both of which function as n-3 LC-PUFA sensors and mediate anti-inflammatory properties. PPAR-γ, a transcription factor with an ability to inhibit the expression of inflammatory cytokines (e.g. TNF-α and IL-6) and to direct immune cell differentiation

towards anti-inflammatory phenotypes, suppresses NF-κB activation via physical interference with the translocation of the NF-κB dimer into the nucleus (46,47). Both EPA and DHA have been reported to activate PPAR-γ, but a greater impact of EPA than DHA was shown in human THP-1 cells (48). Upon binding of ligands including n-3 LC-PUFA, GPR120 has been reported to repress macrophage-mediated tissue inflammation by inhibiting mitogen-activated protein kinase kinase kinase 7 (TAK1) activation and downstream pro-inflammatory signaling to NF-κB and activator protein 1 (AP-1) (45). Of note, the inhibitory actions of n-3 LC-PUFA on these inflammatory signaling cascades may vary by the type of fatty acids.

Long-chain N-3 Polyunsaturated Fatty Acids and Resolution of Inflammation

A new chapter in the field of PUFA and inflammation has opened since the discovery of specialized pro-resolving lipid mediators (SPM), novel lipid mediators mostly produced from EPA and DHA (19). The study of these mediators with pro-resolving capacity has provided evidence that the resolution of inflammation is an active and programmed process, and not a passive process of diluting pro-inflammatory mediators (Figure 3). SPM include not only resolvins derived from EPA (E-series resolvins, RvE) and DHA (D-series resolvins, RvD), protectins (PD) and maresins (MaR) produced from DHA but also lipoxins (LX) produced from AA by the eicosanoid-producing COX and LOX pathways (Figure 2). In addition to their anti-inflammatory properties of competing with AA and thus reducing pro-inflammatory eicosanoids, n-3 LC-PUFA may also serve as substrates for the production of SPM to resolve inflammatory. The synthesis of SPM including resolvins has been shown to increase with increased intake of n-3 LC-PUFA in both animals models of inflammation (49) and humans (50–53). The bioactions of SPM

and some other intermediate precursors have been demonstrated in *in vitro* and in animal models of inflammation. For example, RvE1, RvD1, and PD1 have been shown to inhibit neutrophil recruitment/infiltration into the site of inflammation; counterregulate pro-inflammatory cytokines; and promote macrophage phagocytosis of debris and apoptotic cells (19). DHA-derived RvD1 (54) and MaR1 (55) have been shown to stimulate conversion of macrophage phenotype from "pathogen-killing/pro-inflammatory" M1 (or classically activated) to "regenerating/anti-inflammatory" M2 (or alternatively activated). More recently discovered DPA-derived SPM $RvD5_{n\text{-}3\ DPA}$ and $PD1_{n\text{-}3\ DPA}$ (56), the analogs of DHA-derived SPM, were found to reduce intestinal inflammation (57). Moreover, there is evidence that not only SPM but their intermediate precursors have their own unique bioactivities. 18-hydroxy-EPA (HEPE), the RvE precursor, was shown to have cardioprotective and anti-inflammatory actions by inhibiting macrophage TNF-α secretion (58) and maladaptive cardiac remodeling (59). 17-hydroxy-DHA (HDHA), the precursor of RvD1 and PD1, has been shown to suppress pro-inflammatory cytokine production from human lymphocytes *in vitro* (60) and to be associated with reduced pain in osteoarthritis patients (61). The bioactivities of SPM are mediated via specific G-protein coupled receptors like ChemR23 for RvE1 (62) and GPR32 for RvD1 (63).

1.3.4 Research Gaps and Significance of this Study

Despite the knowledge that has been accumulated regarding the potential actions of n-3 LC-PUFA on inflammation, we still lack an understanding about the differential effects of EPA and DHA and the mechanisms associated. This may have contributed to the controversy over the benefits of n-3 LC-PUFA on inflammation and cardiovascular

health. The significant reduction in CVD risk observed in two recent large clinical trials that administered highly purified EPA, i.e. the Japan EPA Lipid Intervention Study (JELIS) (64) and the Reduction of Cardiovascular Events with Icosapent Ethyl-Intervention Trial (REDUCE-IT) (65), suggests the importance of characterizing the differential effects of EPA and DHA on inflammation and cardioprotection. Evidence from *in vitro* studies using macrophages and endothelial cells suggests that DHA is more potent than EPA in downregulating the expression of pro-inflammatory cytokines TNF-α and IL-1β and/or VCAM-1 (66,67) and that EPA is more effective than DHA in modulating the balance between pro- and anti-inflammatory cytokines (68). In humans, recent studies have examined the independent effects of EPA and DHA supplementation on plasma inflammatory markers (69) and inflammatory gene expression in whole blood (70) and PBMC (71), however, their effects on plasma lipidome of PUFA derivatives including SPM have not been systematically compared. Moreover, a potential composite role of SPM and their intermediates on inflammation has not been studied in humans.

Successful completion of this research will provide critical information on the independent effects of EPA and DHA on inflammatory mechanisms of n-3 LC-PUFA actions in humans. Furthermore, given the complexity of the n-3 LC-PUFA-mediated actions on inflammatory signaling pathways through gene regulation and SPM production, it will allow for the characterization of transcriptome and lipidome profiles. Our findings add to the literature on the bioactive role of plasma PUFA SPM lipidome in inflammation in humans.

1.3.5 Figures

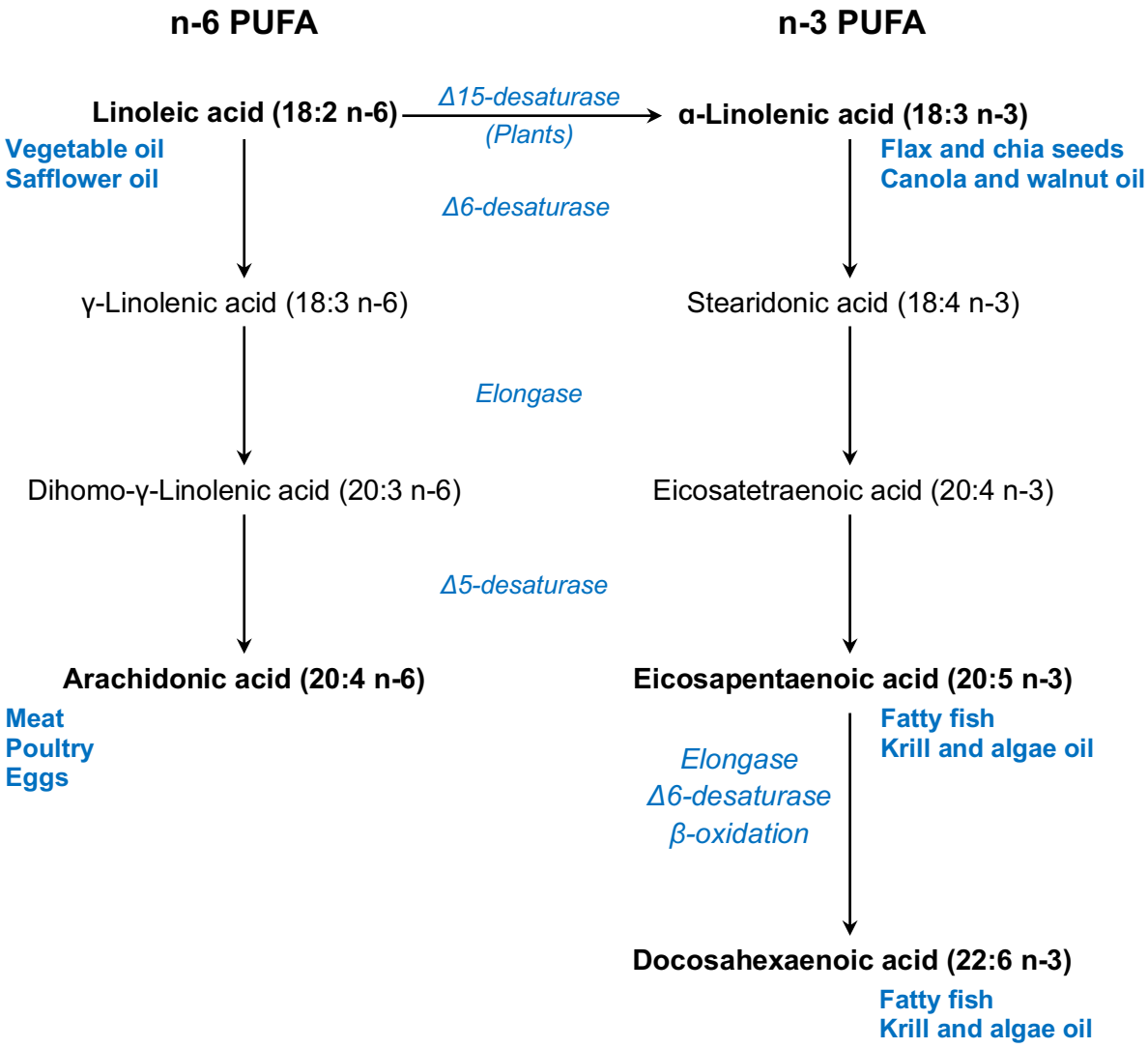


Figure 1.1. Desaturation and elongation of n-6 and n-3 polyunsaturated fatty acids (PUFA) (12–14). The conversion of essential fatty acids (i.e. linoleic acid and α-linolenic acid) to their longer, more saturated derivatives occurs in competition between the substrate n-6 and n-3 PUFA for the same desaturases and elongases. The conversion from eicosapentaenoic acid (EPA) to docosahexaenoic acid (DHA) involves multiple steps, including β-oxidation in peroxisomes. Dietary sources of those PUFA are also listed.

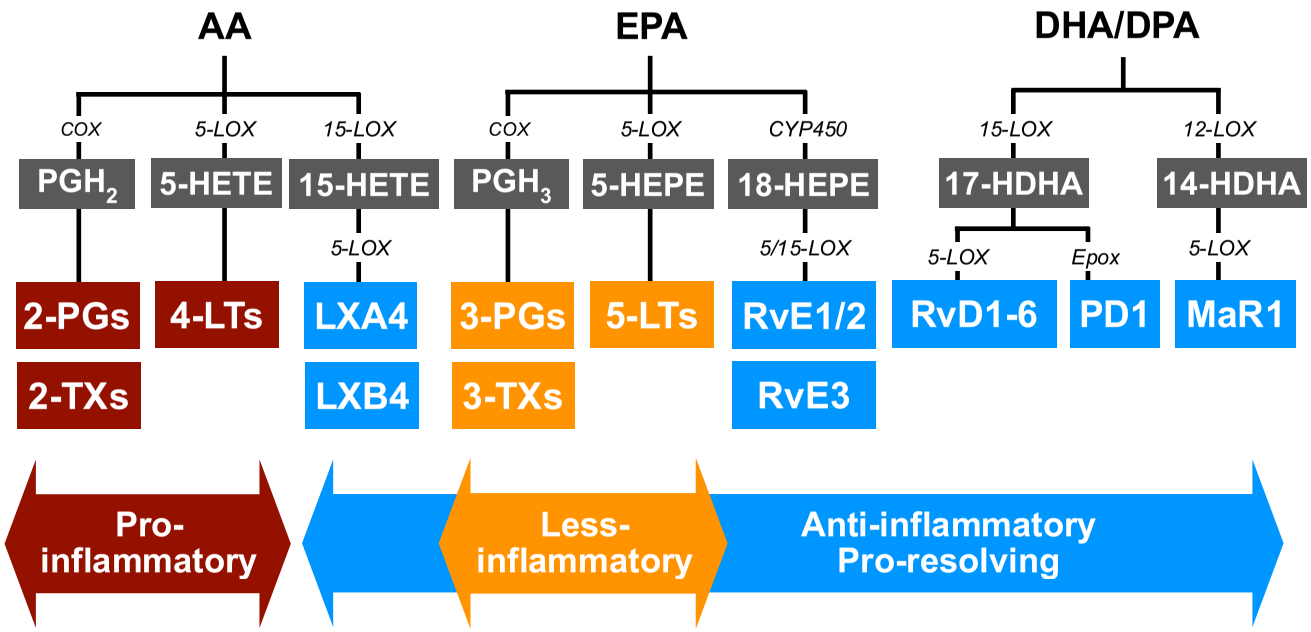


Figure 1.2. The biosynthetic pathways of polyunsaturated fatty acid (PUFA)-derived eicosanoids and specialized pro-resolving lipid mediators (SPM). Figure generated from our interpretation of multiple reviews and research articles (2,19,56). As for the DPA-derived resolvins, only $RvD1_{n\text{-}3\ DPA}$, $RvD2_{n\text{-}3\ DPA}$ and $RvD5_{n\text{-}3\ DPA}$ have been described. Colors indicate known biologic characteristics: red, pro-inflammatory; orange, less-inflammatory; blue, anti-inflammatory and pro-resolving. AA, arachidonic acid; COX, cyclooxygenase; CYP450, cytochrome P450; DPA, docosapentaenoic acid; Epox, epoxygenase; HDHA, hydroxy-docosahexaenoic acid; HEPE, hydroxy-eicosapentaenoic acid; HETE, hydroxy-eicosatetraenoic acid; LOX, lipoxygenase; LT, leukotriene; LX, lipoxin; MaR1, maresin 1; PG, prostaglandin; PD1, protectin 1; RvD, D-series resolvin; RvE, E-series resolvin; TX, thromboxane.

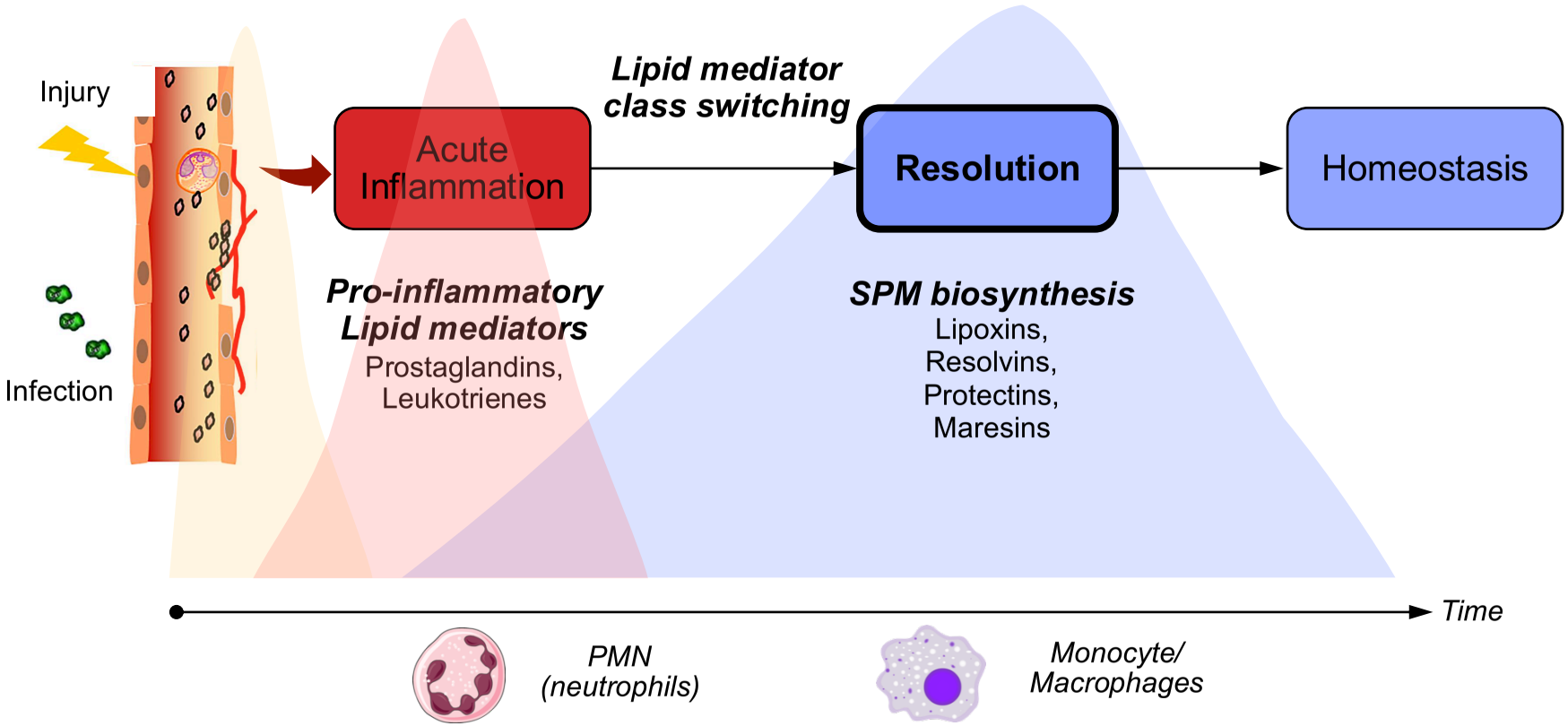


Figure 1.3. Resolution processes of inflammation. Figure adapted from review articles (19,72). Following insults like injury or infection, acute inflammation develops within minutes to hours with bioactions of pro-inflammatory lipid mediators like prostaglandins (PG) and leukotrienes (LT) which activate polymorphonuclear leukocytes (PMN), the first responders to the local site of inflammation. Through a switch of lipid mediators to specialized pro-resolving lipid mediators (SPM), the resolution of inflammation occurs to regain homeostasis by engaging monocyte/macrophages for clearance of debris and apoptotic cells and tissue regeneration.

1.3.6 References

1. Kotas ME, Medzhitov R. Homeostasis, Inflammation, and Disease Susceptibility. Cell. 2015;160:816–27.
2. Calder PC. Omega-3 polyunsaturated fatty acids and inflammatory processes: Nutrition or pharmacology? Br J Clin Pharmacol. 2013;75:645–62.
3. Cotran RS, Kumar V, Collins T, Robbins SL. Robbins Pathologic Basis of Disease. 6th ed. Saunders. Philadelphia, PA; 1999.
4. Libby P, Ridker PM, Hansson GK. Inflammation in Atherosclerosis. From Pathophysiology to Practice. J Am Coll Cardiol. 2009;54:2129–38.
5. Demoruelle MK, Deane KD, Holers VM. When and where does inflammation begin in rheumatoid arthritis? Curr Opin Rheumatol. 2014;26:64–71.
6. Amor S, Peferoen LAN, Vogel DYS, Breur M, van der Valk P, Baker D, Van Noort JM. Inflammation in neurodegenerative diseases - an update. Immunology. 2014;142:151–66.
7. Suzuki K. Chronic inflammation as an immunological abnormality and effectiveness of exercise. Biomolecules. 2019;9.
8. Rubin DC, Shaker A, Levin MS. Chronic intestinal inflammation: Inflammatory bowel disease and colitis-associated colon cancer. Front Immunol. 2012;3:107.
9. Myers GL, Rifai N, Tracy RP, Roberts WL, Alexander RW, Biasucci LM, Catravas JD, Cole TG, Cooper GR, Khan B V., et al. CDC/AHA Workshop on Markers of Inflammation and Cardiovascular Disease: Application to Clinical and Public Health Practice: report from the laboratory science discussion group. Circulation. 2004;110.
10. Food and Nutrition Board; Institute of Medicine. Dietary Reference Intakes for Energy, Carbohydrate, Fiber, Fat, Fatty Acids, Cholesterol, Protein, and Amino Acids (Macronutrients). Washington, D.C.: National Academies Press; 2002. 422–541 p.
11. Meyer BJ, Mann NJ, Lewis JL, Milligan GC, Sinclair AJ, Howe PRC. Dietary intakes and food sources of omega-6 and omega-3 polyunsaturated fatty acids. Lipids. 2003;38:391–8.
12. Gerster H. Can adults adequately convert α-linolenic acid (18:3n-3) to eicosapentaenoic acid (20:5n-3) and docosahexaenoic acid (22:6n-3)? Int J Vitam Nutr Res. 1998;68:159–73.
13. Spector AA. Essentiality of fatty acids. Lipids. 1999;34:S1–3.
14. Nakamura MT, Nara TY. Structure, function, and dietary regulation of Δ6, Δ5, and Δ9 desaturases. Annu Rev Nutr. 2004;24:345–76.
15. Sprecher HW, Luthria DL, Mohammed BS, Baykousheva SP. Reevaluation of the pathways for the biosynthesis of polyunsaturated fatty acids. J Lipid Res. 1995;
16. Calder PC. Omega-3: The good oil. Nutr Bull. 2017;42:132–40.

17. Bang HO, Dyerberg J, Nielsen AB o. ndu. Plasma lipid and lipoprotein pattern in Greenlandic West-coast Eskimos. Lancet. 1971;297:1143–6.

18. Kagawa Y, Nishizawa M, Suzuki M, Miyatake T, Hamamoto T, Goto K, Motonaga E, Izumikawa H, Hirata H, Ebihara A. Eicosapolyenoic Acids of Serum Lipids of Japanese Islanders with Low Incidence of Cardiovascular Diseases. J Nutr Sci Vitaminol. 1982;28:441–53.

19. Serhan CN. Pro-resolving lipid mediators are leads for resolution physiology. Nature. 2014;510:92–101.

20. Stillwell W, Wassall SR. Docosahexaenoic acid: Membrane properties of a unique fatty acid. Chem Phys Lipids. 2003;126:1–27.

21. Jump DB, Tripathy S, Depner CM. Fatty Acid–Regulated Transcription Factors in the Liver. Annu Rev Nutr. 2013;33:249–69.

22. Yaqoob P, Pala HS, Cortina-Borja M, Newsholme EA, Calder PC. Encapsulated fish oil enriched in α-tocopherol alters plasma phospholipid and mononuclear cell fatty acid compositions but not mononuclear cell functions. Eur J Clin Invest. 2000;30:260–74.

23. Lewis RA, Austen KF, Soberman RJ. Leukotrienes and Other Products of the 5-Lipoxygenase Pathway: Biochemistry and Relation to Pathobiology in Human Diseases. N Engl J Med. N Engl J Med; 1990;323:645–55.

24. Tilley SL, Coffman TM, Koller BH. Mixed messages: modulation of inflammation and immune responses by prostaglandins and thromboxanes. J Clin Invest. 2001;108:15–23.

25. Kalinski P. Regulation of Immune Responses by Prostaglandin E 2. J Immunol. 2012;188:21–8.

26. Rees D, Miles EA, Banerjee T, Wells SJ, Roynette CE, Wahle KW, Calder PC. Dose-related effects of eicosapentaenoic acid on innate immune function in healthy humans: a comparison of young and older men 1-3. 2006;

27. Browning LM, Walker CG, Mander AP, West AL, Madden J, Gambell JM, Young S, Wang L, Jebb SA, Calder PC. Incorporation of eicosapentaenoic and docosahexaenoic acids into lipid pools when given as supplements providing doses equivalent to typical intakes of oily fish. Am J Clin Nutr. 2012;96:748–58.

28. Obata T, Nagakura T, Masaki T, Maekawa K, Yamashita K. Eicosapentaenoic acid inhibits prostaglandin D2 generation by inhibiting cyclo-oxygenase-2 in cultured human mast cells. Clin Exp Allergy. 1999;29:1129–35.

29. Wada M, DeLong CJ, Hong YH, Rieke CJ, Song I, Sidhu RS, Yuan C, Warnock M, Schmaier AH, Yokoyama C, et al. Enzymes and receptors of prostaglandin pathways with arachidonic acid-derived versus eicosapentaenoic acid-derived substrates and products. J Biol Chem. 2007;282:22254–66.

30. Norris PC, Dennis EA. Omega-3 fatty acids cause dramatic changes in TLR4 and purinergic eicosanoid signaling. Proc Natl Acad Sci U S A. 2012;109:8517–22.

31. Calder PC. Marine omega-3 fatty acids and inflammatory processes: Effects, mechanisms and clinical relevance. Biochim Biophys Acta - Mol Cell Biol Lipids. 2015;1851:469–84.

32. De Caterina R, Cybulsky MI, Clinton SK, Gimbrone MA, Libby P. The omega-3 fatty acid docosahexaenoate reduces cytokine-induced expression of proatherogenic and proinflammatory proteins in human endothelial cells. Arterioscler Thromb Vasc Biol. 1994;14:1829–36.

33. Khalfoun B, Thibault G, Bardos P, Lebranchu Y. Docosahexaenoic and eicosapentaenoic acids inhibit in vitro human lymphocyte-endothelial cell adhesion. Transplantation. 1996;62:1649–57.

34. Lo CJ, Chiu KC, Fu M, Lo R, Helton S. Fish oil decreases macrophage tumor necrosis factor gene transcription by altering the NFκB activity. J Surg Res. 1999;82:216–21.

35. Babcock TA, Novak T, Ong E, Jho DH, Helton WS, Espat NJ. Modulation of lipopolysaccharide-stimulated macrophage tumor necrosis factor-α production by ω-3 fatty acid is associated with differential cyclooxygenase-2 protein expression and is independent of interleukin-10. J Surg Res. 2002;107:135–9.

36. Novak TE, Babcock TA, Jho DH, Helton WS, Espat NJ. NF-κB inhibition by ω-3 fatty acids modulates LPS-stimulated macrophage TNF-α-transcription. Am J Physiol - Lung Cell Mol Physiol. 2003;284.

37. Myhrstad MCW, Retterstøl K, Telle-Hansen VH, Ottestad I, Halvorsen B, Holven KB, Ulven SM. Effect of marine n-3 fatty acids on circulating inflammatory markers in healthy subjects and subjects with cardiovascular risk factors. Inflamm Res. 2011;60:309–19.

38. Rangel-Huerta OD, Aguilera CM, Mesa MD, Gil A. Omega-3 long-chain polyunsaturated fatty acids supplementation on inflammatory biomakers: a systematic review of randomised clinical trials. Br J Nutr. 2012;107:S159–70.

39. Hughes DA, Pinder AC, Piper Z, Johnson IT, Lund EK. Fish oil supplementation inhibits the expression of major histocompatibility complex class II molecules and adhesion molecules on human monocytes13. Am J C/in Nuir. 1996;63:267–72.

40. Luu NT, Madden J, Calder PC, Grimble RF, Shearman CP, Chan T, Tull SP, Dastur N, Rainger GE, Nash GB. Comparison of the pro-inflammatory potential of monocytes from healthy adults and those with peripheral arterial disease using an in vitro culture model. Atherosclerosis. 2007;193:259–68.

41. Yamada H, Yoshida M, Nakano Y, Suganami T, Satoh N, Mita T, Azuma K, Itoh M, Yamamoto Y, Kamei Y, et al. In vivo and in vitro inhibition of monocyte adhesion to endothelial cells and endothelial adhesion molecules by eicosapentaenoic acid. Arterioscler Thromb Vasc Biol. 2008;28:2173–9.

42. Lawrence T. The nuclear factor NF-kappaB pathway in inflammation. Cold Spring Harb Perspect Biol. 2009;1.

43. Wong SW, Kwon MJ, Choi AMK, Kim HP, Nakahira K, Hwang DH. Fatty acids

modulate toll-like receptor 4 activation through regulation of receptor dimerization and recruitment into lipid rafts in a reactive oxygen species-dependent manner. J Biol Chem. 2009;284:27384–92.

44. Adkins Y, Kelley DS. Mechanisms underlying the cardioprotective effects of omega-3 polyunsaturated fatty acids. J Nutr Biochem. 2010;21:781–92.

45. Oh DY, Talukdar S, Bae EJ, Imamura T, Morinaga H, Fan W, Li P, Lu WJ, Watkins SM, Olefsky JM. GPR120 is an omega-3 fatty acid receptor mediating potent anti-inflammatory and insulin-sensitizing effects. Cell. 2010;142:687–98.

46. Forman BM, Chen J, Evans RM. Hypolipidemic drugs, polyunsaturated fatty acids, and eicosanoids are ligands for peroxisome proliferator-activated receptors α and δ. Proc Natl Acad Sci U S A. 1997;94:4312–7.

47. Kliewer SA, Sundseth SS, Jones SA, Brown PJ, Wisely GB, Koble CS, Devchand P, Wahli W, Willson TM, Lenhard JM, et al. Fatty acids and eicosanoids regulate gene expression through direct interactions with peroxisome proliferator-activated receptors α and γ. Proc Natl Acad Sci U S A. 1997;94:4318–23.

48. Mullen A, Loscher CE, Roche HM. Anti-inflammatory effects of EPA and DHA are dependent upon time and dose-response elements associated with LPS stimulation in THP-1-derived macrophages. J Nutr Biochem. 2010;21:444–50.

49. Hong S, Gronert K, Devchand PR, Moussignac RL, Serhan CN. Novel docosatrienes and 17S-resolvins generated from docosahexaenoic acid in murine brain, human blood, and glial cells: Autacoids in anti-inflammation. J Biol Chem. 2003;278:14677–87.

50. Mas E, Croft KD, Zahra P, Barden A, Mori TA. Resolvins D1, D2, and other mediators of self-limited resolution of inflammation in human blood following n-3 fatty acid supplementation. Clin Chem. 2012;58:1476–84.

51. Mas E, Barden A, Burke V, Beilin LJ, Watts GF, Huang RC, Puddey IB, Irish AB, Mori TA. A randomized controlled trial of the effects of n-3 fatty acids on resolvins in chronic kidney disease. Clin Nutr. 2016;35:331–6.

52. Polus A, Zapala B, Razny U, Gielicz A, Kiec-Wilk B, Malczewska-Malec M, Sanak M, Childs CE, Calder PC, Dembinska-Kiec A. Omega-3 fatty acid supplementation influences the whole blood transcriptome in women with obesity, associated with pro-resolving lipid mediator production. Biochim Biophys Acta - Mol Cell Biol Lipids. 2016;1861:1746–55.

53. Souza PR, Marques RM, Gomez EA, Colas RA, De Matteis R, Zak A, Patel M, Collier DJ, Dalli J. Enriched Marine Oil Supplements Increase Peripheral Blood Specialized Pro-Resolving Mediators Concentrations and Reprogram Host Immune Responses: A Randomized Double-Blind Placebo-Controlled Study. Circ Res. 2020;126:75–90.

54. Titos E, Rius B, González-Périz A, López-Vicario C, Morán-Salvador E, Martínez-Clemente M, Arroyo V, Clària J. Resolvin D1 and Its Precursor Docosahexaenoic Acid Promote Resolution of Adipose Tissue Inflammation by Eliciting Macrophage Polarization toward an M2-Like Phenotype. J Immunol.

2011;187:5408–18.

55. Dalli J, Zhu M, Vlasenko NA, Deng B, Haeggström JZ, Petasis NA, Serhan CN. The novel 13S,14S-epoxy-maresinis converted by human macrophages to maresin 1 (MaR1), inhibits leukotriene A4 hydrolase (LTA4H), and shifts macrophage phenotype. FASEB J. 2013;27:2573–83.

56. Dalli J, Colas RA, Serhan CN. Novel n-3 immunoresolvents: Structures and actions. Sci Rep. 2013;3.

57. Gobbetti T, Dalli J, Colas RA, Canova DF, Aursnes M, Bonnet D, Alric L, Vergnolle N, Deraison C, Hansen T V., et al. Protectin D1n-3 DPA and resolvin D5n-3 DPA are effectors of intestinal protection. Proc Natl Acad Sci U S A. 2017;114:3963–8.

58. Weylandt KH, Krause LF, Gomolka B, Chiu C-Y, Bilal S, Nadolny A, Waechter SF, Fischer A, Rothe M, Kang JX. Suppressed liver tumorigenesis in fat-1 mice with elevated omega-3 fatty acids is associated with increased omega-3 derived lipid mediators and reduced TNF-α. Carcinogenesis. 2011;32:897–903.

59. Endo J, Sano M, Isobe Y, Fukuda K, Kang JX, Arai H, Arita M. 18-HEPE, an n-3 fatty acid metabolite released by macrophages, prevents pressure overload-induced maladaptive cardiac remodeling. J Exp Med. 2014;211:1673–87.

60. Neuhofer A, Zeyda M, Mascher D, Itariu BK, Murano I, Leitner L, Hochbrugger EE, Fraisl P, Cinti S, Serhan CN, et al. Impaired local production of proresolving lipid mediators in obesity and 17-HDHA as a potential treatment for obesity-associated inflammation. Diabetes. American Diabetes Association; 2013;62:1945–56.

61. Valdes AM, Ravipati S, Menni C, Abhishek A, Metrustry S, Harris J, Nessa A, Williams FMK, Spector TD, Doherty M, et al. Association of the resolvin precursor 17-HDHA, but not D-or E-series resolvins, with heat pain sensitivity and osteoarthritis pain in humans. Sci Rep. 2017;7.

62. Arita M, Bianchini F, Aliberti J, Sher A, Chiang N, Hong S, Yang R, Petasis NA, Serhan CN. Stereochemical assignment, antiinflammatory properties, and receptor for the omega-3 lipid mediator resolvin E1. J Exp Med. 2005;201:713–22.

63. Krishnamoorthy S, Recchiuti A, Chiang N, Yacoubian S, Lee CH, Yang R, Petasis NA, Serhan CN. Resolvin D1 binds human phagocytes with evidence for proresolving receptors. Proc Natl Acad Sci U S A. 2010;107:1660–5.

64. Yokoyama M, Origasa H, Matsuzaki M, Matsuzawa Y, Saito Y, Ishikawa Y, Oikawa S, Sasaki J, Hishida H, Itakura H, et al. Effects of eicosapentaenoic acid on major coronary events in hypercholesterolaemic patients (JELIS): a randomised open-label, blinded endpoint analysis. Lancet. 2007;369:1090–8.

65. Bhatt DL, Steg PG, Miller M, Brinton EA, Jacobson TA, Ketchum SB, Doyle RT, Juliano RA, Jiao L, Granowitz C, et al. Cardiovascular risk reduction with icosapent ethyl for hypertriglyceridemia. N Engl J Med. 2019;380:11–22.

66. De Caterina R, Liao JK, Libby P. Fatty acid modulation of endothelial activation.

Am J Clin Nutr. 2000;71.

67. Weldon SM, Mullen AC, Loscher CE, Hurley LA, Roche HM. Docosahexaenoic acid induces an anti-inflammatory profile in lipopolysaccharide-stimulated human THP-1 macrophages more effectively than eicosapentaenoic acid. J Nutr Biochem. 2007;18:250–8.

68. Serini S, Bizzarro A, Piccioni E, Fasano E, Rossi C, Lauria A, R.M. Cittadini A, Masullo C, Calviello G. EPA and DHA Differentially Affect In Vitro Inflammatory Cytokine Release by Peripheral Blood Mononuclear Cells from Alzheimer's Patients. Curr Alzheimer Res. 2012;9:913–23.

69. Allaire J, Couture P, Leclerc M, Charest A, Marin J, Lépine M-C, Talbot D, Tchernof A, Lamarche B. A randomized, crossover, head-to-head comparison of eicosapentaenoic acid and docosahexaenoic acid supplementation to reduce inflammation markers in men and women: the Comparing EPA to DHA (ComparED) Study. Am J Clin Nutr. 2016;104:280–7.

70. Vors C, Allaire J, Marin J, Lépine MC, Charest A, Tchernof A, Couture P, Lamarche B. Inflammatory gene expression in whole blood cells after EPA vs. DHA supplementation: Results from the ComparED study. Atherosclerosis. 2017;257:116–22.

71. Tsunoda F, Lamon-Fava S, Asztalos BF, Iyer LK, Richardson K, Schaefer EJ. Effects of oral eicosapentaenoic acid versus docosahexaenoic acid on human peripheral blood mononuclear cell gene expression. Atherosclerosis. 2015;241:400–8.

72. Hansen TV, Vik A, Serhan CN. The protectin family of specialized pro-resolving mediators: Potent immunoresolvents enabling innovative approaches to target obesity and diabetes. Front Pharmacol. 2019;9:1582.

Chapter 2 Effects of EPA and DHA on Monocyte Inflammatory Response and Plasma PUFA Lipidome

EPA and DHA differentially modulate monocyte inflammatory response in subjects with chronic inflammation in part via plasma specialized pro-resolving lipid mediators: a randomized, double-blind, crossover study

Jisun So[1], Dayong Wu[2], Alice H Lichtenstein[1], Albert K Tai[3], Nirupa R Matthan[1], Krishna Rao Maddipati[4], Stefania Lamon-Fava[1]*

Affiliations: [1]Cardiovascular Nutrition Laboratory and [2]Nutritional Immunology Laboratory, Jean Mayer USDA Human Nutrition Research Center on Aging at Tufts University, Boston, MA; [3]Department of Immunology, Tufts University School of Medicine, Boston, MA; [4]Department of Pathology, Lipidomics Core Facility, Wayne State University, Detroit, MI

Short title: Differential effects of EPA and DHA on inflammation and SPM

Clinical Trial Registry: ClinicalTrials.gov, NCT02670382

***Corresponding Author:**
Stefania Lamon-Fava, MD PhD
Cardiovascular Nutrition Laboratory,
Jean Mayer USDA Human Nutrition Research Center on Aging,
Tufts University
711 Washington Street,
Boston, MA 02111
Tel: +1 617 556 3105
E-mail: stefania.lamon-fava@tufts.edu

2.1 Abstract

Background and aims: The independent effects of EPA and DHA on inflammation through their downstream lipid mediators, including the specialized pro-resolving lipid mediators (SPM), remain unstudied. Therefore, we compared the effects of EPA and DHA supplementation on monocyte inflammatory response and plasma PUFA SPM lipidome.

Methods: After a 4-week lead-in phase (baseline), 9 men and 12 postmenopausal women (50-75 yrs) with chronic inflammation received two phases of 10-week supplementation with 3 g/day EPA and DHA in a random order, separated by a 10-week washout.

Results: Compared with baseline, EPA and DHA supplementation differently modulated LPS-stimulated monocyte cytokine expression. EPA lowered *TNFA* (p <0.001) whereas DHA reduced *TNFA* (p <0.001), *IL6* (p <0.02), *MCP1* (p <0.03), and *IL10* (p <0.01). DHA also lowered *IL10* expression relative to EPA (p=0.03). Relative to baseline, EPA, but not DHA, decreased the ratios of *TNFA/IL10* and *MCP1/IL10* (both p <0.01). EPA and DHA also significantly changed plasma PUFA SPM lipidome by replacing n-6 AA derivatives with their respective derivatives including 18-hydroxy-EPA (+5 fold by EPA) and 17- and 14-hydroxy-DHAs (+3 folds by DHA). However, DHA showed a wider effect than EPA by also significantly increasing EPA derivatives and DPA-derived SPM at a greater expense of AA derivatives. Different groups of PUFA derivatives mediated the differential effects of EPA and DHA on monocyte cytokine expression.

Conclusions: While DHA mostly inhibited pro-inflammatory cytokines, EPA was more effective in balancing pro-and anti-inflammatory cytokines. These differential effects were potentially mediated by different groups of PUFA derivatives, suggesting immunomodulatory activities of SPM and their intermediates.

2.2 Introduction

Very-long-chain n-3 polyunsaturated fatty acids (n-3 LC-PUFA) eicosapentaenoic acid (EPA, 20:5 n-3) and docosahexaenoic acid (DHA, 22:6 n-3) found in fish oil have long been considered to be protective against cardiovascular disease (CVD) and other inflammatory disorders (1,2) in part through their anti-inflammatory effects (3,4). *In vitro* studies have suggested that n-3 LC-PUFA suppress the production of inflammatory proteins such as cytokines/chemokines and adhesion molecules by directly affecting multiple transcription factors including nuclear factor-kappa B (NF-κB) (5,6) and peroxisome proliferator-activated receptors (PPAR) (7,8). In addition, n-3 LC-PUFA may counterbalance the pro-inflammatory eicosanoids produced from n-6 arachidonic acid (AA, 20:4 n-6), the major constituent of the membrane phospholipids, by replacing AA in the membrane and producing their downstream eicosanoids with less potency (4). Recent studies are suggesting that n-3 LC-PUFA may also reinforce the resolution processes of inflammation through the synthesis of specialized pro-resolving lipid mediators (SPM) (9). These include resolvins derived from EPA (E-series resolvins, RvE) and DHA (D-series resolvins, RvD), and protectins (PD) and maresins (MaR) derived from DHA. In studies conducted in cell culture and in animal models of inflammation, SPM have been shown to limit neutrophil infiltration into inflammatory sites, enhance phagocytosis of apoptotic cells and promote tissue regeneration, however, these effects remain to be demonstrated in humans (9).

Though the cardioprotective benefits of fish oils containing EPA and DHA have been questioned in some clinical trials (10), significant reductions of CVD risk have been reported in two large clinical trials that administered highly purified EPA (i.e. the Japan

EPA Lipid Intervention Study [JELIS] (11) and the Reduction of Cardiovascular Events with Icosapent Ethyl-Intervention Trial [REDUCE-IT] (12)). Therefore, there is a need to better understand the independent and differential effects of EPA and DHA (13). A greater potency of DHA than EPA in lowering the pro-inflammatory cytokines tumor necrosis factor (TNF)-α, interleukin (IL)-1β and 6, and/or adhesion molecule vascular cell molecule 1 has been reported in studies using macrophages and endothelial cells (14,15). In humans, a few recent studies have compared EPA and DHA supplementation on plasma inflammatory markers (16) and peripheral blood mononuclear cell (PBMC) gene expression (17,18), but the role of SPM on these effects has not been systematically investigated.

We aimed to assess, in individuals with chronic inflammation, the common and differential effects of high-dose (3 g/day) supplementation with purified EPA and DHA on systemic inflammation, monocyte inflammatory response after *ex vivo* LPS stimulation, and plasma PUFA lipidome including SPM. We also examined whether and how much such changes in inflammatory markers during each supplementation were mediated by plasma PUFA SPM lipidome profiles.

2.3 Patients and Methods

Study design

The effects of EPA and DHA supplementation on inflammation and plasma PUFA SPM lipidome were investigated in a 34-week randomized, double-blind, crossover trial (ClinicalTrials.gov, NCT02670382, Supplementary Fig. 2.1). Participants met with a registered dietician at the beginning of the study and at each study visit and were instructed to follow a low-saturated fat (< 7% of total energy) and low-cholesterol (< 200 mg/day) diet throughout the study. We have previously shown that stabilization occurs by 4 weeks (19). After a 4-week lead-in phase during which participants were given 3 g/day of high oleic acid sunflower oil, they were randomly assigned to two sequential 10-week supplementation phases with 3 g/day of pure EPA or pure DHA with a 10-week washout phase in between. The 4th-week visit at the end of the lead-in phase was considered as baseline. The random allocation sequence was generated by REDCap. Study participants, coordinators, and laboratory technicians were blinded to treatment codes throughout the study. The ethyl ester forms of EPA and DHA were provided in capsules containing 750 mg/capsule with a purity > 97%. High oleic acid sunflower oil (> 80% oleic acid) was also provided in identical capsules containing 750 mg/capsule. Participants were instructed to take two capsules each with morning and evening meals. Compliance was assessed by counting the returned capsules and > 80%.

The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Tufts Health Sciences Institutional Review Board.

Study participants

Men and postmenopausal women aged 50–75 years were recruited through the Metabolic Research Unit at the Jean Mayer USDA Human Nutrition Research Center of Aging at Tufts University. Inclusion criteria were: 1) chronic inflammation, defined as serum high-sensitivity C-reactive protein (hs-CRP) ≥ 2 μg/mL, 2) fasting plasma triglyceride (TG) concentrations 90–500 mg/dL, and 3) at least one of the following criteria for the modified definition of metabolic syndrome: i) waist circumference ≥ 102 cm in men and ≥ 89 cm in women, ii) blood pressure ≥ 130/80 mmHg or use of anti-hypertensive medications, and iii) fasting plasma glucose concentrations ≥ 100 mg/dL. Exclusion criteria were: 1) smoking, 2) high alcohol consumption (> 7 drinks/week), 3) consumption of a high-fish diet (> 2 fish meals/week), fish oil or EPA/DHA-containing supplements during the previous 6 months, 4) allergy to sardines and/or sunflower oil, 5) regular use of anti-inflammatory medications (e.g. NSAID, COX inhibitors), 6) anticoagulant therapy, and 7) any disease that might affect metabolic status (uncontrolled thyroid dysfunction, altered coagulation, type 2 diabetes mellitus, kidney or liver disease, or any other major acute/chronic diseases). All participants signed written informed consent.

Sample size was calculated based on the primary outcomes of the trial, the changes in plasma TNF-α, IL-6, and low-density lipoprotein cholesterol (LDL-C) concentrations. No previous data being available to predict differences between EPA and DHA on inflammatory cytokines, we had hypothesized that DHA would have ¼ of the effect of EPA on TNF-α and IL-6 with post-treatment mean ratios of 0.79 and 0.52, respectively (20,21). For LDL-C, the difference between EPA and DHA was predicted as 6 mg/dL

(22). As a result, 24, 21, and 20 participants were needed, respectively, to achieve 80% power using a two-sided 0.05 significance level. From April 2016 to November 2017, 24 participants were enrolled in the study (Supplementary Fig. 2.2). Nine men and 12 postmenopausal women (N=21) completed the study with a dropout rate of < 15%. Three participants dropped out during the lead-in phase due to health or family reasons.

Plasma/serum marker measurements

Twelve-hour fasting venous blood was collected at baseline and the end of each supplementation (Supplementary Fig. 2.1). Plasma samples were obtained by immediate centrifugation of EDTA tubes (Beckton Dickinson, Franklin Lakes, NJ) and stored at –80 °C until analyses. Serum samples were obtained by centrifugation after the blood was allowed to clot at room temperature for 20 min and then stored at –80 °C until examination.

Plasma hs-CRP concentrations were assessed by an immunoturbidometric method, and serum concentrations of TNF-α, IL-6, monocyte chemoattractant protein 1 (MCP-1) and IL-10 were measured using V-PLEX electrochemiluminescence (ECL) assays (Meso Scale Diagnostics LLC, Rockville, MD). The fatty acid profile of plasma phospholipids was measured by gas chromatography as previously described (23) and expressed as a relative proportion (molar percentage, mol %) of the total phospholipid fatty acids.

Targeted PUFA SPM lipidome profiling

Plasma concentrations of PUFA-derived lipid mediators including SPM were assessed by liquid chromatography-mass spectrometry (LC-MS) as previously described with minor modifications(24). Briefly, aliquots of plasma samples (100 µl) were spiked with 5

ng each of prostaglandin (PG) E_1-d_4, RvD2-d_5, leukotriene (LT) B_4-d_4, and 15S-hydroxy-eicosatetraenoic acid (HETE)-d_8 as internal standards for analyte recovery and quantitation. Samples were then extracted for fatty acyl lipid metabolites with C18 extraction columns and subjected to LC-MS analysis. All samples were batch-analyzed at the end of the study.

Isolation of peripheral blood monocyte and ex vivo cytokine expression analysis

PBMC were isolated from fasting venous blood collected in sodium citrate Vacutainer Cell Preparation Tubes (Becton Dickinson, Franklin Lakes, NJ) at baseline and the end of each supplementation. Monocytes were further isolated by negative selection (Miltenyi Biotec, Bergisch Gladbach, Germany). Monocytes ($1x10^6$) were plated in culture medium in the presence or absence of LPS 10 ng/mL. After 4 hours, monocyte cell pellets and culture media were separately collected after centrifugation and stored at –80 °C until examination.

Total RNA was isolated using QIAshredder and RNeasy Mini kit (Qiagen, Hilden, Germany), and reverse transcription was performed on 0.1–0.2 μg total RNA. Real-time PCR assays were performed using gene-specific primers (*TNFA*, QT00104006; *IL6*, QT00098875; *MCP1*/*CCL2*, QT00212730; *IL10*, QT00041685; all from Qiagen, Hilden, Germany) and QuantiFast SYBR Green PCR Master Mix (Qiagen, Hilden, Germany) on an Applied Biosystems 7300 (Applied Biosystems, Waltham, MA). Relative gene expression was calculated by the $2^{-\Delta\Delta Ct}$ method using glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) as endogenous control. Concentrations of TNF-α, IL-6, MCP-1, and IL-10 in cell culture supernatants were quantified by the ECL assays also used for serum cytokine assessment.

Data analyses

Data were assessed for normal distribution and, when skewed, a logarithmic transformation was applied before analysis. Data are presented as means ± SEM or medians [25^{th}, 75^{th}]. We performed an intention-to-treat analysis, followed by sensitivity analysis if necessary. Differences between changes in study outcomes during EPA versus DHA supplementation were assessed using linear mixed models for repeated measures (*lme4* package) in R 3.5.2 with RStudio IDE (www.rstudio.com) with fixed effects of treatment, phase, and sequence of supplementation, a random subject effect, and baseline values as a covariate. Significance of the change from baseline for each treatment was tested by the *lsmeansLT* function of the *lmerTest* package, which resembles the LSMEANS statement in the MIXED procedure. When a significant sequence effect was found, we analyzed only the first supplementation period using ANCOVA with a baseline covariate for comparing EPA and DHA and paired *t* tests for comparing each treatment to baseline.

Five monocyte samples (one man; four women) with low purity were identified based on the expression of other leukocytes specific markers (i.e. *CD3*, *CD8*, *CD19*, and *NCAM1*) and excluded for sensitivity analysis but the exclusion of these samples did not change the results in terms of significance levels except for a newly appeared reduced MCP-1 secretion (p=0.04) after EPA supplementation (data not shown).

In the analysis of plasma PUFA SPM lipidome data, a total of 24 lipid mediators detected in >80% of the samples were included. Sub-threshold values were imputed with the quantitation limit (5 pg) or the lowest concentration detected for each mediator [1 pg for $MaR1_{n\text{-}3\ DPA}$ and 11-hydroxy-EPA (HEPE); 2 pg for 5S,12S-diHETE and 12S-

hydroxy-heptadecatrienoic acid (HHTrE); 4 pg for lipoxin (LX) B4]. Principal Component Analysis (PCA) was performed using MetaboAnalyst 4.0 (www.metaboanalyst.ca). Concentrations of the 24 mediators at baseline and the end of supplementation phases were cube-root transformed, centered to mean, and divided by the SD of each mediator for normalization. Principal component (PC) scores were calculated for each participant for the top three PC explaining > 10% of the overall variance. A lipid mediator with a loading >|0.2| was considered to have a major contribution to the given PC. In the additional analysis examining a potential composite role of plasma PUFA SPM lipidome in mediating the effects of EPA and DHA supplementation on monocyte gene expression, a series of linear mixed models were used to assess the effects of either supplementation on 1) the outcome (i.e. total effect) and 2) a select PC, 3) the association between the PC and the outcome, and 4) the effect of supplementation on the outcome when adjusted for the given PC (i.e. direct effect). Upon the significance of 1)-3), the presence of a potential mediation was determined if the total effect of supplementation was abolished or weakened after adjusting for PC in terms of coefficient size, and the amount of mediation (i.e. indirect effect) was calculated as [(total effect–direct effect)/total effect *100 (%)] (25,26).

2.4 Results

Characteristics of participants at screening visit

As per the screening criteria, all participants had chronic inflammation as demonstrated by a high median hs-CRP concentration (Table 2.1). Also, they had a mean waist circumference of the abdominal obesity (men > 102 cm; women > 88 cm) (27) and

borderline high fasting plasma TG and glucose concentrations. Forty-three percent of the completers (44% of men; 42% of women) had metabolic syndrome according to the International Diabetes Federation definition (28) (data not shown).

Inflammation-related markers

Relative to baseline, EPA and DHA supplementation did not affect serum concentrations of hs-CRP and cytokines (Table 2.2A). There was no significant difference between the EPA- and DHA-driven changes. The LPS-stimulated inflammatory response of monocytes was significantly affected by both EPA and DHA but in a distinct manner (Fig. 2.1A, C, E, and G). Compared with baseline, EPA lowered only *TNFA* expression (median % change: -20%, p <0.001), whereas DHA lowered the expression of pro-inflammatory *TNFA* (-45%, p <0.001), *IL6* (-51%, p <0.02) and *MCP1* (-28%, p <0.03) as well as anti-inflammatory *IL10* (-33%, p <0.01). Relative to EPA, DHA significantly lowered *IL10* expression (p=0.03). When examining the pro- to anti-inflammatory cytokine ratios, indicative of overall inflammatory profile, EPA, but not DHA, was effective in reducing the ratios of *TNFA/IL10* (p=0.005) and *MCP1/IL10* (p=0.006) compared with baseline (Fig. 2.1B, D, and F). Regarding the LPS-induced monocyte cytokine secretion, DHA lowered the monocyte secretion of TNF-α (-41%, p=0.007), IL-6 (-35%, p <0.04) and MCP-1 (-29%, p=0.005) (Supplementary Fig. 2.3).

Plasma PUFA SPM lipidome profile

The main PUFA lipidomes along the biosynthetic pathways of eicosanoids and SPM are displayed in Supplementary Fig. 2.4. It includes docosapentaenoic acid (DPA, 22:5 n-3)-derived SPM that were more recently discovered as analogs of DHA-derived SPM (29).

The profiles of plasma PUFA SPM lipidomes at baseline and after supplementations are presented in Table 2.2B. Relative to baseline, EPA supplementation significantly increased plasma EPA derivatives including 18-HEPE (+4.9 fold) and 5-HEPE (+3.5 fold), consistent with the increased proportion of EPA in plasma phospholipids (Supplementary Table 2.1). Despite the marked increase in 18-HEPE, RvEs remained undetected with our methodology. Concomitantly, EPA supplementation significantly reduced the AA derivatives 15-HETE and 5-HETE, but not DPA or DHA derivatives.

In contrast, DHA supplementation affected a wider range of plasma PUFA-derived mediators. Relative to baseline, DHA significantly increased DHA-derived hydroxy-DHAs (HDHA) including 17-HDHA (+3.3 fold), a precursor of RvD and PD1, and 14-HDHA (+3.2 fold), a MaR1 pathway marker, and also EPA derivatives (Table 2.2B). In addition, relative to baseline, DHA significantly lowered more AA derivatives, including PGD_2, PGE_2, and thromboxane (TX) B_2, than EPA. This is in agreement with the greater reduction in plasma phospholipid AA during DHA than EPA supplementation (Supplementary Table 2.1). Notably, plasma DPA-derived $RvD5_{n\text{-}3\ DPA}$ and $MaR1_{n\text{-}3\ DPA}$ were significantly increased during DHA supplementation, in contrast with the reduced plasma phospholipid DPA.

PCA confirmed the differential effects of supplementation with EPA versus DHA on plasma PUFA SPM lipidome showing clustering by phase (Fig. 2.2A). The top three PC displayed distinct signatures, representing the lipidomes from DHA/DPA, AA, and EPA, respectively (Fig. 2.2B). PC1 was mostly and positively associated with DHA/DPA derivatives, including DPA-derived SPM but not RvD1, and significantly increased after both EPA and DHA supplementations (Table 2.3, Supplementary Table 2.2), as indicated

by their elevated concentrations in plasma. PC2 and PC3 were mostly and negatively associated with AA- and EPA-derived mediators, respectively. Of note, the AA-derived SPM LXB4, unlike the other AA derivatives, did have little contribution to the PC2 with a loading of 0.08. The PC2 score was significantly increased only by DHA supplementation, confirming the stronger lowering effects of DHA on AA derivatives than EPA. As indicated by the enhanced profile of plasma EPA derivatives during both EPA and DHA supplementation, the PC3 score was significantly decreased by 3.69 and 0.64, respectively (Table 2.3, Supplementary Table 2.2).

Composite role of plasma PUFA SPM lipidome in inflammation

To gain insight into potential pro-resolving or anti-inflammatory actions of SPM and other PUFA derivatives, we examined whether the plasma PUFA SPM lipidome signatures mediated the effects of each EPA or DHA supplementation on *ex vivo* monocyte cytokine expression. Potential mediation was identified as present if the total effect of supplementation on the outcome was abolished or partially reduced in the magnitude of coefficient after adjusting for the specific signature of plasma PUFA SPM lipidome (Table 2.3). We found that the *TNFA*-lowering by EPA supplementation was weakened by 44% with adjustment for PC3, but not other PC, which suggests a strong indirect effect of EPA supplementation via multiple EPA derivatives. In contrast, the *TNFA*-lowering effect of DHA supplementation was attenuated by adjusting for PC2 (19%) and PC3 (8%), indicating a stronger influence of the reduction in AA derivatives than the increase in EPA derivatives. Notable is the lack of potential mediation via PC1 (i.e. DHA/DPA derivatives) despite the great increase in the PC1 score (+4.13, p <0.001) during DHA supplementation. Regarding the anti-inflammatory effects of DHA on *IL6*

and *MCP1* expression, partial mediations through PC1 (39%) and PC2 (67%), respectively, were suggested. Finally, the suppressing effect of DHA supplementation on the anti-inflammatory *IL10* expression seemed to be partially through PC2 (28%) and PC1 (7%).

2.5 Discussion

To the best of our knowledge, this is the first study to compare the independent effects of high-dose (3 g/day) EPA and DHA supplementation on plasma n-3 LC-PUFA-derived pro-resolving SPM and other downstream lipid mediators as well as the inflammatory response of peripheral blood monocytes. Our results indicate that differential effects of EPA and DHA in attenuating *ex vivo* LPS-stimulated monocyte expression of inflammation-related genes may be partly due to their differential modulations of plasma PUFA SPM lipidome profiles.

The cardioprotective effect of n-3 LC-PUFA has been partly explained by their anti-inflammatory actions through the inhibition of NF-κB signaling via PPARγ and/or G-protein coupled receptor 120 activation (3,4,6). However, clinical studies have not consistently demonstrated anti-inflammatory effects of n-3 LC-PUFA, possibly due to large variations in the supplement composition of EPA and DHA and diverse participant populations ranging from healthy individuals to CVD patients (30,31). To address some of these issues, in the present study we administered high-dose purified EPA and DHA with a crossover design in participants with chronic inflammation. As for serum inflammatory markers, we found no effect of EPA and DHA supplementation, as also reported by some other clinical studies (30,31). However, we did find a distinct effect of

EPA and DHA supplementation in regulating *ex vivo* cytokine expression in LPS-stimulated peripheral monocytes, with DHA lowering the expression of a wider range of genes than EPA. While both EPA and DHA lowered *TNFA* expression, DHA also attenuated the expression of *IL6* and *MCP1*. This is consistent with the previous findings of a greater anti-inflammatory potency of DHA compared to EPA from studies using stimulated *THP-1* macrophages (15) and PBMC from Alzheimer's disease patients (32). Notably, DHA did not enhance but rather suppressed the expression of the anti-inflammatory cytokine *IL10*, unlike EPA. Previous studies have reported that both EPA and DHA increase *IL10 in vitro,* possibly via PPARγ (33,34), however, DHA was found to be less efficient than EPA in balancing the production of pro-inflammatory cytokines and anti-inflammatory IL-10 in human PBMC (32). This is consistent with our results showing a significant reduction in *TNFA/IL10* and *MCP1/IL10* with EPA, but not DHA.

One of the purported mechanisms of n-3 LC-PUFA action against inflammation is through their downstream lipid mediators, including the novel families of pro-resolving SPM generated by enzymatic reactions (9,35). SPM play an important role in the resolution of inflammation, an active process that is crucial in repairing tissue and regaining homeostasis, thereby preventing the deleterious consequences of unresolved inflammation (9). Compared to the growing evidence of the bioactions of SPM and other intermediates obtained from *in vitro* and animal studies, little is known of their role in humans. In addition, it is important to characterize the impact of the overall alterations in plasma PUFA SPM lipidome, and not just the individual lipid mediators. As indicated by the PCA plot, EPA and DHA supplementation significantly altered the lipidome profiles from baseline, but differently. Some of these changes consisted in the replacement of n-6

AA derivatives with n-3 LC-PUFA derivatives. In addition, supplementation with EPA and DHA enhanced their respective downstream pathways of SPM biosynthesis, substantially increasing the intermediates 18-HEPE versus 17-HDHA and 14-HDHA, respectively. SPM, i.e. resolvins, remained unchanged or undetected, possibly due to concentrations below the detection limits of our assays. Low SPM detection in our participants with chronic inflammation may also be due to depressed SPM biosynthesis, the so-called "resolution deficit", as documented in animal models of atherosclerosis and several chronic inflammatory diseases (9,36,37). In that respect, the increases in DPA-derived SPMs $RvD5_{n\text{-}3\ DPA}$ and $MaR1_{n\text{-}3\ DPA}$ during DHA supplementation were unexpected given the lower DPA content in plasma phospholipids. The lower DPA content may be explained by enhanced retro-conversion from DPA to EPA and/or sparing of EPA from further conversion to DPA and DHA (38–40). Since EPA supplementation had no impact on DPA-derived SPM despite the higher plasma phospholipid DPA content, it is likely that DPA metabolism is less dependent on DPA availability and accelerated by other factors; we speculate that the activation of enzymatic metabolic pathways for DHA, shared with DPA, by DHA supplementation would drive DPA metabolism concomitantly.

As the plasma PUFA SPM lipidome was altered in a complex way by EPA and DHA supplementation, it was of great interest to take into consideration the combined effects of all lipid mediators. Moreover, even though several n-3 LC-PUFA studies have repetitively reported improvements in plasma concentrations of SPM intermediates (36,41,42), their bioactivities have been less investigated compared with SPM. Therefore, we have extracted key signatures of plasma PUFA SPM lipidome and examined how

they may have contributed to the differential impacts of EPA and DHA supplementation on monocyte-associated inflammation. Our results suggest that the anti-inflammatory effects of EPA and DHA supplementation on circulating monocytes may be partly due to the combined effect of different groups of plasma PUFA SPM lipid mediators. Specifically, the reduction in *TNFA* expression by EPA and DHA was mostly explained by EPA and/or AA derivatives, but not DHA/DPA derivatives. In animals, 18-HEPE, one of the most prominent EPA-derived mediators in PC3, has been shown to reduce macrophage TNF-α secretion (43), neutrophil infiltration (44), and macrophage-mediated maladaptive cardiac remodeling (45). In contrast, the *IL6* and *MCP1*-lowering effects of DHA appeared to be mediated by DHA/DPA and AA derivatives that were represented by PC1 and PC2, respectively. Anti-inflammatory actions of 17-HDHA (46) and DPA-derived $RvD5_{n\text{-}3\ DPA}$ and $MaR1_{n\text{-}3\ DPA}$ (29) have been reported *in vitro* and in animal models. Particularly in human B cells *in vitro*, 17-HDHA lowered the production of IL-6 and IL-10 but not TNF-α (47), which is in agreement with our results where PC1, representing mostly DHA-derived lipid mediators, partly explained the reductions in *IL-6* and *IL-10* not *TNFA*. The PC2-mediated monocyte *MCP1* suppression is also in accordance with the key role of AA products such as 12-HETE (48) and PGE_2 (49) in monocyte/macrophage migration via MCP-1.

Taken together, our results show that supplementation with EPA and DHA have distinct effects on *ex vivo* monocyte inflammatory response to LPS by differently regulating cytokine expression. DHA appeared more potent in inhibiting individual pro-inflammatory cytokines whereas EPA was more effective in balancing pro- and anti-inflammatory cytokines. The potential mediating effects of plasma PUFA SPM lipidome

signatures on these differential effects support the immunomodulatory actions of SPM and their intermediates in humans. More studies are needed to confirm the mediating effects of different subgroups of lipid mediators on different target genes. Our sample size was small and our assays had limited ability to detect some SPM. However, our findings add to the literature on the comparison between EPA and DHA actions on inflammation, which will further provide the mechanistic foundation for explaining the cardioprotective benefits of EPA from the recent JELIS and REDUCE-IT trials amid the n-3 LC-PUFA controversy.

2.6 References

1. Bang HO, Dyerberg J, Nielsen AB o. ndu. Plasma lipid and lipoprotein pattern in Greenlandic West-coast Eskimos. Lancet. 1971;297:1143–6.

2. Simopoulos AP. Omega-3 fatty acids in inflammation and autoimmune diseases. J Am Coll Nutr. 2002;21:495–505.

3. Calder PC. Long chain fatty acids and gene expression in inflammation and immunity. Curr Opin Clin Nutr Metab Care. 2013;16:425–33.

4. Calder PC. Marine omega-3 fatty acids and inflammatory processes: Effects, mechanisms and clinical relevance. Biochim Biophys Acta - Mol Cell Biol Lipids. 2015;1851:469–84.

5. Lo CJ, Chiu KC, Fu M, Lo R, Helton S. Fish oil decreases macrophage tumor necrosis factor gene transcription by altering the NFκB activity. J Surg Res. 1999;82:216–21.

6. Oh DY, Talukdar S, Bae EJ, Imamura T, Morinaga H, Fan W, Li P, Lu WJ, Watkins SM, Olefsky JM. GPR120 is an omega-3 fatty acid receptor mediating potent anti-inflammatory and insulin-sensitizing effects. Cell. 2010;142:687–98.

7. Forman BM, Chen J, Evans RM. Hypolipidemic drugs, polyunsaturated fatty acids, and eicosanoids are ligands for peroxisome proliferator-activated receptors α and δ. Proc Natl Acad Sci U S A. 1997;94:4312–7.

8. Martin H. Role of PPAR-gamma in inflammation. Prospects for therapeutic intervention by food components. Mutat Res - Fundam Mol Mech Mutagen. 2010;690:57–63.

9. Serhan CN. Pro-resolving lipid mediators are leads for resolution physiology. Nature. 2014;510:92–101.

10. Aung T, Halsey J, Kromhout D, Gerstein HC, Marchioli R, Tavazzi L, Geleijnse JM, Rauch B, Ness A, Galan P, et al. Associations of omega-3 fatty acid supplement use with cardiovascular disease risks meta-analysis of 10 trials involving 77 917 individuals. JAMA Cardiol. American Medical Association; 2018;3:225–34.

11. Yokoyama M, Origasa H, Matsuzaki M, Matsuzawa Y, Saito Y, Ishikawa Y, Oikawa S, Sasaki J, Hishida H, Itakura H, et al. Effects of eicosapentaenoic acid on major coronary events in hypercholesterolaemic patients (JELIS): a randomised open-label, blinded endpoint analysis. Lancet. 2007;369:1090–8.

12. Bhatt DL, Steg PG, Miller M, Brinton EA, Jacobson TA, Ketchum SB, Doyle RT,

Juliano RA, Jiao L, Granowitz C, et al. Cardiovascular risk reduction with icosapent ethyl for hypertriglyceridemia. N Engl J Med. 2019;380:11–22.

13. Mozaffarian D, Wu JHY. (n-3) Fatty Acids and Cardiovascular Health: Are Effects of EPA and DHA Shared or Complementary? J Nutr. 2012;142:614S-625S.

14. De Caterina R, Liao JK, Libby P. Fatty acid modulation of endothelial activation. Am J Clin Nutr. 2000;71.

15. Weldon SM, Mullen AC, Loscher CE, Hurley LA, Roche HM. Docosahexaenoic acid induces an anti-inflammatory profile in lipopolysaccharide-stimulated human THP-1 macrophages more effectively than eicosapentaenoic acid. J Nutr Biochem. 2007;18:250–8.

16. Allaire J, Couture P, Leclerc M, Charest A, Marin J, Lépine M-C, Talbot D, Tchernof A, Lamarche B. A randomized, crossover, head-to-head comparison of eicosapentaenoic acid and docosahexaenoic acid supplementation to reduce inflammation markers in men and women: the Comparing EPA to DHA (ComparED) Study. Am J Clin Nutr. 2016;104:280–7.

17. Tsunoda F, Lamon-Fava S, Asztalos BF, Iyer LK, Richardson K, Schaefer EJ. Effects of oral eicosapentaenoic acid versus docosahexaenoic acid on human peripheral blood mononuclear cell gene expression. Atherosclerosis. 2015;241:400–8.

18. Vors C, Allaire J, Marin J, Lépine MC, Charest A, Tchernof A, Couture P, Lamarche B. Inflammatory gene expression in whole blood cells after EPA vs. DHA supplementation: Results from the ComparED study. Atherosclerosis. 2017;257:116–22.

19. Schaefer EJ, Lichtenstein AH, Lamon-Fava S, Contois JH, Li Z, Goldin BR, Rasmussen H, McNamara JR, Ordovas JM. Effects of National Cholesterol Education Program Step 2 diets relatively high or relatively low in fish-derived fatty acids on plasma lipoproteins in middle-aged and elderly subjects. Am J Clin Nutr. 1996;63:234–41.

20. Moertl D, Hammer A, Steiner S, Hutuleac R, Vonbank K, Berger R. Dose-dependent effects of omega-3-polyunsaturated fatty acids on systolic left ventricular function, endothelial function, and markers of inflammation in chronic heart failure of nonischemic origin: A double-blind, placebo-controlled, 3-arm study. Am Heart J. 2011;161:915.e1-915.e9.

21. Zhao YT, Shao L, Teng LL, Hu B, Luo Y, Yu X, Zhang DF, Zhang H. Effects of n-3 polyunsaturated fatty acid therapy on plasma inflammatory markers and N-terminal pro-brain natriuretic peptide in elderly patients with chronic heart failure.

J Int Med Res. 2009;37:1831–41.

22. Wei MY, Jacobson TA. Effects of eicosapentaenoic acid versus docosahexaenoic acid on serum lipids: A systematic review and meta-analysis. Curr Atheroscler Rep. 2011;13:474–83.

23. Matthan NR, Ooi EM, Van Horn L, Neuhouser ML, Woodman R, Lichtenstein AH. Plasma phospholipid fatty acid biomarkers of dietary fat quality and endogenous metabolism predict coronary heart disease risk: a nested case-control study within the Women's Health Initiative observational study. J Am Heart Assoc. 2014;3.

24. Markworth JF, Kaur G, Miller EG, Larsen AE, Sinclair AJ, Maddipati KR, Cameron-Smith D. Divergent shifts in lipid mediator profile following supplementation with n-3 docosapentaenoic acid and eicosapentaenoic acid. FASEB J. 2016;30:3714–25.

25. Baron RM, Kenny DA. The Moderator-Mediator Variable Distinction in Social Psychological Research. Conceptual, Strategic, and Statistical Considerations. J Pers Soc Psychol. 1986;51:1173–82.

26. Shrout PE, Bolger N. Mediation in Experimental and Nonexperimental Studies: New Procedures and Recommendations. 2002;

27. Grundy SM, Cleeman JI, Daniels SR, Donato KA, Eckel RH, Franklin BA, Gordon DJ, Krauss RM, Savage PJ, Smith SC, et al. Diagnosis and management of the metabolic syndrome: An American Heart Association/National Heart, Lung, and Blood Institute scientific statement. Circulation. 2005;112:2735–52.

28. Alberti KGMM, Zimmet P, Shaw J. The metabolic syndrome - A new worldwide definition. Lancet. 2005;366:1059–62.

29. Dalli J, Colas RA, Serhan CN. Novel n-3 immunoresolvents: Structures and actions. Sci Rep. 2013;3.

30. Myhrstad MCW, Retterstøl K, Telle-Hansen VH, Ottestad I, Halvorsen B, Holven KB, Ulven SM. Effect of marine n-3 fatty acids on circulating inflammatory markers in healthy subjects and subjects with cardiovascular risk factors. Inflamm Res. 2011;60:309–19.

31. Rangel-Huerta OD, Aguilera CM, Mesa MD, Gil A. Omega-3 long-chain polyunsaturated fatty acids supplementation on inflammatory biomakers: a systematic review of randomised clinical trials. Br J Nutr. 2012;107:S159–70.

32. Serini S, Bizzarro A, Piccioni E, Fasano E, Rossi C, Lauria A, R.M. Cittadini A, Masullo C, Calviello G. EPA and DHA Differentially Affect In Vitro

Inflammatory Cytokine Release by Peripheral Blood Mononuclear Cells from Alzheimer's Patients. Curr Alzheimer Res. 2012;9:913–23.

33. Draper E, Reynolds CM, Canavan M, Mills KH, Loscher CE, Roche HM. Omega-3 fatty acids attenuate dendritic cell function via NF-κB independent of PPARγ. J Nutr Biochem. 2011;22:784–90.

34. Satoh-Asahara N, Shimatsu A, Sasaki Y, Nakaoka H, Himeno A, Tochiya M, Kono S, Takaya T, Ono K, Wada H, et al. Highly purified eicosapentaenoic acid increases interleukin-10 levels of peripheral blood monocytes in obese patients with dyslipidemia. Diabetes Care. 2012;

35. Schmitz G, Ecker J. The opposing effects of n-3 and n-6 fatty acids. Prog Lipid Res. 2008;47:147–55.

36. Grenon SM, Owens CD, Nosova E V, Hughes-Fulford M, Alley HF, Chong K, Perez S, Yen PK, Boscardin J, Hellmann J, et al. Short-Term, High-Dose Fish Oil Supplementation Increases the Production of Omega-3 Fatty Acid-Derived Mediators in Patients With Peripheral Artery Disease (the OMEGA-PAD I Trial). J Am Heart Assoc. 2015;4:e002034.

37. Merched AJ, Ko K, Gotlinger KH, Serhan CN, Chan L. Atherosclerosis: evidence for impairment of resolution of vascular inflammation governed by specific lipid mediators. FASEB J. 2008;22:3595–606.

38. Plourde M, Chouinard-Watkins R, Vandal M, Zhang Y, Lawrence P, Brenna JT, Cunnane SC. Plasma incorporation, apparent retroconversion and β-oxidation of 13C-docosahexaenoic acid in the elderly. Nutr Metab (Lond). 2011;8:5.

39. Miller E, Kaur G, Larsen A, Loh SP, Linderborg K, Weisinger HS, Turchini GM, Cameron-Smith D, Sinclair AJ. A short-term n-3 DPA supplementation study in humans. Eur J Nutr. 2013;52:895–904.

40. Metherel AH, Irfan M, Klingel SL, Mutch DM, Bazinet RP. Compound-specific isotope analysis reveals no retroconversion of DHA to EPA but substantial conversion of EPA to DHA following supplementation: a randomized control trial. Am J Clin Nutr. 2019;110:823–31.

41. Barden AE, Mas E, Croft KD, Phillips M, Mori TA. Specialized proresolving lipid mediators in humans with the metabolic syndrome after n–3 fatty acids and aspirin. Am J Clin Nutr. 2015;102:1357–64.

42. See VHL, Mas E, Prescott SL, Beilin LJ, Burrows S, Barden AE, Huang R-C, Mori TA. Effects of prenatal n -3 fatty acid supplementation on offspring resolvins at birth and 12 years of age: a double-blind, randomised controlled clinical trial. Br J Nutr. 2017;118:971–80.

43. Weylandt KH, Krause LF, Gomolka B, Chiu C-Y, Bilal S, Nadolny A, Waechter SF, Fischer A, Rothe M, Kang JX. Suppressed liver tumorigenesis in fat-1 mice with elevated omega-3 fatty acids is associated with increased omega-3 derived lipid mediators and reduced TNF-α. Carcinogenesis. 2011;32:897–903.

44. Krishnamurthy VR, Dougherty A, Haller CA, Chaikof EL. Total Synthesis and Bioactivity of 18(R)-Hydroxyeicosapentaenoic Acid. J Org Chem. 2011;76:5433–7.

45. Endo J, Sano M, Isobe Y, Fukuda K, Kang JX, Arai H, Arita M. 18-HEPE, an n-3 fatty acid metabolite released by macrophages, prevents pressure overload-induced maladaptive cardiac remodeling. J Exp Med. 2014;211:1673–87.

46. Neuhofer A, Zeyda M, Mascher D, Itariu BK, Murano I, Leitner L, Hochbrugger EE, Fraisl P, Cinti S, Serhan CN, et al. Impaired local production of proresolving lipid mediators in obesity and 17-HDHA as a potential treatment for obesity-associated inflammation. Diabetes. American Diabetes Association; 2013;62:1945–56.

47. Ramon S, Gao F, Serhan CN, Phipps RP. Specialized Proresolving Mediators Enhance Human B Cell Differentiation to Antibody-Secreting Cells. J Immunol. The American Association of Immunologists; 2012;189:1036–42.

48. Wen Y, Gu J, Vandenhoff GE, Liu X, Nadler JL. Role of 12/15-lipoxygenase in the expression of MCP-1 in mouse macrophages. Am J Physiol Hear Circ Physiol. 2008;294:1933–8.

49. Osma-Garcia IC, Punzón C, Fresno M, Díaz-Muñoz MD. Dose-dependent effects of prostaglandin E2 in macrophage adhesion and migration. Eur J Immunol. 2016;46:677–88.

50. Serhan CN. Pro-resolving lipid mediators are leads for resolution physiology. Nature. 2014;510:92–101.

2.7 Tables and Figures

Table 2.1 Characteristics of study participants at screening visit.

	Men (n=9)	Women (n=12)
Age, years	59 ± 2	64 ± 2
Body mass index, kg/m^2	32.2 ± 1.9	32.2 ± 2.2
Waist circumference, cm	111 ± 4	100 ± 4
Systolic blood pressure, mmHg	132 ± 3	128 ± 6
Diastolic blood pressure, mmHg	87 ± 3	73 ± 3
Hs-CRP†, μg/mL	4.7 [4.2, 7.4]	4.5 [3.7, 7.0]
Fasting TG, mg/dL	129 ± 10	155 ± 15
Fasting blood glucose, mg/dL	101 ± 4	99 ± 3

Data are presented as means ± SEM or †medians [25th, 75th]. Hs-CRP, high-sensitivity C-reactive protein; TG, triglycerides.

Table 2.2 Serum/plasma concentrations of inflammation-related markers and PUFA-derived lipid mediators at baseline and after supplementation with EPA and DHA.

	Baseline	EPA	ΔEPA[a]	DHA	ΔDHA[b]	*p*-ΔEPA vs. ΔDHA[c]
(A) Serum markers						
Pro-inflammatory markers						
Hs-CRP, μg/mL	2.82 [2.33, 3.70]	2.92 [1.92, 3.75]	0.16 [-0.89, 0.64]	2.80 [1.89, 4.38]	0.05 [-1.00, 0.73]	0.93
TNF-α, pg/mL	2.31 [2.04, 2.65]	2.46 [2.00, 2.90]	0.07 [-0.08, 0.22]	2.35 [1.97, 2.89]	-0.03 [-0.09, 0.12]	0.83
IL-6, pg/mL	0.76 [0.60, 1.13]	0.85 [0.62, 1.35]	0.07 [-0.04, 0.18]	0.77 [0.59, 1.00]	-0.03 [-0.06, 0.02]	0.44
MCP-1[d], pg/mL	302 [244, 331]	274 [232, 300]	11 [-31, 27]	309 [255, 351]	7 [-17, 36]	0.98
Anti-inflammatory marker						
IL-10, pg/mL	0.30 [0.23, 0.40]	0.30 [0.25, 0.43]	0.03 [-0.02, 0.08]	0.31 [0.26, 0.45]	-0.02 [-0.04, 0.05]	0.75
(B) Plasma PUFA-derived lipid mediators, pg/mL						
AA derivatives						
12S-HHTrE	16 [9, 24]	10 [8, 20]	-3 [-12, 4]*	10 [5, 14]	-6 [-14, 4]†	0.32
15-HETE[d]	289 [243, 368]	219 [156, 268]	-110 [-142, -46]**	228 [207, 283]	-54 [-138, 37]	0.08
12-HETE	2580 [1472, 3939]	1680 [1071, 4601]	164 [-1505, 1021]	1700 [1107, 2246]	-87 [-2025, 197]	0.37
11-HETE	255 [222, 467]	283 [196, 320]	-49 [-130, 24]*	240 [191, 310]	-64 [-173, -1]**	0.71
5-HETE	270 [242, 355]	249 [196, 290]	-50 [-110, -10]***	252 [194, 311]	-59 [-104, -31]***	0.82
PGD_2	45 [24, 54]	49 [32, 51]	0 [-19, 23]	32 [5, 37]	-6 [-42, 12]*	**0.027**
PGE_2	126 [80, 204]	111 [61, 154]	-14 [-86, 21]	69 [38, 118]	-46 [-81, 6]***	**<0.05**
TXB_2	439 [213, 677]	293 [227, 568]	-9 [-351, 199]	247 [137, 396]	-95 [-402, 91]*	0.23
LXB4	10 [5, 13]	10 [7, 15]	1 [-4, 6]	9 [7, 14]	1 [-5, 3]	0.96
EPA derivatives						
18-HEPE	26 [21, 32]	128 [82, 170]	103 [64, 143]***	58 [48, 70]	31 [19, 40]***	**<0.001**
15S-HEPE	22 [19, 33]	85 [68, 132]	71 [37, 98]***	39 [30, 52]	15 [3, 28]***	**<0.001**

12-HEPE	131 [91, 231]	730 [581, 1599]	584 [389, 1444]***	227 [148, 318]	93 [-45, 194]*	**<0.001**
11-HEPE	8 [5, 12]	52 [25, 68]	46 [20, 59]***	17 [14, 23]	9 [6, 11]***	**<0.001**
5-HEPE	38 [27, 57]	133 [98, 166]	72 [48, 121]***	103 [78, 135]	57 [32, 88]***	0.17
RvEs	ND	ND	-	ND	-	-
DPA derivatives						
$RvD5_{n\text{-}3\ DPA}$	17 [13, 21]	17 [15, 24]	2 [-3, 4]	40 [30, 55]	25 [15, 37]***	**<0.001**
$MaR1_{n\text{-}3\ DPA}$	4 [2, 5]	4 [3, 5]	0 [-2, 0]	10 [7, 12]	7 [4, 9]***	**<0.001**
DHA derivatives						
17-HDHA[d]	15 [10, 22]	13 [10, 15]	-2 [-9, 1]	49 [35, 83]	40 [24, 62]***	**<0.001**
14-HDHA	271 [208, 388]	323 [150, 525]	86 [-24, 179]	866 [614, 1251]	602 [242, 1005]***	**0.001**
13-HDHA	35 [25, 43]	40 [36, 58]	7 [2, 15]	143 [90, 167]	93 [55, 136]***	**<0.001**
7-HDHA	14 [11, 18]	16 [14, 21]	3 [0, 6]*	64 [40, 80]	48 [26, 58]***	**<0.001**
4-HDHA	62 [46, 90]	73 [55, 88]	12 [-1, 20]	250 [155, 299]	178 [93, 201]***	**<0.001**
RvD1	80 [42, 128]	78 [41, 115]	4 [-28, 31]	59 [47, 114]	-2 [-35, 25]	0.33

Data are presented as unadjusted medians [25^{th}, 75^{th}]. Data were log-transformed before analysis. AA, arachidonic acid; DPA, docosapentaenoic acid; HDHA, hydroxy-DHA; HEPE, hydroxy-EPA; HETE, hydroxy-eicosatetraenoic acid; HHTrE, hydroxy-heptadecatrienoic acid; Hs-CRP, High-sensitivity C-reactive protein; IL, interleukin; LXB4, lipoxin B4; $MaR1_{n\text{-}3\ DPA}$, maresin $1_{n\text{-}3\ DPA}$; MCP-1, monocyte chemoattractant protein 1; ND, not detected; PG, prostaglandin; RvD1, resolvin D1; $RvD5_{n\text{-}3\ DPA}$, resolvin $D5_{n\text{-}3\ DPA}$; RvEs, E-series resolvins; TNF-α, tumor necrosis factor-alpha; TXB_2, thromboxane B_2.

[a] ΔEPA = EPA – baseline, * p <0.05; ** p <0.01; *** p <0.001.

[b] ΔDHA = DHA – baseline, * p <0.05; * p <0.01; *** p <0.001.

[c] p value, comparison between ΔEPA and ΔDHA.

[d] Due to the significant sequence effect (p=0.0195 for serum MCP-1; p=0.0349 for plasma 15-HETE; and p=0.0237 for plasma 17-HDHA), data only from the first supplementation period were analyzed. Significant difference between ΔEPA and ΔDHA was tested using ANCOVA with the baseline concentrations as a covariate. Significance of ΔEPA or ΔDHA was tested using paired t tests.

Table 2.3 Mediation of plasma PUFA SPM profiles on monocyte cytokine mRNA expression.

	PC1[a] *DHA/DPA derivatives*		PC2[b] *AA derivatives*		PC3[c] *EPA derivatives*	
	EPA supplementation (Δ = +1.81)***	DHA supplementation (Δ = +4.13)***	EPA supplementation (Δ = +0.09)	DHA supplementation (Δ = +2.97)***	EPA supplementation (Δ = -3.69)***	DHA supplementation (Δ = -0.64)***
TNFA	-	-	-	19%	44%	8%
IL6	-	39%	-	-	-	-
MCP1	-	-	-	67%	-	-
IL10	-	7%	-	28%	-	-

Data are presented as % of total effect of EPA/DHA supplementation that was mediated by each principal component of plasma PUFA-derived lipid mediator profiles (PC1, PC2, or PC3). The presence of potential mediation by each PC was determined using sequential linear mixed models if the significant effect of EPA or DHA supplementation on the cytokine mRNA expression was weakened or abolished with the adjustment for the PC as covariate. The amount of mediation was estimated by the comparison between β coefficients for the supplementation effect before and after the adjustment, and calculated as a percentage of the total effect that was mediated by the PC. Significant changes in PC scores during either supplementation compared to baseline, *** $p \leq 0.001$. *IL*, Interleukin; *MCP1*, monocyte chemoattractant protein 1; PC, principal component; *TNFA*, tumor necrosis factor-alpha.

[a] The PC1 score was primarily influenced by plasma concentrations of DHA and DPA derivatives with positive associations.

[b] The PC2 score was primarily influenced by plasma concentrations of AA derivatives with negative associations.

[c] The PC3 score was primarily influenced by plasma concentrations of EPA derivatives with negative associations.

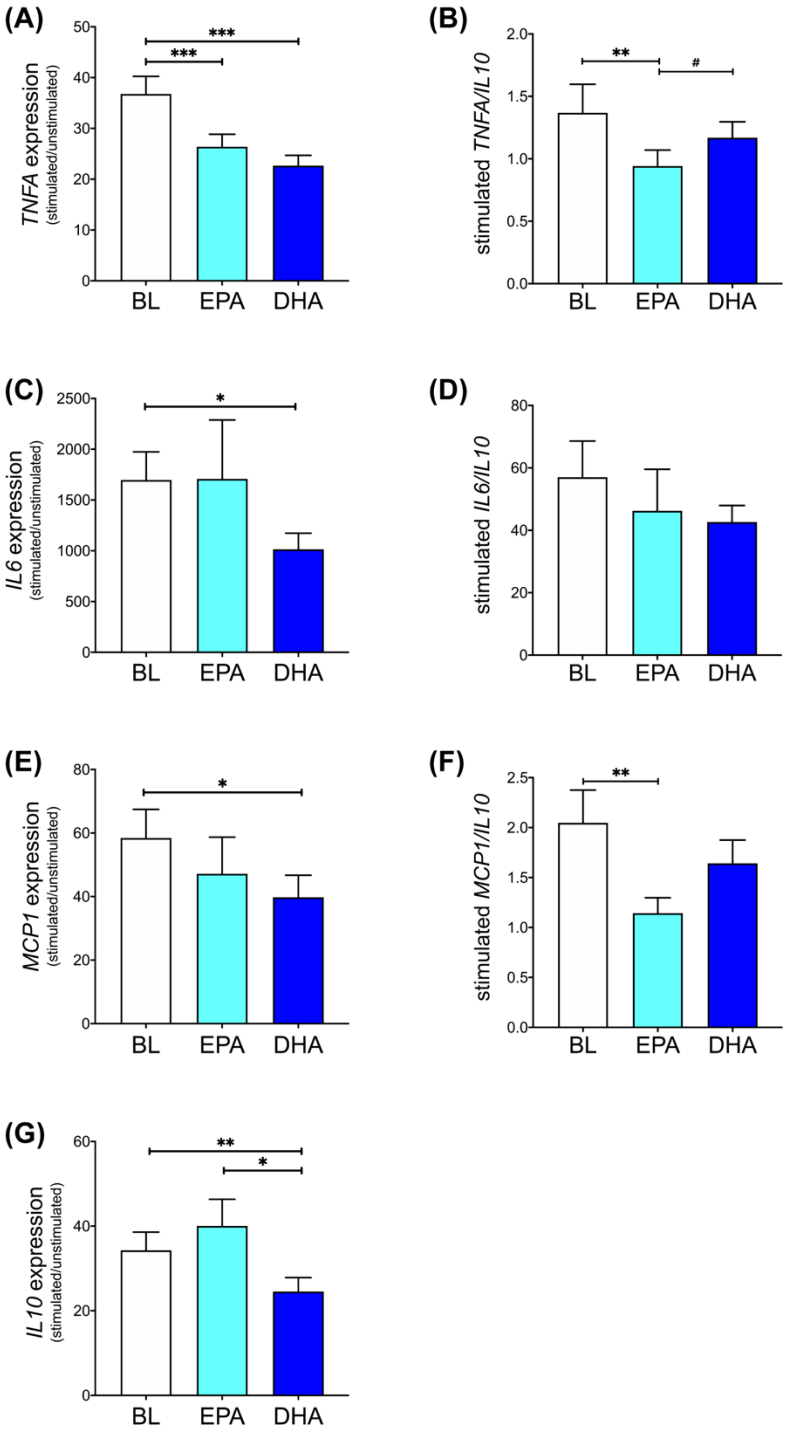


Figure 2.1. Differential regulation of cytokines by EPA and DHA in monocytes. **(A, C, E, G)** Monocyte gene expression of pro- and anti-inflammatory cytokines and **(B, D, F)** the ratios of each pro-inflammatory cytokine expression to anti-inflammatory *IL10* expression at baseline (BL) and after supplementation. Data are presented as means [SEM]. # p <0.06, * p <0.05, ** p <0.01, *** p <0.001. *IL*, interleukin; *MCP1*, monocyte chemoattractant protein 1; *TNFA*, tumor necrosis factor alpha

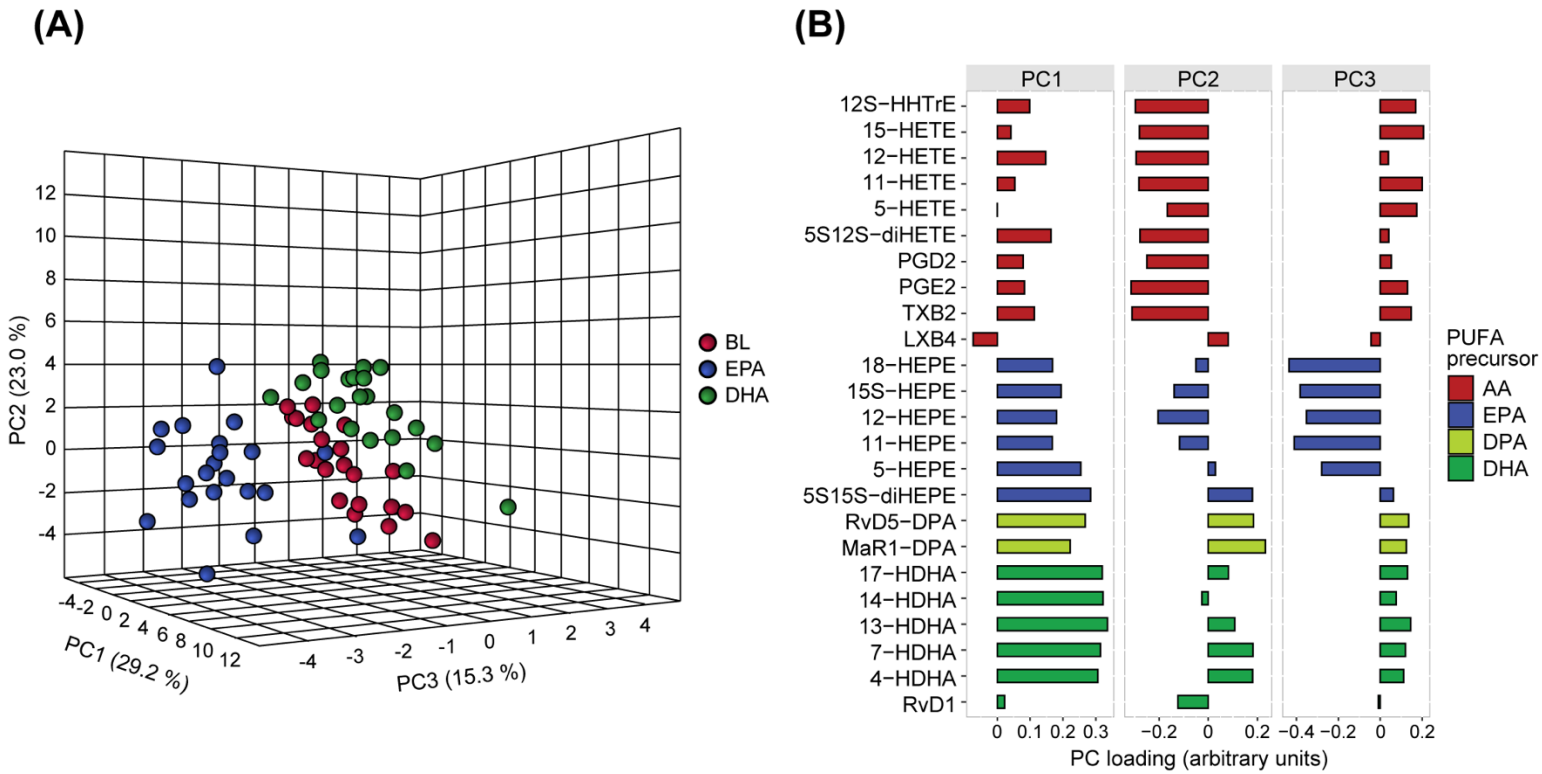


Figure 2.2. PCA of the plasma PUFA SPM lipidome at baseline (BL) and after supplementation with EPA and DHA. **(A)** 3-dimensional score plot. red, baseline; blue, EPA; green, DHA. **(B)** Loading scores of 24 lipid mediators in the top three identified components in the PCA.

2.8 Supplementary Material

Supplementary Table 2.1 Fatty acid profile (mol %) of plasma phospholipid at baseline and after supplementation with EPA and DHA.

	Baseline	EPA	ΔEPA[a]	DHA	ΔDHA[b]	*p*-ΔEPA vs. ΔDHA[c]
SFA	39.1 [38.3, 40.4]	40.0 [39.6, 40.3]	1.01 [0.59, 1.43]***	40.2 [39.6, 41.2]	0.82 [0.36, 2.01]***	0.39
10:0	0.02 [0.01, 0.02]	0.02 [0.01, 0.02]	0 [-0.004, 0.004]	0.02 [0.01, 0.02]	0.001 [-0.002, 0.004]	0.32
12:0	0.08 [0.05, 0.10]	0.08 [0.04, 0.09]	-0.003 [-0.012, 0.007]	0.08 [0.03, 0.10]	-0.004 [-0.012, 0.001]	0.83
14:0[d]	0.48 [0.44, 0.57]	0.54 [0.46, 0.58]	0.02 [-0.03, 0.11]	0.43 [0.34, 0.46]	-0.06 [-0.15, -0.03]**	**0.018**
15:0	0.25 [0.21, 0.3]	0.24 [0.19, 0.27]	0 [-0.03, 0.01]	0.24 [0.21, 0.31]	0.01 [-0.01, 0.02]	0.055
16:0	34.7 [33.9, 35.7]	35.6 [35.0, 35.9]	0.92 [0.54, 1.36]***	35.5 [35.1, 36.8]	0.75 [0.33, 1.5]***	0.54
18:0	1.67 [1.57, 1.77]	1.69 [1.63, 1.75]	0.05 [-0.05, 0.08]	1.62 [1.56, 1.71]	-0.07 [-0.09, 0.05]*	**0.017**
20:0	0.33 [0.31, 0.36]	0.35 [0.34, 0.38]	0.02 [0, 0.05]***	0.38 [0.35, 0.40]	0.05 [0.02, 0.06]***	**0.029**
22:0	0.93 [0.82, 1.05]	0.94 [0.85, 0.97]	0.02 [-0.11, 0.11]	1.00 [0.92, 1.10]	0.1 [0.01, 0.18]**	**0.033**
24:0	0.67 [0.62, 0.75]	0.71 [0.65, 0.75]	0 [-0.02, 0.08]	0.77 [0.67, 0.80]	0.08 [0.02, 0.14]**	0.27
MUFA	13.5 [12.7, 14.3]	12.4 [12.0, 13.4]	-1.17 [-1.75, -0.54]***	12.1 [11.7, 13.1]	-1.26 [-1.74, -0.53]***	0.92
16:1 n-9	0.16 [0.14, 0.17]	0.13 [0.10, 0.16]	-0.02 [-0.04, -0.01]***	0.13 [0.11, 0.15]	-0.03 [-0.03, -0.01]***	0.67
16:1 n-7[d]	0.70 [0.51, 0.77]	0.72 [0.57, 0.75]	-0.001 [-0.09, 0.04]	0.46 [0.40, 0.62]	-0.14 [-0.19, -0.06]**	**<0.001**
18:1 n-9	9.98 [9.0, 10.90]	8.99 [8.38, 9.89]	-0.99 [-1.91, -0.32]***	8.65 [8.46, 9.42]	-1.16 [-1.55, -0.39]***	0.94
18:1 n-7	1.39 [1.30, 1.54]	1.38 [1.25, 1.51]	-0.04 [-0.12, 0.10]	1.37 [1.24, 1.47]	-0.04 [-0.14, 0.06]	0.50
20:1 n-9[d]	0.11 [0.10, 0.13]	0.10 [0.09, 0.11]	-0.018 [-0.02, -0.01]**	0.10 [0.09, 0.13]	-0.013 [-0.02, 0.01]	0.054
22:1 n-9	0.01 [0.01, 0.01]	0.01 [0.01, 0.01]	-0.002 [-0.003, 0]	0.01 [0.01, 0.01]	-0.002 [-0.004, 0.001]*	0.80
24:1 n-9	1.03 [0.87, 1.25]	1.11 [0.95, 1.21]	0.05 [-0.03, 0.19]	1.23 [1.02, 1.31]	0.121 [0.05, 0.19]**	**0.035**

n-6 PUFA	41.3 [40.4, 42.2]	35.6 [33.4, 37.2]	-6.28 [-7.36, -3.74]***	36.2 [34.6, 38.0]	-5.89 [-6.58, -3.62]***	0.09
18:2 n-6 LA	24.3 [21.9, 25.9]	22.0 [18.7, 23.9]	-2.78 [-4.09, -1.47]***	23.4 [21.5, 25.7]	-1.44 [-2.51, 0.28]*	**0.014**
18:3 n-6	0.10 [0.08, 0.16]	0.05 [0.04, 0.10]	-0.05 [-0.08, -0.01]***	0.05 [0.03, 0.08]	-0.05 [-0.08, -0.03]***	0.11
20:2 n-6	0.29 [0.28, 0.34]	0.26 [0.23, 0.29]	-0.06 [-0.07, -0.03]***	0.27 [0.24, 0.32]	-0.03 [-0.07, 0]**	**0.037**
20:3 n-6	3.64 [3.04, 4.14]	2.54 [1.98, 3.07]	-1.12 [-1.36, -0.50]***	2.45 [2.26, 3.11]	-1.03 [-1.30, -0.45]***	0.42
20:4 n-6 AA	11.5 [9.50, 13.8]	10.5 [9.50, 11.1]	-1.20 [-2.57, -0.41]**	9.40 [8.50, 10.7]	-1.95 [-2.94, -1.05]***	**0.010**
22:2 n-6	0.010 [0.01, 0.01]	0.008 [0.01, 0.01]	-0.001 [-0.003, 0]*	0.010 [0.01, 0.01]	0 [-0.001, 0.001]	**0.007**
22:4 n-6	0.40 [0.32, 0.44]	0.22 [0.19, 0.28]	-0.16 [-0.22, -0.13]***	0.18 [0.16, 0.22]	-0.20 [-0.27, -0.16]***	**<0.001**
22:5 n-6	0.28 [0.23, 0.34]	0.14 [0.11, 0.17]	-0.14 [-0.22, -0.09]***	0.10 [0.08, 0.12]	-0.16 [-0.26, -0.12]***	**<0.001**
n-3 PUFA	4.91 [4.56, 5.48]	11.4 [9.61, 13.6]	6.47 [5.28, 7.82]***	10.4 [9.38, 10.6]	5.64 [4.51, 6.14]***	**0.036**
18:3 n-3 ALA	0.24 [0.21, 0.27]	0.24 [0.21, 0.27]	0 [-0.06, 0.06]*	0.24 [0.17, 0.32]	0 [-0.10, 0.03]	0.98
18:4 n-3 SDA[d,e]	0.12 [0.10, 0.15]	0.13 [0.10, 0.16]	-0.005 [-0.01, 0.05]	0.10 [0.09, 0.13]	-0.02 [-0.05, -0.01]*	0.054
20:5 n-3 EPA	0.72 [0.57, 0.94]	5.34 [4.75, 7.51]	4.67 [3.66, 6.32]***	1.55 [1.16, 2.02]	0.80 [0.43, 1.27]***	**<0.001**
22:5 n-3 DPA	0.86 [0.82, 0.98]	2.10 [1.79, 2.52]	1.35 [0.89, 1.65]***	0.59 [0.51, 0.65]	-0.29 [-0.36, -0.21]***	**<0.001**
22:6 n-3 DHA	2.80 [2.58, 3.22]	3.08 [2.50, 3.59]	0.36 [-0.24, 0.78]	7.66 [6.69, 8.06]	4.77 [4.21, 5.62]***	**<0.001**
Trans	0.78 [0.68, 0.95]	0.78 [0.66, 0.87]	-0.04 [-0.16, 0.02]	0.82 [0.66, 0.96]	-0.01 [-0.14, 0.09]	0.26
16:1 n-9t	0.04 [0.03, 0.04]	0.03 [0.03, 0.04]	-0.004 [-0.007, 0]††	0.03 [0.03, 0.04]	-0.01 [-0.01, -0.004]***	0.72
16:1 n-7t	0.22 [0.20, 0.24]	0.21 [0.16, 0.24]	-0.001 [-0.02, 0.02]	0.23 [0.18, 0.27]	0 [-0.024, 0.03]	0.46
18:1 n-9t	0.19 [0.16, 0.25]	0.21 [0.17, 0.22]	0.013 [-0.03, 0.04]	0.19 [0.16, 0.22]	-0.008 [-0.06, 0.025]	0.66
18:1 n-7t	0.16 [0.13, 0.2]	0.16 [0.12, 0.20]	-0.01 [-0.03, 0.02]	0.19 [0.15, 0.24]	0.021 [-0.04, 0.062]	0.057
18:1 n-12t	0.12 [0.10, 0.15]	0.11 [0.09, 0.14]	-0.007 [-0.02, 0.003]	0.12 [0.10, 0.14]	-0.005 [-0.016, 0.01]	0.20
18:2 t[d]	0.03 [0.02, 0.03]	0.02 [0.02, 0.02]	-0.004 [-0.01, -0.001]	0.02 [0.02, 0.02]	-0.01 [-0.02, -0.002]**	0.36
18:2 CLA[e]	0.02 [0.01, 0.02]	0.02 [0.01, 0.02]	0.001 [-0.007, 0.005]	0.02 [0.01, 0.02]	0.001 [-0.006, 0.005]	0.87

Data are presented as unadjusted median [25th, 75th]. N = 21 for all phases. Data were log-transformed for the analysis due to the skewness of their distributions. AA, arachidonic acid; ALA, alpha-linolenic acid; CLA, conjugated linoleic acid; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; EPA, eicosapentaenoic acid; LA, linoleic acid; MUFA, monounsaturated fatty acids; n-3, omega 3; n-6, omega 6; PUFA, polyunsaturated fatty acids; SDA, stearidonic acid; SFA, saturated fatty acids; *trans*, trans fatty acids.

[a] ΔEPA = post EPA – baseline, * p <0.05; ** p <0.01; *** p <0.001.

[b] ΔDHA = post DHA – baseline, * p <0.05; ** p <0.01; *** p <0.001.

[c] p value of comparison between ΔEPA and ΔDHA.

[d] Due to the significant sequence effect, data only from the first supplementation period were analyzed. Significant difference between ΔEPA and ΔDHA was tested using ANCOVA with the baseline concentrations as a covariate. Significance of ΔEPA or ΔDHA was tested using paired *t* tests.

[e] N = 20 for baseline.

Supplementary Table 2.2 Matrices of β coefficients identified along the steps of determining potential mediation by PC on the effect of EPA or DHA supplementation on monocyte *ex vivo* cytokine gene expression.

	EPA/DHA → outcome [Total effect][a]	EPA/DHA → PC[b]	PC ↔ outcome[c]	EPA/DHA → outcome [Direct effect][d]
(A) EPA supplementation				
PC1				
TNFA	-0.341 (0.014)	1.810 (0.001)	-0.023 (0.523)	-0.384 (0.015)
IL6	-0.245 (0.423)	1.810 (0.001)	-0.022 (0.784)	-0.269 (0.445)
MCP1	-0.362 (0.123)	1.810 (0.001)	-0.028 (0.697)	-0.472 (0.107)
IL10	0.015 (0.927)	1.810 (0.001)	0.012 (0.809)	-0.011 (0.955)
PC2				
TNFA	-0.341 (0.014)	0.090 (0.875)	-0.042 (0.228)	-0.337 (0.014)
IL6	-0.245 (0.423)	0.090 (0.875)	0.003 (0.975)	-0.245 (0.429)
MCP1	-0.362 (0.123)	0.090 (0.875)	-0.093 (0.198)	-0.354 (0.136)
IL10	0.015 (0.927)	0.090 (0.875)	-0.001 (0.986)	0.015 (0.928)
PC3				
TNFA[e]	-0.341 (0.014)	-3.688 (<0.000)	0.079 (0.014)	-0.190 (0.448)
IL6	-0.245 (0.423)	-3.688 (<0.000)	0.124 (0.085)	0.802 (0.171)
MCP1	-0.362 (0.123)	-3.688 (<0.000)	0.101 (0.071)	0.059 (0.908)
IL10	0.015 (0.927)	-3.688 (<0.000)	0.008 (0.844)	0.210 (0.566)
(B) DHA supplementation				
PC1				
TNFA	-0.481 (0.0001)	4.127 (<0.0001)	-0.061 (0.011)	-0.516 (0.003)
IL6[e]	-0.507 (0.050)	4.127 (<0.0001)	-0.089 (0.058)	-0.311 (0.407)
MCP1	-0.457 (0.033)	4.127 (<0.0001)	-0.062 (0.120)	-0.505 (0.132)

IL10[e]	-0.403 (0.013)	4.127 (<0.0001)	-0.057 (0.052)	-0.373 (0.108)
PC2				
TNFA[e]	-0.481 (0.0001)	2.975 (<0.0001)	-0.100 (0.001)	-0.389 (0.010)
IL6	-0.507 (0.050)	2.975 (<0.0001)	-0.091 (0.133)	-0.470 (0.186)
MCP1[e]	-0.457 (0.033)	2.975 (<0.0001)	-0.131 (0.014)	-0.152 (0.609)
IL10[e]	-0.403 (0.013)	2.975 (<0.0001)	-0.084 (0.027)	-0.290 (0.174)
PC3				
TNFA[e]	-0.481 (0.0001)	-0.643 (0.001)	0.175 (0.045)	-0.441 (0.001)
IL6	-0.507 (0.050)	-0.643 (0.001)	0.269 (0.140)	-0.419 (0.142)
MCP1	-0.457 (0.033)	-0.643 (0.001)	0.284 (0.100)	-0.372 (0.128)
IL10	-0.403 (0.013)	-0.643 (0.001)	0.170 (0.128)	-0.364 (0.042)

Data are presented as β coefficients (*p* values). Outcome variables were log-transformed before analysis. *IL*, interleukin; *MCP1*, monocyte chemoattractant protein 1; PC, principal component; *TNFA*, tumor necrosis factor alpha.

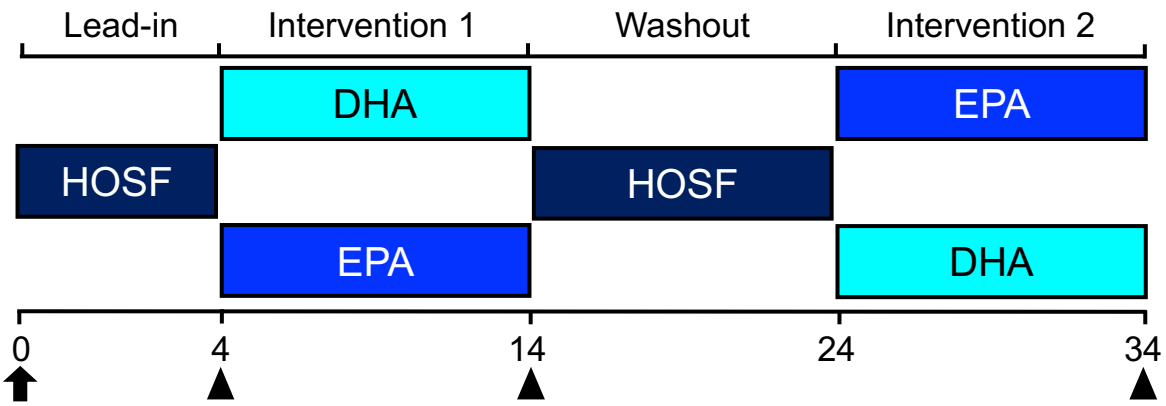
[a] The effect of EPA/DHA supplementation on the outcome (i.e. total effect).

[b] The effect of EPA/DHA supplementation on the PC.

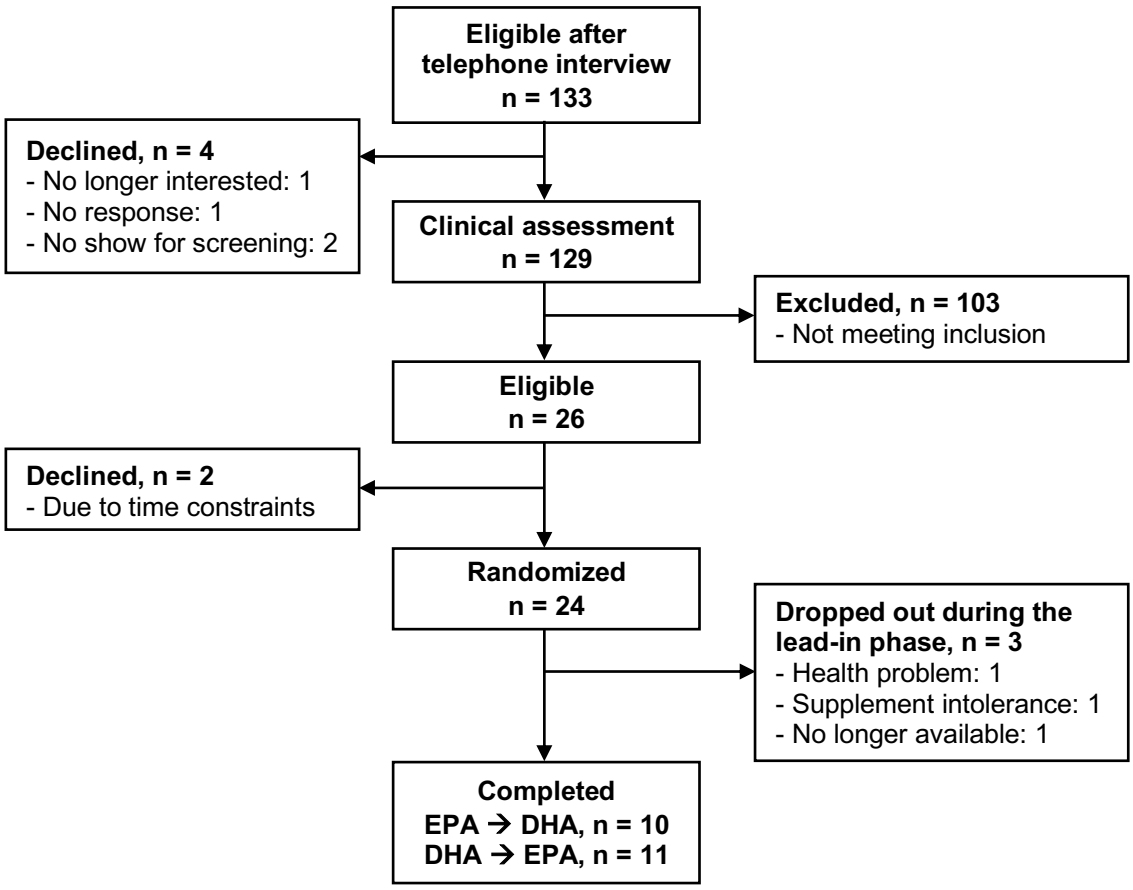
[c] The association between the PC and the outcome.

[d] The effect of EPA/DHA supplementation on the outcome with adjustment for the PC (i.e. direct effect).

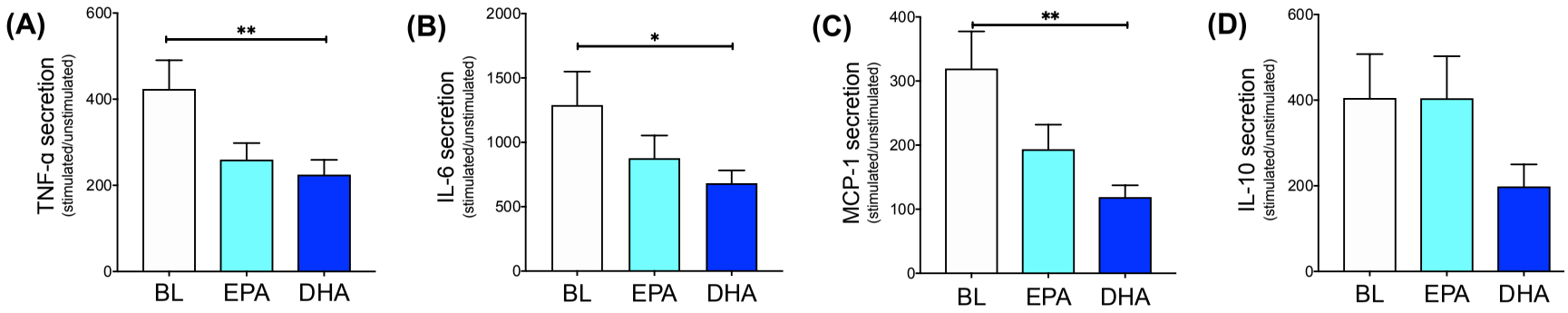
[e] Potential presence of mediating effect (i.e. indirect effect) of the PC on the effect of EPA/DHA supplementation on the outcome.



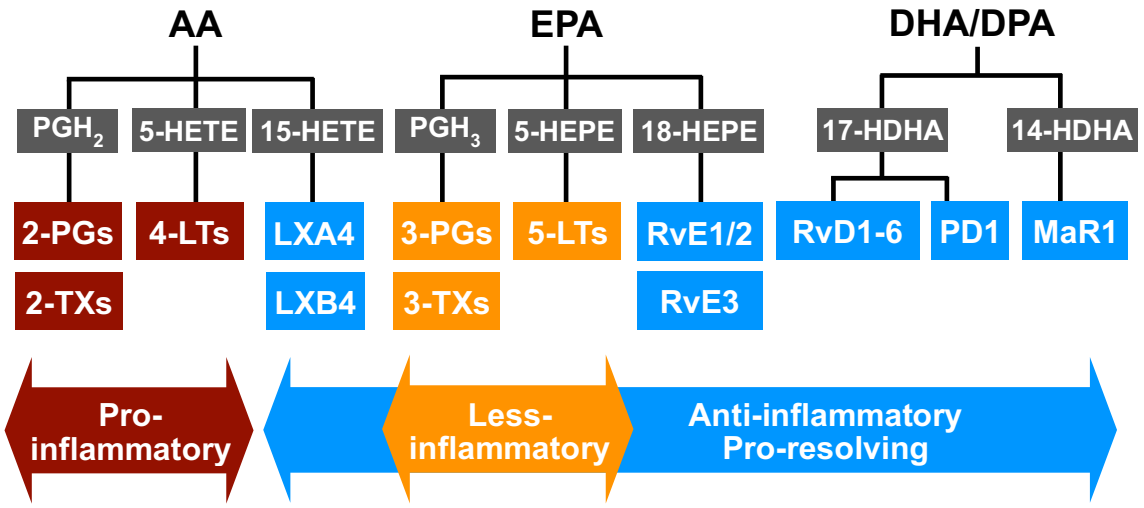
Supplementary Figure 2.1. Study design.
The black arrow represents the time point of subject randomization while the black triangles represent time points of blood collection. HOSF, high oleic acid sunflower oil.



Supplementary Figure 2.2. The Consolidated Standards of Reporting Trials (CONSORT) chart of study participants.



Supplementary Figure 2.3. Differential effects of EPA and DHA on *ex vivo* cytokine production in LPS-stimulated monocytes. Monocyte secretion of pro-inflammatory cytokines **(A)** TNF-α, **(B)** IL-6, and **(C)** MCP-1 and **(D)** anti-inflammatory cytokine IL-10 at baseline (BL) and after supplementation. Data are presented as means [SEM]. ** p <0.01. IL, interleukin; MCP-1, monocyte chemoattractant protein 1; TNF-α, tumor necrosis factor-alpha.



Supplementary Figure 2.4. Overview of the biosynthetic pathways of eicosanoids and SPMs derived from four different PUFAs – AA, EPA, DHA, and DPA.
Figure generated from our interpretation of multiple reviews and research articles (4,29,50). As for the DPA-derived resolvins, only $RvD1_{n\text{-}3\ DPA}$, $RvD2_{n\text{-}3\ DPA}$ and $RvD5_{n\text{-}3\ DPA}$ have been described. Colors indicate known biologic characteristics: red, pro-inflammatory; orange, less-inflammatory; blue, anti-inflammatory and pro-resolving. AA, arachidonic acid; DPA, docosapentaenoic acid; HDHA, hydroxy-docosahexaenoic acid; HEPE, hydroxy-eicosapentaenoic acid; HETE, hydroxy-eicosatetraenoic acid; LT, leukotriene; LX, lipoxin; MaR1, maresin 1; PG, prostaglandin; PD1, protectin 1; RvD, D-series resolvin; RvE, E-series resolvin; SPM, specialized pro-resolving lipid mediators; TX, thromboxane.

Chapter 3 Sex Dimorphism of Monocyte Transcriptome

Sexual dimorphism of monocyte transcriptome in individuals with chronic low-grade inflammation

Jisun So[1], Albert K Tai[2], Alice H Lichtenstein[1], Dayong Wu[3], Stefania Lamon-Fava[1]*

Affiliations: [1]Cardiovascular Nutrition Laboratory, Jean Mayer USDA Human Nutrition Research Center on Aging at Tufts University, Boston, MA; [2]Department of Immunology, Tufts University School of Medicine, Boston, MA; [3]Nutritional Immunology Laboratory, Jean Mayer USDA Human Nutrition Research Center on Aging at Tufts University, Boston, MA

Short title: Transcriptional sex dimorphism in monocytes

***Corresponding Author:**
Stefania Lamon-Fava, MD PhD
Cardiovascular Nutrition Laboratory
Jean Mayer USDA Human Nutrition Research Center on Aging
Tufts University
711 Washington Street
Boston, MA 02111
Phone: +1 617 556 3105
E-mail: stefania.lamon-fava@tufts.edu

3.1 Abstract

Sexual dimorphism in the immune system is evidenced by a higher prevalence of autoimmune diseases in women and higher susceptibility to infectious diseases in men. However, the molecular basis of these sex-based differences is not fully understood. We have characterized the transcriptome profiles of peripheral blood monocytes from males and postmenopausal females with chronic low-grade inflammation. We identified 41 sexually differentially expressed genes [adjusted *p* value (FDR) <0.1], including genes involved in immune cell activation (e.g., *CEACAM1*, *FCGR2B*, and *SLAMF7*) and antigen presentation (e.g., *AIM2*, *CD1E*, and *UBA1*), with a higher expression in females than males. Moreover, signaling pathways of immune or inflammatory responses, including interferon (IFN) signaling [z-score=2.45, -log(*p*)=3.88], were found to be more upregulated in female versus male monocytes, based on a set of genes exhibiting sex-biased expression (*p* <0.03). The contribution of IFN signaling to the sexual transcriptional differences was further confirmed by direct comparisons of the monocyte sex-biased genes with IFN signature genes (ISGs) that were previously curated in mouse macrophages. ISGs showed a greater overlap with female-biased genes than male-biased genes and a higher overall expression in female than male monocytes, particularly for the genes of antiviral and inflammatory responses to IFN. Given the role of IFN in immune defense and autoimmunity, our results suggest that sexual dimorphism in immune functions may be associated with better priming of steady-state innate immune pathways in female than male monocytes. These findings highlight the role of sex on the human immune transcriptome.

3.2 Introduction

Sex dimorphism is one of the critical factors contributing to immunological variability among individuals and has been under-appreciated in the majority of immunology studies (1,2). Phenotypic differences in immune-related diseases between the sexes provide evidence of this dimorphism: while men have a higher susceptibility to a variety of pathogens leading to an increased frequency of infectious diseases (3), women have a higher rate of autoimmune diseases (4). Females, compared to males, show a stronger humoral and cell-mediated immunity (5,6), as demonstrated by higher levels of immunoglobulins (7) as well as stronger antibody responses to viral vaccines (8). Systemic lupus erythematosus (SLE), the autoimmune disease with the most striking female-biased prevalence (9), is characterized by overproduction of autoantibodies, resulting in inflammation and organ damage (10).

There are several hypotheses explaining the sex differences in immune function. Differences in sex steroid hormone concentrations (11) and sex chromosomes (12) play a prevailing role in genomic regulation of the immune system. Upon receptor binding, sex steroid hormones exert biological effects on immune cells by influencing signaling pathways such as nuclear factor kappa B (NF-κB), c-Jun, and interferon regulatory factors (IRF) in various lymphoid tissues as well as circulating immune cells (13). Genes unique to the Y chromosome or additional copies of X chromosome genes that escape X-inactivation result in differential gene expression between males and females (12). For example, some immune-related genes located on the X chromosome such as *TLR7* and *CD40LG* are expressed at higher levels in females than males (14,15), possibly as a result of imperfect X-silencing. Particularly, duplication of *TLR7* has been suggested as an

underlying mechanism for the higher susceptibility to SLE in mice (14). Also, sex-specific genetic polymorphisms on autosomal genes and epigenetic controls may contribute to sexual differential gene expression (2).

Transcriptome analyses have provided a foundation for a better understanding of the molecular basis of the phenotypic immunological differences between the sexes (16–19). Sexually differential gene expression has been documented in whole blood and peripheral blood mononuclear cells (PBMC) (20–23). However, due to limitations in the ability to control for different proportions of immune cell subsets between the sexes (21–23), there is an incomplete understanding of the transcriptional sex dimorphism manifested by different bloodborne cell types. Data from mouse microglial cells indicate only a limited number of sex chromosome genes differentially expressed between the sexes (24). In contrast, mouse bone marrow-derived macrophages (BMDM) displayed a moderate sex-dependent effect in a large number of genes (25). Using samples from the Immunological Genome Project (ImmGen, www.immgen.org), immune transcriptome profiling of 11 different murine immune cell types at baseline and after immune challenge demonstrated that only macrophages exhibited sex dimorphism (19). In particular, unstimulated macrophages showed a female-biased expression of interferon (IFN)-responsive genes that was further increased upon IFN stimulation, accompanied by stronger antiviral and inflammatory responses (19). The greater activation of IFN pathways upon stimulation in these cells suggests that females may be equipped with a more vigilant immune defense system that exerts a more vigorous immune response against external or internal challenges. However, considering the strong correlation between IFN overexpression and autoimmune diseases such as SLE (26), elevated expression of genes in these pathways in

females in the absence of a clear threat signal is suggested to be one of the major factors contributing to a higher prevalence of autoimmunity in females compared to males.

In light of the transcriptional sex dimorphism observed in mice, it is of interest to explore cell-type-specific sex effects on basal immune transcriptomes in humans. While conducting a study on the effects of omega-3 fatty acid supplementation on inflammation in individuals with chronic low-grade inflammation, we observed evidence of sex dimorphism in monocyte transcriptome. Therefore, the aim of this study, as a secondary analysis of the parent study, was to identify differentially expressed genes on the basis of sex in unstimulated human monocytes obtained from peripheral blood at baseline and to characterize the biological pathways, particularly immune-related pathways, associated with the transcriptional sex dimorphism.

3.3 Materials and Methods

Study participants

This study is part of a randomized, double-blind, crossover clinical trial (registered at ClinicalTrials.gov as NCT02670382) assessing the individual effects of eicosapentaenoic acid (EPA, 20:5 n-3) and docosahexaenoic acid (DHA, 22:5 n-3) on inflammation in individuals with chronic low-grade inflammation (manuscript in revision). Briefly, in this crossover study, after a 4-week lead-in control phase during which participants received 3 g/day of high oleic acid sunflower oil, they were randomly assigned to receive 3 g/day EPA or DHA supplementation for 10 weeks, followed by a 10-week washout phase before switching to the other supplementation for another 10 weeks. Data for the current study was obtained at the end of the lead-in control phase. Participant inclusion criteria

were: age 50–75 years, if women, postmenopausal, serum high-sensitivity C-reactive protein (hs-CRP) concentration ≥ 2 μg/mL, fasting plasma triglyceride concentration between 90 and 500 mg/dL, and in addition, having at least one of the following characteristics: abdominal obesity (waist circumference ≥ 102 cm in men and ≥ 89 cm in women), hypertension (blood pressure ≥ 130/80 mmHg or use of anti-hypertensive medications), or fasting plasma glucose concentrations ≥ 100 mg/dL, but otherwise healthy. Twenty-one participants completed the study, and 19 (9 men and 10 women) who had monocyte samples with high purity were included in the current study for determining the transcriptional sexual dimorphism in peripheral blood monocytes.

Blood collection, monocyte isolation, and RNA extraction

Venous blood was drawn after a 12-hour overnight fast in sodium citrate Vacutainer Cell Preparation Tubes (Beckton Dickinson, Franklin Lakes, NJ) for PBMC isolation. All subtypes of monocytes including classical ($CD14^{++}/CD16^{-}$), non-classical ($CD16^{++}/CD14^{+}$), and intermediate ($CD16^{+}/CD14^{++}$) were further isolated from the PBMC fraction by a negative selection method using antibody-coupled magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany). The flow-through cells were centrifuged at 300 x *g* for 10 min at 22 °C and stored at -80 °C until further analyses. Total RNA was isolated using QIAshredder and RNeasy Mini kit (both from Qiagen, Hilden, Germany) according to the manufacturer's instructions. Isolated RNA was treated on-column with RNase-free DNase (Qiagen, Hilden, Germany) to eliminate genomic DNA contamination. RNA quality was assessed using a Fragment Analyzer (Agilent, Santa Clara, CA).

RNA sequencing data generation, processing, and analysis

The extracted RNA samples that passed quality checks were used as input to prepare RNA-Seq library using Illumina TruSeq stranded mRNA kit (Illumina, San Diego, CA) per manufacturer instruction. Briefly, mRNA was enriched via polyA selection from input total RNA. Enriched mRNA was then fragmented, followed by first cDNA synthesis with random priming, and second-strand cDNA synthesis with dUTP. The 3' adenylates were added to the double-stranded cDNA, followed by adaptor ligation and second strand removal and amplification. The molar concentration and size distribution of resultant libraries were assessed on a Fragment Analyzer (Agilent, Santa Clara, CA). Libraries were sequenced on an Illumina HiSeq 2500 sequencer (Illumina, San Diego, CA) with High Output V4 chemist and 50 base-pair single-end reads format. Raw data in FASTQ format were processed for quality control using FastQC (www.bioinformatics.babraham.ac.uk/projects/fastqc/) and then mapped to the human genome (USC hg38) using HISAT v2.1 (www.ccb.jhu.edu/software/hisat/index.shtml). The mapped reads to genes were quantified by featureCounts (subread.sourceforge.net), and raw count data were normalized by the median of ratios method using DESeq2 package from Bioconductor (27). For principal component analysis (PCA) and heatmap presentation, the normalized counts were variance stabilized using a regularized log transformation.

Monocyte purity was assessed by cell-type-specific gene expression, which concluded the exclusion of two female participants who displayed abnormally high counts of *CD3* and *CD8* (markers for T cells), *CD19* (a marker for B cells), and *NCAM1* (a marker for

natural killer, or NK cells). The count data were re-normalized based on the remaining 19 samples.

Differential gene expression between the sexes was compared using Wald tests of the DEseq2 package followed by the Benjamini-Hochberg false discovery rate (FDR) correction for multiple comparisons (28). The difference in fold change was calculated as a ratio of expression values in females versus males and then $\log_2$ transformed. Genes with FDR of 0.1 or less were termed as differentially expressed genes. The analysis was performed using R 3.6.2.

Pathway analysis

To determine canonical signaling pathways that contained the differentially expressed genes, we performed a pathway analysis using Ingenuity Pathway Analysis (IPA; v 9.0, Mountain View, CA). The z-score, which is to infer the activation state of implicated biological pathway/function, was determined by the observed gene regulation ("up" or down") and a literature-derived direction of effect of the gene to the pathway ("activating" or "inhibiting"). Pathways with absolute |z-score| ≥ 1 and $p < 0.05$ (calculated by a right-tailed Fisher's Exact Test) were considered significant.

Interferon signature genes (ISGs)

Mostafavi et al. (29) charted the transcriptional responses induced by IFNα across a range of mouse immune cell lineages from the ImmGen and identified 975 ISGs that were upregulated in at least one cell type with > 2-fold increase and FDR <0.1. Of those, the genes that were significantly upregulated in macrophages were named MF-ISGs (19). To compare human monocyte sex-biased genes identified in the present study with the

mouse MF-ISGs, all mouse gene symbols of the MF-ISGs were translated to their human orthologs using ENSEMBL BioMart data mining tool (30). The fold change distribution of the human MF-ISG orthologs was compared to that of all other mapped genes by two-sided Welch's *t* test.

We also compared our monocyte sex-biased genes to the expanded list of 628 ISGs constituting an IFN transcriptional regulatory network that was computationally built based on 1,398 human and mouse datasets by Mostafavi et al. (29). The direction of fold changes of the ISGs from our monocytes was tested by a one-sample two-sided *t* test with the Bonferroni correction (31).

3.4 Results

Participant characteristics

Consistent with the eligibility criteria, study participants had chronic low-grade inflammation as indicated by a median hs-CRP concentration greater than 3 μg/mL (Table 3.1). Participant characteristics and measures of inflammation were similar between females and males, with the exception of diastolic blood pressure which was lower in females than in males (two-sided *t* test $p < 0.02$). Similarly, monocytes, as a percent of total leukocytes in peripheral blood, were similar between the sexes.

Monocyte-specific sex signature genes

Monocyte transcriptional sex dimorphism was clearly observed, as shown in the separation of the samples based on sex in the PCA plot (Fig. 3.1A). Of the 41 sexually differentially expressed genes identified, the expression of approximately half (22 genes, 54%) was higher in females than males. Of the 22 genes, 7 were autosomal and 15 genes

were X-linked. These genes are involved in immune cell activation (*CEACAM1*, *FCGR2B*, and *SLAMF7*) and antigen-recognition or presentation (*AIM2*, *CD1E*, and *UBA1*), suggesting enhanced activation of innate and adaptive immune responses in monocytes from females compared to males. Expression of 19 genes (46%) was higher in males than females. These included 3 autosomal genes (*SERPINB2*, *BNIP3*, and *EBPL*) and 16 Y-linked genes. The overall median absolute $\log_2$ fold difference in females versus males was 1.38 (Fig. 3.1B, Supplementary Table 3.1).

Increased activation of immune responses in monocytes

To gain further insight into the biological pathways that are associated with the transcriptional sexual dimorphism in monocytes, we selected genes that were significant at $p < 0.03$ for the sexual difference (360 female-biased and 205 male-biased genes; Fig. 3.1C, Online Appendix 1) and mapped them to the canonical pathways of the IPA database. Out of the 11 canonical pathways that were significantly upregulated in females (z-score ≥1, -log(p) >1.30 by Fisher's exact test), most pathways (8/11, 73%) were involved in immune or inflammatory responses (Table 3.2). IFN signaling was the top (z-score=2.45, -log(p)=3.88) among the 11 female-biased pathways, consistent with its documented association with female-biased autoimmunity (23,32). Genes involved in IFN signaling (IFN-induced protein with tetratricopeptide repeats [*IFIT*] 1-3, IFN-induced transmembrane protein 1 [*IFITM1*], Janus kinase 2 [*JAK2*], signal transducer and activator of transcription 1 [*STAT1*]), antigen-presentation (CD1 molecules [*CD1B, CD1C*], major histocompatibility complex, class II, DO alpha [*HLA-DOA*], major histocompatibility complex class II, DR beta 5 [*HLA-DRB5*]), and immune cell activation (C-C motif chemokine receptor 5 [*CCR5*], CD274 molecule [*CD274*], CD40 molecule

[*CD40*], Fc fragment of IgG receptor IIb [*FCGR2B*]) were principally implicated in the other significant pathways (i.e., T helper type 1 (Th1) pathway, dendritic cell maturation, crosstalk between dendritic cells and NK cells, and SLE in B cell signaling pathway) as well. In addition to the 11 significant female-biased pathways, we identified two male-biased pathways with borderline significance: pathways related to programmed cell death protein 1 (PD-1), programmed cell death-ligand1 (PD-L1) cancer immunotherapy (z-score=-0.82, -log(p)=2.01) and p53 signaling (z-score=-0.45, -log(p)=1.64).

Comparison of IFN signature and regulatory pathways

Immune transcriptional sex dimorphism has been documented in unstimulated mouse macrophages, showing higher IFN responsiveness in females (19). Based on this evidence and our pathway analysis results, we sought to further examine how the IFN pathways differ between males and females in human monocyte transcriptome. To this end, we first compared the monocyte sex-biased genes with p <0.03 to a set of genes that were recently demonstrated to be upregulated by IFN in mouse macrophages (i.e., MF-ISGs, 601 genes). Of the 498 out of 601 MF-ISGs that have human orthologs, 485 genes were expressed in our monocyte samples (Online Appendix 2). Out of the 360 female-biased genes in monocytes, 48 were also MF-ISGs (13.3%). In contrast, only 6 of the 205 (2.9%) male-biased genes were MF-ISGs (Fig. 3.2, Supplementary Table 3.2). Next, we assessed the expression of the entire set of MF-ISG human orthologs in our monocytes. The $\log_2$ fold change distribution of MF-ISGs was skewed toward females (mean = 0.09) and was significantly different from the symmetrical distribution of all other genes (20,602 genes, mean = 0.003) (two-sided t test $p = 2.9 \times 10^{-4}$; Fig. 3.3). Consistent with

the pathway analysis, these results suggest that upregulation of IFN-response genes is more frequently observed in female than male monocytes under unstimulated conditions.

Despite conserved transcriptomic signatures of immune cell lineages between human and mouse, specific differences exist due to divergent evolutionary paths (33). Moreover, monocytes have limited contribution to tissue-resident macrophages (34). Thus, we applied a larger set of genes (623 genes), instead of MF-ISGs, that had been validated in various immune cells both from mouse and human and that cover more complicated IFN responses (29). These genes are from an IFN transcriptional network consisting of 92 predicted regulators and 628 ISGs that was built based on co-expression in human and mouse responses (29). Similar to the comparison with MF-ISGs, we assessed the expression of target ISGs in our monocytes. We identified expression of 623 genes out of the 628 ISGs in unstimulated monocytes and their $\log_2$ fold change distribution was significantly skewed to the female side (mean = 0.11), compared to that of all other genes (20,464 genes, mean = 0.002) (two-sided *t* test $p = 2.2 \times 10^{-16}$; Supplemental Fig. 3.1). We further examined sexual bias in expression of those ISGs based on the five clusters (C1–C5) that were parsed within the network and characterized by distinct functionalities; C1 and C2 enriched for RNA processing, C3 for antiviral effectors, C4 for metabolic regulation, and C5 for inflammation mediators or regulators (29). While most of the clusters, except for C1, displayed significantly higher expression in females, the antiviral cluster C3 showed the strongest positive $\log_2$ fold change (median = 0.28, one sample two-sided *t* test Bonferroni-adjusted $p < 2.2 \times 10^{-26}$; Fig. 4), followed by the C5 cluster of inflammation (median = 0.13, Bonferroni-adjusted $p = 7.6 \times 10^{-6}$; Fig. 3.4) (Online Appendix 3). These results further confirm the higher baseline expression of

IFN-responsive genes observed in female monocytes, particularly for the genes involved in antiviral and inflammatory responses, which may contribute to the phenotypic sex differences in autoimmunity.

3.5 Discussion

Sex dimorphism in immune system is evidenced by differences in the prevalence and intensity of infectious diseases between the sexes (3) and the strong female-biased incidence of autoimmune diseases (4). Compared to the marked phenotypic differences, sex differences at DNA sequence level are limited to the sex chromosomes and not apparent in autosomes (35). This observation suggests an important role of higher-level molecular regulations such as transcriptional and epigenetic processes. In humans, a difference in transcriptome between males and females has been reported in the liver (17) and skeletal muscle (18) but also in whole blood (21,23) or PBMC (20). However, as peripheral blood contains several immune cell types, it is not clear whether the sex difference in the peripheral blood transcriptome results from differential immune cell type frequencies or different patterns of transcriptome in specific cell populations present between the sexes. Here, we compared transcriptional profiles of unstimulated peripheral blood monocytes between male and postmenopausal female participants with chronic low-grade inflammation. To the best of our knowledge, this is the first study to identify monocyte-specific transcriptional sex differences in humans. Consistent with the observations in mouse macrophages (19), sex-biased differential gene expression was detected in our untreated monocytes.

Among the 22 female-biased differentially expressed genes, the majority were involved in essential features of innate immunity, immune cell activation and antigen-processing/presentation. These included *FCGR2B*, a member of Fc gamma (Fcγ) receptors. Those receptors are expressed on the surface of antigen-presenting cells (APC) such as monocytes, and mediate immune cell activation, phagocytosis, and production of inflammatory cytokines upon binding to immunoglobulin G (36). Their functions are determined by the balance between activating and inhibitory Fcγ receptors (36). Of note, not only the inhibitory *FCGR2B*, but also activating Fcγ receptors such as *FCGR1A* and *FCGR1C* tended to be expressed at higher levels in female monocytes, suggesting overall higher activation levels of female monocytes in the steady state prior to pathogen invasion. This is consistent with the immune sexual dimorphism observed in murine macrophages (19). The higher expression levels of *UBA1* and *CD1E*, which processes antigens for the ubiquitin-proteasome system (37,38) and presents lipid molecules as a major histocompatibility complex (MHC)-like protein (39), respectively, suggest a greater ability of female monocytes to present antigens, once infected by viruses, actively linking innate and adaptive immune systems. Higher antigen-presenting capability of APC has been reported in mouse splenocytes, possibly due to differential control of sex hormones (40).

The pathway analysis using IPA revealed that several immune-related pathways were upregulated in females in the unstimulated state, including IFN signaling as the top pathway for the sex differences. This may be indicative of a more vigilant innate immune defense in females under unstimulated condition. Interestingly, though the pathways identified by IPA need to be carefully interpreted in the context of cell type, most of the

female-biased pathways include components involved in IFN signaling, suggesting an important role of IFN signaling in immune sexual dimorphism. For example, the female-biased Th1 pathway was characterized by upregulation of the genes related to MHC-II and IFN-STAT1 signaling, implying a propensity of female monocytes to foster Th1 differentiation (41). This sex-based difference may account for the more robust Th1 immune responses observed in females than males in line with suppressive effects of androgens on key nodes of Th1 differentiation pathway including IL-12 and IFNγ production (42). In contrast, the genes in the PD-1, PD-L1 pathway, which halts the development of T cells to minimize inappropriate autoimmune inflammation as an immune checkpoint (43), tended to be expressed higher in male than female monocytes (z-score=-0.82). Taken together, monocytes from males and females may be in a differential state under resting conditions with regard to the balance between immune defense and its negative feedback system.

IFN, which plays a central role in initiating immune responses especially with antiviral effects (44), is also a key player implicated in the pathogenesis of a variety of autoimmune disorders such as SLE (26), the most female-biased disease with a 9:1 ratio of females to males (45). In contrast to early SLE studies centered on the adaptive immune system, the paradigm has shifted with recent advances in the field of innate immunity, suggesting a crucial role of monocyte/macrophage abnormalities in the development of autoimmune responses (46). To better understand how immune sex dimorphism in monocytes potentially contributes to the sex-biased susceptibility to autoimmunity, we further looked into IFN signaling. Using the gene set responding to IFN stimulation in mouse macrophages (i.e., MF-ISGs), which was recently published as

part of the ImmGen project (29), we assessed sex-biased expression of human homologous genes in our unstimulated monocytes. The overall expression of MF-ISGs was higher in females than males, as shown by the appreciable overlap with the female-biased genes and skewed fold changes toward females. In terms of functionality of ISGs (29), the most significant difference between the sexes was in genes of antiviral effectors, followed by genes of inflammatory mediators or regulators. These observations are largely consistent with the prior work that assessed the sexually differential expression of the same MF-ISG profiles in murine macrophages, where the ISGs for antiviral responses showed significant upregulation in female macrophages in both baseline and IFN-induced states while expression of the inflammation-regulating ISGs became significantly higher in females only upon IFN stimulation (19). Our results suggest a better-primed steady-state innate immunity in females, especially in antiviral responses. In addition, our results of female-biased upregulation of Th1 pathway and male-biased PD-1, PD-L1 pathway suggest a greater transcriptional alertness of female monocytes to foster adaptive immune response. This may be a signature characteristic of human peripheral blood monocytes that is partially shared by murine macrophages from peritoneal cavity but not by those from the spleen, and microglia from the central nervous system (19).

The sex dimorphism in immune system, displaying a stronger IFN response of female monocyte/macrophages, appears to be conserved across a variety of species, including birds (19,47). Confirmation of human-mouse conservation of transcriptional sex dimorphism has previously been obtained through the significant overlap between the sex-biased genes of human $CD14^{+}$ monocytes and murine macrophages (19). Our results further confirm that human monocytes also exhibit the conserved sex dimorphic

expression of IFN-responsive genes. Despite the small sample size, we observed an upregulation of the IFN signaling pathway in female monocytes using two different approaches. Due to the recruitment criteria of the study participants, it is unlikely that the immune sex dimorphism observed is confounded by other characteristics such as age (21) and menopausal status (20). The frequencies of monocytes and other immune cell types were similar between males and females; therefore, our results may better reflect *in vivo* sex dimorphism of monocyte transcriptome. Further studies are needed to better characterize sex-based differences in other monocyte immune pathways. We observed higher expression of *TIFAB* and *CEACAM1* in females than males. *TIFAB* is an inhibitor of tumor necrosis factor (TNF) receptor-associated factor (TRAF) mediated signal transduction down to NF-κB (48), and *CEACAM1* is a negative regulator of IL-6 signaling in response to LPS (49). Reported in the parent study of our clinical trial, we found no differential expression between male and female monocytes of *TNFA*, *IL6,* and *MCP1*, the genes coding for cytokines and chemokines that are regulated by the TNF receptor pathways in both baseline and LPS-stimulated conditions (Supplementary Table 3). These data suggest that other immune-related pathways may not have the transcriptional sex dimorphism as demonstrated in IFN signaling.

In summary, sexual transcriptional differences in the steady-state immune system are present in unstimulated human monocytes and are primarily associated with IFN-related signaling pathways. Our results support monocyte/macrophage IFN signaling being an important molecular effector of sex dimorphism in immune pathology.

3.6 References

1. Beery AK, Zucker I. Sex bias in neuroscience and biomedical research. Neurosci Biobehav Rev. 2011;35:565–72.

2. Klein SL. Immune cells have sex and so should journal articles. Endocrinology. 2012;153:2544–50.

3. McClelland EE, Smith JM. Gender specific differences in the immune response to infection. Arch Immunol Ther Exp (Warsz). 2011;59:203–13.

4. Whitacre CC, Reingold SC, O'Looney PA. A gender gap in autoimmunity. Science. 1999;283:1277–8.

5. Cook IF. Sexual dimorphism of humoral immunity with human vaccines. Vaccine. 2008;26:3551–5.

6. Hewagama A, Patel D, Yarlagadda S, Strickland FM, Richardson BC. Stronger inflammatory/cytotoxic T-cell response in women identified by microarray analysis. Genes Immun. 2009;10:509–16.

7. Butterworth M, McClellan B, Allansmith M. Influence of sex in immunoglobulin levels. Nature. 1967;214:1224–5.

8. Klein SL, Jedlicka A, Pekosz A. The Xs and Y of immune responses to viral vaccines. Lancet Infect Dis. 2010;10:338–49.

9. Ortona E, Pierdominici M, Maselli A, Veroni C, Aloisi F, Shoenfeld Y. Sex-based differences in autoimmune diseases. Ann Ist Super Sanita. 2016;52:205–12.

10. Yu C, Gershwin ME, Chang C. Diagnostic criteria for systemic lupus erythematosus: A critical review. J Autoimmun. 2014;48–49:10–3.

11. Cutolo M, Sulli A, Capellino S, Villaggio B, Montagna P, Seriolo B, Straub RH. Sex hormones influence on the immune system: Basic and clinical aspects in autoimmunity. Lupus. 2004;13:635–8.

12. Libert C, Dejager L, Pinheiro I. The X chromosome in immune functions: When a chromosome makes the difference. Nat Rev Immunol. 2010;10:594–604.

13. Kovats S, Carreras E, Agrawal H. Sex steroid receptors in immune cells. Sex Hormones and Immunity to Infection. Springer-Verlag Berlin Heidelberg; 2010. p. 53–91.

14. Pisitkun P, Deane JA, Difilippantonio MJ, Tarasenko T, Satterthwaite AB, Bolland S. Autoreactive B cell responses to RNA-related antigens due to TLR7 gene duplication. Science (80-). 2006;312:1669–72.

15. Lu Q, Wu A, Tesmer L, Ray D, Yousif N, Richardson B. Demethylation of CD40LG on the Inactive X in T Cells from Women with Lupus. J Immunol. 2007;179:6352–8.

16. Yang X, Schadt EE, Wang S, Wang H, Arnold AP, Ingram-Drake L, Drake TA, Lusis AJ. Tissue-specific expression and regulation of sexually dimorphic genes in mice. Genome Res. 2006;16:995–1004.

17. Zhang Y, Klein K, Sugathan A, Nassery N, Dombkowski A, Zanger UM, Waxman DJ. Transcriptional profiling of human liver identifies sex-biased genes associated with polygenic dyslipidemia and coronary artery disease. PLoS One. 2011;6:e23506.

18. Welle S, Tawil R, Thornton CA. Sex-related differences in gene expression in human skeletal muscle. PLoS One. 2008;3:e1385.

19. Gal-Oz ST, Maier B, Yoshida H, Seddu K, Elbaz N, Czysz C, Zuk O, Stranger BE, Ner-Gaon H, Shay T. ImmGen report: sexual dimorphism in the immune system transcriptome. Nat Commun. 2019;10:1–14.

20. Jansen R, Batista S, Brooks AI, Tischfield JA, Willemsen G, Van Grootheest G, Hottenga JJ, Milaneschi Y, Mbarek H, Madar V, et al. Sex differences in the human peripheral blood transcriptome. BMC Genomics. 2014;15:33.

21. Piasecka B, Duffy D, Urrutia A, Quach H, Patin E, Posseme C, Bergstedt J, Charbit B, Rouilly V, MacPherson CR, et al. Distinctive roles of age, sex, and genetics in shaping transcriptional variation of human immune responses to microbial challenges. Proc Natl Acad Sci U S A. 2018;115:E488–97.

22. Bongen E, Lucian H, Khatri A, Fragiadakis GK, Bjornson ZB, Nolan GP, Utz PJ, Khatri P. Sex Differences in the Blood Transcriptome Identify Robust Changes in Immune Cell Proportions with Aging and Influenza Infection. Cell Rep. 2019;29:1961-1973.e4.

23. Panousis NI, Bertsias GK, Ongen H, Gergianaki I, Tektonidou MG, Trachana M, Romano-Palumbo L, Bielser D, Howald C, Pamfil C, et al. Combined genetic and transcriptome analysis of patients with SLE: distinct, targetable signatures for susceptibility and severity. Ann Rheum Dis. 2019;78:1079–89.

24. Morgane Sonia Thion A, Low D, Silvin A, Pettersson S, Ginhoux F, Garel S. Microbiome Influences Prenatal and Adult Microglia in a Sex-Specific Manner. Cell. 2018;172:500-507.e16.

25. Bhasin JM, Chakrabarti E, Peng D-Q, Kulkarni A, Chen X, Smith JD. Sex specific gene regulation and expression QTLs in mouse macrophages from a strain intercross. PLoS One. 2008;3:e1435.

26. Banchereau J, Pascual V. Review Type I Interferon in Systemic Lupus

Erythematosus and Other Autoimmune Diseases. Immunity. 2006;25:383–92.

27. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. 2014;15:550.

28. Benjamini Y, Hochberg Y. Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. J R Stat Soc Ser B. 1995;57:289–300.

29. Mostafavi S, Yoshida H, Moodley D, Leboité H, Rothamel K, Raj T, Ye CJ, Chevrier N, Zhang SY, Feng T, et al. Parsing the Interferon Transcriptional Network and Its Disease Associations. Cell. 2016;164:564–78.

30. Kinsella RJ, Kä Hä Ri A, Haider S, Zamora J, Proctor G, Spudich G, Almeida-King J, Staines D, Derwent P, Kerhornou A, et al. Ensembl BioMarts: a hub for data retrieval across taxonomic space.

31. Jean Dunn O. Multiple comparisons among means. J Am Stat Assoc. 1961;56:52–64.

32. Chasset F, Arnaud L. Targeting interferons and their pathways in systemic lupus erythematosus. Autoimmun Rev. 2018;17:44–52.

33. Shay T, Jojic V, Zuk O, Rothamel K, Puyraimond-Zemmour D, Feng T, Wakamatsu E, Benoist C, Koller D, Regev A. Conservation and divergence in the transcriptional programs of the human and mouse immune systems. Proc Natl Acad Sci U S A. 2013;110:2946–51.

34. Yona S, Kim KW, Wolf Y, Mildner A, Varol D, Breker M, Strauss-Ayali D, Viukov S, Guilliams M, Misharin A, et al. Fate Mapping Reveals Origins and Dynamics of Monocytes and Tissue Macrophages under Homeostasis. Immunity. 2013;38:79–91.

35. Boraska V, Jerončić A, Colonna V, Southam L, Nyholt DR, Rayner NW, Perry JRB, Toniolo D, Albrecht E, Ang W, et al. Genome-wide meta-analysis of common variant differences between men and women. Hum Mol Genet. 2012;21:4805–15.

36. Bournazos S, Wang TT, Ravetch J V. The Role and Function of Fcγ Receptors on Myeloid Cells. Microbiol Spectr. American Society for Microbiology; 2016;4.

37. Loureiro J, Ploegh HL. Antigen Presentation and the Ubiquitin-Proteasome System in Host-Pathogen Interactions. Adv Immunol. 2006;92:225–305.

38. Groen EJN, Gillingwater TH. UBA1: At the Crossroads of Ubiquitin Homeostasis and Neurodegeneration. Trends Mol Med. 2015;21:622–32.

39. Brigl M, Brenner MB. CD1: Antigen Presentation and T Cell Function. Annu Rev Immunol. 2004;22:817–90.

40. Weinstein Y, Ran S, Segal S. Sex-associated differences in the regulation of immune responses controlled by the MHC of the mouse. J Immunol. 1984;132.

41. Zhu J, Yamane H, Paul WE. Differentiation of Effector CD4 T Cell Populations. Annu Rev Immunol. 2010;28:445–89.

42. Zhang MA, Ahn JJ, Zhao FL, Selvanantham T, Mallevaey T, Stock N, Correa L, Clark R, Spaner D, Dunn SE. Antagonizing Peroxisome Proliferator-Activated Receptor α Activity Selectively Enhances Th1 Immunity in Male Mice. J Immunol. 2015;195:5189–202.

43. Dai S, Jia R, Zhang X, Fang Q, Huang L. The PD-1/PD-Ls pathway and autoimmune diseases. Cell Immunol. 2014;290:72–9.

44. Aricò E, Belardelli F. Interferon-α as antiviral and antitumor vaccine adjuvants: mechanisms of action and response signature. J Interferon Cytokine Res. 2012;32:235–47.

45. Beeson PB. Age and sex associations of 40 autoimmune diseases. Am J Med. 1994;96:457–62.

46. Li Y, Lee PY, Reeves WH. Monocyte and macrophage abnormalities in systemic lupus erythematosus. Arch Immunol Ther Exp (Warsz). 2010;58:355–64.

47. Garcia-Morales C, Nandi S, Zhao D, Sauter KA, Vervelde L, McBride D, Sang HM, Clinton M, Hume DA. Cell-Autonomous Sex Differences in Gene Expression in Chicken Bone Marrow–Derived Macrophages. J Immunol. 2015;194:2338–44.

48. Matsumura T, Semba K, Azuma S, Ikawa S, Gohda J, Akiyama T, Inoue JI. TIFAB inhibits TIFA, TRAF-interacting protein with a forkhead-associated domain. Biochem Biophys Res Commun. 2004;317:230–4.

49. Zhang Z, La Placa D, Nguyen T, Kujawski M, Le K, Li L, Shively JE. CEACAM1 regulates the IL-6 mediated fever response to LPS through the RP105 receptor in murine monocytes. BMC Immunol. 2019;20:7.

3.7 Tables and Figures

Table 3.1 Characteristics of study participants.

	Men (n=9)	Women (n=10)	*p*
Age, years	59 ± 6	63 ± 6	0.20
Body mass index, kg/m^2	31.6 ± 5.3	33.6 ± 7.5	0.52
Waist circumference, cm	111 ± 13	101 ± 17	0.17
Systolic blood pressure, mmHg	129 ± 7	127 ± 24	0.76
Diastolic blood pressure, mmHg	85 ± 6	73 ± 12	0.02
Total leukocyte count, 1000/uL	6.5 ± 1.3	5.7 ± 1.7	0.28
Lymphocyte proportion, % of leukocytes	28 ± 5	31 ± 8	0.24
Monocyte proportion, % of leukocytes	7.9 ± 1.8	7.9 ± 1.9	0.99
Serum inflammatory markers[a]			
Hs-CRP, μg/mL	3.39 ± 2.43	3.18 ± 1.93	0.55
TNF-α, pg/mL	2.04 ± 0.68	2.54 ± 0.75	0.12
IL-6, pg/mL	0.83 ± 0.68	0.75 ± 0.49	0.72
MCP-1, pg/mL	288 ± 136	302 ± 74	0.59

Data are presented as means ± SD or †medians ± interquartile ranges. Hs-CRP, high-sensitivity C-reactive protein.

[a] Wilcoxon test was used due to the skewness of data.

Table 3.2 Top IPA biological pathways (|z-score| ≥1, p <0.05) of sex-biased genes (n=565, p <0.03) in peripheral blood monocytes.

Top Pathways	Z-score[a]	Molecules	Functions & Diseases	p
Interferon Signaling	Up	*IFIT1, IFIT3, IFITM1, JAK2, MX1, STAT1*	Cellular Immune Response; Cytokine Signaling	1.33E-04
PI3K Signaling in B Lymphocytes	Up	*CD180, CD40, CD79A, DAPP1, FCGR2B, IRS2, ITPR2, PIK3CG, PLCH1, PLCH2*	Cellular Immune Response	1.10E-03
Sperm Motility	Up	*ABL2, AXL, EPHB3, FLT1, GUCY1A1, ITPR2, JAK2, MAP2K6, MAP3K11, PLAAT4, PLAAT5, PLCH1, PLCH2*	Organismal Growth and Development	1.60E-03
Th1 Pathway	Up	*CCR5, CD274, CD40, HLA-DOA, HLA-DRB5, JAK2, MAP2K6, PIK3CG, STAT1*	Cellular Growth and Proliferation and Development; Cellular Immune Response; Cytokine Signaling; Pathogen-Influenced Signaling	1.60E-03
Dendritic Cell Maturation	Up	*CD1B, CD1C, CD40, FCGR2B, HLA-DOA, HLA-DRB5, JAK2, PIK3CG, PLCH1, PLCH2, STAT1*	Cellular Immune Response; Cytokine Signaling; Pathogen-Influenced Signaling	2.83E-03
Type I Diabetes Mellitus Signaling	Up	*FAS, HLA-DOA, HLA-DRB5, ICA1, JAK2, MAP2K6, STAT1*	Apoptosis; Disease-Specific Pathways	1.24E-02
Crosstalk between Dendritic Cell and Natural Killer Cells	Up	*CD40, FAS, HLA-DRB5, KIR3DL2, TLN2, TNFSF10*	Cellular Immune Response	1.48E-02
UVA-Induced MAPK Signaling	Up	*PARP12, PARP9, PIK3CG, PLCH1, PLCH2, STAT1*	Cellular Stress and Injury	2.27E-02
Adrenomedullin Signaling Pathway	Up	*ADCY5, GUCY1A1, ITPR2, MAP2K6, PIK3CG, PLCH1, PLCH2, RAMP3, SOX15*	Cardiovascular Signaling; Cellular Growth, Proliferation and Development; Cellular Stress and Injury	3.35E-02

Pancreatic Adenocarcinoma Signaling	Up	*CDK2, E2F2, E2F6, JAK2, PIK3CG, STAT1*	Cancer; Disease-Specific Pathways	3.57E-02
Systemic Lupus Erythematosus in B Cell Signaling Pathway	Up	*CD40, CD79A, FCGR2B, IFIT2, IFIT3, JAK2, LILRA6, PIK3CG, PLAAT4, STAT1, TNFSF10*	Cellular Immune Response; Disease-Specific Pathways	4.59E-02
PD-1, PD-L1 Cancer Immunotherapy Pathway[b]	Down (z = -0.82)	*CD274, CDK2, HLA-DOA, HLA-DRB5, JAK2, PDCD1LG2, PIK3CG*	Cancer; Cellular-Immune Response	9.74E-03
p53 signaling[b]	Down (z = -0.45)	*CDK2, FAS, PIK3CG, PML, THBS1, TP53I3*	Cancer; Ingenuity Toxicity List Pathways	2.27E-02

[a] Z-score is calculated as females relative to males.

[b] The most affected among the pathways downregulated ($-1 <$ z-score < 0, $p < 0.05$) in females relative to males.

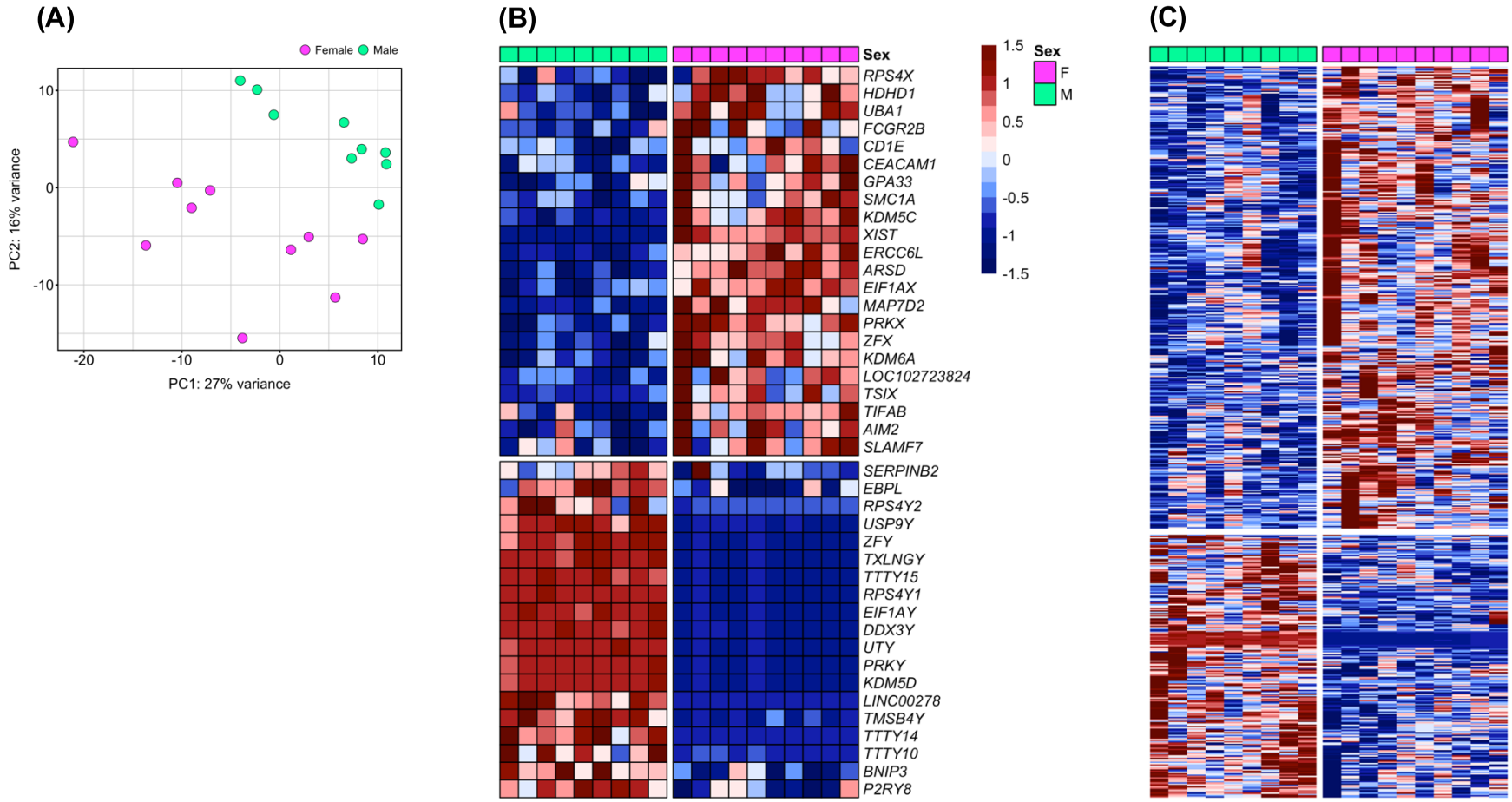


Figure 3.1. Monocyte transcriptional sex dimorphism. (**A**) Principal component analysis (PCA) of peripheral blood monocytes of males and females (green and pink) based on 26,485 genes. Heatmaps of relative expression of (**B**) 41 sexually differentially expressed genes (FDR <0.1) and (**C**) 565 sex-biased genes (p <0.03) in peripheral blood monocytes from male (green) and female (pink) participants. A horizontal gap separates female- from male-biased genes, and a vertical gap separates male and female participants. Expression values are trimmed to range [-1.5, 1.5].

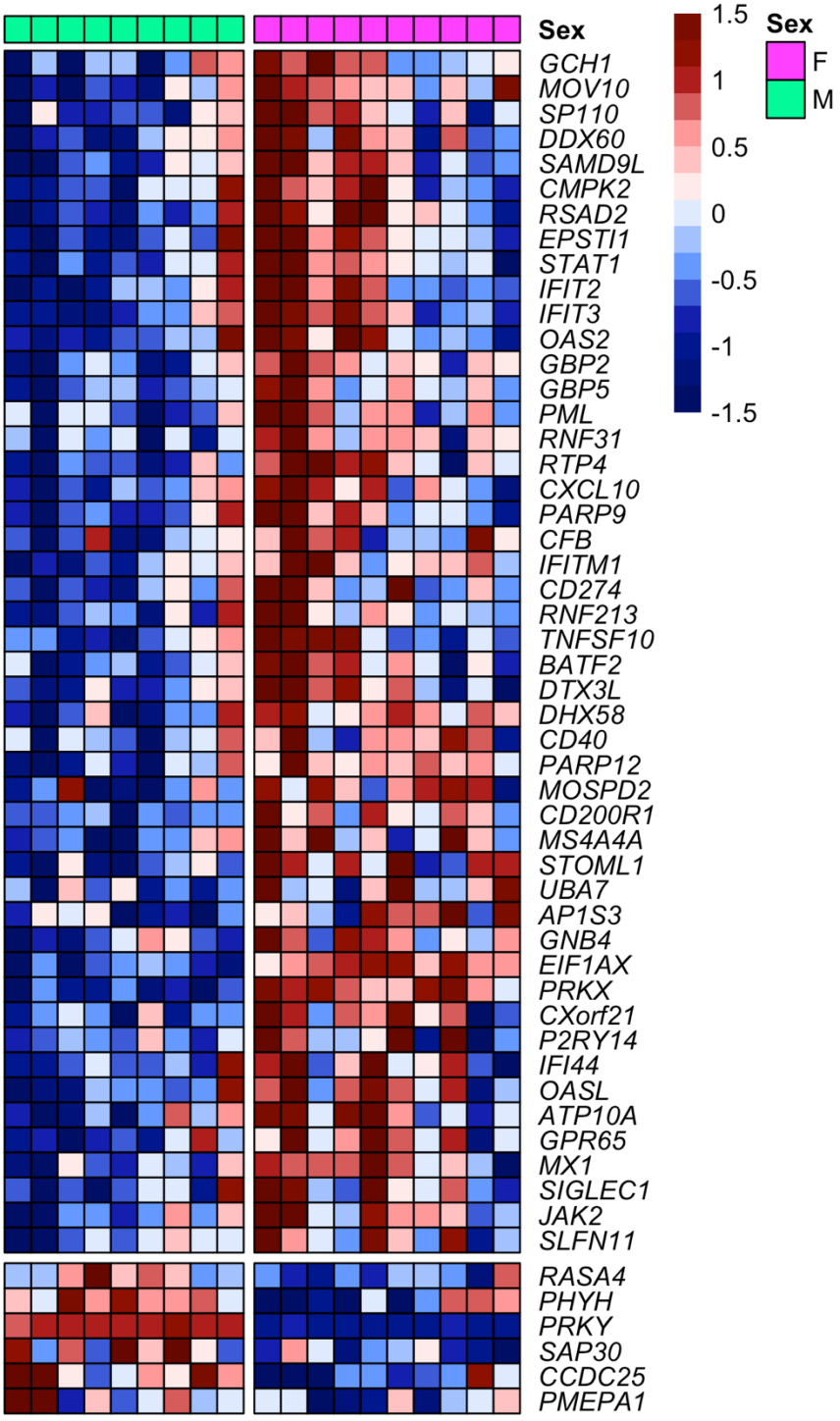


Figure 3.2. Heatmap of relative expression of 54 macrophage-specific IFN signature genes (MF-ISGs) that are also identified as sex-biased genes in peripheral blood monocytes. A horizontal gap separates female- from male-biased genes, and a vertical gap separates male and female participants. Expression values are trimmed to range [-1.5, 1.5].

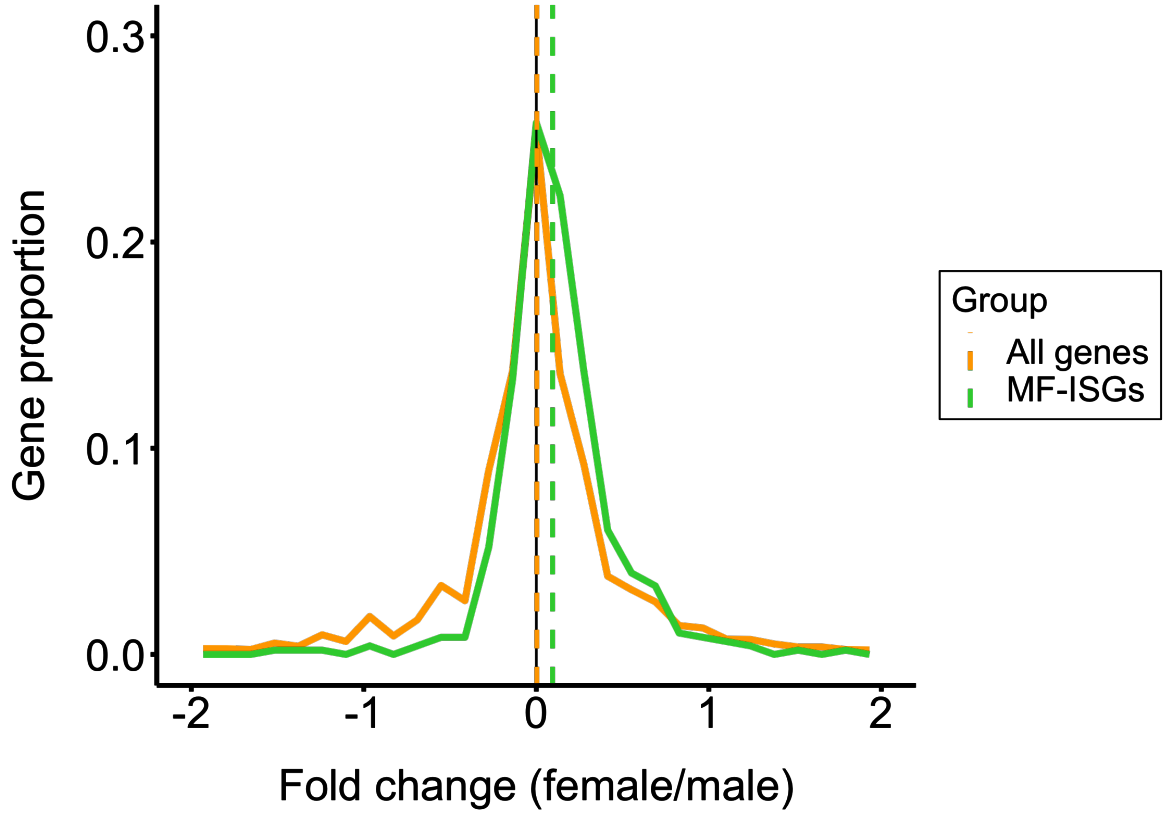


Figure 3.3. Female/male fold change (log_2) distribution of genes related to IFN pathway from peripheral blood monocytes. Human orthologs of macrophage-specific IFN signature genes (MF-ISGs; 485 genes, green) and all other genes (20,602 genes, yellow). p=0.00029 between the distributions (Welch's two sample t test).

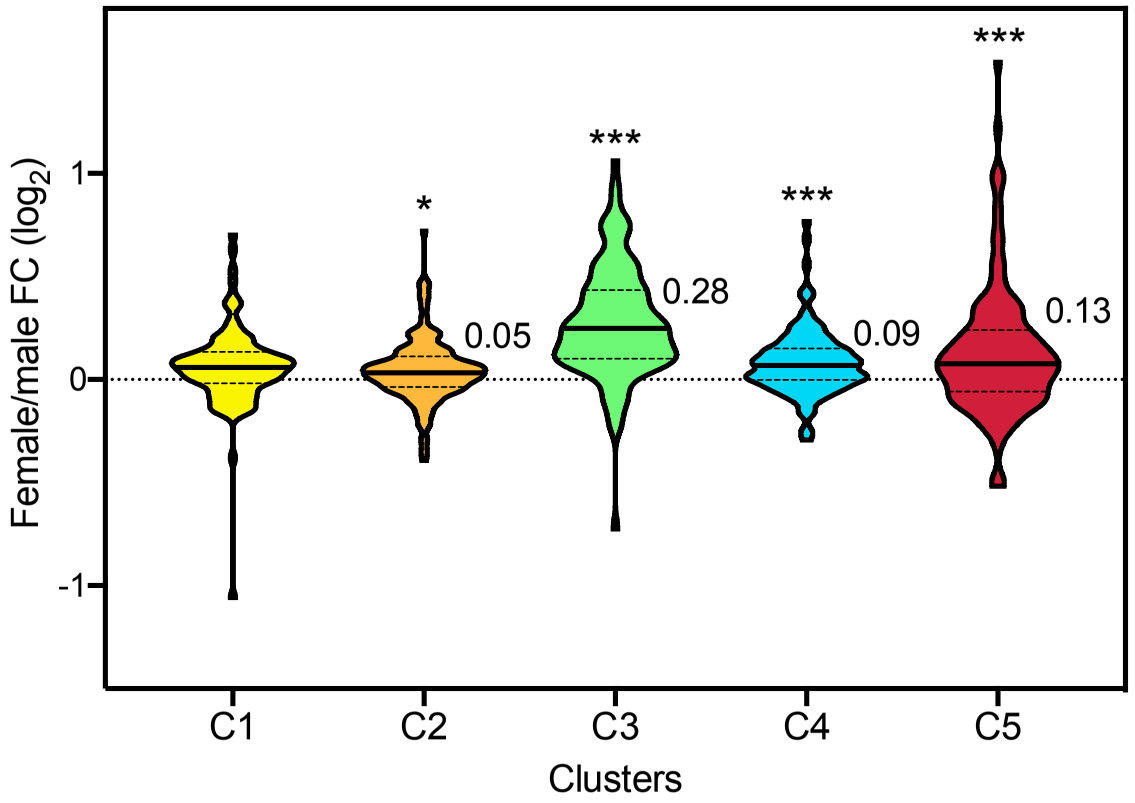


Figure 3.4. Violin plot of female/male fold change (log2) distribution of the genes in C1-C5 clusters of the IFN regulatory network constructed by Mostafavi et al. from peripheral blood monocytes. Each cluster denotes distinct function: C1-2 [RNA processing], C3 [antiviral effectors], C4 [metabolic regulation], and C5 [inflammation mediators or regulators]. The central line of each cluster indicates the median, and the bottom and top lines indicate the 25th and 75th percentiles, respectively. The clusters having mean fold changes significantly different from zero (one sample two-sided *t* test with Bonferroni correction) are marked with graded asterisks. * Adjusted p <0.05; *** Adjusted p <0.001.

3.8 Supplementary Material

Supplementary Table 3.1 Sexually-based differentially expressed genes (SDEGs) in peripheral blood monocytes (n=41, FDR <0.1).

Gene Symbol	Gene Name	log_2FC[a]	*p*	FDR
	Female-biased genes (n=22)			
XIST	X inactive specific transcript	12.09	5.06E-46	1.33E-42
TSIX	TSIX transcript, XIST antisense RNA	4.53	3.85E-07	3.53E-04
MAP7D2	MAP7 domain containing 2	3.34	2.60E-09	2.74E-06
ERCC6L	ERCC excision repair 6 like, spindle assembly checkpoint helicase	3.12	9.68E-10	1.07E-06
LOC102723824	*uncharacterized*	2.08	3.37E-05	2.29E-02
TIFAB[b]	TIFA inhibitor	1.38	7.23E-05	4.12E-02
CEACAM1[b]	CEA cell adhesion molecule 1	1.37	7.15E-05	4.12E-02
GPA33[b]	glycoprotein A33	1.27	2.80E-05	1.97E-02
FCGR2B[b]	Fc fragment of IgG receptor Iib	1.25	2.36E-05	1.72E-02
CD1E[b]	CD1e molecule	1.13	1.24E-04	6.70E-02
AIM2[b]	absent in melanoma 2	0.99	6.10E-05	3.68E-02
SLAMF7[b]	SLAM family member 7	0.78	1.77E-04	9.13E-02
KDM6A	lysine demethylase 6A	0.71	1.99E-08	2.00E-05
PRKX	protein kinase X-linked	0.70	2.16E-14	2.85E-11
ARSD	arylsulfatase D	0.70	1.45E-18	2.04E-15
EIF1AX	eukaryotic translation initiation factor 1A X-linked	0.62	6.23E-10	7.29E-07
KDM5C	lysine demethylase 5C	0.60	7.23E-11	8.97E-08
ZFX	zinc finger protein X-linked	0.58	4.99E-08	4.78E-05
SMC1A	structural maintenance of chromosomes 1A	0.52	6.05E-05	3.68E-02
HDHD1	pseudouridine 5'-phosphatase	0.51	7.15E-06	6.03E-03
RPS4X	ribosomal protein S4 X-linked	0.30	1.58E-04	8.34E-02
UBA1	ubiquitin like modifier activating enzyme 1	0.28	4.54E-05	2.99E-02
	Male-biased genes (n=19)			
RPS4Y1	ribosomal protein S4 Y-linked 1	-14.02	1.67E-102	3.51E-98
KDM5D	lysine demethylase 5D	-12.51	1.17E-81	6.16E-78
EIF1AY	eukaryotic translation initiation factor 1A Y-linked	-11.21	2.71E-65	1.14E-61
UTY	ubiquitously transcribed tetratricopeptide repeat containing,	-11.02	6.28E-63	2.21E-59

	Y-linked			
TXLNGY	taxilin gamma pseudogene, Y-linked	-10.83	2.62E-60	7.91E-57
DDX3Y	DEAD-box helicase 3 Y-linked	-9.94	7.46E-89	5.25E-85
USP9Y	ubiquitin specific peptidase 9 Y-linked	-9.14	6.45E-42	1.51E-38
ZFY	zinc finger protein Y-linked	-8.99	1.17E-41	2.46E-38
PRKY	protein kinase Y-linked (pseudogene)	-8.63	4.35E-97	4.58E-93
TTTY15	testis-specific transcript, Y-linked 15	-8.29	1.16E-35	2.23E-32
LINC00278	long intergenic non-protein coding RNA 278	-6.78	8.70E-21	1.41E-17
TTTY14	testis-specific transcript, Y-linked 14	-6.53	4.10E-19	6.18E-16
RPS4Y2	ribosomal protein S4 Y-linked 2	-4.78	8.30E-07	7.29E-04
TTTY10	testis-specific transcript, Y-linked 10	-4.24	1.90E-05	1.49E-02
TMSB4Y	thymosin beta 4 Y-linked	-4.03	3.75E-29	6.59E-26
SERPINB2[b]	serpin family B member 2	-1.01	1.28E-05	1.04E-02
BNIP3[b]	BCL2 interacting protein 3	-0.67	2.30E-05	1.72E-02
EBPL[b]	EBP like	-0.50	5.12E-05	3.27E-02
P2RY8	P2Y receptor family member 8	-0.34	9.79E-05	5.43E-02

[a] Fold change (FC) is calculated as a ratio of expression values in females relative to males.
[b] Autosomal genes.

Supplementary Table 3.2 The overlap of monocyte sex-biased genes with IFN signature genes that were previously identified in mouse macrophages (MF-ISGs)[a].

Gene Symbol	Gene Name	log_2FC[b]	*p*	FDR
	Female-biased (n=48)			
CD274	CD274 molecule	1.22	2.11E-02	0.999740
CD200R1	CD200 receptor 1	1.07	3.68E-04	1.55E-01
GBP5	Guanylate binding protein 5	1.06	1.16E-03	3.58E-01
CXCL10	C-X-C motif chemokine ligand 10	1.00	1.07E-02	9.60E-01
SIGLEC1	Sialic acid binding Ig like lectin 1	0.94	2.25E-02	0.999740
IFIT3	Interferon induced protein with tetratricopeptide repeats 3	0.88	1.37E-03	3.86E-01
BATF2	Basic leucine zipper ATF-like transcription factor 2	0.86	6.54E-03	8.31E-01
RSAD2	Radical S-adenosyl methionine domain containing 2	0.76	3.59E-04	1.54E-01
IFITM1	Interferon induced transmembrane protein 1	0.73	3.56E-04	1.54E-01
SAMD9L	Sterile alpha motif domain containing 9 like	0.73	1.04E-03	3.44E-01
MX1	MX dynamin like GTPase 1	0.72	4.17E-03	7.16E-01
CFB	Complement factor B	0.71	1.45E-02	0.999740
PRKX	Protein kinase X-linked	0.70	2.16E-14	2.85E-11
P2RY14	Purinergic receptor P2Y14	0.67	3.00E-02	0.999740
EPSTI1	Epithelial stromal interaction 1	0.65	2.58E-03	5.83E-01
AP1S3	Adaptor related protein complex 1 subunit sigma 3	0.64	9.46E-03	9.41E-01
EIF1AX	Eukaryotic translation initiation factor 1A X-linked	0.62	6.23E-10	7.29E-07
CMPK2	Cytidine/uridine monophosphate kinase 2	0.60	1.95E-02	0.999740
RTP4	Receptor transporter protein 4	0.59	3.04E-04	1.49E-01
DDX60	DExD/H-box helicase 60	0.58	7.24E-03	8.57E-01
CD40	CD40 molecule	0.56	1.85E-02	0.999740
STAT1	Signal transducer and activator of transcription 1	0.56	5.79E-03	8.30E-01
IFI44	Interferon induced protein 44	0.56	2.59E-02	0.999740
GBP2	Guanylate binding protein 2	0.54	1.16E-03	3.58E-01
ATP10A	ATPase phospholipid transporting 10A (putative)	0.54	1.08E-02	9.60E-01
IFIT2	Interferon induced protein with tetratricopeptide repeats 2	0.54	1.53E-02	0.999740
MOSPD2	Motile sperm domain containing 2	0.53	1.42E-02	0.999740

OASL	2'-5'-oligoadenylate synthetase like	0.51	5.65E-03	8.17E-01
MS4A4A	Membrane spanning 4-domains A4A	0.50	3.83E-03	6.91E-01
PARP9	Poly(ADP-ribose) polymerase family member 9	0.46	1.25E-02	0.999740
OAS2	2'-5'-oligoadenylate synthetase 2	0.43	1.64E-02	0.999740
MOV10	Mov10 RISC complex RNA helicase	0.43	3.54E-04	1.54E-01
TNFSF10	TNF superfamily member 10	0.41	2.79E-02	0.999740
RNF213	Ring finger protein 213	0.39	1.67E-02	0.999740
JAK2	Janus kinase 2	0.36	6.93E-03	8.47E-01
PARP12	Poly(ADP-ribose) polymerase family member 12	0.34	1.43E-03	3.97E-01
STOML1	Stomatin like 1	0.32	6.10E-03	8.31E-01
GCH1	GTP cyclohydrolase 1	0.31	1.62E-02	0.999740
DHX58	DExH-box helicase 58	0.31	2.52E-03	5.83E-01
SLFN11	Schlafen family member 11	0.30	2.90E-02	0.999740
DTX3L	Deltex E3 ubiquitin ligase 3L	0.29	2.67E-02	0.999740
SP110	SP110 nuclear body protein	0.29	1.32E-02	0.999740
GPR65	G protein-coupled receptor 65	0.28	9.69E-03	9.47E-01
CXorf21	Chromosome X open reading frame 21	0.22	2.78E-02	0.999740
PML	Promyelocytic leukemia	0.22	1.38E-02	0.999740
RNF31	Ring finger protein 31	0.19	1.19E-02	9.87E-01
GNB4	G protein subunit beta 4	0.19	1.75E-02	0.999740
UBA7	Ubiquitin like modifier activating enzyme 7	0.17	2.92E-02	0.999740
	Male-biased (n=6)			
PRKY	Protein kinase Y-linked (pseudogene)	-8.63	4.35E-97	4.58E-93
RASA4	RAS p21 protein activator 4	-1.47	4.37E-03	7.18E-01
PMEPA1	Prostate transmembrane protein, androgen induced 1	-0.93	1.82E-02	0.999740
SAP30	Sin3A associated protein 30	-0.57	2.32E-03	5.45E-01
PHYH	Phytanoyl-CoA 2-hydroxylase	-0.39	6.83E-03	8.42E-01
CCDC25	Coiled-coil domain containing 25	-0.21	1.11E-02	9.60E-01

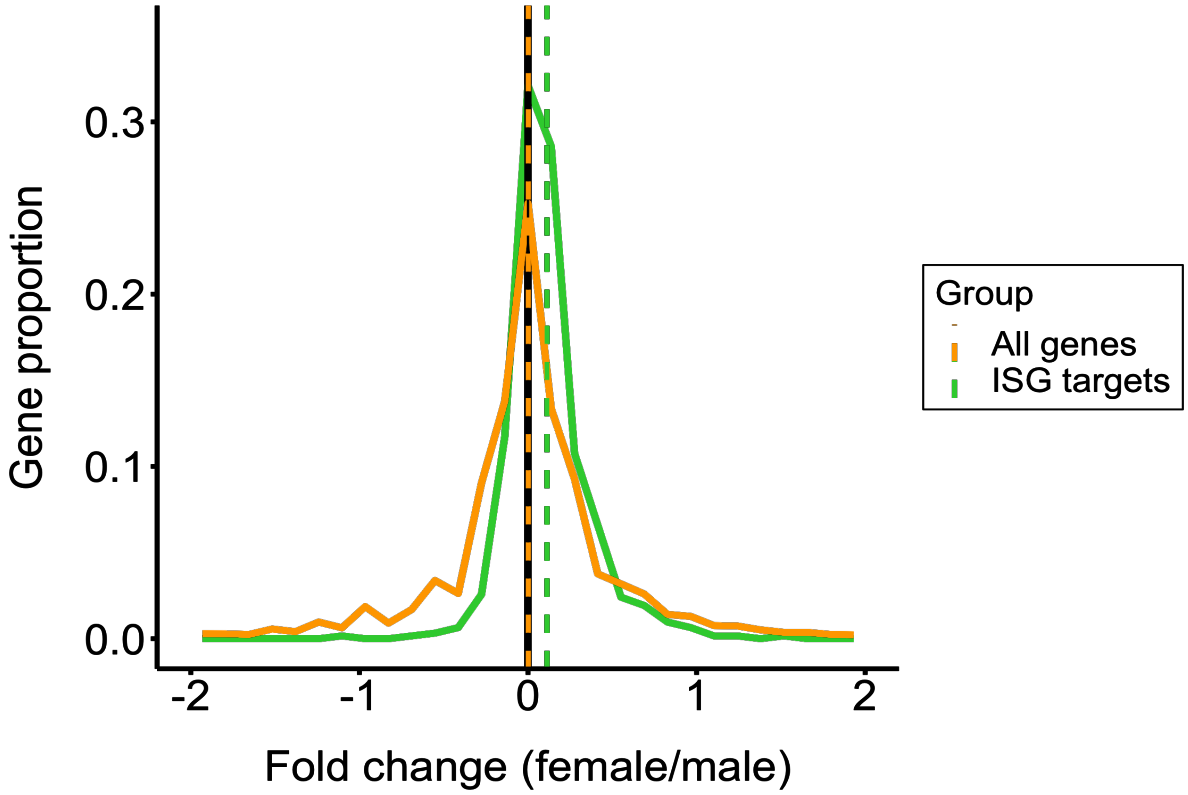
[a] Human orthologs of the 601 MF-ISGs were compared to the sex-biased genes.

[b] Fold change is calculated as a ratio of expression values in females relative to males.

Supplementary Table 3.3 mRNA expression and secretion of inflammatory cytokine/chemokine in LPS-stimulated peripheral blood monocytes.

	Men (n=9)	Women (n=10)	*p*
mRNA expression			
Unstimulated monocytes (relative expression compared to males)			
TNF-α	1 ± 0.47	1.33 ± 0.45	0.42
IL-6	1 ± 0.93	1.75 ± 2.53	0.17
MCP-1	1 + 0.95	1.20 ± 0.99	0.29
Stimulated monocytes (relative expression compared to males)			
TNF-α	1 ± 0.59	1.35 ± 0.86	0.47
IL-6	1 ± 0.46	1.17 ± 0.71	0.35
MCP-1	1 ± 0.79	1.34 ± 1.06	0.52
Ratio of stimulated/unstimulated			
TNF-α	33 ± 19	36 ± 18	0.82
IL-6	1385 ± 2062	1259 ± 1246	0.26
MCP-1	57 ± 55	50 ± 70	0.62
Secretion			
Unstimulated monocytes (pg/mL)			
TNF-α	13 ± 15	15 ± 9	0.87
IL-6	13 ± 8	13 ± 8	0.43
MCP-1	13 ± 12	11 ± 29	> 0.99
Stimulated monocytes (pg/mL)			
TNF-α	3773 ± 558	4021 ± 2820	0.39
IL-6	9885 ± 5744	9103 ± 7665	0.59
MCP-1	2334 ± 1460	3246 ± 2541	0.68
Ratio of stimulated/unstimulated			
TNF-α	223 ± 380	326 ± 167	0.90
IL-6	892 ± 506	1126 ± 942	0.47
MCP-1	247 ± 274	200 ± 209	0.78

Data are presented as medians ± interquartile ranges. Log-transformed data were used due to skewed distributions of values.



Supplementary Figure 3.1. Female/male fold change (log_2) distribution of target genes of the IFN regulatory network, built by Mostafavi et al., from peripheral blood monocytes. The ISG target genes (623 genes, green) and all other genes (20,464 genes, yellow). p = 2.2 x 10-16 between the distributions (Welch's two sample *t* test).

Chapter 4 Effects of EPA and DHA on Gene Expression in Peripheral Blood Monocytes

Eicosapentaenoic acid and docosahexaenoic acid supplementation differentially modulate monocyte transcriptome

Jisun So[1], Albert K Tai[2], Dayong Wu[3], Alice H Lichtenstein[1], and Stefania Lamon-Fava[1]*

Affiliations: [1]Cardiovascular Nutrition Laboratory, Jean Mayer USDA Human Nutrition Research Center on Aging at Tufts University, Boston, MA; [2]Department of Immunology, Tufts University School of Medicine, Boston, MA; [3]Nutritional Immunology Laboratory, Jean Mayer USDA Human Nutrition Research Center on Aging at Tufts University, Boston, MA.

***Corresponding Author:**
Stefania Lamon-Fava, MD PhD
Cardiovascular Nutrition Laboratory,
Jean Mayer USDA Human Nutrition Research Center on Aging, Tufts University
711 Washington Street,
Boston, MA 02111
Tel: +1 617 556 3105
E-mail: stefania.lamon-fava@tufts.edu

Short title: Sex-specific effects of EPA and DHA in monocytes

Clinical Trial Registry: ClinicalTrials.gov, NCT02670382

4.1 Abstract

Background and aims: Though the anti-inflammatory effect of n-3 eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) has been well documented *in vitro*, their individual *in vivo* effects have not been fully characterized in human monocytes, an important therapeutic target for inflammation-related diseases. We determined the whole transcriptome profile of peripheral blood monocytes isolated from individuals with chronic inflammation at baseline and after supplementation with EPA and DHA.

Methods: In a randomized, double-blind, crossover study, 8 men and 8 postmenopausal women aged (50-75 years) with chronic inflammation received control capsules (3 g/day high oleic acid sunflower oil) during a 4-week lead-in phase followed by two sequential 10-week supplementation phases with 3 g/day EPA and DHA in random order, separated by a 10-week washout phase. Monocyte transcriptomics and pathway analyses were performed after the lead-in (baseline) and both supplementation phases in men and women separately.

Results: EPA supplementation upregulated the pathways involved in cell proliferation/survival as well as differentiation in men but not in women. The differentially expressed genes shared by these pathways suggest the promotion of protein kinase B and/or cyclic AMP pathways. In contrast, in women, EPA downregulated the gene involved in mitochondrial oxidative phosphorylation (OXPHOS). The downregulation of OXPHOS was also observed after DHA supplementation in both men and women. DHA also upregulated the biological pathways with antioxidant function in both men and women, including the nuclear factor erythroid 2-related factor 2 and aryl hydrocarbon receptor signaling.

Conclusions: Our results indicate different immunomodulatory effects of EPA in monocytes from men and women by promoting pathways involved in cell proliferation and differentiation in men and lowering OXPHOS in women. In contrast, DHA promoted metabolic reprogramming in both men and women by lowering OXPHOS as well as enhancing pathways with antioxidant functions.

4.2 Introduction

Very-long-chain n-3 polyunsaturated fatty acids (hereafter, n-3 LC-PUFA) such as eicosapentaenoic acid (EPA, 20:5 n-3) and docosahexaenoic acid (DHA, 22:6 n-3) are well documented for their immunomodulating effects on monocytes and macrophages. Extensive *in vitro* work has demonstrated the ability of n-3 LC-PUFA to alter monocyte/macrophage gene expression and inflammatory function, with some evidence of differential effects of EPA and DHA (1,2). N-3 LC-PUFA can interfere with the nuclear factor kappa B-mediated inflammatory signaling pathway (3) via toll-like receptors (4), peroxisomal proliferator-activated receptors (PPAR) (5) and/or G protein-coupled receptor GPR120 (6), leading to decreased production of pro-inflammatory cytokines.

Monocytes and monocyte-derived macrophages are essential components of the innate immune system, protecting against pathogens and maintaining tissue homeostasis. They exhibit remarkable phenotype plasticity in response to environmental stimuli to execute a diverse range of immune functions. M1 macrophages, classically activated by lipopolysaccharides (LPS) or interferon (IFN) γ, have enhanced microbicidal capacity by producing reactive oxygen species (ROS) and pro-inflammatory cytokines, whereas M2 macrophages, alternatively activated by interleukin (IL)-4, display anti-inflammatory properties and promote tissue remodeling for resolution of inflammation (7). Metabolic transitions in monocytes and macrophages can dictate their inflammatory responses by producing energy and substrates to fulfill their functional requirements in response to the environment (8,9). Altered or dysregulated monocyte/macrophage metabolism and function are associated with chronic inflammation, elevated oxidative stress, and a

variety of metabolic diseases such as atherosclerosis and obesity (10–12). *In vitro* and animal studies have demonstrated the effect of n-3 LC-PUFA in reversing macrophage defects in inflammation-associated pathological conditions by improving oxidative stress status (13) and phagocytosis of apoptotic cells (14,15).

However, clinical studies examining the effect of n-3 LC-PUFA supplementation on targeted serum inflammatory markers and their gene expression in peripheral blood have generated inconsistent results, and the underlying mechanisms of the *in vivo* effects have yet to be fully characterized (16,17). Several studies have assessed the effect of combinations of n-3 LC-PUFA (i.e. EPA + DHA) in peripheral blood mononuclear cells (PBMC) using transcriptomic approaches and suggested their immunomodulatory role in oxidative stress, cell cycle, and inflammation (18–21). We have previously shown in individuals with chronic inflammation that 3 g/day supplementation with EPA and DHA differentially modulated inflammatory response of monocytes that were isolated from peripheral blood and stimulated with LPS *in vitro* (manuscript in revision; chapter 2). To gain a more comprehensive insight of the monocyte cellular processes modulated by EPA and DHA, we performed transcriptomic analysis on freshly isolated monocytes sampled at baseline and after 10 weeks of supplementation with EPA and DHA. Based on the monocyte sex dimorphism previously described on the baseline samples (manuscript submitted; chapter 3), sex-specific gene alterations by EPA and DHA were assessed.

4.3 Patients and Methods

Study design and participants

This study is part of a randomized, double-blind, crossover clinical trial (registered at ClinicalTrials.gov as NCT02670382) designed to assess the individual effects of EPA and DHA on inflammation in individuals with chronic low-grade inflammation (Chapter 2, manuscript submitted). Briefly, after a 4-week lead-in phase during which participants were given 3 g/day high oleic acid sunflower oil, they were randomly assigned to receive either 3 g/day pure EPA (> 97%) or pure DHA (> 97%) for 10 weeks and then switched to the other supplementation for another 10 weeks, with a 10-week washout phase in between. The high oleic acid sunflower oil was given during the lead-in phase with the dual purpose of assessing the compliance of participants and excluding a potential bias resulting from taking 3 g/day of fatty acids in addition to their usual diet. The 4th-week at the end of the lead-in phase was considered as baseline. Participants were men and postmenopausal women aged 50-75 years with chronic inflammation, defined as serum high-sensitivity C-reactive protein (hs-CRP) ≥ 2 μg/mL, fasting plasma triglyceride (TG) concentrations 90-500 mg/dL, and at least one of the following characteristics: i) abdominal obesity (waist circumference ≥ 102 cm in men and ≥ 89 cm in women), ii) hypertension (blood pressure ≥ 130/80 mmHg or use of anti-hypertensive drugs), and iii) fasting plasma glucose concentrations ≥ 100 mg/dL. Participants i) taking fish oil- or EPA/DHA-containing supplements or consuming a high-fish diet, ii) taking anti-inflammatory or anticoagulant medications, or iii) having any major metabolic disease (e.g. type 2 diabetes, kidney or liver disease, cardiovascular disease [CVD], etc.) were excluded. Nine men and 12 postmenopausal women completed the study with a dropout rate of < 15%. All participants signed written informed consent, and the study protocol was approved by the Tufts Health Sciences Institutional Review Board.

Blood collection, monocyte isolation, and RNA extraction

Twelve-hour fasting venous blood was collected in sodium citrate Vacutainer Cell Preparation Tubes (Becton Dickinson, Franklin Lakes, NJ) for PBMC isolation at baseline and the end of each supplementation. Monocytes were further isolated by a negative selection method using antibody-coupled magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany). Total RNA was extracted from monocytes using QIAshredder and RNeasy Mini kits, followed by on-column DNA digestion using RNase-free DNase (all from Qiagen, Hilden, Germany). The quality of RNA was assessed using a Fragment Analyzer (Agilent, Santa Clara, CA).

RNA sequencing data generation, processing, and analysis

The RNA samples were used for RNA-Seq library using Illumina TruSeq stranded mRNA kit (Illumina, San Diego, CA). Briefly, mRNA was enriched by polyA selection, fragmented, and subjected to cDNA synthesis. The 3' adenylates were added to the double-stranded cDNA, followed by adaptor ligation, second strand removal, and amplification. The size and molar concentration of resultant libraries were assessed using a Fragment Analyzer (Agilent, Santa Clara, CA). Then, libraries were sequenced on an Illumina HiSeq 2500 sequencer (Illumina, San Diego, CA) with High Output V4 chemist and 50 base-pair single-end reads format. Raw FASTQ data were processed for quality control using FastQC (www.bioinformatics.babraham.ac.uk/projects/fastqc) and mapped to the human genome (USC hg38) using HISAT v2.1 (www.ccb.jhu.edu/software/hisat/index.shtml). The mapped reads were counted by featureCounts (subread.sourceforge.net).

Monocyte purity was assessed by cell-type-specific gene expression, leading to the exclusion of 1 male and 4 female participants who displayed abnormally high counts of

CD3 and *CD8* (markers for T cells), *CD19* (a marker for B cells), and *NCAM1* (a marker for natural killer, or NK cells) in at least one of their samples. Therefore, samples from 8 men and 8 women were included in the analysis.

Differential expression analysis was performed in DESeq2, a Bioconductor package based on a negative binomial distribution (22). Raw count data were filtered based on a minimum of 10 counts at least in 16 samples in each sex group and normalized using the median of ratios method. At baseline and the end of each supplementation, sexually differentially expressed genes were identified using Wald tests comparing the expression of genes in women versus men with adjustment for phase, followed by the Benjamini-Hochberg false discovery rate (FDR) method for multiple testing corrections (23). Within each sex, differential expression attributable to EPA or DHA supplementation was determined by the Wald tests with FDR correction comparing the expression of genes at the end of each supplementation with baseline with adjustment for subject effect. Fold change was calculated as a ratio of expression values i) in women versus men or ii) after each supplementation versus baseline and then log2 transformed. Genes with FDR <0.1 were considered as significantly differentially expressed genes. The analysis was performed using R 3.6.2.

Pathway analysis

To determine the biological pathways effected by i) sex difference and ii) EPA or DHA supplementation, we performed pathway analysis using Ingenuity Pathway Analysis (IPA; v 9.0, Mountain View, CA). The z-score was calculated for each pathway by matching the observed gene expression (up or down) and the literature-derived direction of effect of the genes to the pathway (activating or inhibiting). Pathways with

absolute z-score ≥2 and p <0.05, calculated by a right-tailed Fisher's Exact Test, were considered significant. Pathways with p <0.05 but with absolute z-score between 1 and 2 were also investigated.

Comparison with interferon signature genes

To examine how the IFN signaling pathways differed between male and female monocytes at baseline and after EPA and DHA supplementation, we compared the monocyte sex-biased genes (p <0.05) to a set of genes that were recently demonstrated to be upregulated by IFN in mouse macrophages (MF-ISGs, n=601) (24,25). Briefly, among the genes induced by IFNα with >2-fold increase and FDR <0.1 in at least one cell type of mouse immune cell lineages obtained from the ImmGen project (24), the genes that were upregulated (>1-fold, FDR <0.1) in macrophages, in particular, were defined as macrophage-specific ISGs (25). Before comparisons, these genes were translated to the human orthologs using ENSEMBL BioMart data mining tool (26). Among 489 MF-ISGs that had human orthologs, 437 genes were expressed in our monocyte samples.

4.4 Results

Characteristics of study participants at baseline

As per the inclusion criteria, all participants had chronic low-grade inflammation as demonstrated by a median hs-CRP concentration of 3 μg/mL (Table 4.1). Based on their mean BMI and waist circumference, participants were also at high risk for diabetes and cardiovascular disease (CVD) (27). Baseline characteristics, including inflammatory markers and immune cell indices, were similar between men and women, except for diastolic blood pressure which was lower in women than men (p <0.02).

Modulation of monocyte transcriptional sex dimorphism by EPA and DHA

We have previously shown a significant sex dimorphism of the monocyte transcriptome at baseline, including the upregulation of genes involved in IFN signaling, in monocytes isolated from women relative to men (N=19, chapter 3). After excluding 3 additional participants due to the inclusion of other blood-borne cell types into their monocyte samples, the transcriptional sex dimorphism was still present at baseline and was maintained after supplementation with EPA and DHA (Fig. 4.1). At baseline and after EPA and DHA supplementation, the number of genes differentially expressed by sex at FDR <0.1 was 30, 27, and 23, respectively (Supplementary Table 4.1). When genes were selected based on p <0.05, 726, 815, and 684 genes were differentially expressed at baseline and after EPA and DHA supplementation, respectively (sex-biased genes; Fig. 4.2A, Online Appendix 4). While most of the sexually differentially expressed genes (FDR <0.1) found across the phases were X- or Y-linked except for Fc fragment of IgG receptor IIb [*FCGR2B*], more than 90% of the sex-biased genes (p <0.05) were autosomal (93.4%, 93.3%, and 93.4% at baseline and the end of EPA and DHA supplementation, respectively). This suggests that the sex difference in monocyte transcriptome may be broader than that resulting from sex chromosome-linked genes.

IPA pathway analysis of the sex-biased genes showed that the female bias in monocyte IFN signaling previously observed at baseline remained after EPA, but not DHA supplementation (Chapter 3, Supplementary Table 4.2). Moreover, when the sex-biased genes were compared with the previously identified macrophage-specific IFN signature genes (i.e. MF-ISGs, n=601) (25), the MF-ISG genes that were sex-biased were 61 (61/726=8.4%) at baseline and 62 (62/815=7.6%) after EPA supplementation, but a

smaller number of MF-ISGs overlapped with the sex-biased genes after DHA supplementation (32/684=4.7%).

Sex-specific alteration in monocyte gene expression profile caused by EPA and DHA

Given the significant influence of sex on monocyte transcriptome, we performed DESeq2 differential expression analysis (FDR <0.1) in men and women separately, comparing the expression of genes after each supplementation with baseline. One gene was significantly downregulated after DHA supplementation in women (*CD300A*, $\log_2$ fold change= -0.24, FDR=0.048), while no genes were differentially expressed after EPA supplementation in either sex.

To better understand the biological pathways and/or functions affected by overall changes in the whole monocyte transcriptome during each supplementation phase, we used IPA analysis on the sets of genes that were differentially expressed at p <0.05 post EPA and DHA supplementation, relative to baseline (Online Appendix 5). As shown in the Venn diagrams comparing the numbers of differentially expressed genes (p <0.05) by fatty acid and sex (i.e., EPA in men, n=602; EPA in women, n=422; DHA in men, n=439; and DHA in women, n=615), both EPA and DHA supplementation had substantially differential effects in men and women with only a small number of genes shared by both sexes (Fig. 4.2B). In addition, EPA and DHA had both common and distinct effects in men and women (Fig. 4.2C).

IPA analysis showed that EPA supplementation, in men but not women, significantly altered the expression of genes involved in cell survival and proliferation, relative to the chronic inflammatory status at baseline, by upregulating P2Y purinergic receptor signaling, relaxin signaling, G beta gamma signaling, ephrin receptor signaling, and

AMP-activated protein kinase (AMPK) signaling as well as downregulating ceramide signaling. The affected pathways share a core set of differentially expressed genes that comprise G protein signaling pathways mediated by protein kinase B (PKB, or Akt) and protein kinase A (PKA): AKT serine/threonine kinase 3 [*AKT3*], cyclic AMP (cAMP) responsive element binding protein 1 [*CREB1*], Fos proto-oncogene [*FOS*], G protein subunit beta 5 [*GNB5*], G protein subunit gamma 11 [*GNG11*], protein kinase cAMP-dependent type 1 and 2 regulatory subunit beta [*PRKAR1B; PRKAR2B*], and protein kinase C theta [*PRKCQ*] (Table 4.2). In contrast, in women, EPA significantly downregulated the expression of genes associated with mitochondrial oxidative phosphorylation (OXPHOS), relative to baseline (Table 4.2).

The downregulation of monocyte OXPHOS was also observed after DHA supplementation in both men and women (Table 4.3, Fig. 4.3), with a lowered expression of genes encoding respiratory complexes of the OXPHOS system. DHA also upregulated, albeit with 1< z-score <2, the expression of genes involved in xenobiotic metabolism aryl hydrocarbon receptor (AHR) signaling in men, and the nuclear factor erythroid 2-related factor 2 (NFE2L2, also called NRF2)-mediated oxidative stress response and sirtuin signaling in women, both of which are related to antioxidant mechanisms against oxidative stress (Table 4.3). In addition, there was a trend for DHA downregulating genes related to protein translation: eukaryotic initiation factor 2 (eIF2) signaling in men and tRNA charging in women (Table 4.3). In women only, DHA downregulated the expression of genes related to the liver X receptor/retinoid X receptor (LXR/RXR) pathway that regulates cholesterol metabolism (Table 4.3).

4.5 Discussion

We have investigated the changes in transcriptome profile of peripheral blood monocytes effected by EPA and DHA supplementation in a randomized, double-blind, crossover trial in individuals with chronic inflammation. While there have been several investigations on the combined effect of n-3 LC-PUFA (i.e. EPA + DHA) on the PBMC transcriptome (18–21), this is the first study comparing the effects of EPA and DHA on the gene expression in monocytes, the key cells involved in systemic inflammation and CVD pathogenesis. Our findings indicate that 3 g/day supplementation with EPA or DHA for 10 weeks differentially affects monocyte gene expression, and some of these changes are sex-specific.

The sex dimorphism of monocyte transcriptome previously reported at baseline remained after supplementation with both EPA and DHA. However, while the female bias in IFN signaling observed at baseline was conserved after EPA supplementation, it was no longer present after DHA supplementation. This was mostly explained by a reduced expression of the female-biased IFN signature genes in women after DHA supplementation (data not shown). N-3 LC-PUFA have been reported to impair IFNγ receptor signaling *in vivo* in mouse peritoneal macrophages and splenocytes (28). In particular, DHA has been shown to suppress lupus flares in mice by impeding IFN- and chemokine-related gene expression (29).

Changes in the monocyte transcriptome profile indicated that EPA supplementation induces differential effects in men versus women. EPA, in men but not women, affected a variety of pathways that are involved in cell proliferation, survival, and apoptosis as well as differentiation, specifically in monocytes and macrophages. In monocytes and

macrophages, some of these pathways (e.g. P2Y purinergic receptor-, relaxin-, and ephrin-mediated signaling) have been demonstrated, mostly *in vitro*, to improve chemotactic activity during differentiation and thus, the clearance of apoptotic cells (30–33). In terms of inflammation, the EPA-induced modulations appear to have conflicting implications: both the pathways generally known pro-inflammatory (i.e. thrombin and G beta gamma signaling) and those with anti-inflammatory function (i.e. relaxin and AMPK signaling) were upregulated while pro-inflammatory ceramide signaling was downregulated. However, the common set of differentially expressed genes shared among these affected pathways characterizes G protein signaling mediated by Akt and cAMP/PKA that has been shown to regulate macrophage functional phenotype with anti-inflammatory and/or pro-resolution properties (34–37). Taken together, the *in vivo* impact of these EPA-induced changes in monocytes in the context of chronic inflammation in men needs further investigation.

Significant changes in the expression of genes encoding mitochondrial OXPHOS, characterized by lower expression of the respiratory complex subunits of the electron transfer chain (ETC), were observed after supplementation with EPA in women only and after DHA in both men and women (Table 4.2 and 4.3, Fig. 4.3). OXPHOS is the mitochondrial metabolic process that produces adenosine triphosphate (ATP) through a transfer of electrons from nicotinamide and flavin adenine dinucleotide (NADH and $FADH_2$) to oxygen (38). Increases in OXPHOS and subsequent excess ROS production have been documented in *in vivo* immune cells under pro-inflammatory conditions such as bone marrow cells from the atherosclerosis-susceptible *ApoE*$^{-/-}$ mice (39) and monocytes isolated from CVD patients (40), relative to their respective controls. Based

on these observations and the fact that our participants had chronic inflammation with a high risk of diabetes and CVD, it is likely that their monocyte OXPHOS was over-activated at baseline. Therefore, the downregulation of OXPHOS after EPA and DHA supplementation may suggest normalization of monocyte metabolism, suggestive of reduced inflammatory status.

We also showed a trend towards upregulation in the genes related to antioxidant defense systems, including NRF2, sirtuin, or AHR signaling, by DHA, but not EPA, in both men and women. These results are in agreement with the recent findings of enhanced NRF2- or PPARα-mediated oxidative stress response (19,20) and antioxidant gene expression (41) in PBMC from individuals at high CVD risk following n-3 LC-PUFA supplementation. Also, the N-formylmethionine-leucyl-phenylalanine (fMLP) signaling that is involved in pro-inflammatory chemokine signaling and ROS production (42) was downregulated by DHA in men. The upregulation of the pathways with antioxidant functions, synergistically with the downregulation of OXPHOS, suggests an ability of DHA to control monocyte ROS levels, and thus potentially regulate inflammatory signaling. Moreover, DHA reduced the expression of genes related to protein translation: eIF2 signaling in men and tRNA charging in women. The downregulation of protein synthesis, especially via eIF2 signaling suppression, could be another mechanism of regulating oxidative stress as part of the unfolded protein response (43) by alleviating endoplasmic reticulum (ER) stress through reduction of protein load and prevention of protein misfolding in the ER (44–46). However, we cannot exclude the possibility that the downregulation of protein synthesis was the consequence of lowered

energy metabolism, as shown by the OXPHOS downregulation, in response to the reduced energy demands for pro-inflammatory monocyte activities (47,48).

In addition, the downregulation of the LXR/RXR pathway by DHA in women, with a reduction in the expression of acetyl-CoA carboxylase [*ACACA*], phospholipid transfer protein [*PLTP*], and stearoyl-CoA desaturase [*SCD*], is possibly due to direct binding of DHA to LXR (49–51) or activation of PPAR by DHA that competes with LXR for heterodimer formation with RXR (52–54), both leading to LXR inhibition.

Overall, our findings indicate that supplementation with EPA, but not DHA, induced differential effects on monocyte gene expression in men and women: EPA showed a unique effect in men by modulating a distinct set of pathways compared with the EPA effect in women of downregulating mitochondrial OXPHOS. Sex differences in the effect of EPA have also been reported in two large clinical trials using EPA alone, the Japan EPA Lipid Intervention Study (JELIS) (55) and the Reduction of Cardiovascular Events with Icosapent Ethyl-Intervention Trial (REDUCE-IT) (56). Both trials have reported a significant CVD risk reduction following EPA supplementation but indicated greater effectiveness of the supplementation in men than women.

In summary, monocytes are an essential component of innate immunity and precursors of macrophages that are involved in a variety of pathological inflammatory diseases, hence, are an important therapeutic target. Despite the exploratory approach and small sample size, a strength of the present study is the analysis of monocyte- and sex-specific transcriptomic changes resulting from EPA and DHA supplementation. Our data suggest that EPA may have different immunomodulatory effects in monocytes in men and women by promoting cell proliferation and differentiation/polarization in men but

reducing OXPHOS in women. In contrast, DHA has a relatively similar pattern of monocyte gene modulation in men and women, implying an improvement of ROS control through downregulating OXPHOS and increasing antioxidant capacity. Future studies are needed to further examine the sex-dependent effect of EPA and DHA in monocytes and macrophages with a focus on metabolic reprogramming and characterization of monocyte phenotype and function.

4.6 References

1. Gutiérrez S, Svahn SL, Johansson ME. Effects of omega-3 fatty acids on immune cells. Int J Mol Sci. 2019;20.

2. Allam-Ndoul B, Guénard F, Barbier O, Vohl MC. A study of the differential effects of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) on gene expression profiles of stimulated thp-1 macrophages. Nutrients. 2017;9.

3. Castrillo A, Tontonoz P. Nuclear receptors in macrophage biology: at the crossroads of lipid metabolism and inflammation. Annu Rev Cell Dev Biol. 2004;20:455–80.

4. Lee JY, Plakidas A, Lee WH, Heikkinen A, Chanmugam P, Bray G, Hwang DH. Differential modulation of Toll-like receptors by fatty acids: Preferential inhibition by n-3 polyunsaturated fatty acids. J Lipid Res. 2003;44:479–86.

5. Kliewer SA, Sundseth SS, Jones SA, Brown PJ, Wisely GB, Koble CS, Devchand P, Wahli W, Willson TM, Lenhard JM, et al. Fatty acids and eicosanoids regulate gene expression through direct interactions with peroxisome proliferator-activated receptors α and γ. Proc Natl Acad Sci U S A. 1997;94:4318–23.

6. Oh DY, Talukdar S, Bae EJ, Imamura T, Morinaga H, Fan W, Li P, Lu WJ, Watkins SM, Olefsky JM. GPR120 is an omega-3 fatty acid receptor mediating potent anti-inflammatory and insulin-sensitizing effects. Cell. 2010;142:687–98.

7. Mantovani A, Sica A, Sozzani S, Allavena P, Vecchi A, Locati M. The chemokine system in diverse forms of macrophage activation and polarization. Trends in Immunology. 2004.

8. Andrejeva G, Rathmell JC. Similarities and Distinctions of Cancer and Immune Metabolism in Inflammation and Tumors. Cell Metab. 2017;26:49–70.

9. Langston PK, Shibata M, Horng T. Metabolism supports macrophage activation. Front Immunol. 2017;8.

10. Bryan S, Baregzay B, Spicer D, Singal PK, Khaper N. Redox-inflammatory synergy in the metabolic syndrome. Can J Physiol Pharmacol. 2013;91:22–30.

11. Wang Y, Wang GZ, Rabinovitch PS, Tabas I. Macrophage mitochondrial oxidative stress promotes atherosclerosis and nuclear factor-κB-mediated inflammation in macrophages. Circ Res. 2014;114:421–33.

12. Geeraerts X, Bolli E, Fendt SM, Van Ginderachter JA. Macrophage metabolism as therapeutic target for cancer, atherosclerosis, and obesity. Front Immunol. 2017;8.

13. Meital LT, Windsor MT, Perissiou M, Schulze K, Magee R, Kuballa A, Golledge J, Bailey TG, Askew CD, Russell FD. Omega-3 fatty acids decrease oxidative stress and inflammation in macrophages from patients with small abdominal aortic aneurysm. Sci Rep. 2019;9:1–11.

14. Li S, Sun Y, Liang C-P, Thorp EB, Han S, Jehle AW, Saraswathi V, Pridgen B, Kanter JE, Li R, et al. Defective Phagocytosis of Apoptotic Cells by Macrophages in Atherosclerotic Lesions of ob/ob Mice and Reversal by a Fish Oil Diet. Circ Res. 2009;105:1072.

15. Chang HY, Lee H-N, Kim W, Surh Y-J. Docosahexaenoic acid induces M2 macrophage polarization through peroxisome proliferator-activated receptor γ activation. Life Sci. 2015;120:39–47.

16. Myhrstad MCW, Retterstøl K, Telle-Hansen VH, Ottestad I, Halvorsen B, Holven KB, Ulven SM. Effect of marine n-3 fatty acids on circulating inflammatory markers in healthy subjects and subjects with cardiovascular risk factors. Inflamm Res. 2011;60:309–19.

17. Ulven SM, Myhrstad MC, Holven KB. Marine n-3 Fatty Acids and Gene Expression in Peripheral Blood Mononuclear Cells. Curr Cardiovasc Risk Rep. 2014;8:1–10.

18. Bouwens M, Van De Rest O, Dellschaft N, Bromhaar MG, De Groot LCPGM, Geleijnse JM, Müller M, Afman LA. Fish-oil supplementation induces antiinflammatory gene expression profiles in human blood mononuclear cells. Am J Clin Nutr. 2009;90:415–24.

19. Rudkowska I, Ponton A, Jacques H, Lavigne C, Holub BJ, Marette A, Vohl MC. Effects of a supplementation of n-3 polyunsaturated fatty acids with or without fish gelatin on gene expression in peripheral blood mononuclear cells in obese, insulin-resistant subjects. J Nutrigenet Nutrigenomics. 2011;4:192–202.

20. Rudkowska I, Paradis AM, Thifault E, Julien P, Tchernof A, Couture P, Lemieux S, Barbier O, Vohl MC. Transcriptomic and metabolomic signatures of an n-3 polyunsaturated fatty acids supplementation in a normolipidemic/normocholesterolemic Caucasian population. J Nutr Biochem. 2013;24:54–61.

21. Myhrstad MCW, Ulven SM, Günther CC, Ottestad I, Holden M, Ryeng E, Borge GI, Kohler A, Brønner KW, Thoresen M, et al. Fish oil supplementation induces expression of genes related to cell cycle, endoplasmic reticulum stress and apoptosis in peripheral blood mononuclear cells: A transcriptomic approach. J Intern Med. 2014;276:498–511.

22. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. 2014;15:550.

23. Benjamini Y, Hochberg Y. Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. J R Stat Soc Ser B. 1995;57:289–300.

24. Mostafavi S, Yoshida H, Moodley D, Leboité H, Rothamel K, Raj T, Ye CJ, Chevrier N, Zhang SY, Feng T, et al. Parsing the Interferon Transcriptional Network and Its Disease Associations. Cell. 2016;164:564–78.

25. Gal-Oz ST, Maier B, Yoshida H, Seddu K, Elbaz N, Czysz C, Zuk O, Stranger BE, Ner-Gaon H, Shay T. ImmGen report: sexual dimorphism in the immune system transcriptome. Nat Commun. 2019;10:1–14.

26. Kinsella RJ, Kä Hä Ri A, Haider S, Zamora J, Proctor G, Spudich G, Almeida-King J, Staines D, Derwent P, Kerhornou A, et al. Ensembl BioMarts: a hub for data retrieval across taxonomic space.

27. Jensen MD, Ryan DH, Donato KA, Apovian CM, Ard JD, Comuzzie AG, Hu FB, Hubbard VS, Jakicic JM, Kushner RF, et al. Executive summary: Guidelines (2013) for the management of overweight and obesity in adults. Obesity. 2014;22:S5–39.

28. Irons R, Fritsche KL. Omega-3 Polyunsaturated Fatty Acids Impair In Vivo Interferon-γ Responsiveness via Diminished Receptor Signaling. J Infect Dis. 2005;191:481–6.

29. Benninghoff AD, Bates MA, Chauhan PS, Wierenga KA, Gilley KN, Holian A, Harkema JR, Pestka JJ. Docosahexaenoic Acid Consumption Impedes Early Interferon- and Chemokine-Related Gene Expression While Suppressing Silica-Triggered Flaring of Murine Lupus. Front Immunol. 2019;10:2851.

30. Desai BN, Leitinger N. Purinergic and calcium signaling in macrophage function and plasticity. Front Immunol. 2014;5.

31. Bhagavathi Perumal M, Dhanasekaran S. Relaxin: A missing link in the pathomechanisms of Systemic Lupus Erythematosus? Mod Rheumatol. 2014;24:547–51.

32. Mukai M, Suruga N, Saeki N, Ogawa K. EphA receptors and ephrin-A ligands are upregulated by monocytic differentiation/maturation and promote cell adhesion and protrusion formation in HL60 monocytes. BMC Cell Biol [Internet]. BioMed Central Ltd.; 2017 [cited 2020 Aug 18];18 (1). Available from: https://pubmed-ncbi-nlm-nih-gov.ezproxy.library.tufts.edu/28851287/

33. Pasquale EB. Eph-Ephrin Bidirectional Signaling in Physiology and Disease. Cell. 2008;133:38–52.

34. Vergadi E, Ieronymaki E, Lyroni K, Vaporidi K, Tsatsanis C. Akt Signaling Pathway in Macrophage Activation and M1/M2 Polarization. J Immunol. 2017;198:1006–14.

35. Serezani CH, Ballinger MN, Aronoff DM, Peters-Golden M. Cyclic AMP: Master regulator of innate immune cell function. Am J Respir Cell Mol Biol. 2008;39:127–32.

36. Bystrom J, Evans I, Newson J, Stables M, Toor I, Van Rooijen N, Crawford M, Colville-Nash P, Farrow S, Gilroy DW. Resolution-phase macrophages possess a unique inflammatory phenotype that is controlled by cAMP. Blood. 2008;112:4117–27.

37. Wan S, Sun H. Glucagon-like peptide-1 modulates RAW264.7 macrophage polarization by interfering with the JNK/STAT3 signaling pathway. Exp Ther Med. 2019;17:3573.

38. Saraste M. Oxidative phosphorylation at the fin de siecle. Science (80-). 1999;283:1488–93.

39. Sarrazy V, Viaud M, Westerterp M, Ivanov S, Giorgetti-Peraldi S, Guinamard R, Gautier EL, Thorp EB, De Vivo DC, Yvan-Charvet L. Disruption of Glut1 in Hematopoietic Stem Cells Prevents Myelopoiesis and Enhanced Glucose Flux in Atheromatous Plaques of ApoE -/- Mice. Circ Res. 2016;118:1062–77.

40. Shirai T, Nazarewicz RR, Wallis BB, Yanes RE, Watanabe R, Hilhorst M, Tian L, Harrison DG, Giacomini JC, Assimes TL, et al. The glycolytic enzyme PKM2 bridges metabolic and inflammatory dysfunction in coronary artery disease. J Exp Med. 2016;213:337–54.

41. Schmidt S, Stahl F, Mutz KO, Scheper T, Hahn A, Schuchardt JP. Transcriptome-based identification of antioxidative gene expression after fish oil supplementation in normo- and dyslipidemic men. Nutr Metab. 2012;9.

42. Zhu L, He P. fMLP-stimulated release of reactive oxygen species from adherent leukocytes increases microvessel permeability. Am J Physiol Circ Physiol. 2006;290:H365–72.

43. Chong WC, Shastri MD, Eri R. Endoplasmic reticulum stress and oxidative stress: A vicious nexus implicated in bowel disease pathophysiology. Int J Mol Sci. 2017;18.

44. Harding HP, Zhang Y, Zeng H, Novoa I, Lu PD, Calfon M, Sadri N, Yun C, Popko B, Paules R, et al. An integrated stress response regulates amino acid metabolism and resistance to oxidative stress. Mol Cell. 2003;11:619–33.

45. Shimizu Y, Hendershot LM. Oxidative folding: cellular strategies for dealing with the resultant equimolar production of reactive oxygen species. Antioxidants Redox Signal. 2009;11:2317–31.

46. Krishnamoorthy J, Tenkerian C, Gupta J, Ghaddar N, Wang S, Darini C, Staschke KA, Ghosh A, Gandin V, Topisirovic I, et al. Downregulation of PERK activity and eIF2α serine 51 phosphorylation by mTOR complex 1 elicits pro-oxidant and pro-death effects in tuberous sclerosis-deficient cells article. Cell Death Dis. 2018;9.

47. Liu L, Luc Y, Martinez J, Bi Y, Lian G, Wang T, Milasta S, Wang J, Yang M, Liu G, et al. Proinflammatory signal suppresses proliferation and shifts macrophage metabolism from Myc-dependent to HIF1α-dependent. Proc Natl Acad Sci U S A. 2016;113:1564–9.

48. Lachmandas E, Boutens L, Ratter JM, Hijmans A, Hooiveld GJ, Joosten LAB, Rodenburg RJ, Fransen JAM, Houtkooper RH, Van Crevel R, et al. Microbial

stimulation of different Toll-like receptor signalling pathways induces diverse metabolic programmes in human monocytes. Nat Microbiol. 2016;2.

49. Ou J, Tu H, Shan B, Luk A, DeBose-Boyd RA, Bashmakov Y, Goldstein JL, Brown MS. Unsaturated fatty acids inhibit transcription of the sterol regulatory element-binding protein-1c (SREBP-1c) gene by antagonizing ligand-dependent activation of the LXR. Proc Natl Acad Sci U S A. 2001;98:6027–32.

50. Yoshikawa T, Shimano H, Yahagi N, Ide T, Amemiya-Kudo M, Matsuzaka T, Nakakuki M, Tomita S, Okazaki H, Tamura Y, et al. Polyunsaturated fatty acids suppress sterol regulatory element-binding protein 1c promoter activity by inhibition of liver X receptor (LXR) binding to LXR response elements. J Biol Chem. 2002;277:1705–11.

51. Jump DB, Tripathy S, Depner CM. Fatty Acid–Regulated Transcription Factors in the Liver. Annu Rev Nutr. 2013;33:249–69.

52. Ide T, Shimano H, Yoshikawa T, Yahagi N, Amemiya-Kudo M, Matsuzaka T, Nakakuki M, Yatoh S, Iizuka Y, Tomita S, et al. Cross-Talk between Peroxisome Proliferator-Activated Receptor (PPAR) α and Liver X Receptor (LXR) in Nutritional Regulation of Fatty Acid Metabolism. II. LXRs Suppress Lipid Degradation Gene Promoters through Inhibition of PPAR Signaling. Mol Endocrinol. 2003;17:1255–67.

53. Matsusue K, Miyoshi A, Yamano S, Gonzalez FJ. Ligand-activated PPARβ efficiently represses the induction of LXR-dependent promoter activity through competition with RXR. Mol Cell Endocrinol. 2006;256:23–33.

54. Moreno M, Lombardi A, Silvestri E, Senese R, Cioffi F, Goglia F, Lanni A, De Lange P. PPARs: Nuclear receptors controlled by, and controlling, nutrient handling through nuclear and cytosolic signaling. PPAR Res. 2010;

55. Yokoyama M, Origasa H, Matsuzaki M, Matsuzawa Y, Saito Y, Ishikawa Y, Oikawa S, Sasaki J, Hishida H, Itakura H, et al. Effects of eicosapentaenoic acid on major coronary events in hypercholesterolaemic patients (JELIS): a randomised open-label, blinded endpoint analysis. Lancet. 2007;369:1090–8.

56. Bhatt DL, Steg PG, Miller M, Brinton EA, Jacobson TA, Ketchum SB, Doyle RT, Juliano RA, Jiao L, Granowitz C, et al. Cardiovascular risk reduction with icosapent ethyl for hypertriglyceridemia. N Engl J Med. 2019;380:11–22.

4.7 Tables and Figures

Table 4.1 Baseline characteristics of study participants.

	Men (n=8)	Women (n=8)	*p*
Age, years	59 ± 6	64 ± 5.5	0.12
Body mass index, kg/m^2	30.2 ± 3.1	32.9 ± 7.7	0.36
Waist circumference, cm	108 ± 10	96 ± 16	0.08
Systolic blood pressure, mmHg	129 ± 7	126 ± 28	0.79
Diastolic blood pressure, mmHg	86 ± 4	73 ± 14	0.02
Fasting TG, mg/dL	134 ± 50	144 ± 54	0.72
Fasting blood glucose, mg/dL	99 ± 9	101 ± 8	0.57
Total leukocyte count, 1000/μL	6.1 ± 0.8	5.6 ± 1.8	0.48
Lymphocyte proportion, % of leukocytes	28 ± 6	31 ± 8	0.41
Monocyte proportion, % of leukocytes	8.0 ± 1.9	7.5 ± 1.9	0.70
Serum inflammatory markers			
Hs-CRP†[a], μg/mL	3.0 ± 2.3	3.1 ± 2.5	0.14
TNF-α, pg/mL	2.10 ± 0.44	2.53 ± 0.61	0.13
IL-6†[a], pg/mL	0.83 ± 0.51	0.73 ± 0.61	0.79
MCP-1, pg/mL	288 ± 98	264 ± 82	0.60

Data are presented as means ± SD or †medians ± interquartile ranges. Hs-CRP, high-sensitivity C-reactive protein; TG, triglycerides.

[a] Log-transformed data were used due to the skewed distribution of the values.

Table 4.2 Top IPA biological pathways of EPA-driven differentially expressed genes (p <0.05) in peripheral blood monocytes in men and women.

Top Pathways	Z-score[a]	Molecules	p
Men			
P2Y Purinergic Receptor Signaling	+2.33	*AKT3, CREB1, FOS, GNB5, GNG11, ITGB3, P2RY1, PLCH1, PRKAR1B, PRKAR2B, PRKCQ, RASD1*	6.36 E-05
Relaxin Signaling	+2.12	*AKT3, APEX1, CREB1, FOS, GNAZ, GNB5, GNG11, GUCY1A1, GUCY1B1, PDE5A, PRKAR1B, PRKAR2B*	3.08 E-04
G Beta Gamma Signaling	+2.12	*AKT3, CACNA2D3, CAV2, GNAZ, GNB5, GNG11, PRKAR1B, PRKAR2B, PRKCQ, RASD1*	7.95 E-04
Ephrin Receptor Signaling	+2.53	*ACP1, AKT3, CREB1, CRK, CRKL, EGF, GNAZ, GNB5, GNG11, ITGB1, RASD1, WIPF1*	1.55 E-03
eNOS Signaling	+2.83	*AKT3, AQP10, AQP3, CASP8, GUCY1A1, GUCY1B1, LPAR5, PRKAB2, PRKAR1B, PRKAR2B, PRKCQ*	1.79 E-03
Thrombin Signaling	+2.65	*AKT3, CREB1, EGF, F2R, GATA1, GNAZ, GNB5, GNG11, PLCH1, PPP1R12A, PRKCQ, RASD1, RHOBTB1*	1.79 E-03
AMPK Signaling	+2.50	*AKT3, CREB1, MLST8, MLYCD, PPM1J, PPP2R5D, PRKAB2, PRKAR1B, PRKAR2B, RAB11A, RAB3A, RAB6A, TBC1D1*	2.30 E-03
Ceramide Signaling	-2.24	*AKT3, CREB1, FOS, GNB5, GNG11, ITGB3, P2RY1, PLCH1, PRKAR1B, PRKAR2B, PRKCQ, RASD1*	2.03 E-02
Women			
Oxidative Phosphorylation	-2.45	*ATP5MF, ATP5PO, COX5B, COX7A2, NDUFA9, NDUFB2*	1.20 E-02

[a] Z-score is based on the expression of genes after EPA supplementation relative to baseline.

Table 4.3 Top IPA biological pathways of DHA-driven differentially expressed genes (p <0.05) in peripheral blood monocytes in men and women.

Top Pathways	Z-score[a]	Molecules	p
Men			
Oxidative Phosphorylation	-2.83	*ATP5PB, COX7A2, COX7A2L, COX8A, NDUFA4, NDUFAB1, NDUFB5, VPS9D1*	7.27 E-04
EIF2 Signaling[b]	-1.63	*EIF2S2, EIF3F, EIF3G, PIK3CA, RAP1A, RPL21, RPL22L1, RPL35A, RPLP0, RPS10, RPS27A, RPS3A*	6.95 E-04
fMLP Signaling in Neutrophils[b]	-1.34	*ARPC2, CDC42, FPR1, GNAI3, PIK3CA, RAP1A*	1.74 E-02
Coronavirus Pathogenesis Pathway	+2.00	*CASP8, E2F3, EEF1A1, MAVS, RPS10, RPS27A, RPS3A, SERPINE1*	5.39 E-03
Xenobiotic Metabolism AHR Signaling[b]	+1.34	*AHR, ARNT, HSP90AB1, NCOA2, PTGES3*	1.76 E-02
Women			
LXR/RXR Activation	-2.45	*A1BG, ABCA1, ACACA, APOL1, CCL2, MYLIP, PLTP, SCD, SERPINA1*	3.79 E-03
Oxidative Phosphorylation	-2.12	*ATP5F1D, COX5B, NDUFB2, NDUFB6, NDUFB7, NDUFS8, SURF1, UQCRC1*	6.70 E-03
tRNA Charging	-2.00	*CARS2, TARS2, VARS2, WARS1*	1.68 E-02
Sirtuin Signaling[b]	+1.27	*ABCA1, APP, ATP5F1D, E2F1, HIF1A, NDUFB2, NDUFB6, NDUFB7, NDUFS8, NFE2L2, POLR3D, SLC25A6, TIMM10, ZBTB14*	1.74 E-02
NRF2-mediated Oxidative Stress Response[b]	+1.41	*CBR1, DNAJB4, EIF2AK3, GSTZ1, HERPUD1, MAFK, MAP2K2, MAP3K1, NFE2L2, PRKCI*	2.31 E-02

[a] Z-score is based on the expression of genes after DHA supplementation relative to baseline.

[b] The pathways affected at p <0.05 with |z-score| between 1 and 2.

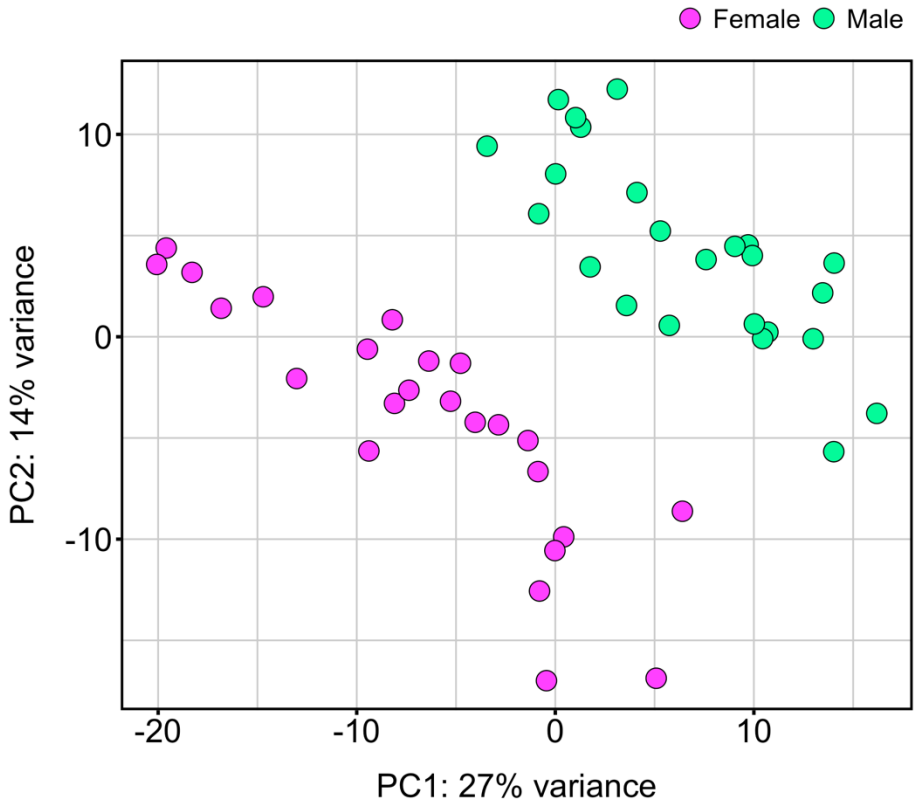


Figure 4.1. Sex dimorphism in monocyte transcriptome. Principal component analysis (PCA) of male and female peripheral blood monocytes of (n=24 each; green and pink) obtained at baseline and after supplementation phases.

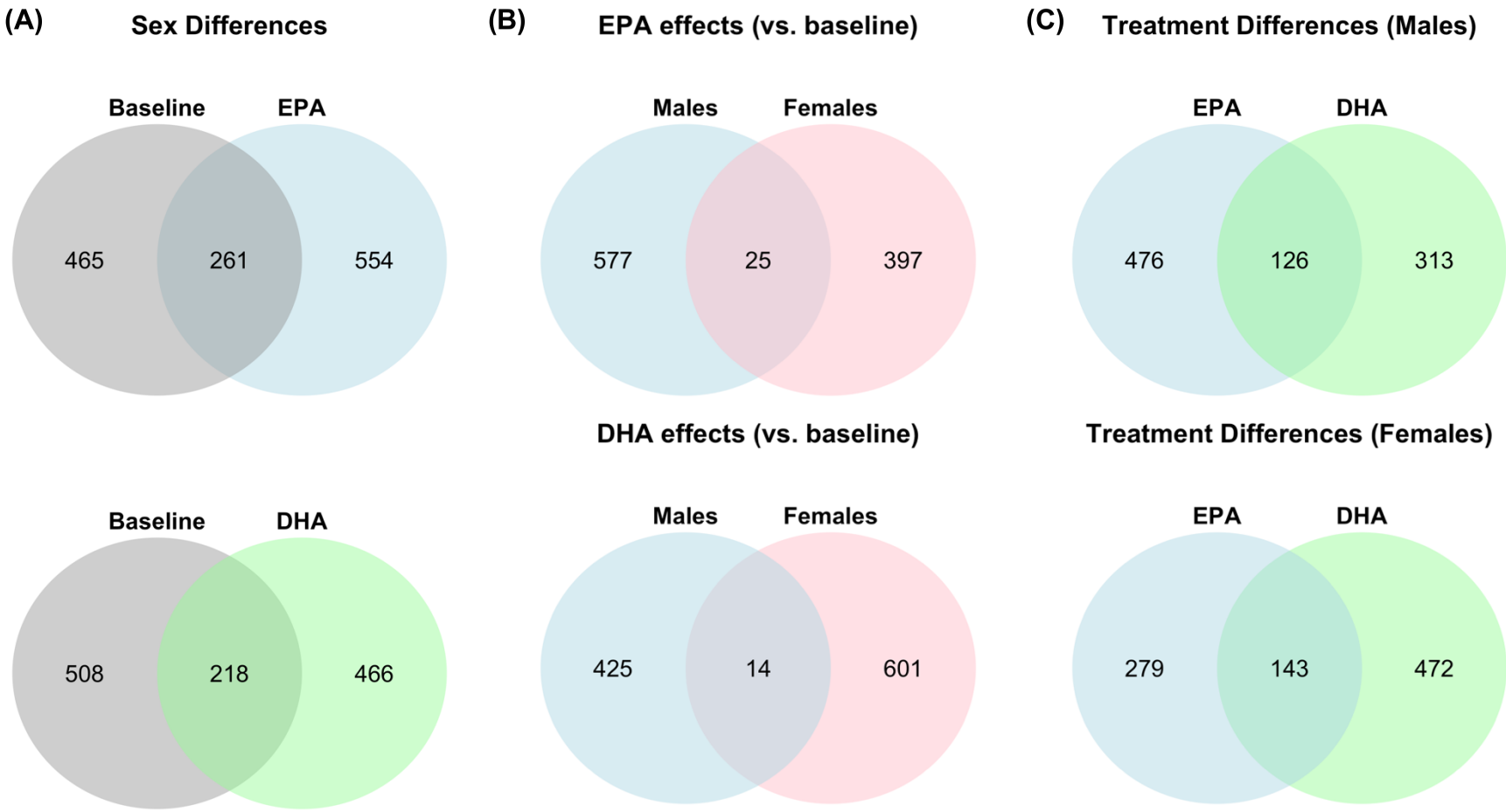


Figure 4.2. Transcriptional sex differences in monocytes at baseline and in their responses to EPA and DHA supplementation. The Venn diagrams show the numbers of differentially expressed genes (p <0.05) for **(A)** the sex differences at baseline and the end of EPA and DHA supplementation phases and **(B)** the sex-specific effects of EPA and DHA supplementation. **(C)** presents the genes differentially expressed (p <0.05) in each sex by EPA and DHA supplementation in common and uniquely.

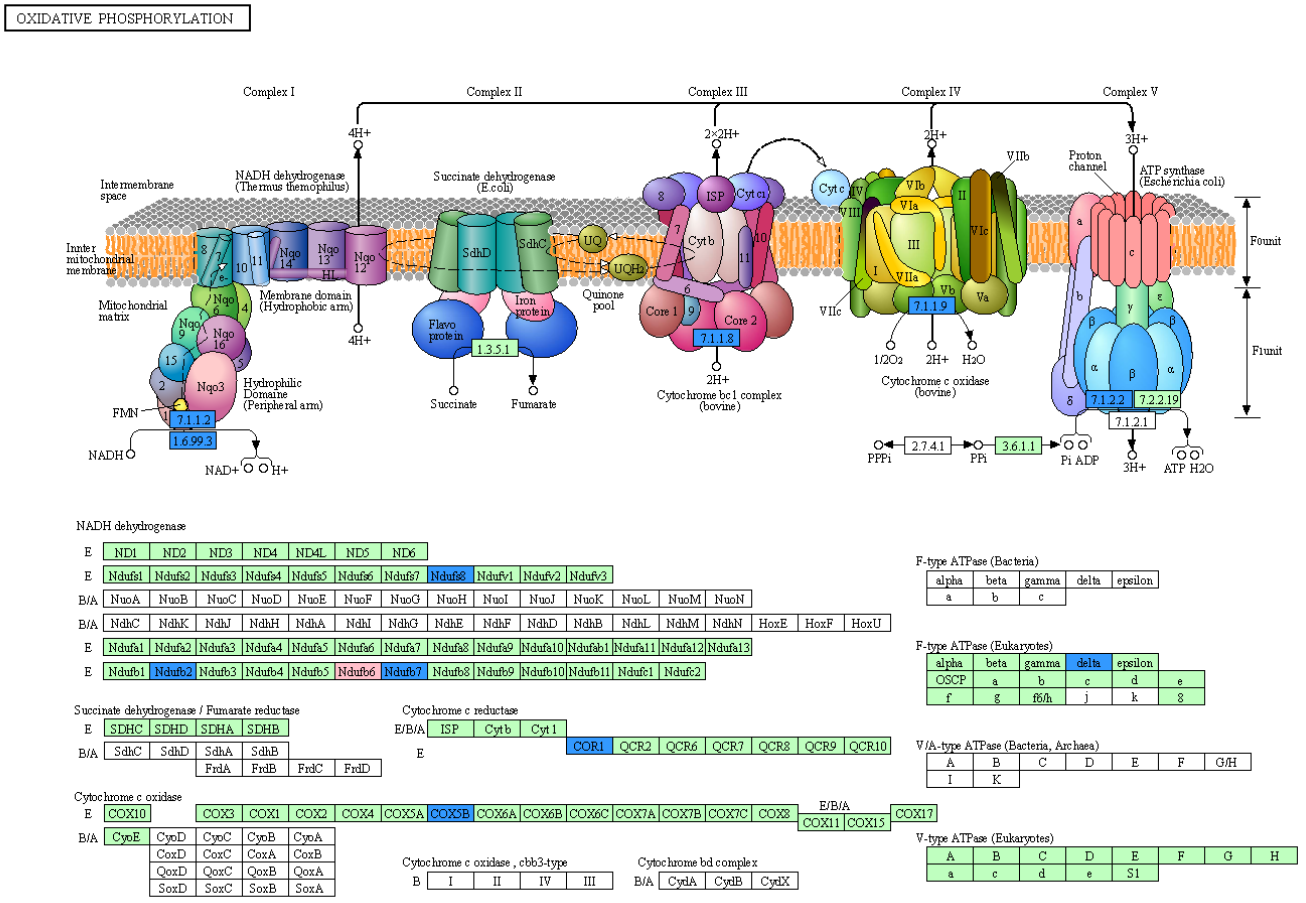


Figure 4.3. KEGG pathway diagram including 7 oxidative phosphorylation (OXPHOS) genes differentially expressed after DHA supplementation in women. **(Up)** Five electron transfer chain complexes including ATP synthase located in inner mitochondrial membrane. **(Bottom)** Rectangles represent the subunits of each complex, and the subunits encoded by differentially expressed genes are colored (down-regulated genes in blue; up-regulated genes in pink).

4. 8 Supplementary Material

Supplementary Table 4.1 Sexually differentially expressed genes (FDR <0.1) in peripheral blood monocytes at baseline and the end of EPA and DHA supplementation.

Gene Symbol	Gene Name	Baseline			EPA			DHA		
		log_2FC[a]	*p*	FDR	log_2FC[a]	*p*	FDR	log_2FC[a]	*p*	FDR
Male-biased genes										
DDX3Y	DEAD-box Helicase 3 Y-linked	-9.81	6.30E-77	3.85E-73	-10.31	1.00E-53	3.04E-50	-10.85	4.18E-52	1.01E-48
EIF1AY	Eukaryotic Translation Initiation Factor 1A Y-linked	-11.16	3.45E-52	8.42E-49	-10.64	2.69E-47	6.53E-44	-10.95	4.07E-50	8.22E-47
JDP2[b]	Jun Dimerization Protein 2	-0.50	8.38E-05	0.044	-0.42	0.015	0.637	-0.34	0.046	0.880
KDM5D	Lysine Demethylase 5D	-12.63	7.15E-67	2.91E-63	-12.15	8.31E-60	3.37E-56	-12.23	1.22E-62	4.93E-59
MRPL45[b]	Mitochondrial Ribosomal Protein L45	-0.29	4.73E-03	0.503	-0.23	0.073	0.770	-0.36	1.36E-04	0.075
NUDT14[b]	Nudix Hydrolase 14	-0.26	0.118	0.917	-0.26	0.159	0.817	-0.56	1.57E-04	0.083
P2RY8	P2Y Receptor Family Member 8	-0.36	1.41E-04	0.066	-0.23	0.023	0.697	-0.33	1.63E-03	0.366
PRKY	Protein Kinase Y-linked (pseudogene)	-8.98	4.65E-66	1.42E-62	-8.21	1.45E-68	8.82E-65	-8.41	5.29E-68	3.21E-64
RPS4Y1	Ribosomal Protein S4 Y-linked 1	-14.15	8.91E-84	1.09E-79	-12.79	2.59E-99	3.14E-95	-13.63	8.96E-78	1.09E-73
SERPINB2[b]	Serpin Family B Member 2	-1.01	1.55E-04	0.068	0.02	0.977	0.999	0.62	0.233	0.942
SNAI3[b]	Snail Family Transcriptional Repressor 3	-0.25	0.258	0.974	-0.40	0.034	0.734	-0.66	4.73E-05	0.027
TMSB4Y	Thymosin Beta 4 Y-Linked	-3.85	2.38E-22	2.42E-19	-4.18	2.81E-20	2.85E-17	-4.04	1.54E-28	1.70E-25
TTTY15	Testis-specific Transcript Y-linked 15	-8.24	3.62E-28	4.02E-25	-7.98	2.88E-25	3.19E-22	-8.01	2.26E-25	2.28E-22

TXLNGY	Taxilin Gamma Pseudogene Y-linked	-10.71	1.51E-47	2.63E-44	-10.17	1.25E-41	2.53E-38	-10.93	8.67E-48	1.50E-44
USP9Y	Ubiquitin Specific Peptidase 9 Y-linked	-9.04	7.90E-33	1.07E-29	-9.10	1.09E-31	1.47E-28	-9.11	2.58E-33	3.13E-30
UTY	Ubiquitously Transcribed Tetratricopeptide Repeat Containing Y-linked	-10.99	2.61E-50	5.30E-47	-9.31	1.48E-38	2.56E-35	-9.15	1.20E-44	1.81E-41
ZFY	Zinc Finger Protein Y-linked	-8.87	1.39E-32	1.70E-29	-8.69	7.93E-30	9.63E-27	-9.20	1.52E-34	2.04E-31

Gene Symbol	Gene Name	Baseline			EPA			DHA		
		log_2FC[a]	*p*	FDR	log_2FC[a]	*p*	FDR	log_2FC[a]	*p*	FDR
Female-biased genes										
AIM2[b]	Absent In Melanoma 2	1.06	1.18E-04	0.057	0.49	0.104	0.790	0.84	8.97E-04	0.282
ARSD	Arylsulfatase D	0.70	1.15E-14	1.08E-11	0.53	2.33E-08	1.77E-05	0.56	6.64E-08	4.73E-05
DES[b]	Desmin	1.05	8.52E-03	0.641	1.10	1.78E-05	0.010	0.95	0.015	0.818
EFHC2	EF-Hand Domain Containing 2	1.33	1.56E-04	0.068	1.22	1.98E-03	0.348	0.93	0.012	0.804
EIF1AX	Eukaryotic Translation Initiation Factor 1A X-linked	0.61	2.51E-07	1.92E-04	0.69	2.80E-13	2.43E-10	0.81	1.10E-09	8.32E-07
EML4[b]	EMAP Like 4	0.40	2.26E-03	0.354	0.52	1.24E-04	0.066	0.38	3.58E-03	0.563
EPB41L2[b]	Erythrocyte Membrane Protein Band 4.1 Like 2	0.55	0.022	0.760	0.57	2.14E-04	0.096	0.54	7.75E-04	0.277
FCGR2B[b]	Fc fragment of IgG Receptor IIb	1.44	4.66E-06	2.99E-03	1.32	2.13E-06	1.52E-03	1.31	1.18E-05	7.13E-03
HDHD1	Pseudouridine 5'-Phosphatase	0.52	1.98E-05	0.012	0.59	1.77E-08	1.43E-05	0.68	2.02E-13	1.75E-10
IFITM3[b]	Interferon Induced Transmembrane Protein 3	0.69	2.21E-03	0.350	0.82	1.33E-04	0.067	0.65	9.92E-03	0.738
KDM5C	Lysine Demethylase 5C	0.56	7.44E-08	6.06E-05	0.51	9.82E-06	5.96E-03	0.41	4.38E-06	2.80E-03

KDM6A	Lysine Demethylase 6A	0.71	1.36E-06	9.79E-04	0.79	3.14E-06	2.12E-03	0.83	9.07E-11	7.33E-08
MRC1[b]	Mannose Receptor C-Type 1	1.70	1.07E-04	0.055	0.93	0.018	0.663	0.59	0.123	0.926
MTRNR2L1[b]	MT-RNR2 Like 1	4.32	3.14E-05	0.017	-1.70	0.251	0.842	-1.93	0.198	0.939
PRKX	Protein Kinase X-linked	0.70	1.49E-11	1.30E-08	0.83	2.63E-17	2.46E-14	0.73	6.24E-15	5.82E-12
PTK2[b]	Protein Tyrosine Kinase 2	0.66	1.53E-05	9.32E-03	0.51	0.010	0.562	0.42	0.043	0.880
SLAMF7[b]	SLAM Family Member 7	0.82	2.31E-04	0.094	0.78	2.16E-03	0.350	0.69	4.10E-03	0.589
SLC28A3[b]	Solute Carrier Family 28 Member 3	0.60	0.041	0.826	1.19	1.42E0-4	0.069	0.79	0.039	0.880
STS	Steroid Sulfatase	0.25	0.044	0.835	0.53	1.91E-04	0.089	0.26	0.055	0.885
UBA1	Ubiquitin Like Modifier Activating Enzyme 1	0.29	1.87E-04	0.079	0.22	0.024	0.701	0.20	8.29E-03	0.675
ZC3H12D[b]	Zinc Finger CCCH-Type Containing 12D	0.46	0.020	0.745	0.78	5.94E-06	3.80E-03	0.47	0.035	0.875
XIST	X Inactive Specific Transcript	12.08	1.04E-36	1.58E-33	13.27	1.75E-35	2.65E-32	11.84	1.03E-53	3.12E-50
ZFX	Zinc Finger Protein X-linked	0.58	4.38E-06	2.97E-03	0.74	6.77E-05	0.037	0.68	1.25E-06	8.42E-04

[a] Fold change (FC) is calculated as a ratio of expression values in females relative to males.

[b] Autosomal genes.

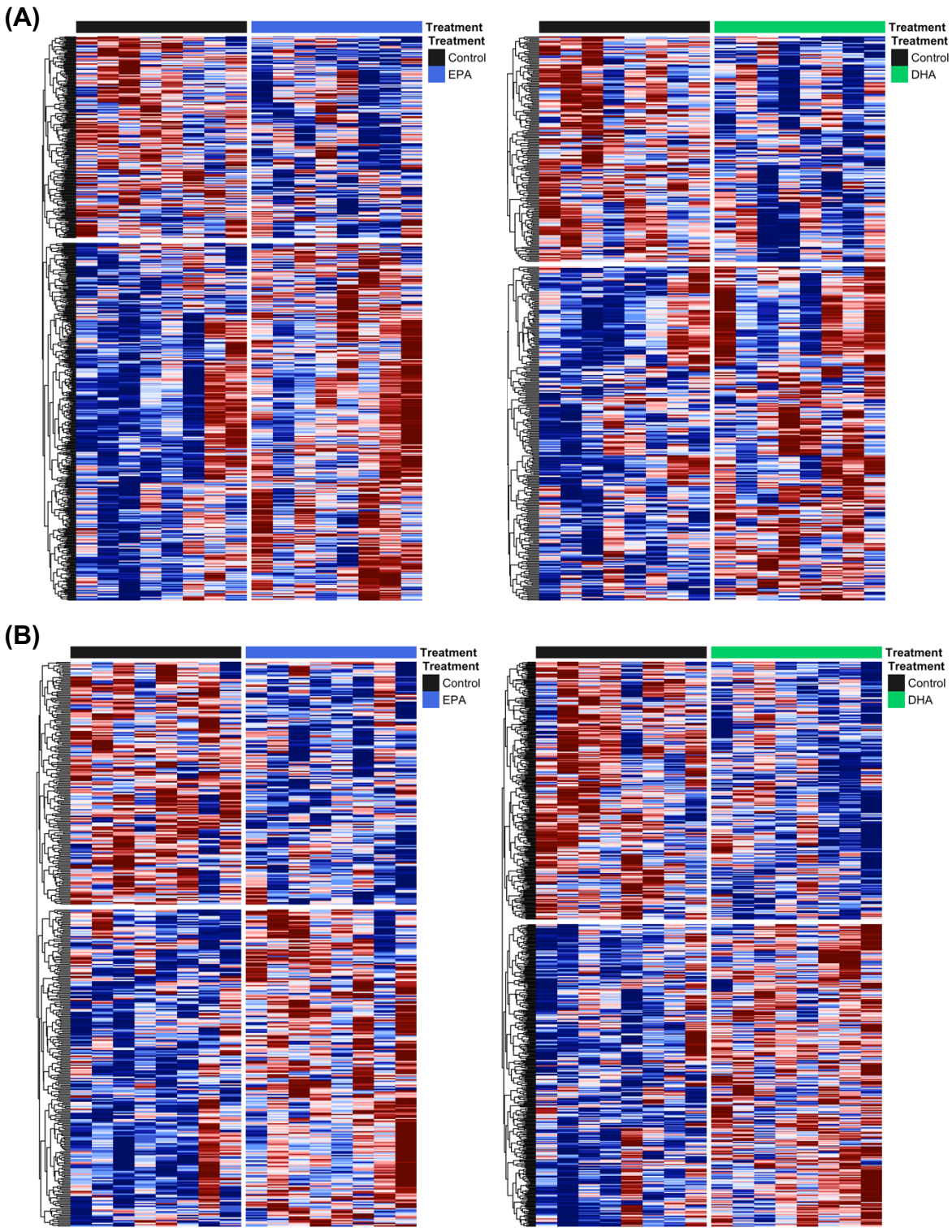
Supplementary Table 4.2 Top IPA biological pathways of sex-biased genes (p <0.05) in peripheral blood monocytes at baseline and after supplementation with EPA and DHA.

Top Pathways	Z-score[a]	Molecules	p
Baseline			
Interferon Signaling	+2.83	*IFIT1, IFIT3, IFITM1, IFITM2, IFITM3, JAK2, MX1, STAT1*	8.76 E-06
Ephrin Receptor Signaling	+3.16	*EFNB1, EGF, EPHB3, GNAO1, GNAZ, GNB4, GNG11, GRIN3A, JAK2, NCK2, PDGFA, PDGFB, PIK3CG, PTK2, RAP1B, VEGFA, VEGFC*	3.00 E-05
Thrombin Signaling	+2.71	*ARHGEF12, EGF, F2R, F2RL3, GATA1, GNAO1, GNAZ, GNB4, GNG11, ITPR2, PIK3CG, PLCH1, PLCH2, PRKCA, PTK2, RAP1B, RHOBTB1*	1.80 E-04
PDGF Signaling	+2.33	*JAK2, PDGFA, PDGFB, PIK3CG, PRKCA, RAP1B, SPHK2, STAT1, SYNJ2*	1.06 E-03
Role of NFAT in Regulation of Immune Response	+2.71	*FCER1A, FCGR1A, FCGR2B, GNAO1, GNAZ, GNB4, GNG11, HLA-DOA, HLA-DRB5, IKBKE, ITPR2, LAT, PIK3CG, RAP1B*	1.12 E-03
Dendritic Cell Maturation	+2.50	*CD1B, CD1C, CD40, FCGR1A, FCGR2B, HLA-DOA, HLA-DRB5, IKBKE, JAK2, LEPR, PIK3CG, PLCH1, PLCH2, STAT1*	1.24 E-03
p53 Signaling[b]	-1.13	*ADGRB1, E2F1, FAS, PIK3CG, PMAIP1, PML, THBS1*	2.75 E-02
After EPA supplementation			
Interferon Signaling	+2.83	*IFIT1, IFIT3, IFITM1, IFITM3, IRF1, JAK2, MX1, STAT1*	2.48 E-05
Coronavirus Pathogenesis Pathway	+2.32	*DDX58, E2F1, EEF1A1, PA2G4, RPS11, RPS13, RPS15A, RPS18, RPS23, RPS27, RPS28, RPS29, RPS3A, RPS4Y1, RPS7, STAT1*	6.37 E-05
Oncostatin M Signaling	+2.24	*JAK2, MRAS, PLAU, RAP2A, STAT1*	1.57 E-02
EIF2 Signaling	-3.13	*EIF1AX, EIF1AY, EIF2S2, EIF3G, EIF3H, EIF3L, MRAS, MYC, PIK3C2A, PIK3R3, RAP2A, RPL10, RPL11, RPL15, RPL18A,*	9.22 E-18

		RPL19, RPL21, RPL23, RPL23A, RPL26L1, RPL27, RPL29, RPL3, RPL30, RPL4, RPL5, RPL6, RPL7, RPLP0, RPS11, RPS13, RPS15A, RPS18, RPS23, RPS27, RPS28, RPS29, RPS3A, RPS4Y1, RPS7	
Fatty Acid β-oxidation I	-2.24	*ACAA2, ECI1, ECI2, HADHA, HADHB*	4.47 E-03
After DHA supplementation			
Angiopoietin Signaling	+2.12	*AKT3, IKBKE, MRAS, NFKBIA, PAK4, PIK3R2, PTK2, RAP1B, RAP2B*	2.43 E-04
Glioma Invasiveness Signaling	+2.12	*F2R, MRAS, PIK3R2, PTK2, RAP1B, RAP2B, RHOU, TIMP2*	1.08 E-03
Thrombin Signaling	+2.71	*ADCY4, AKT3, F2R, GATA3, GNAQ, MPRIP, MRAS, MYL12A, PIK3R2, PRKCI, PTK2, RAP1B, RAP2B, RHOU*	2.29 E-03
α-Adrenergic Signaling	+2.24	*ADCY4, GNAQ, MRAS, PRKAG1, PRKAR2B, PRKCI, RAP1B, RAP2B*	5.55 E-03
G Beta Gamma Signaling	+2.12	*AKT3, GNAQ, MRAS, PRKAG1, PRKAR2B, PRKCI, RAP1B, RAP2B*	2.16 E-02
P70S6K Signaling	+2.12	*AKT3, F2R, GNAQ, MRAS, PIK3R2, PRKCI, RAP1B, RAP2B*	2.89 E-02
HIF1α Signaling	+2.11	*AKT3, CCNG2, EIF4EBP1, JUN, MRAS, PIK3R2, PRKCI, RACK1, RAP1B, RAP2B, SERPINE1*	3.02 E-02
TNFR2 Signaling	-2.24	*FOS, IKBKE, JUN, NFKBIA, TNFAIP3, XIAP*	1.58 E-04
TNFR1 Signaling	-2.45	*FOS, IKBKE, JUN, NFKBIA, PAK4, TNFAIP3, XIAP*	4.60 E-04
Unfolded protein response	-2.24	*BCL2, CEBPD, INSIG1, P4HB, PPP1R15A, XBP1*	4.67 E-03

[a] Z-score is based on the expression of genes in women relative to men.

[b] The most affected among the pathways downregulated with borderline significance ($-2 < \text{z-score} < -1$, $p < 0.05$) in females relative to males.



Supplementary Figure 4.1. Sex-specific effects of EPA and DHA supplementation on monocyte transcriptome. Heatmaps of **(A)** male-specific differentially expressed genes (p <0.05) by EPA (left; n=602) and DHA (right; n=415) and **(B)** female-specific differentially expressed genes (p <0.05) by EPA (left; n=422) and DHA (right; n=615).

Chapter 5 Summary

5.1 Research Summary

The very-long-chain n-3 polyunsaturated fatty acids (n-3 LC-PUFA) eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), have long been believed to have anti-inflammatory and cardioprotective properties based on evidence from some epidemiological and mechanistic studies (1–3). The purported mechanisms include the ability of n-3 LC-PUFA to function as signaling molecules *per se*, altering gene expression via membrane-associated and/or intracellular receptors like peroxisome proliferator-activated receptors (PPAR) (4) and G protein-coupled receptor (GPR) 120 (5). In addition, n-3 LC-PUFA-derived lipid mediators can counterbalance the activities of pro-inflammatory mediators derived from n-6 FA (6), with particular subsets of these mediators, called specialized pro-resolving lipid mediators (SPM), promoting the resolutive process of inflammation (7). However, the anti-inflammatory and cardioprotective effects of n-3 LC-PUFA have been challenged in some studies that have predominantly used a mixture of EPA and DHA in varying doses and ratios (8,9). The significantly reduced CVD risks shown in recent two large clinical trials evaluating EPA-only therapies (i.e. the Japan EPA Lipid Intervention Study [JELIS] (10) and the Reduction of Cardiovascular Events with Icosapent Ethyl-Intervention Trial [REDUCE-IT] (11)) underscores the need to investigate the unique effects of EPA and DHA and their respective mechanisms of action. Some *in vitro* studies have shown higher efficacy of DHA than EPA in suppressing pro-inflammatory cytokines (12) and differential regulation of transcriptome by EPA and DHA in THP-1 monocytes (13). We have also previously reported that, in peripheral blood mononuclear cells (PBMC), distinct biological pathways are regulated after supplementation with EPA and DHA (14).

The overall objective of this thesis was to determine the common and distinct effects of EPA and DHA supplementation in modulating both systemic and cellular inflammation, specifically in blood monocytes, the key cell type involved in the pathogenesis of atherosclerosis, and identify the mechanisms involved. This objective was addressed in a randomized, double-blind, crossover study where participants with chronic inflammation received 3 g/day purified EPA and DHA for 10 weeks, separated by a washout phase. Systemic markers of inflammation and monocyte inflammatory response to lipopolysaccharides (LPS) were assessed at baseline and the end of each supplementation phase (**Aim 1, Chapter 2**). To better understand the underlying mechanisms, the effects of EPA and DHA on plasma PUFA-derived lipidome profile, including specialized pro-resolving lipid mediators (SPM) (**Aim 2, Chapter 2**), and monocyte transcriptome (**Aim 3, Chapter 4**) were examined.

Initially, we assessed the effects of EPA and DHA on high-sensitivity C-reactive protein (hs-CRP), pro-inflammatory cytokines tumor necrosis factor (TNF)-a, interleukin (IL)-6, and monocyte chemoattractant protein 1 (MCP-1), and anti-inflammatory cytokine IL-10 (**Chapter 2**). We found that serum concentrations of these markers, relative to baseline, were not significantly affected by EPA and DHA (Table 2.2A). However, EPA and DHA significantly modulated the LPS-stimulated monocyte inflammatory response, but differently (Fig. 2.1; Supplementary Fig. 2.2): EPA lowered only TNF-α expression whereas DHA suppressed the expression of all pro-inflammatory cytokines but also the anti-inflammatory IL-10. This resulted in EPA lowering the gene expression ratios of TNF-α/IL-10 and MCP-1/IL-10, compared with baseline, with no significant effect of DHA on these ratios. To examine a potential role of lipidome

changes in mediating the differential effects of EPA and DHA on the monocyte inflammatory response, we measured plasma concentrations of the lipid mediators and SPMs derived from EPA, DPA, DHA and AA at baseline and the end of the supplementation phases (Table 2.2B). Despite overall low detection of SPM end products, we found that EPA enhanced the EPA-derived lipidome containing the E-series resolvin (RvE) precursor 18-hydroxy EPA (HEPE) and lowered some of the n-6 AA-derived mediators. DHA had a wider effect by increasing not only DHA-derived lipid mediators but also those derived from the other n-3 LC-PUFAs, EPA and DPA, and reducing a greater number of n-6 AA-derived mediators. Signatures of plasma PUFA SPM lipidome, each of which represents the lipidome derived from DHA/DPA, AA, and EPA, respectively, were extracted by principal component analysis (PCA) (Fig. 2.2). An exploratory mediation analysis showed that changes in distinct PUFA lipidome signatures during EPA or DHA supplementation, at least in part, mediated the supplementation effect on LPS-induced monocyte cytokine expression (Table 2.3). The TNF-α-lowering effect of EPA was partly (44%) mediated by the significant increases in the EPA-derived mediators, whereas the TNF-α suppression by DHA was, at least in part (19%), mediated by the reductions in AA-derived mediators, rather than the increases in DHA/DPA or EPA derivatives. In contrast, the IL-6 and MCP-1 suppression by DHA were partly mediated by the changes in DHA/DPA (39%) and AA (67%), respectively. *Taken together these data suggest that EPA and DHA not only differentially mitigate monocyte inflammatory response in subjects with chronic inflammation, but also that they exert, at least in part, these effects on monocytes by differentially changing the plasma lipidome.*

We then sought to investigate the effects of EPA and DHA supplementation in monocytes by determining changes in the transcriptome and identifying biological pathways affected. During our analysis of the data, we observed clear evidence of sex dimorphism of the monocyte transcriptome at baseline, as evidenced by PCA (**Chapter 3**). Forty-one genes were found to be differentially expressed (false discovery rate [FDR] < 0.1) between men and women at baseline (Fig. 3.1). The genes with higher expression in monocytes from women than men (n=22) include genes involved in immune cell activation (*CEACAM1, FCGR2B*, and *SLAMF7*) and antigen recognition/presentation (*AIM2*, *CD1E*, and *UBA1*), suggesting that the steady-state innate immune system is more primed in women than men. Pathway analysis of the genes with sex-biased expression (p <0.03, n=565) identified 11 pathways upregulated in women relative to men, most of which were involved in the immune and inflammatory response (Table 3.2). Of these, female bias in interferon (IFN) signaling was the most significant. The contribution of IFN signaling to sex differences was further confirmed by the comparison between the monocyte sex-biased genes and the previously-documented macrophage-specific IFN signature genes (MF-ISG) (15,16). Most of the overlapping genes (48/54=89%) were female-biased (Fig. 3.2), and the overall fold change distribution of MF-ISG was skewed toward the female side compared to all other genes (Fig. 3.3). Of particular relevance, the MF-ISG involved in antiviral effector response showed the most significant female bias followed by those encoding inflammatory mediators (Fig. 3.4). *These results indicate a more enhanced monocyte IFN signaling in women than in men with chronic inflammatory status at baseline. These differences in monocyte*

transcriptome possibly explain the well-characterized sex differences in rates of autoimmune diseases (17) and viral infections (18).

Given the observed sex differences at baseline, the effects of EPA and DHA supplementation on monocyte transcriptome were examined separately in men and women (**Chapter 4**). Following identification of sex-biased genes at baseline and at the end of each supplementation, pathway analysis indicated that the upregulation of IFN signaling observed in women compared to men at baseline remained after supplementation with EPA, but not DHA (Supplementary Table 3.2). When we assessed the effect of EPA and DHA supplementation in men and women separately, differential expression analysis comparing post-supplementation with baseline identified only 1 gene that was downregulated (FDR <0.1) by DHA in women, but none by EPA. To better understand the biological processes affected by the changes in the whole transcriptome, we performed exploratory pathway analysis using the sets of genes differentially expressed at p <0.05 by EPA or DHA supplementation, compared with baseline, in men and women (Table 3.2 and 3.3). EPA supplementation showed differential effects in men and women: EPA promoted pathways involved in monocyte/macrophage proliferation and differentiation in men while downregulating mitochondrial oxidative phosphorylation (OXPHOS) in women. The OXPHOS was also downregulated by DHA supplementation in both men and women. DHA, in both men and women, also upregulated intracellular signaling related to antioxidant defense, including the nuclear factor erythroid 2-related factor 2 (or NRF2) and aryl hydrocarbon receptor (AHR) signaling, and downregulated protein translation. *These results suggest that EPA and DHA have distinct*

immunomodulating effects on monocyte transcriptome, and some of these effects are sex-dependent, particularly for EPA.

5.2 Discussion

Collectively, results from this thesis demonstrate that 3 g/day EPA and DHA supplementation for 10 weeks improve monocyte inflammatory status in individuals with chronic inflammation in a distinct manner by affecting different sets of biological pathways. Our findings indicate that some of these effects are possibly accomplished, at least in part, through alterations in the n-3 and n-6 PUFA SPM lipidome.

The monocyte inflammatory profile is dynamically modulated by a balance between pro- and anti-inflammatory responses. Active processes of inflammation resolution following an acute inflammatory response, in addition to suppression of the ongoing inflammatory response (i.e. anti-inflammation), are critical to retain homeostasis and prevent the chronic inflammation associated with various pathological conditions (19). Our findings suggest that EPA supplementation improves monocyte inflammatory status, when LPS-stimulated, by effectively balancing the expression of pro-inflammatory cytokines with that of the anti-inflammatory cytokine IL-10, despite a modest suppression of the pro-inflammatory cytokines. According to the exploratory mediation analysis, this effect may be at least partly mediated by the augmented profile of EPA downstream lipid metabolites including 18-HEPE, the RvE precursor. This supports the anti-inflammatory actions of EPA-derived lipid molecules (e.g. 18-HEPE and 5-HEPE) observed in animal disease models (20–23), further suggesting their composite *in vivo* role in inflammation. However, in freshly isolated, unstimulated monocytes, EPA

modulated the overall gene expression profile toward upregulating proliferation and differentiation in men and downregulating mitochondrial OXPHOS in women, which implies distinct effects of EPA by sex. Especially, EPA induced unique changes in monocytes from men, not shared by DHA. The pathways affected by EPA have common functions related to cell proliferation, survival, and apoptosis and, specifically within the context of monocytes and macrophages, determination of functional phenotypes during differentiation. They have been shown in monocytes and macrophages *in vitro* to enhance adhesive characteristics (24–26), which are essential for differentiation/maturation, and promote phagocytosis of apoptotic cells (24,25) and tissue repair after injury (27). While the EPA-induced changes in some of these pathways may suggest pro-inflammatory effects, it is notable that some of these pathways, e.g., AMPK signaling and P2Y1 purinergic receptor- and relaxin-mediated signaling, have been shown to promote macrophage polarization toward the anti-inflammatory and pro-reparative M2-like functional phenotype (24,25,28,29). However, the *in vivo* influence of these changes in monocytes in the context of chronic inflammation needs to be further studied. The observed sex difference in the EPA effect in modulating monocyte transcriptome is in accordance with clinical observations from the JELIS (10) and REDUCE-IT (11) trials that indicated greater effectiveness of EPA for CVD prevention in men than women. Taken altogether, it can be speculated that EPA is beneficial in high-CVD-risk individuals, especially in men, who possibly have an inflammation resolution deficit by helping monocytes gain a phenotype with anti-inflammatory and pro-resolving activities.

In contrast, DHA supplementation was effective in modulating monocyte-associated inflammation by attenuating LPS-induced inflammatory response through strong

suppression of a wide range of pro-inflammatory cytokines but also resulted in the downregulation of the anti-inflammatory cytokine IL-10. We found that the suppression of multiple distinct cytokines by DHA was partly mediated by the DHA-induced changes in various subgroups of PUFA derivatives. In freshly isolated monocytes, DHA showed similar effects on the transcriptome profile in men and women. Most striking was the downregulation of OXPHOS system in both men and women. Based on the evidence of increased OXPHOS rates in *in vivo* monocyte/macrophages under pro-inflammatory environments shown in other studies (30–32), it can be assumed that our study participants' monocytes had an activated OXPHOS at baseline due to their chronic inflammation. The lowered OXPHOS by DHA in men and women, and by EPA in women, then suggests a ramping-down of the activated monocyte energy metabolism due to the reduction in energy demands required for inflammatory cell proliferation and signaling. In addition, DHA affected pathways related to antioxidant mechanism by upregulating the AHR- or NRF2-mediated signaling that regulates transcription of key antioxidant and detoxifying enzymes that can counteract the deleterious effects of ROS (33). Also, the inhibition of eIF2-mediated protein synthesis by DHA, known as an adaptive stress response, may prevent additional oxidative stress resulting from protein misfolding and spare OXPHOS from energy and ROS production (34,35). The downregulation of OXPHOS, a major source of ROS production, by DHA would work synergistically with the other concomitant changes for a better control of monocyte ROS production related to pro-inflammatory responses. This is to some extent in agreement with the strong effect of DHA in suppressing pro-inflammatory cytokine expression in monocytes when LPS-activated.

In summary, this thesis showed that EPA and DHA exert different immunomodulatory effects on monocytes in subjects with chronic inflammation and that there are potential sex differences in their effectiveness. The cardioprotection by EPA-only supplementation observed in the JELIS and REDUCE-IT trials, which is in contrast to the conflicting findings for the combinations of EPA and DHA (10,11), supports the need for therapeutic approaches differentiating EPA and DHA for disease prevention and more specifically for CVD.

5.3 Considerations and Future Directions

This work is not without limitations. First, the transcriptome and lipidome analyses provide a comprehensive but descriptive assessment of the supplementation effects. Alterations in gene expression do not necessarily translate to changes in protein expression and function. In regard to lipidome assessment, the inability of our assay to detect low-level SPM products limits our understanding of the effect of supplementation on the SPM biosynthetic pathways and the role of SPM on biological pathways. The small sample size may have limited our ability to assess the effects of EPA and DHA. However, the crossover design of our study allowed for a reduction in bias related to genetic and environmental variability among participants.

By integrating the changes induced in plasma PUFA lipidome and monocyte gene expression after each supplementation, our exploratory mediation analysis identified a potential composite role of PUFA lipid mediators on monocyte-associated inflammation. Further *in vitro* work assessing the effect of EPA, DHA and their respective downstream lipid mediators is needed for the mechanistic understanding of our findings. Though our

transcriptomic analysis indicated a strong sex-specific effect of EPA and DHA in monocytes, this sub-analysis was completed on 8 participants in each sex. Future work on EPA or DHA treatment should attempt to consider sex dimorphism and replicate this analysis in a larger cohort. Our work also suggested a close link between mitochondrial energy metabolism and ROS control in determining monocyte phenotype in chronic inflammation. To dissect the mechanisms involved in the EPA or DHA-induced monocyte metabolic reprogramming, additional work should aim to perform a detailed characterization of cell phenotype and function, together with an evaluation of cellular metabolism and redox homeostasis. *Our work provides strong evidence that future clinical studies should consider the sex- and fatty acid-dependent effect of n-3 LC-PUFA to promote health and prevent diseases.*

5.4 References

1. Burr ML, Gilbert JF, Holliday RM, Elwood PC, Fehily AM, Rogers S, Sweetnam PM, Deadman NM. Effects of changes in fat, fish, and fibre intakes on death and myocardial reinfarction: Diet and Reinfarction Trial (DART). Lancet. 1989;334:757–61.

2. Simopoulos AP. Omega-3 fatty acids in inflammation and autoimmune diseases. J Am Coll Nutr. 2002;21:495–505.

3. Mori TA, Beilin LJ. Omega-3 fatty acids and inflammation. Curr Atheroscler Rep. 2004;6:461–7.

4. Kliewer SA, Sundseth SS, Jones SA, Brown PJ, Wisely GB, Koble CS, Devchand P, Wahli W, Willson TM, Lenhard JM, et al. Fatty acids and eicosanoids regulate gene expression through direct interactions with peroxisome proliferator-activated receptors α and γ. Proc Natl Acad Sci U S A. 1997;94:4318–23.

5. Oh DY, Talukdar S, Bae EJ, Imamura T, Morinaga H, Fan W, Li P, Lu WJ, Watkins SM, Olefsky JM. GPR120 is an omega-3 fatty acid receptor mediating potent anti-inflammatory and insulin-sensitizing effects. Cell. 2010;142:687–98.

6. Calder PC. Marine omega-3 fatty acids and inflammatory processes: Effects, mechanisms and clinical relevance. Biochim Biophys Acta - Mol Cell Biol Lipids. 2015;1851:469–84.

7. Serhan CN. Pro-resolving lipid mediators are leads for resolution physiology. Nature. 2014;510:92–101.

8. Siscovick DS, Barringer TA, Fretts AM, Wu JHY, Lichtenstein AH, Costello RB, Kris-Etherton PM, Jacobson TA, Engler MB, Alger HM, et al. Omega-3 Polyunsaturated Fatty Acid (Fish Oil) Supplementation and the Prevention of Clinical Cardiovascular Disease: A Science Advisory from the American Heart Association. Circulation. 2017;135:e867–84.

9. Aung T, Halsey J, Kromhout D, Gerstein HC, Marchioli R, Tavazzi L, Geleijnse JM, Rauch B, Ness A, Galan P, et al. Associations of omega-3 fatty acid supplement use with cardiovascular disease risks meta-analysis of 10 trials involving 77 917 individuals. JAMA Cardiol. American Medical Association; 2018;3:225–34.

10. Yokoyama M, Origasa H, Matsuzaki M, Matsuzawa Y, Saito Y, Ishikawa Y, Oikawa S, Sasaki J, Hishida H, Itakura H, et al. Effects of eicosapentaenoic acid on major coronary events in hypercholesterolaemic patients (JELIS): a randomised open-label, blinded endpoint analysis. Lancet. 2007;369:1090–8.

11. Bhatt DL, Steg PG, Miller M, Brinton EA, Jacobson TA, Ketchum SB, Doyle RT, Juliano RA, Jiao L, Granowitz C, et al. Cardiovascular risk reduction with

icosapent ethyl for hypertriglyceridemia. N Engl J Med. 2019;380:11–22.

12. Weldon SM, Mullen AC, Loscher CE, Hurley LA, Roche HM. Docosahexaenoic acid induces an anti-inflammatory profile in lipopolysaccharide-stimulated human THP-1 macrophages more effectively than eicosapentaenoic acid. J Nutr Biochem. 2007;18:250–8.

13. Allam-Ndoul B, Guénard F, Barbier O, Vohl MC. A study of the differential effects of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) on gene expression profiles of stimulated thp-1 macrophages. Nutrients. 2017;9.

14. Tsunoda F, Lamon-Fava S, Asztalos BF, Iyer LK, Richardson K, Schaefer EJ. Effects of oral eicosapentaenoic acid versus docosahexaenoic acid on human peripheral blood mononuclear cell gene expression. Atherosclerosis. 2015;241:400–8.

15. Mostafavi S, Yoshida H, Moodley D, Leboité H, Rothamel K, Raj T, Ye CJ, Chevrier N, Zhang SY, Feng T, et al. Parsing the Interferon Transcriptional Network and Its Disease Associations. Cell. 2016;164:564–78.

16. Gal-Oz ST, Maier B, Yoshida H, Seddu K, Elbaz N, Czysz C, Zuk O, Stranger BE, Ner-Gaon H, Shay T. ImmGen report: sexual dimorphism in the immune system transcriptome. Nat Commun. 2019;10:1–14.

17. Whitacre CC, Reingold SC, O'Looney PA. A gender gap in autoimmunity. Science. 1999;283:1277–8.

18. McClelland EE, Smith JM. Gender specific differences in the immune response to infection. Arch Immunol Ther Exp (Warsz). 2011;59:203–13.

19. Serhan CN, Chiang N, Van Dyke TE. Resolving inflammation: Dual anti-inflammatory and pro-resolution lipid mediators. Nat Rev Immunol. 2008;8:349–61.

20. Weylandt KH, Krause LF, Gomolka B, Chiu C-Y, Bilal S, Nadolny A, Waechter SF, Fischer A, Rothe M, Kang JX. Suppressed liver tumorigenesis in fat-1 mice with elevated omega-3 fatty acids is associated with increased omega-3 derived lipid mediators and reduced TNF-α. Carcinogenesis. 2011;32:897–903.

21. Krishnamurthy VR, Dougherty A, Haller CA, Chaikof EL. Total Synthesis and Bioactivity of 18(R)-Hydroxyeicosapentaenoic Acid. J Org Chem. 2011;76:5433–7.

22. Wang C, Liu W, Yao L, Zhang X, Zhang X, Ye C, Jiang H, He J, Zhu Y, Ai D. Hydroxyeicosapentaenoic acids and epoxyeicosatetraenoic acids attenuate early occurrence of nonalcoholic fatty liver disease. Br J Pharmacol. 2017;174:2358–72.

23. Onodera T, Fukuhara A, Shin J, Hayakawa T, Otsuki M, Shimomura I.

Eicosapentaenoic acid and 5-HEPE enhance macrophage-mediated Treg induction in mice. Sci Rep. 2017;7:4560.

24. Desai BN, Leitinger N. Purinergic and calcium signaling in macrophage function and plasticity. Front Immunol. 2014;5.

25. Bhagavathi Perumal M, Dhanasekaran S. Relaxin: A missing link in the pathomechanisms of Systemic Lupus Erythematosus? Mod Rheumatol. 2014;24:547–51.

26. Mukai M, Suruga N, Saeki N, Ogawa K. EphA receptors and ephrin-A ligands are upregulated by monocytic differentiation/maturation and promote cell adhesion and protrusion formation in HL60 monocytes. BMC Cell Biol [Internet]. BioMed Central Ltd.; 2017 [cited 2020 Aug 18];18 (1). Available from: https://pubmed-ncbi-nlm-nih-gov.ezproxy.library.tufts.edu/28851287/

27. Pasquale EB. Eph-Ephrin Bidirectional Signaling in Physiology and Disease. Cell. 2008;133:38–52.

28. Sag D, Carling D, Stout RD, Suttles J. Adenosine 5′-Monophosphate-Activated Protein Kinase Promotes Macrophage Polarization to an Anti-Inflammatory Functional Phenotype. J Immunol. 2008;181:8633–41.

29. Qing L, Fu J, Wu P, Zhou Z, Yu F, Tang J. Metformin induces the m2 macrophage polarization to accelerate the wound healing via regulating ampk/ mtor/nlrp3 inflammasome singling pathway. Am J Transl Res. 2019;11:655–68.

30. Sarrazy V, Viaud M, Westerterp M, Ivanov S, Giorgetti-Peraldi S, Guinamard R, Gautier EL, Thorp EB, De Vivo DC, Yvan-Charvet L. Disruption of Glut1 in Hematopoietic Stem Cells Prevents Myelopoiesis and Enhanced Glucose Flux in Atheromatous Plaques of ApoE -/- Mice. Circ Res. 2016;118:1062–77.

31. Shirai T, Nazarewicz RR, Wallis BB, Yanes RE, Watanabe R, Hilhorst M, Tian L, Harrison DG, Giacomini JC, Assimes TL, et al. The glycolytic enzyme PKM2 bridges metabolic and inflammatory dysfunction in coronary artery disease. J Exp Med. 2016;213:337–54.

32. Serbulea V, Upchurch CM, Schappe MS, Voigt P, DeWeese DE, Desai BN, Meher AK, Leitinger N. Macrophage phenotype and bioenergetics are controlled by oxidized phospholipids identified in lean and obese adipose tissue. Proc Natl Acad Sci U S A. 2018;115:E6254–63.

33. Dietrich C. Antioxidant Functions of the Aryl Hydrocarbon Receptor. 2016;

34. Shimizu Y, Hendershot LM. Oxidative folding: cellular strategies for dealing with the resultant equimolar production of reactive oxygen species. Antioxidants Redox Signal. 2009;11:2317–31.

35. Venditti P, Di Stefano L, Di Meo S. Mitochondrial metabolism of reactive oxygen species. Mitochondrion. 2013;13:71–82.

Appendix

A.1 Detailed Methods

A.1.1 PBMC and Monocyte Isolation and Monocyte Culture

Materials and Equipment:

- Cell Preparation Tubes (CPT) (BD, 362761)
- 12 well plates, (Costar #3513)
- Corning Disposable 500 mL sterile filter system (Fisher, 357105)
- Refrigerated centrifuge (Sorvall Legend Mach 1.6R)
- Microcentrifuge
- CO_2 cell incubator
- Water bath
- Microscope
- Automated cell counter (Moxi)
- Sterile Hood (The Baker Company, SG403A)
- Vacuum with pump
- Carbon Dioxide (Airgas, CD USP 50)

Reagents:

- 1xPBS-1%BSA buffer
- Cell culture media (RPMI, FBS, P/S, HEPES)
- Monocyte isolation buffer (1xPBS-0.5%BSA)
- Freezing buffer (90% FBS, 10% DMSO)
- LPS from *E. coli* O111:B4

Pre-Preparation: CO_2 incubator

Ensure that humidity tray is filled with copper sulfate solution.

Check CO_2 tank to ensure gas will be adequate for the duration of the experiment.

Morning Protocol: Disinfection

Wipe Down with 70% Ethanol

Sterile hood
Drawer handles
Refrigerator/Freezer handles
Microscope
Centrifuge buttons and lid
Incubator door
Water Bath buttons and lid
Any racks, plastic containers, thermometers that will go into water bath
Any bench tops that will be used to place tubes containing the cells
Centrifuge buckets and inserts.

Put down new bench-coat by the centrifuge before each new experiment.

Clean water bath.

Pre-Experiment:

1. Turn off UV light. Wipe the following with 70% alcohol using kimwipe
    - Interior surfaces of sterile hood
    - (6) 15 mL conical tubes. (Label tubes with HNRC#, then #1-#6)
    - (4) 50 mL conical tubes (for 1X PBS)
    - Large bottle for waste
    - Pipet Aid
    - Microcentrifuge tube rack
2. Set water bath to 37ºC.
3. Set the centrifuge to 22°C.
4. Place 50 mL aliquots of PBS-1% BSA at room temperature. Return tubes to sterile hood when at room temperature.

PBMC Isolation (USE STERILE TECHNIQUE)

Retrieve 9 CPT tubes from MRU immediately after blood draw. Ensure that the CPT tubes have been inverted 8-10 times prior to centrifugation.

Note "Start Time" when blood tubes are brought into lab (see Testing Logs):

Preparation of cell layers for wash:

1. Let tubes sit on lab bench next to centrifuge **10-15 minutes** until no longer warm to the touch. Must be ambient temperature.
2. Take out 1X PBS-1% BSA to reach room temperature.
3. **Invert CPT tubes again 8-10 times prior to centrifugation.**
4. Centrifuge at **1600 x g** for **20 min** at 22ºC.

   If cell layer is not completely separated, the CPT tubes may be spun up to additional 10 minutes (DO NOT exceed a 30 minute total spin).
5. Bring CPTs into the sterile hood.
6. Aspirate and discard approximately 5 ml of the top layer (plasma) from all CPT tubes using the Pipet Aid and a 10 mL serological pipet, without disturbing the cell layer (off-white, just above the gel).
7. Collect the PBMC layer above the gel from 8 CPT tubes as follows: collect PBMC layer from 2 CPT tubes using P1000 micropipette, and transfer into a single sterile 50 ml conical tube. Repeat for the six remaining CPT tubes. Total = 4 x 50ml conical tubes for first wash. For tube 9, collect PBMC Layer into 1 15 mL tube.

First PBMC wash:

8. Bring volume of 50 ml tubes up to **30 mL** with room temperature sterile 1X PBS-1% BSA using Pipet Aid and 10 mL serological pipet. Bring volume of 15 ml tube up to **15 mL** with room temperature sterile 1X PBS-1% BSA.
9. Pipette up and down using Pipet Aid and 10 mL serological pipet to mix, cap tubes, invert 5 times.
10. Centrifuge tubes at **200 x g** for **10 min** at 22ºC.

Second PBMC wash:

11. Using the Pipet Aid and a 10 mL serological pipette, discard as much supernatant as possible from tube without disturbing pellet. Repeat this step with P1000 pipet and discard any remaining supernatant.
12. Using a P1000 pipet add 1 mL sterile 1XPBS-1%BSA to tube and pipet up and down to re-suspend cells in 50 mL tubes. Combine cells from 2 50 mL tubes into 1 50 mL tube . Total = 2 x 50 mL tubes.
13. Using the P10 micropipette, remove **10 µL** from each tube and dilute 1:30 (in 290 µL of cell culture media) in order to get a cell count for each of the 4 50mL. conical tubes.

    Using the cell counter, count cells in the middle square and any four other squares. To get the total number of cells in the 50 mL conical tube, calculate the mean number of cells in one square, multiply by 10,000, then multiply by dilution factor (30), and lastly multiply by the total volume that the cells are suspended in. **Alternatively, use the Moxi automated cell counter. To get the total number of cells in the 50mL conical tube, place 75µL. of cells on cassette and measure. The number Moxi provides will need to be multiplied by the dilution factor (30) and the volume of cells.**
14. Add sterile 1XPBS -1% BSA to bring volume to **30 mL.** Mix 5 times.
15. Resuspend pellet in 15 mL tube with 1 mL sterile 1XPBS-1%BSA and bring to 15 mL final volume with sterile 1XPBS-1%BSA.
16. Centrifuge at **200 x g** for **10 min** at 22ºC.
17. Re-suspend the pellet of 50 mL tubes in the appropriate volume of Monocyte isolation BSA buffer. To determine the appropriate amount to re-suspend the pellet in, calculate 30 uL of buffer for each 10^7 cells, and multiply that by 2. Re-suspend the pellet in tube 1 in calculated amount, transfer its entirety into tube 2, and mix.
18. Resuspend cell pellet of 15 mL tube into 100 µL of 1XPBS-1%BSA and aliquot in to 3 1.5 ml tubes (1= 40 µL for future RNA isolation [freeze as is]), and 2 tubes of 30 µL for FACS [in 90% serum and1 10% DMSO).
19. Take out LPS stock solution and sterile PBS from freezer.
    Prepare ice for keeping monocyte isolation BSA buffer.

Monocyte Isolation (USE STERILE TECHNIQUE)

Follow Miltenyi's procedure for pan monocyte isolation (use the buffer provided by Miltenyi (PBS-0.5% BSA). Buffers and reagents should be at 4 ºC.

1. Wet the pre-separation filter with 500 uL of buffer and discard buffer.
2. Place cells in the filter reservoir and collect cell flow through in conical tube.
3. Remove filter reservoir from conical tube.
4. Add 10 uL FcR Blocking Reagent for each 10^7 cells by using 200µL pipette and pipetting directly into cell flow though.
5. Add 10 uL of Biotin-Antibody Cocktail for each 10^7 cells by using 200µL pipette and pipetting directly into cell flow though.
6. Mix well and incubate for 5 min in refrigerator.
7. Add 30 uL of buffer for each 10^7 cells by using 200µL pipette and pipetting directly into cell flow though.
8. Add 20 ul of Anti-Biotin Microbeads for each 10^7 cells by using 200µL pipette and pipetting directly into cell flow though.
9. Mix well and incubate for 10 min in refrigerator.
10. During this time, prepare LPS+ media and control media.

    A. Obtain LPS stock solution (stock solution is 1 mg/mL in endotoxin-free sterile water; made several aliquots of 100 µL each and stored at -20 ºC). Prepare similar aliquots of endotoxin-free sterile water without LPS to use as control. **Always use the same LPS and control for the same subject.**

    B. **Dilute** LPS stock (1 mg/mL) 1:100 to 10 µg/mL (e.g. add 10 µL of LPS stock to 990 µL of sterile PBS, mix).

    C. Make "LPS+" media (in a 15 mL conical tube):
        i. 2.0 mL cell culture media
        ii. 2.66 uL diluted LPS (use filtered pipette tips)

    D. Make "Control" media (n a 15 mL conical tube):
        iii. 2.0 mL cell culture media
        iv. 2.66 uL sterile PBS (use filtered pipette tips)

11. Place MS column on magnetic field of MACS separator, with wings facing the front.
12. Rinse column with 500 uL buffer and discard buffer.
13. Apply cells suspension to column and collect the flow-through.
14. Wash the column 3 X with 500 uL buffer and collect flow-through.

15. Count cells (dilution 1:5 (Cells: PBS) using Moxi. If Moxi reads "Error; High Concentration", re-dilute the cells 1:10.
16. Calculate the volume needed for 2.1 x 10^6 cells and centrifuge at 300 x g for 10 min at 22 C. Re-suspend pellet cell culture media to a final volume of 525 mL.
17. The remaining cells should be divided into 2 tubes: 1 with 2 x 10^6 cells and the other with remaining cells, Both tubes should be centrifuged at **1,500 RPM** and the supernatant discarded. The tube with 2 x 10^6 cells should be immediately frozen for future RNA isolation and the other <u>resuspended</u> in 90% FBS-10% DMSO and immediately frozen for future analysis (make note of number of cells on the vial).

Monocyte Culture (USE STERILE TECHNIQUE)

<u>Cell Culture Media Preparation</u> – keep sterile, store at 4°C; use within one week.

**Media is enough for 7-8 patient samples.*

Into a sterile 50 mL conical tube, add:

- 40 mL RPMI media
- 4.456 mL heat-inactivated Fetal Bovine Serum (FBS)
- 0.456 mL HEPES buffer (1M)
- 0.456 mL Pen-Strep
- Label tube "PUFA LPS Media" on cap and on tube. Initial and date the tube.

<u>Note</u>: *RPMI has a built in color indicator. When the solution changes color to a harsh pink, the pH has changed and the solution is bad. Only use RPMI that has a red-orange color. Discard bad media and do not use.*

<u>Cell Culture Procedure</u>

1. In the cell culture hood, label 1 of the wells of a12-well culture plate as control and 1well as LPS+, and include patient/visit information, e.g.:

Control	Patient ID Visit # (empty)	LPS +

2. Add 750 µL of "Control" media to the "Control" well.
3. Add 750 µL of the "LPS+" media to the "LPS+" wells.
4. Add 250 µL of the 525 mL of cells prepared above (step 6c) to each well for a final volume of 1 mL per well (use filtered pipettes; change pipettes between each well).
5. Gently move side to side and backward to forward plate to mix. Place in incubator for **4 hours** +/- 5 minutes.

4 hour Post Test

1. Collect supernatant from cell culture plate (No longer need to keep sterile).
2. Remove culture plate from incubator, gently swirl the plate to mix, place on ice.
3. Scraper the bottom of each well with a cell scraper.
4. With a transfer pipette, collect the entire sample (~1ml) from each well into a fresh labeled 1.5 mL chilled eppendorf tube.
5. With **additional 100 µL of 1XPBS**, collect the cells remaining on the plate.
6. Label each tube with the patient ID number, and label the tubes containing LPS with a "+" on the side and on the cap.
7. Spin tubes at **1000-1500 rpm** for 5 minutes at 4 °C.
8. Aliquot ~500 µL of supernatant samples into four chilled (2 tubes for control and 2 for LPS, cell medium should be divided equally between the tubes), 1.5 ml twist top storage tubes. Use pre-printed labels on tubes.
9. Collect cell pellet into in RNAase free microfuge tubes. Use pre-printed labels on microcentrifuge tubes. Freeze immediately at -80°C.
10. Freeze supernatant immediately, upright in -80°C freezer.

A.1.2 RNA Extraction from Monocytes with DNA Digestion

Kits/Reagents:

- RNeasy Mini Kit (Qiagen 74104)
- QIAshredder (Qiagen 79654)
- RNase-Free DNase Set (Qiagen 79254)

Pre-Experiment:

1. Clean working area and all tools/instruments with RNase Zap or 70% ethanol.
2. Keep samples on **ice**.

Purification of Total RNA

1. Loosen the cell pellet thoroughly by flicking the tube. Add 350 µL of Buffer RLT (added 10 µL β-ME per 1 ml Buffer RLT). Vortex or pipet to mix.
2. Homogenize the lysate by pipetting the lysate directly into a QIAshredder spin column placed in a 2 ml collection tube, and centrifuge for 2 min at full speed.
3. Discard the QIAshredder spin column and keep the homogenized lysate. Add 350 µL of 70% ethanol to the homogenized lysate, and mix well by pipetting. Do not centrifuge.
4. Transfer up to 700 µL of the sample, including any precipitate that may have formed, to an RNeasy spin column placed in a 2 ml collection tube (supplied). Close the lid gently, and centrifuge for 15 s at ≥ 8,000 x g (10,000 rpm). Discard the flow-through. Reuse the collection tube.

   Note: After centrifugation, carefully remove the RNeasy spin column from the collection tube so that the column does not contact the flow-through. Be sure to empty the collection tube completely.
5. Add 700 µL Buffer RW1 to the RNeasy spin column. Close the lid gently, and centrifuge for 15 s at ≥ 8,000 x g (≥ 10,000 rpm) to wash the spin column membrane. Discard the flow-through. Reuse the collection tube in the following step.
6. Add 500 µL Buffer RPE to the RNeasy spin column. Close the lid gently, and centrifuge for 15 s at ≥ 8,000 x g (≥ 10,000 rpm) to wash the spin column membrane. Discard the flow-through. Reuse the collection tube in the following step.
7. Add 500 µL Buffer RPE to the RNeasy spin column. Close the lid gently, and centrifuge for 2 min at ≥ 8,000 x g (≥ 10,000 rpm) to wash the spin column membrane.

   Note: Ensure that no ethanol is carried over during RNA elution. Residual ethanol may interfere with downstream reactions.
8. Place the RNeasy spin column in a new 1.5 ml collection tube (supplied). Add 35

µL RNase-free water directly to the spin column membrane. Close the lid gently, and centrifuge for 1 min at ≥ 8,000 x g (≥ 10,000 rpm) to elute the RNA.

9. Measure RNA concentration using the Nanodrop.

On-Column DNA Digestion

Instead of the step 5 of total RNA purification:

1. Add 350 µl Buffer RW1 to RNeasy column, close lid, centrifuge for 15 s at ≥ 8,000 x g (10,000 rpm). Discard flow-through.
2. Note: DO NOT VORTEX the reconstituted DNase I.
3. Add 10 µl DNase I stock solution to 70 µl Buffer RDD. Mix by gently inverting the tube. Centrifuge briefly.
4. Add DNase I incubation mix (80 µl) directly to RNeasy column membrane, and place on benchtop (20-30°C) for 15 min.
5. Add 350 µl Buffer RW1 to RNeasy column, close lid, centrifuge for 15 s at ≥ 8,000 x g (10,000 rpm). Discard flow-through.

A.1.3 Reverse Transcriptase PCR

Materials and Equipment:

- Sterile PCR tubes
- 96-well PCR plate
- Optical Adhesive Film
- Thermal Cycler (AB 9700)
- PCR instrument (AB 7300)
- 1.5ml tube

Kits/Reagents:

- SuperScript™ III First-Strand Synthesis System (Invitrogen 18080051)
  - 10 mM dNTP mix
  - Random hexamers (50 ng/uL)
  - DEPC-treated water
  - 10xRT buffer
  - 25 mM magnesium chloride
  - 0.1 M DTT
  - RNaseOUT (40 U/uL)
  - SuperScript III RT (200 U/uL)
  - *E. coli* RNase H (2 U/uL)
- Primers
- 1x Tris-EDTA
- SYBR Green PCR Master Mix (Qiagen)
- RNase-free water

Reverse Transcription

1. Determine an amount of RNA to use: (total RNA amount)/(a number of samples): 0.2 or 0.3 ug RNA
2. Prepare *RNA/Primer mixture* in sterile PCR tubes as follows:
   (Mix and briefly spin each component before use)

RNA (0.3 ug)	300 ÷ X (ng/ul) = XX ul
10 mM dNTP	1 ul
Random hexamer	1 ul
DEPC H_2O	Up to 10 ul
Total	10 ul

3. **[cDNA–1] in AB9700**: Incubate at 65 °C for 5 minutes, then place in ice for 1 minute

4. Prepare *Reaction mix* as follows: for each sample * a number of samples (X)

10xRT buffer	2 ul * X
25 mM $MgCl_2$	4 ul * X
0.1 M DTT	2 ul * X
RNase OUT	1 ul * X
Superscript III RT	1 ul * X
Total	10 ul * X

** Keep 'Superscript III RT' and 'RNase OUT' in a freezer as long as possible.*

5. Add *10 ul of the Reaction mix* to each RNA/Primer mixture. Mix gently and spin briefly.

6. **[exp003] in AB9700**: Incubate at 25 °C for 10 minutes and then, at 42 °C for 2 minutes.

7. Add *1 ul of RNase H* to each tube (now, 21 ul in each tube)

8. **[cDNA–3] in AB9700**:

9. Proceed to PCR or freeze at – 20 °C.

Real-time PCR

1. Reconstitute a 10x QuantiTect Primer Assay:
   a) Briefly centrifuge/spin the vial
   b) Add 1.1 ml TE (pH 8.0) and mix by vortexing the vial

   cf. TE = H_2O 10 ml
   10 mM Tris (pH 8) 100 ul
   1 mM 0.5 EDTA 20 ul

2. Set up for a 96-well plate: for each type of primer * X (a number of wells to use)

Reaction mix	12.5 ul * X
RNase-free H_2O	5 ul * X
cDNA (diluted)	5 ul * X
Primers	2.5 ul * X
Total	25 ul * X

** cDNA 1:5 dilution – 21 ul cDNA + 80 ul H_2O*
** X = 3 (for triplicate) * [n+1] (including NTR) +1*

3. Seal the plate with optical (PCR grade) adhesive film and centrifuge at 1000 x g for 1 minute a 4°C.
4. Run the plate.

A.2 Abstracts

A.2.1 Differential Effects of DHA and EPA Supplementation on Serum Inflammatory Markers and Blood Monocyte Inflammatory Response in Subjects with Chronic Inflammation

Jisun So[1,2], Dayong Wu[2,3], Alice H. Lichtenstein[1,2], and Stefania Lamon-Fava[1,2]

[1]Cardiovascular Nutrition Laboratory, JM USDA HNRCA, Tufts University, Boston, MA; [2]Friedman School of Nutrition Science and Policy, Tufts University; [3]Nutritional Immunology Laboratory, JM USDA HNRCA, Tufts University, Boston, MA.

Presented in Vascular Biology, Boston, MA, May 14, 2019
Published in Arteriosclerosis, Thrombosis, and Vascular Biology 2019;39:A227

Background: The anti-inflammatory effects of fish oil containing docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) have been extensively studied. However, the distinct mechanisms by which DHA and EPA modulate inflammation are not well characterized. The aim of this study was to compare the effects of DHA and EPA supplementation on serum concentrations of inflammatory cytokines and on the inflammatory response of peripheral blood monocytes in subjects with chronic inflammation.

Methods: Twenty-one subjects (9 men and 12 women, age 50-75 y) with chronic inflammation (hsCRP>2 μg/mL) were enrolled in a randomized, controlled crossover trial consisting of a 4-week lead-in control phase (high oleic acid sunflower oil, 3 g/d) followed by two sequential 10-week supplementation phases with EPA or DHA (3 g/d each) separated by a 10-week washout phase. Serum concentrations of pro- and anti-inflammatory cytokines were determined by electrochemiluminescence (ECL) assay. Following blood monocyte isolation by indirect magnetic labeling and LPS stimulation, gene expression and secretion of cytokines were assessed by qPCR and ECL, respectively.

Results: Relative to the control phase, DHA supplementation reduced the LPS-stimulated gene expression of pro-inflammatory *TNFA* (median % change: -45%, $P<0.001$), *IL6* (-51%, $P=0.049$), *MCP1* (-28%, $P=0.03$) as well as anti-inflammatory *IL10* (-33%, $P=0.012$) and secretion of TNF-α (-41%, $P=0.02$), MCP-1 (-29%, $P=0.004$), and IL-10 (-47%, $P=0.046$) in monocytes. On the other hand, EPA supplementation increased serum concentrations of IL-10 (+14%, $P=0.046$) and lowered only *TNFA* expression (-20%, $P=0.03$) in monocytes. When compared to EPA supplementation, DHA decreased serum concentrations of MCP-1 ($P=0.03$) and MCP-1 secretion ($P=0.047$) in monocytes, while lowering *IL10* expression ($P=0.03$).

Conclusions: High-dose supplementation with EPA and DHA differently modulate the balance between pro- and anti-inflammatory cytokines in serum and blood monocytes in subjects with chronic inflammation. While DHA inhibits a broader range of pro- and anti-inflammatory cytokines, EPA has a relatively minor role in lowering pro-inflammatory cytokines but preserves the anti-inflammatory IL-10.

A.2.2 Effect of EPA and DHA Supplementation on Plasma Concentrations of Specialized Pro-resolving Lipid Mediators and their Association with Blood Monocyte Inflammatory Response in Subjects with Chronic Inflammation

Jisun So[1,2], Nirupa R. Matthan[1,2], Krishna Rao Maddipati[3], Alice H. Lichtenstein[1,2], Dayong Wu[2,4], and Stefania Lamon-Fava[1,2].

[1]Cardiovascular Nutrition Laboratory, JM USDA HNRCA, Tufts University, Boston, MA; [2]Friedman School of Nutrition Science and Policy, Tufts University; [3]Department of Pathology, Lipidomics Core Facility, Wayne State University, Detroit, MI; [4]Nutritional Immunology Laboratory, JM USDA HNRCA, Tufts University, Boston, MA.

Presented in Vascular Biology, Boston, MA, May 14, 2019
Published in Arteriosclerosis, Thrombosis, and Vascular Biology 2019;39:A607

Background: N-3 fatty acid-derived specialized pro-resolving mediators (SPMs), including the recently discovered docosapentaenoic acid (DPA) metabolites, have been shown to promote the resolution of inflammation in animal studies. However, their role in inflammation in humans has not been determined. We evaluated the effect of EPA and DHA supplementation on plasma SPMs and the resulting impact on the inflammatory response of peripheral blood monocytes.

Methods: Twenty-one subjects (9 men and 12 women, 50-75 y) with chronic inflammation (hsCRP>2 μg/mL) were enrolled in a randomized, controlled, crossover trial consisting of a 4-week lead-in control phase (high oleic acid sunflower oil, 3 g/d) followed by two sequential 10-week supplementation phases with EPA or DHA (3 g/d each) separated by a 10-week washout phase. Plasma phospholipid (PL) fatty acid profile and SPMs, including their precursors, were determined by gas chromatography and liquid chromatography-tandem mass spectrometry, respectively. The LPS-induced inflammatory response of blood monocytes was assessed by qPCR.

Results: EPA increased PL EPA (P<0.001) and plasma concentrations of 18-HEPE, the precursor of the E-series resolvins (RvEs) (P<0.001). The increase in plasma 18-HEPE concentration was associated with the increase in PL EPA (β=14.9 pg/ml, P=0.003). However, RvEs were undetectable. DHA increased PL DHA and plasma concentrations of 17-HDHA and 14-HDHA, the precursors of DHA-derived SPMs (P<0.001). DHA also significantly increased PL EPA and 18-HEPE (P<0.001), suggesting some DHA retroconversion to EPA. DHA also increased the DPA-derived $RvD5_{n\text{-}3\ DPA}$ and $MaR1_{n\text{-}3\ DPA}$ (P<0.001) despite a reduction in PL DPA (P<0.001). In monocytes, both EPA and DHA lowered the LPS-induced expression of *TNFA* (P=0.03 and P<0.001, respectively), and *TNFA* expression was inversely correlated with plasma $MaR1_{n\text{-}3\ DPA}$ (ρ=-0.32, P=0.036).

Conclusions: Relative to EPA, DHA supplementation results in the increase of a broader range of SPMs, including those derived from DPA. Higher plasma concentrations of $MaR1_{n\text{-}3\ DPA}$ following EPA and DHA supplementation are associated with an attenuated inflammatory response in blood monocytes, suggesting a potential role of this SPM in reducing inflammation.

A.3.3 Docosahexaenoic Acid and Eicosapentaenoic Acid Supplementation Differentially Modulate Pro- and Anti-inflammatory Cytokines in Subjects with Chronic Inflammation

Jisun So[1,2], Dayong Wu[2,3], Alice H. Lichtenstein[1,2], and Stefania Lamon-Fava[1,2]

[1]Cardiovascular Nutrition Laboratory, JM USDA HNRCA, Tufts University, Boston, MA; [2]Friedman School of Nutrition Science and Policy, Tufts University; [3]Nutritional Immunology Laboratory, JM USDA HNRCA, Tufts University, Boston, MA.

Presented in Nutrition 2019, Baltimore, MD. June 11, 2019
Published in Current Developments in Nutrition, June 2019, nzz031.OR29-02-19

Objectives: Despite extensive works on the cardioprotective effects of fish oil containing docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), the distinct mechanisms by which DHA and EPA modulate inflammation and plasma lipids are not well characterized. We compared the effects of DHA and EPA supplementation on serum cytokines and blood monocyte inflammatory response and on blood lipids in subjects with chronic inflammation.

Methods: Twenty-one subjects (9 men and 12 women, 50-75 y) with chronic inflammation (CRP>2 μg/mL) were enrolled in a randomized, controlled crossover trial consisting of a 4-week lead-in control phase (high oleic sunflower oil, 3 g/d) followed by two sequential 10-week supplementation phases with pure DHA or EPA (3 g/d each) with a 10-week washout in between. Serum concentrations of cytokines were determined by electrochemiluminescence (ECL) assay. Following lipopolysaccharides (LPS) stimulation of blood monocytes, gene expression and secretion of cytokines were assessed by qPCR and ECL. Plasma lipid concentrations were measured by enzymatic assays.

Results: Relative to the control phase, DHA reduced the LPS-induced gene expression of pro-inflammatory *TNFA* (median % change: -45%, $P<0.001$), *IL6* (-51%, $P<0.05$), *MCP1* (-28%, $P<0.04$) as well as anti-inflammatory *IL10* (-33%, $P<0.02$) and the secretion of TNF-α (-41%, $P<0.02$), MCP-1 (-29%, $P<0.01$), and IL-10 (-47%, $P<0.05$) in monocytes. On the other hand, EPA increased serum concentrations of IL-10 (+14%, $P<0.05$) and lowered only *TNFA* expression (-20%, $P<0.03$) in monocytes. When compared to EPA supplementation, DHA decreased serum concentrations of MCP-1 ($P<0.03$), and monocyte MCP-1 secretion ($P<0.05$) and *IL10* expression ($P<0.04$). Regarding plasma concentrations of lipids, relative to the control phase, both DHA (-16%, $P<0.001$) and EPA decreased triglycerides (-22%, $P<0.001$) while only DHA increased LDL-cholesterol (+7%, $P<0.02$).

Conclusions: DHA and EPA differently modulate the balance between pro- and anti-inflammatory cytokines in serum and blood monocytes in subjects with chronic inflammation. While DHA inhibits a broad range of pro- and anti-inflammatory cytokines, EPA has a relatively minor role in lowering pro-inflammatory cytokines but preserves the anti-inflammatory IL-10. DHA, but not EPA, increases LDL-cholesterol.

A.3.4 Effect of EPA and DHA Supplementation on Plasma Specialized Proresolving Lipid Mediators and Blood Monocyte Inflammatory Response in Subjects with Chronic Inflammation

Jisun So[1,2], Nirupa R. Matthan[1,2], Krishna Rao Maddipati[3], Alice H. Lichtenstein[1,2], Dayong Wu[2,4], and Stefania Lamon-Fava[1,2].

[1]Cardiovascular Nutrition Laboratory, JM USDA HNRCA, Tufts University, Boston, MA; [2]Friedman School of Nutrition Science and Policy, Tufts University; [3]Department of Pathology, Lipidomics Core Facility, Wayne State University, Detroit, MI; [4]Nutritional Immunology Laboratory, JM USDA HNRCA, Tufts University, Boston, MA.

Presented in Nutrition 2019, Baltimore, MD. June 11, 2019
Published in Current Developments in Nutrition, June 2019, nzz031.OR29-01-19

Objectives: The role of n-3 fatty acid-derived specialized pro-resolving mediators (SPMs), including the novel docosapentaenoic acid (DPA) products, in reducing inflammation in humans has not been determined. We evaluated the differential effects of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) supplementation on plasma SPMs and the resulting impact on the inflammatory response of peripheral blood monocytes.

Methods: In a randomized, controlled, crossover trial, 21 subjects (9 men and 12 women, 50-75 y) with chronic inflammation (C-reactive protein> 2 μg/mL) entered a 4-week lead-in control phase (high oleic sunflower oil, 3 g/d) and then two sequential 10-week supplementation phases with pure EPA or DHA (3 g/d each), separated by a 10-week washout phase. Plasma phospholipid (PL) fatty acid composition and SPMs, including their precursors, were measured at the end of each phase. Following lipopolysaccharides (LPS) stimulation, the inflammatory response of blood monocytes was assessed by inflammatory gene expression.

Results: EPA increased PL EPA (P<0.001) and plasma concentrations of 18-HEPE, the precursor of the E-series resolvins (RvEs) (P<0.001). The increase in plasma 18-HEPE concentrations, was associated with the increase in PL EPA (β=14.9 pg/ml, P<0.01). However, RvEs were undetectable. EPA increased PL DPA but not DPA-derived SPMs. DHA increased PL DHA and plasma concentrations of 17-HDHA and 14-HDHA, the precursors of DHA-derived SPMs (P<0.001). DHA also significantly increased PL EPA and 18-HEPE (P<0.001), suggesting some DHA retroconversion to EPA. Interestingly, DHA lowered PL DPA (P<0.001) but increased the DPA-derived SPMs $RvD5_{n\text{-}3\ DPA}$ and $MaR1_{n\text{-}3\ DPA}$ (P<0.001). In monocytes, while both EPA and DHA lowered the LPS-induced expression of *TNFA* (P<0.03 and P<0.001, respectively), *TNFA* expression was inversely correlated with plasma concentrations of $MaR1_{n\text{-}3\ DPA}$ (ρ=-0.32, P<0.04).

Conclusions: Relative to EPA, DHA supplementation increases a broader range of SPMs, with EPA and DHA differentially affecting PL DPA and DPA-derived SPMs. Plasma concentrations of $MaR1_{n\text{-}3\ DPA}$ following EPA and DHA supplementation are associated with an attenuated inflammatory response in blood monocytes, suggesting a potential role of this SPM in reducing inflammation in humans.