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IMMUNOMETABOLISM OF LONG-CHAIN
OMEGA-3 FATTY ACIDS IN HUMANS

A Dissertation in
Nutritional Sciences

by

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ABSTRACT

Over the last couple decades, there has been a surge in research describing the role of fatty acids in immune function due in large part to advances in molecular and biochemistry techniques. Long-chain omega-3 polyunsaturated fatty acids (n-3 PUFAs), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), have emerged as potent modulators of metabolic and immune processes, which has led to their increased use as a potential therapeutic option for the prevention and treatment of inflammatory conditions. Mechanisms underlying the immunometabolic effects of EPA and DHA are multifaceted and require further investigation. Questions regarding the appropriate dose, form, and delivery of n-3 PUFA need to be examined in order to provide effective n-3 PUFA recommendations for individuals seeking approaches to ameliorate inflammation and/or support immune health.

The objective of the first study was to model the incorporation of supplemental EPA+DHA intakes within dietary ranges into red blood cell (RBC) membranes of healthy adults, and identify factors that modify this response. This was a randomized, placebo-controlled, double-blind, parallel study conducted in 125 healthy adults comparing 0, 300, 600, 900, and 1,800 mg/d of EPA+DHA given as fish oil supplements for approximately 5 months. The RBC membrane content of EPA+DHA (Omega-3 Index [O3I]) increased in a dose-dependent manner ($p < 0.0001$), with the dose of EPA+DHA alone accounting for 68% (quadratic, $p < 0.0001$) of the variability in response. Dose adjusted per unit body weight accounted for 70% (linear, $p < 0.0001$) of the change. Additional factors that improved prediction of treatment response were baseline O3I, age, sex, and physical activity. Collectively, these explained 78% of the response variability ($p < 0.0001$).

The objective of the second study was to determine the effects of supplemental EPA+DHA on serum inflammatory marker concentrations (i.e., TNF- α , IL-6, and CRP) in healthy adults, and secondly, to evaluate the associations between RBC membrane fatty acid content and inflammatory markers. There were no significant differences in IL-6, TNF- α or CRP concentrations between the 5 groups following supplementation; however, a marginally significant treatment effect on TNF- α was observed ($p < 0.08$). Higher quartiles of DHA content

in RBC membranes were associated with lower TNF- α at baseline ($p = 0.001$), whereas the lowest quartile of docosapentaenoic acid (DPA) content had significantly higher CRP ($p < 0.001$). Increased arachidonic acid (AA) content was associated with higher TNF- α and IL-6 ($p < 0.001$); however, increased linoleic acid (LA) content was associated with lower IL-6 ($p < 0.05$). There were no significant associations between changes in RBC content of n-3 or n-6 PUFA and changes in inflammatory marker concentrations. Our findings indicate that EPA+DHA intake, across a range of nutritionally-achievable doses, has no dose-response effect on circulating TNF- α , IL-6, or CRP in healthy adults after 5 months of supplementation. However, observed baseline associations warrant further investigation.

The objective of the third study was to evaluate changes in gene expression following a low-dose *in vivo* lipopolysaccharide (LPS) challenge in healthy adult men, and secondly, to explore the relationship between subject characteristics, RBC fatty acids, and gene expression. Eight healthy adult males with diverse O3I profiles were intravenously administered sterile protein-free LPS (0.6 ng/kg body weight). Peripheral blood mononuclear cells (PBMC) collected at 0, 2, 4, and 24 hours post-LPS injection were assessed for changes in inflammatory gene expression. Increased pro-inflammatory gene expression occurred 2 hours after LPS administration, whereas anti-inflammatory gene expression increased at 4 hours. RBC content of docosapentaenoic acid (DPA), but not EPA or DHA, was associated with increased IL-1 β expression at 4 hours as well as larger reductions in IL-1 β expression between the 4 and 24 hour time points ($p < 0.01$).

In conclusion, consuming EPA+DHA in the form of fish oil supplementation dose-dependently increases the O3I and several factors in addition to dose (i.e., body weight, baseline O3I, age, physical activity, and sex) further explained the variability in O3I response. These results can be used to estimate an individual's required supplemental intake for achieving target cell membrane levels of n-3 PUFA. Higher intakes of EPA+DHA (i.e., $\geq 1,800$ mg/d) and increased cell membrane content of n-3 PUFA may provide anti-inflammatory benefits as evident by lower circulating inflammatory markers; however, additional clinical studies are needed to assess the effect of higher cell membrane content of n-3 PUFA, including DPA, on inflammatory responses.

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ABBREVIATIONS

AA - arachidonic acid	MAPK - mitogen-activated protein kinase
AD - Alzheimer's disease	MUFA - monounsaturated fatty acids
A/G - albumin/globulin	MyD88 - myeloid differentiation primary response gene 88
AI - adequate intake	NF-κB - nuclear factor - kappa B
ALA - alpha-linolenic acid	NSAID - non-steroidal anti-inflammatory drug
AMDR - acceptable macronutrient distribution range	n-3 - omega-3 fatty acids
COX - cyclooxygenase	n-6 - omega-6 fatty acids
CRP - c-reactive protein	O3I - Omega-3 Index
CVD - cardiovascular disease	PBMC - peripheral blood mononuclear cell
DGA - Dietary Guidelines for Americans	PG - prostaglandin
DHA - docosahexaenoic acid	PPAR - peroxisome proliferator-activated receptor
DRI - dietary reference intake	PUFA - polyunsaturated fatty acids
EAR - estimated average requirement	RBC - red blood cell
EPA - eicosapentaenoic acid	RCT - randomized controlled trial
ER - endoplasmic reticulum	RDA - recommended daily allowance
FA - fatty acid	RNA - ribonucleic acid
GPR - G protein-coupled receptor	RXR - retinoid X receptor
HDL-C - high-density lipoprotein-cholesterol	SCD1 - stearoyl-CoA desaturase 1
IKKβ - inhibitor of kappa B kinase beta	SDA - stearidonic acid
IKK - I κ B kinase	SFA - saturated fatty acids
IL - Interleukin	SREBP - sterol regulatory-binding protein
IOM - Institute of Medicine	TAK1 - TGF β -activated kinase 1
JNK - c-Jun N-terminal kinase	TICAM1 - toll/IL-1R domain containing adaptor molecule 1
LA - linoleic acid	TG - triglycerides
LDL-C - low-density lipoprotein-cholesterol	TLR - toll-like receptor
LOX - lipoxygenase	TNF - tumor necrosis factor
LPS - lipopolysaccharide	TX - thromboxanes
LT - leukotrienes	
LXR - liver X receptor	

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CHAPTER 1

INTRODUCTION

1.1. BACKGROUND

Accumulating evidence in both animal and human studies indicate that long-chain omega-3 (n-3) polyunsaturated fatty acids (PUFA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), have important bioactive properties (1-6). The mechanisms by which EPA and DHA impart their beneficial effects is quite complex and involves multiple processes. Great strides have been made in the past couple decades to understand the effects of consuming n-3 PUFA in the diet (2). Varying intakes of EPA+DHA can change the structure and function of cell membranes, which has significant implications for human health and disease, including cardiovascular disease (CVD) risk and inflammation (7-9). EPA and DHA affect a myriad of metabolic pathways both directly and indirectly via their secondary metabolites. Furthermore, it is known that EPA and DHA induce changes in the expression of several genes involved in metabolism, cell differentiation, eicosanoid production, and immune responses (10-13).

Despite relatively strong evidence indicating beneficial effects of increasing intake of long-chain n-3 PUFA on cardiovascular and immune health, there remain limitations for making dietary recommendations. Individual variability in response to EPA+DHA consumption has made it difficult to determine appropriate intake recommendations. Moreover, few clinical studies have assessed the effects of consuming EPA+DHA with respect to inflammation and gene expression. Research addressing these limitations would provide a more robust evidence base for making dietary recommendations.

1.2. PREMISE OF DISSERTATION

The purpose of this dissertation was to investigate the effects of consuming varying amounts of supplemental EPA+DHA in modifying cell membrane fatty acid content, circulating inflammatory markers, as well as the expression of genes involved in the inflammatory response. This research approach involved a dietary intervention strategy in which healthy adults not regularly consuming fish or taking EPA+DHA supplements received varying doses of EPA+DHA for a 5 month period. These results of adding EPA+DHA to a Westernized diet that is typically low in long chain n-3 PUFA can be translated to the general public.

1.3. CONCLUSION

The studies presented in this dissertation contribute to the knowledge of how nutrition interventions of n-3 PUFA can modify cell membrane structure and function, established cardiovascular risk factors, and inflammatory processes. The following chapter reviews the literature as a background to the studies of n-3 PUFA for their effects on intermediate cardiovascular risk factors and inflammation. In Chapter 3, the results of a dose-response n-3 PUFA supplementation study on red blood cell (RBC) membrane composition and blood lipids levels are presented. Chapter 4 presents the effects n-3 PUFA supplementation on circulating inflammatory markers as well as the relationships between RBC n-3 and n-6 PUFA content and inflammatory status. Chapter 5 studies changes in gene expression following an inflammatory challenge in healthy adult males. Chapter 6 is a research summary and discussion of future directions.

CHAPTER II:

LITERATURE REVIEW

Considerable progress has been made over the past decade to better understand the biological effects of dietary fatty acids. Omega-3 polyunsaturated fatty acids (n-3 PUFAs), specifically eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), modulate both metabolic and immune processes, and confer health benefits on cardiovascular disease (CVD) and neurodevelopment. Alpha-linolenic acid (ALA, 18:3 n-3) is present in a variety of plant-based foods. In contrast, EPA (20:5 n-3) and DHA (22:6 n-3) are highly unsaturated fatty acids (≥ 20 carbons and ≥ 3 double bonds) that are found in marine sources, mainly fish, especially oily fish. Clinical trials, animals studies, and observational studies have demonstrated that fish and fish oil improve different inflammatory pathologies (14). Numerous mechanistic details about how EPA and DHA modulate chronic disease have been reported in the literature (15).

2.1. OMEGA-3 FATTY ACIDS

2.1.1 Structure and Metabolism of n-3 PUFA

The most common PUFAs in nature are those of the n-3 and n-6 families, in which the first double bond is positioned either 3 or 6 carbons from the omega-end, respectively. Linoleic acid (LA, 18:2 n-6) and ALA are essential fatty acids (EFA) because humans lack the desaturase enzyme that inserts a double bond into the n-6 and n-3 position of PUFAs (**Figure 2.1**). In humans, endogenous synthesis of EPA and DHA from ALA is minimal, with between 0.01 and 8% of ALA being converted to EPA and less to DHA (16, 17); thus plasma and tissue levels are determined largely by direct consumption. Even large amounts of dietary ALA have a negligible effect on plasma DHA (18), although in studies with rat fed diets with linoleic acid (LA) + ALA at about 3% energy, substantial amounts of DHA can be synthesized (19).

Some recent studies though have shown that daily supplementation with 2.4-3.7g of ALA for 12 weeks significantly increase erythrocyte EPA and docosapentaenoic acid (DPA),

but not DHA (20-23). Thus, dietary ALA is a source of EPA and DHA although the conversion efficiency is very limited (16, 17). ALA conversion also is affected by age, gender, genetics, and dietary composition, competing for the same enzymes responsible for the conversion of LA to arachidonic acid (AA). Due to the high intake of LA in the average American diet, a greater conversion of LA to form AA often occurs, hindering the conversion of ALA to EPA and DHA (24). Therefore, the traditional recommendation to increase EPA and DHA levels by ALA consumption generally includes the concomitant recommendation to decrease LA intake. Liou et al. (25) addressed the effects of lowering the LA/ALA ratio by maintaining a constant ALA intake (1% total energy) while altering the amount of LA consumed. After 4 weeks of intervention, a high LA diet (7.0-15.8% total energy) with approximately a 10:1 LA/ALA ratio reported a lower plasma EPA concentration compared to a low LA diet (2.9-5.2% energy) with a 4:1 ratio. A strong inverse relationship was demonstrated between LA and EPA ($r=-0.729$, $p<0.0001$), predicting that for every 10% increase in LA, EPA decreased by 0.64g/100g fatty acids (25).

All of this raises the question as to whether EPA and DHA also are essential fatty acids, due to the limited conversion from ALA, particularly with LA intakes at 5%-10% of energy as currently recommended. Since EPA and DHA have various metabolic functions not duplicated by other fatty acids, they could be viewed as at least conditionally essential fatty acids. This conclusion is supported by a systemic review reporting that increased intake of EPA+DHA, but not ALA, reduces the rates of all-cause mortality, cardiac and sudden death, and possibly stroke (26) .

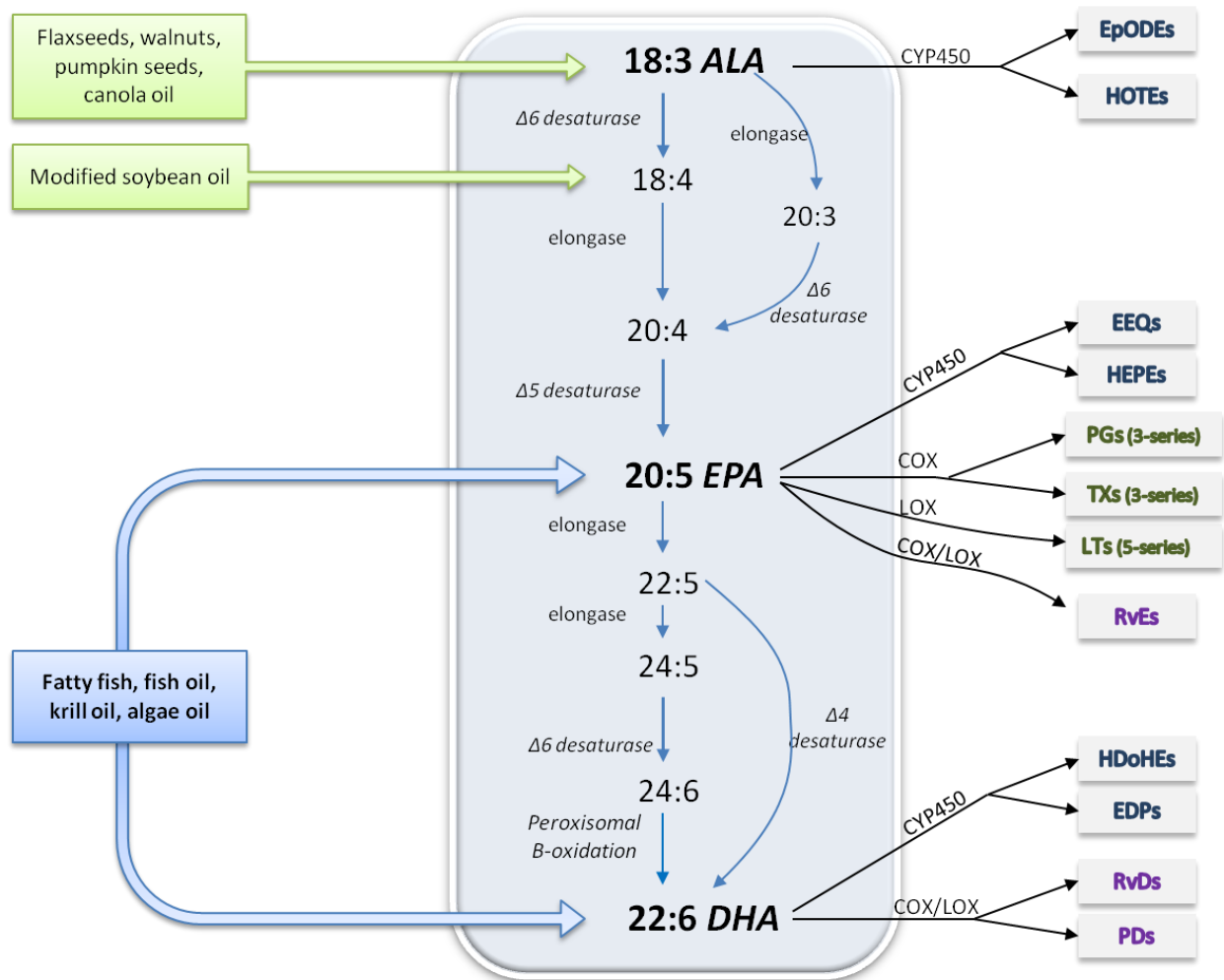


Figure 2.1 Metabolic pathway of n-3 PUFA synthesis via a series of desaturation and elongation reactions in the liver. PUFAs are released from the sn-2 position of membrane phospholipids by the action of phospholipase A2. The released fatty acids are substrates for cytochrome P450 monooxygenases (CYP450), cyclooxygenases (COX), and lipoxygenases (LOX) and generate oxidized, physiologically active fatty acid metabolites. CYP450 enzymes monooxygenate individual PUFAs to unique bioactive hydroxy- and epoxy- metabolites. Cyclooxygenase (COX) and lipoxygenases (LOX) enzymes metabolize EPA to form eicosanoids, including prostaglandins (PG), thromboxanes (TX), and leukotrienes (LT), as well as resolution phase interaction products (resolvins). Resolvins derived from EPA are designated E series (RvE) and those derived from DHA are termed D series (RvDs). Protectins (PDs) are formed from DHA. (Abbreviations: ALA, (alpha)-linolenic acid; DHA, docosahexaenoic acid; EDP, epoxydocosapentaenoic acid; EEQ, epoxyeicosatetraenoic acid; EPA, eicosapentaenoic acid; EpODEs, epoxyeicosatrienoic acid; HDoHE, hydroxydocosahexaenoic acid; HEPE, hydroxyeicosapentaenoic acid; HOTE, hydroxyoctadecatrienoic acid)

2.1.2 Omega-3 Index

A marker of CVD risk involving n-3 PUFA consumption is the Omega-3 Index (O3I), the proportion of EPA and DHA in RBC which reflects the EPA and DHA in cardiac membrane (27). Higher O3I ($\geq 8\%$) have been associated with decreased risk of sudden cardiac death (7, 8, 28). Typically, 1 g/d of EPA and DHA produces an O3I large enough to have a cardioprotective effect ($\geq 8\%$)(7); however, similar to ALA conversion, individual characteristics and dietary variables affect the absorption and metabolism of EPA and DHA. Therefore, individuals consuming identical amounts of EPA and DHA may actually have a different O3I.

In addition to increased EPA and DHA intake, the O3I has been shown to respond favorably to stearidonic acid (SDA) intake, the rate-limiting product of ALA (22, 23). Harris et al. (22) reported that overweight subjects consuming about 24 mL/d of soybean oil enriched with ≈ 3.7 g of SDA for 16 weeks significantly increased the O3I compared to baseline by 19.5%, comparable to the 24.5% increase in O3I following 1 g/d of EPA ($p < 0.05$ for both). Therefore, the authors concluded that SDA-enriched soybean oil may serve as a viable plant-based alternative to provide n-3 PUFA. Another recent study also evaluated the ability of SDA-enriched soybean oil to increase the O3I compared to EPA oil and regular soybean oil (23). Overweight subjects were randomly assigned to receive one of three treatments daily for 12 weeks: 1 g/d of encapsulated soybean oil plus 14.7 g/d liquid soybean oil mixed in food (control group), 1 g/d encapsulated EPA plus 14.6 g/d liquid soybean oil (EPA group), and 1 g/d encapsulated soybean oil and 14.7 g/d SDA acid-enriched soybean (SDA group). The SDA-enriched soybean oil group providing 4.2 g/d SDA significantly increased the mean O3I compared to the regular soybean oil group (4.69 \pm 0.15%, 4.15 \pm 0.12%), not significantly different from the group supplemented with 1.0g/d EPA (4.84 \pm 0.13%). Based on the calculated relative efficiency of RBC incorporation of EPA (17.1%), supplementation of 4.2 g/d SDA was equivalent to ≈ 700 mg/d EPA (23). Therefore, it can be concluded that consumption of ≈ 1.5 g/d SDA in addition to average EPA+DHA consumption in the US diet (135 mg/d (29) or 200 mg/d (30)) would meet the recommended intake of n-3 PUFA (250 mg/d) (1).

2.2 CARDIOPROTECTIVE EFFECTS OF EPA+DHA

A significant body of epidemiological and clinical evidence has examined the cardioprotective benefits of EPA+DHA (**Tables 2.1 - 2.3**). Thus, CVD risk serves as a suitable end point in developing a Dietary Reference Intake (DRI) for EPA+DHA.

2.2.1 Lipids and lipoproteins

A strong body of evidence has demonstrated that long chain n-3 PUFA, EPA and DHA, significantly decrease TG levels. In a review of 36 human studies, consumption of 3-4 g/d of EPA+DHA decreased TG levels in normolipidemic (TG < 2.0 mmol/L) and hypertriglyceridemic patients (TG ≥ 2.0 mmol/L) by 25% and 34%, but also increased LDL-C by 4.5% and 10.8%, respectively (31). EPA+DHA did not significantly affect TC or HDL-C levels. Similarly, Skulas-Ray et al. (32) recently reported in a review of clinical studies that n-3 PUFA (3.4 g/d EPA+DHA) was effective in reducing moderately elevated TG by ≈30%. HDL-C levels often increase as well; however, LDL-C may increase in proportion to the TG lowering effect. For this reason, n-3 PUFA consumption combined with LDL-C lowering therapies would be expected to concurrently reduce both LDL-C and TG levels (32). The consistent TG-lowering effect of long-chain n-3 PUFA generally has not been found at physiologically relevant concentrations of plant sources of n-3 PUFA (i.e., ALA). The Dietary Guidelines for Americans (DGA) 2010 Report concluded that the evidence currently available on ALA is insufficient to make recommendations for increasing n-3 PUFA intake from plant sources to reduce CVD risk (33).

2.2.2 Observational studies

Over the past decade, EPA+DHA have emerged as key modifiers of CVD risk. Numerous epidemiological studies have been conducted over the years, the majority of which have found an inverse relationship between fish intake and a variety of CVD outcomes (**Table 2.1**). Of these studies, 250 mg/d of EPA+DHA was the lowest level where risk of cardiovascular events was significantly reduced. Moreover, a meta-analysis of observational data found that EPA+DHA intakes of 200 mg/d reduced coronary events by 15% and intakes of 500 mg/d reduced risk by 26% (3). The analysis also found that EPA+DHA intakes had to exceed 500 mg/d to be associated with decreased in non-fatal coronary events (3).

Table 2.1 Major epidemiological studies evaluating associations between fish or EPA+DHA and cardiovascular disease

Study	Age	Gender	N	Population	Follow-up, y	Main Finding
Kromhout et al., 1985 (34)	40-59	M	852	The Netherlands; without CHD	20	<i>Inversely</i> related to CHD mortality
Shekelle et al., 1985 (35)	40-55	M	1931	USA; without CVD risk factors	19	<i>Inversely</i> related to CHD mortality
Norell et al., 1986 (36)	n/a	M,F	10966	Sweden	14	<i>Inversely</i> related to CHD mortality
Dolecek, 1992 (37)	35-57	M	6258	USA; High risk of CHD	10.5	<i>Inversely</i> related to CHD mortality
Feskens et al., 1993 (38)	64-87	M,F	272	The Netherlands; CVD risk factors	17	<i>Inversely</i> related to CHD mortality
Keli et al., 1994(39)	50-69	M	552	The Netherlands; without history of stroke	15	<i>Inversely</i> related to stroke incidence
Ascherio et al., 1995(40)	40-84	M	44895	USA; without CHD	6	<i>Not</i> related to CHD or stroke mortality
Kromhout et al., 1995 (41)	64-87	M,F	272	The Netherlands; CVD risk factors	17	<i>Inversely</i> related to CHD mortality
Gillum et al., 1996 (42)	45-74	M,F	5192	USA; without history of stroke	12	<i>Inversely</i> related to stroke incidence
Daviglus et al., 1997 (43)	40-55	M	1822	USA; without CHD	30	<i>Inversely</i> related to CHD mortality
Albert et al., 1998 (44)	40-84	M	20551	USA; without CHD	11	<i>Inversely</i> related to total mortality and SD
Zhang et al., 1999 (45)	45-74	M,F	*	Global, 36 countries	2-30	<i>Inversely</i> related to CHD mortality
Oomen et al., 2000(46)	50-69	M	2713	Finland, Italy, Netherlands; without CHD	20	<i>Inversely</i> related to CHD mortality (only Italy)
Yuan et al., 2001 (47)	45-64	M	18244	China; no history of cancer	12	<i>Inversely</i> related to CHD mortality
Iso et al., 2001 (48)	34-59	F	79839	USA; without CVD	14	<i>Inversely</i> related to stroke incidence
Hu et al., 2002(49)	40-75	100	43671	USA; without CVD	16	<i>Inversely</i> related to stroke incidence
Hu et al., 2002 (49)	34-59	F	84688	USA; without CVD	16	<i>Inversely</i> related to CHD incidence and mortality, non-fatal MI
Mozaffarian et al., 2003	≥66	M,F	3910	USA; without CVD	9.3	<i>Inversely</i> related to CHD mortality and arrhythmic death
Hu et al., 2003(50)	20-55	F	5103	USA; diabetic, without CVD	16	<i>Inversely</i> related to incidence of SHD and total mortality
Erkkila et al., 2003(51)	33-74	M,F	415	USA; with CHD	5	<i>Inversely</i> related to total mortality but <i>not</i> CHD mortality
Mozaffarian et al., 2005(52)	40-75	M	45722	USA; without CVD	14	<i>Inversely</i> related to SD; <i>Not</i> related non-fatal MI or total CHD
Mozaffarian et al., 2005(53)	≥65	M,F	4738	USA; without congestive heart disease	12	<i>Inversely</i> related to incidence of congestive heart disease
Mozaffarian et al., 2005(54)	65-98	M,F	4775	USA; without cerebrovascular disease	12	<i>Inversely</i> related to stroke incidence
Brouwer et al., 2006 (55)	≥55	M,F	5184	The Netherlands	6.4	<i>Not</i> related to the onset of atrial fibrillation
He et al., 2008(56)	45-84	M,F	5488	USA; without CVD	7	<i>Inversely</i> related to prevalence of subclinical atherosclerosis
Sun et al., 2008 (57)	30-55	F	32826	USA; without CVD	6	<i>Inversely</i> related with the risk of non-fatal MI
Bierregaard et al., 2010 (58)	50-64	M,F	57053	Denmark; without CVD	7.6	<i>Inversely</i> related with risk of acute coronary syndrome (in men)

Abbreviations: CHD, coronary heart disease; CVD, cardiovascular disease; MI, myocardial infarction; SD, sudden death; SHD, structural heart disease
 *No total sample size available; ecological study comprising of 36 countries, data obtained from Food and Agriculture Organization and World Health Organization.

Harris et al. (59) pooled results from several observational studies comparing the highest EPA+DHA intake groups with that of the lowest (59); EPA+DHA intake and the relative risk of coronary heart disease (CHD) mortality were significantly associated. These results were derived from over 1.6 million person-years of follow-up, independent of other known risk factors, and dose-dependent (59). The average EPA+DHA intake associated with the greatest reduction in risk of CHD mortality ($\approx 37\%$) was approximately 566 mg/d (59).

2.2.3 Intervention Studies

Evidence supporting EPA+DHA recommendations in adults is based on primary and secondary prevention of CVD. The role of EPA+DHA in reducing sudden cardiac death and CVD risk factors has been the subject of numerous clinical studies (**Tables 2.2 and 2.3**). The 2010 DGAs concluded that consuming two 4 oz servings of seafood per week, providing a total of 250 mg/d of EPA+DHA, reduces mortality from CHD or sudden death in persons with and without CHD (1).

The Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto Miocardico (GISSI) Prevention Study (4), a large prospective clinical trial, tested the efficacy of n-3 PUFA for secondary prevention of CHD. Subjects randomized to the EPA+DHA supplement group (850 mg/d of n-3 PUFA ethyl esters) with and without 300 mg/d of vitamin E experienced a 15% reduction in the primary endpoint of death, nonfatal myocardial infarct and nonfatal stroke ($p < 0.02$). All-cause mortality was reduced by 20% ($p = 0.01$) and sudden death was reduced by 45% ($p < 0.001$) compared with the control group (vitamin E provided no benefit). Compared to baseline, no important changes among groups were reported for TC, LDL-C, or HDL-C levels after 6 months; however, compared to controls, TG levels did significantly decrease in patients receiving the EPA+DHA supplement (-3.4% for n-3, -0.9% for n-3 plus vitamin E, $p = 0.0001$) (4).

The GISSI-Heart Failure (GISSI-HF) study (60) also reported a small benefit of EPA+DHA (850 mg/d) in reducing all-cause mortality in chronic heart failure patients; however, more recently, GISSI-3 failed to demonstrate a benefit of EPA+DHA intake (850 mg/d) among a cohort of high risk CVD patients (61). Reasons for the discrepant results are uncertain. The beneficial effect of EPA+DHA in GISSI and GISSI-HF was due largely to a

reduction in sudden deaths from cardiac causes; thus, it is possible that EPA+DHA effects are more noticeable in individuals prone to ventricular arrhythmic events (61).

The Japan EPA Lipid Intervention Study (JELIS)(62) randomly assigned 18,645 patients with elevated TC (>6.5 mmol/L) to receive either a statin or 1,800 mg/d of EPA (no DHA) with a statin. At mean follow up of 4.6 years, the EPA in combination with statin therapy reduced coronary events by 19% compared to statin alone ($p=0.011$). Non-fatal coronary events and unstable angina also were significantly reduced in the EPA group (19% and 24%, respectively); however, the reduced risk did not apply to coronary death or sudden cardiac death (62). In addition, the reduced risk of coronary events in EPA group was similar at different LDL-C levels, indicating that EPA benefits are independent of LDL-C reduction. The Alpha Omega Trial (63) also tested the effect of EPA+DHA in addition to ALA on the rate of CHD events in patients having had a myocardial infarction. A low dose of EPA+DHA (mean of 376 mg/d), ALA (mean of 1.9g/d), or both, in margarines had no significant effect on the occurrence of cardiovascular events after 40 months of supplementation. Improvements in cardioprotective drug treatments, particularly statin therapy, in addition to a low treatment dose may account for the lack of effect (63).

The effects of EPA+DHA intake on CHD outcomes depend on the dose responses and time relationships (See Figure 2.2). Mozaffarian and Rimm (2) demonstrated that anti-arrhythmic effects are strongest in reducing risk of CHD death and sudden death, having an effect within weeks of a modest intake (<750 mg/d EPA+DHA). However, at higher intakes (>750 mg/d EPA+DHA), maximum anti-arrhythmic effects are achieved and TG-lowering begins to have a clinically relevant effect within months to years. Therefore, 1000 mg/d of EPA and DHA was recommended for individuals with CHD to reduce CHD mortality, greater than the 250 mg/d of EPA and DHA recommended for the general population (30).

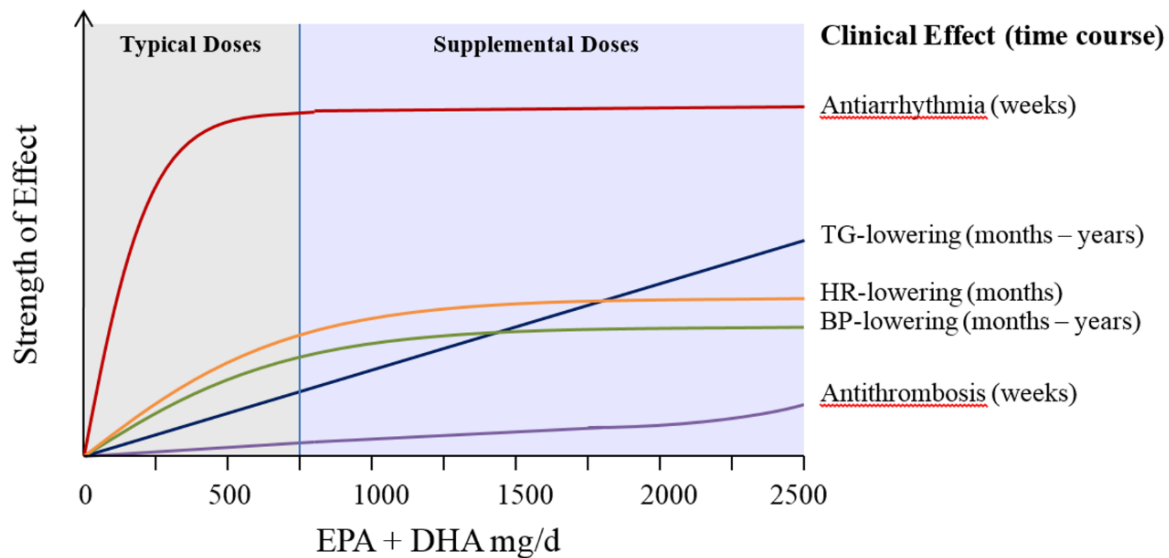


Figure 2.2 Dose responses and time courses for altering clinical events or physiologic effects of EPA+DHA intake. (Adapted from ref. (2))

Several meta-analyses have been published evaluating the potential benefits of EPA+DHA intake in the primary and secondary prevention of cardiac and all-cause mortality (Table 2.2) (64-71). Virtually every meta-analysis has reported beneficial trends for EPA+DHA supplementation (or oily fish intake) for cardiac death, but total mortality, although trending in a beneficial direction, often is not statistically significant. Table 2.3 presents many of the randomized controlled trials (RCTs) included in these meta-analyses. There is a need to determine why some trials show a benefit of EPA+DHA intake whereas others do not. Discrepant findings may be partly explained by inherent differences between baseline populations in primary and secondary designs (i.e., generally healthy subjects compared with individuals with previous vascular disease). Differences in study design, subject population, duration, dosage, as well as the design of the meta-analyses exemplify inconsistencies among primary and secondary prevention trials. A recent meta-analysis of 12 RCTs reported that the pooled relative risk for cerebrovascular disease in EPA+DHA supplemented groups compared with the control group was 1.03 (0.94, 1.12) (72); the pooled relative risk for primary prevention (2 RCTs) was 0.98 (0.89, 1.08) and for secondary prevention (10 RCTs) was 1.17 (0.99, 1.38) (72). Competing risk events, such as CHD and cerebrovascular outcomes, may have limited power analysis by altering the probability of a specific outcome

and thus impeding subsequent events (72). Therefore, more adequately powered intervention data, especially involving healthy populations, is needed.

The Agency for Health Research and Quality published a systematic review and meta-analysis of RCTs and prospective cohort studies evaluating the effects of EPA+DHA on cardiac, cardiovascular, or all-cause mortality (67). Of the 18 eligible RCTs, only 3 were conducted in the United States (13 were done in Europe, one in Japan, and one in India). Supplementation with EPA+DHA ($\approx 0.27 - 6.0$ g/d) reduced the relative risk of cardiac mortality (0.89 [95% CI: 0.83, 0.96]) with no evidence for heterogeneity. Evidence from prospective cohort studies (7 cohorts, 123,122 subjects) showed that EPA+DHA intake up to 200 mg/d was associated with reduced risk of cardiac, cardiovascular, or sudden cardiac mortality (95% CI: 0.45, 0.89). These results suggest that the beneficial effects of EPA+DHA on mortality plateau once reaching a mean intake threshold (67). However, the authors acknowledged that analyzing individual participant data with suitable methodologies and considering other outcomes would help refine this threshold. Regardless, convening an expert panel to set official DRIs for EPA+DHA will help clarify discrepancies in current recommendations and eliminate existing uncertainties. Effects of EPA+DHA in healthy adults (primary prevention) would be the most relevant for DRI development. Despite numerous prospective cohort studies reporting data on outcomes in healthy adults, few RCTs have been published (60, 62, 73, 74). Nonetheless, more well-designed placebo-controlled clinical trials are needed to assess the role of EPA+DHA in the primary prevention of CVD.

Table 2.2 Meta-analyses of randomized controlled trials evaluating the effects of EPA+DHA on cardiac and/or all-cause mortality

Author	Studies	Prevention	Summary Estimate (RR, 95% CI)	
			Cardiac death	All-cause mortality
Bucher et al., 2002 (64)	11	Secondary	0.70 (0.60, 0.80)	0.80 (0.70, 0.90)
Leon et al., 2008 (71)	12	Primary/Secondary	0.80 (0.69, 0.92)	0.92 (0.82, 1.03)
Marik et al., 2009 (70)	11	Primary/Secondary	0.87 (0.79, 0.95)	0.92 (0.85, 0.99)
Hooper et al., 2009 (69)	48	Primary/Secondary	0.90 (0.61, 1.33)	0.98 (0.70, 1.36)
Fillion et al., 2010 (68)	29	Secondary	n/a	0.88 (0.64, 1.03)
Trikalinos et al., 2012 (67)	17	Primary/Secondary	0.89 (0.83, 0.96)	0.95 (0.89, 1.01)
Kwak et al., 2012 (66)	14	Secondary	0.91 (0.84, 0.99)	0.96 (0.90, 1.02)
Rizos et al., 2012 (65)	20	Primary/Secondary	0.91 (0.85, 0.98)	0.96 (0.91, 1.02)

Table 2.3 Randomized controlled trials evaluating the effects of EPA+DHA on cardiac and/or all-cause mortality

Study	Mean age (% males)	N Treat/Control	EPA + DHA g/d	Control	Indication	Duration, y	Summary Estimates RR (95 % CI)	
							Cardiac mortality	All-cause mortality
Burr et al.,1989 (75)	57 (100)	1015/1018	0.24 or 0.86	Non-fish oil diet	Secondary	2	n/a	0.71 (0.54, 0.93)
Sacks et al.,1995 (76)	62 (93)	31/28	6.0	Placebo (olive oil)	Secondary	2.3	0.30 (0.01, 7.13)	0.30 (0.01, 7.13)
Singh et al.,1997 (77)	49 (93)	122/118	1.8	Placebo (non-oil)	Secondary	1	0.52 (0.29, 0.95)	n/a
Leng et al., 1998 (78)	66 (68)	60/60	0.27	Placebo (sunflower seed oil)	Secondary	2	1.00 (0.15, 6.87)	1.00 (0.21, 4.76)
von Schacky et al., 1999 (79)	59 (76)	111/112	3.4 (3 m), 1.7	Placebo (nonmarine fatty acids)	Secondary	2	0.33 (0.01, 8.02)	0.50 (0.05, 5.48)
Johansen et al. , 1999 (80)	60 (78)	250/250	5.1	Placebo (corn oil)	Secondary	0.5	0.33 (0.03, 3.18)	0.33 (0.03, 3.18)
Durrington et al., 2001 (81)	59 (73)	30/29	3.2	Placebo (corn oil)	Secondary	0.9	0.32 (0.01, 7.61)	0.32 (0.01, 7.61)
Nilsen et al., 2001(82)	64 (80)	150/150	3.4	Placebo (corn oil)	Secondary	1.5	1.00 (0.39, 2.59)	1.00 (0.45, 2.24)
GISSI, 2002 (83)	60 (85)	5665/5658	0.85	Vitamin E or no supplement	Secondary	3.5	0.81 (0.68, 0.95)	0.86 (0.77, 0.97)
Burr et al., 2003(84)*	61 (100)	1571/1543	0.34 or 0.86	Non-fish oil diet	Secondary	5	1.26 (1.00,1.58)	1.15 (0.96, 1.36)
Leaf et al., 2005 (85)	66 (83)	200/202	2.6	Placebo (olive oil)	ICD	1	1.01 (0.41, 2.49)	1.09 (0.51, 2.34)
Raitt et al., 2005(86)	63 (86)	100/100	1.3	Placebo (olive oil)	ICD	2	0.40 (0.08, 2.01)	0.40 (0.13, 1.23)
Brouwer et al, 2006 (87)	62 (84)	273/273	0.96	Placebo (sunflower oil) (sunflower oil)	ICD	1	0.46 (0.18, 1.20)	0.57 (0.24, 1.34)
JELIS, 2007 (62)	61 (32)	9326/9319	1.8 ^b	Standard care	Primary/Secondary	4.6	0.93 (0.56, 1.55)	1.08 (0.91, 1.27)
GISSI-HF, 2008 (60)	67 (78)	3494/3481	0.85	Placebo (olive oil)	Primary/Secondary	3.9	0.91 (0.83, 0.99)	0.92 (0.85, 0.99)
Rauch et al., 2010 (88)	64 (74)	1940/1911	1.0	Placebo (olive oil)	Secondary	1	0.95 (0.57, 1.59)	1.24 (0.91, 1.68)
Galan et al., 2010 (89)	61 (80)	1253/1248	0.6	Placebo (paraffin liquid)	Secondary	4.7	n/a	0.98 (0.69, 1.39)
Kromhout et al., 2010 (90)	69 (78)	2404/2433	0.4	Placebo (margarine)	Secondary	3.4	0.99 (0.73, 1.34)	1.02 (0.84, 1.24)
Einvik et al., 2010 (74)	70 (100)	282/281	2.4	Placebo (corn oil)	Primary/Secondary	3	0.94 (0.88, 1.01)	0.58 (0.31, 1.10)
Bosch et al., 2012 (73)	64 (65)	6281/6255	1.0	Placebo (olive oil)	Primary/Secondary	6.2	0.98 (0.87, 1.10)	0.98 (0.89, 1.07)
GISSI-3, 2013 (61)	64 (61)	6244/6269	0.85	Placebo (olive oil)	Secondary	5	1.03 (0.82, 1.30)	n/a

Abbreviations: DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; ICD, implantable cardioverter defibrillators; GISSI or GISSI-HF, Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto miocardio - heart failure; JELIS, Japan EPA Lipid Intervention Study; m, month; RR, relative risk.

* DART-2 has been criticized for its study design and considered by many to be methodologically poor (91)

^bEPA only

2.2.4 Potential Mechanisms

The cardioprotective benefits of EPA+DHA are multifaceted; EPA+DHA have been shown to reduce the susceptibility for cardiac arrhythmias (86, 92-97), stabilize atherosclerotic plaques (98), favorably affect serum TGs (92, 99-102), modestly reduce blood pressure (103-107), produce less aggregatory eicosanoids compared to those from the omega-6 (n-6) PUFA family (108-112), and decrease markers of systemic inflammation and oxidative stress (113-119). The mechanisms by which EPA+DHA exert their effects are also multifaceted. Due to their highly unsaturated nature (5-6 double bonds), they can increase membrane fluidity when incorporated into the phospholipid bilayer. Variation in the reported effects of EPA+DHA on cardiovascular outcomes complicates the interpretation; however, the physiological effects of EPA+DHA on CVD risk factors (i.e. hypertension, arrhythmias, triglycerides) clearly highlight the benefits of EPA+DHA consumption on cardiovascular health.

Mozaffarian and Rimm (2) evaluated evidence in 2006 from RCTs and large prospective studies demonstrating the benefits of modest fish consumption (1-2 servings/wk). In fact, 1-2 servings/wk of fish, particularly those high in of EPA+DHA, reduced the risk of CHD by 36% ($p < 0.001$) and total mortality by 17% ($p = 0.046$). The analysis also demonstrated a dose-response effect of fish intake comparing 0 and 250 mg/d. Anti-arrhythmic effects were reportedly strongest in reducing risk of CHD death and sudden death, having an effect within weeks of a modest intake (< 750 mg/d EPA+DHA). However, at higher intakes (> 750 mg/d EPA+DHA), maximum anti-arrhythmic effects were achieved and TG-lowering was greater. It is well established that EPA+DHA dose-dependently reduce fasting serum TG levels (120). This effect is, in part, attributed to decreased hepatic production as well as increased clearance of TG rich particles (121-123). Nonetheless, 1000 mg/d of EPA+DHA was recommended for individuals with CHD to reduce CHD morbidity and mortality; a dose greater than the 250-500 mg/d of EPA+DHA recommended for the general population for the primary prevention of CHD (1, 30). EPA and DHA are also ligands for certain G-protein-coupled receptors (e.g., GPR-120 (124)) that inhibit pro-inflammatory, cell-signaling pathways. Finally, EPA and DHA oxygenated metabolites (eicosanoids and docosanoids, respectively) are known to have numerous physiological functions.

Increased intake of EPA+DHA results in the incorporation of these fatty acids into the membrane phospholipids of various cells, which can be enzymatically oxidized to generate eicosanoids and various lipid mediators that can modulate signaling events and alter a variety of metabolic activities (**Figures 2.1 and 2.3**). Arachidonic acid (AA) typically is the dominant substrate for eicosanoid synthesis, which includes prostaglandin (PG) PGE₂, PGD₂, leukotrienes (LT), and thromboxanes (TX). These eicosanoids are involved in the development of pro-inflammatory responses, pivotal to the progression of inflammation (125). The discovery of pro-resolution metabolites provides an alternative strategy in reducing inflammation by which EPA and DHA exert pro-resolving properties and reduce inflammatory complications. **Section 2.3.3** and **2.3.4** briefly reviews these EPA and DHA derived metabolites.

The anti-inflammatory effects of EPA+DHA are promising, although results from observational and clinical trials have been somewhat mixed (126). There does appear to be evidence of the efficacy of EPA+DHA in patients with arthritis (126-129); however, clinical studies in other inflammatory conditions, such as asthma, inflammatory bowel disease, and psoriasis, have resulted in conflicting results. (127, 130-136). Moreover, there is little evidence for an effect of EPA+DHA intake on cancer risk which is thought to involve an inflammatory component (137, 138). More clinical studies are needed to assess the therapeutic use of EPA+DHA for the treatment of inflammatory conditions.

2.3 IMMUNOMETABOLIC PROPERTIES OF EPA+DHA

There has been a recent surge in research focused on fatty acid modulation of the immune system, largely due in part to an increased understanding of fatty acid and eicosanoid biology. More and more studies are being published evaluating the extensive role of fatty acid metabolites and lipid mediators in various inflammatory processes. The particular fatty acids currently receiving the most attention are long-chain n-3 PUFA, specifically EPA and DHA. Incorporation of EPA and DHA into the membrane phospholipids of various cells, including adipocytes, can modulate signaling events and alter a variety of metabolic activities (139). Many mechanisms have been proposed regarding the anti-inflammatory and cardioprotective properties of EPA and DHA. The literature on EPA and DHA has grown dramatically over

the past decade; therefore, this section will focus on the direct and indirect role of EPA and DHA in inflammation, regulating transcription factors, resolving inflammation, and potential use as an insulin sensitizing agent.

2.3.1 Inflammatory signaling

EPA and DHA, as well as their oxygenated metabolites, regulate the expression of genes involved in various metabolic processes by interacting with several nuclear receptors and transcription factors. EPA and DHA have been shown to interfere with SFA or LPS induced COX-2 expression through modulation of toll-like receptor (TLR)-mediated signaling pathways, with DHA having the most potent inhibitory effect (140, 141). Some evidence suggests that the inhibitory effect can be attributed to the ability of EPA and DHA to disrupt TLR4 recruitment to lipid rafts (142-144). TLR4 is thought to be translocated to lipid rafts upon stimulation by SFA or LPS, leading to the activation of downstream signaling pathways and thereby increasing pro-inflammatory gene expression (142). However, incorporation of EPA and DHA could alter the lipid composition of lipid rafts typically high in SFA, thereby disrupting the TLR4 recruitment to lipid rafts (142-144). This indicates that the anti-inflammatory properties of EPA and DHA are mediated in part through the inhibition of TLR signaling pathways as well as target gene expression. A recent study showed that parental nutrition formulas enriched in EPA+DHA reduced TLR2 and TLR4 expression in peripheral blood mononuclear cells at both protein and mRNA levels of trauma patients, suggesting that EPA and DHA may suppress transcription of TLR2 and TLR4 genes (145). Even though the inflammation was a result of trauma, these observations represent a potential mechanism by which EPA and DHA inhibit inflammatory responses and downstream signaling.

Nutrients or inflammatory signals can activate a variety of inflammatory kinases, including JNK and IKK, that not only inhibit insulin action but also regulate transcription factors activator protein-1, interferon regulatory factor, and most notably NF- κ B. EPA and DHA are known to inhibit NF- κ B activity, which induces the expression of pro-inflammatory genes encoding for cytokines, chemokines, adhesion molecules, and other effectors of the immune response. Ligand-receptor interaction (e.g., LPS with TLR4) initiates a downstream signal cascade leading to the phosphorylation of I κ B, thereby releasing NF- κ B where it can then translocate to the nucleus and modulate inflammatory genes. There is evidence to

suggest that EPA and DHA can block NF- κ B activity at various stages during the NF- κ B pathway, including the inhibition of TLR-mediated inflammatory signaling (146). Another mechanism is by impeding I κ B phosphorylation and thus preventing NF- κ B translocation to the nucleus (15); however, it is not known which signaling molecules are actually inhibited.

2.3.2 Eicosanoids and pro-resolving metabolites

Fatty acids and inflammation are linked via generation of eicosanoids, 20-carbon fatty acids released from cell-membrane phospholipids (**Figures 2.1 and 2.3**). Cell membranes typically contain low proportions of EPA and DHA relative to AA. Thus, AA is typically the dominant substrate for eicosanoid synthesis, while EPA and DHA prevent the conversion of AA to pro-inflammatory eicosanoids mitigating the inflammatory response. Several eicosanoids derived from AA are involved in the development and synthesis of pro-inflammatory responses, pivotal to the progression of inflammation (125). EPA can be enzymatically converted to eicosanoids very similar in structure to AA-derived eicosanoids, using the same enzymes (e.g., COX-2), yet they exhibit quite contrasting properties. Three- and five-series eicosanoids produced from EPA tend to be more anti-inflammatory than AA-derived eicosanoids (15). EPA can suppress COX-2 and inhibit 5-lipoxygenase (LOX), the enzymes responsible for producing two-series PG and TX and four-series LTs, respectively. In contrast, DHA, a 22-carbon fatty acid, appears to only inhibit COX-2 activity, although DHA supplementation in healthy men decreased production of both PGE₂ and LTB₄ (147). This suggests that DHA may have an inhibitory effect on 5-LOX, similar to EPA, or that EPA is formed by DHA via retroconversion which can then inhibit COX-2 and 5-LOX enzymes. Unfortunately, there is no information currently available to confirm either theory.

Resolvins (resolution phase interaction products), protectins, and maresins are oxygenated metabolites derived from EPA and DHA that possess potent anti-inflammatory and pro-resolving actions. These specialized lipid mediators are not immunosuppressive, but rather activate specific mechanisms to promote resolution of inflammation (148). Mediators derived from EPA are designated E-series resolvins and those biosynthesized from DHA are designated D series resolvins, whereas protectins and maresins are bioactive compounds derived only from DHA.

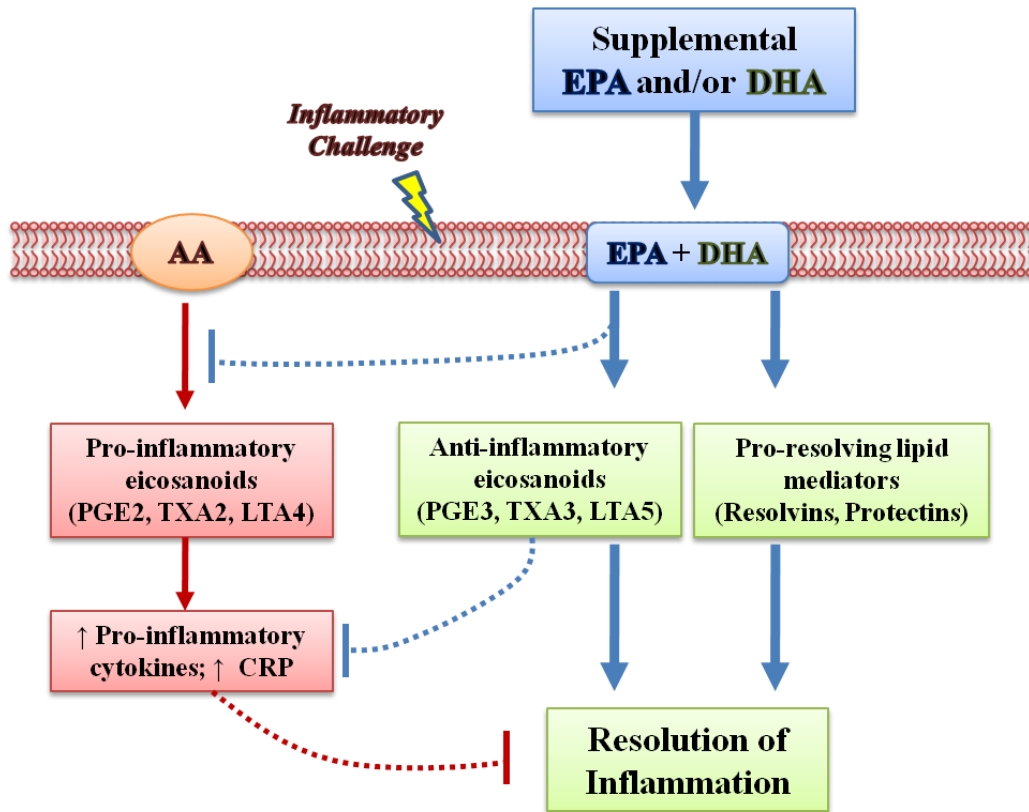


Figure 2.3 EPA and DHA are hypothesized to exert anti-inflammatory effects when incorporated into cellular membranes by decreasing the production of pro-inflammatory eicosanoids derived from arachidonic acid (AA) and by serving as the substrates for anti-inflammatory, pro-solving lipid mediators such as resolvins and protections. PGE, prostaglandin E;TXA, thromboxane A; LTA, leukotriene A; CRP, c-reactive protein.

2.3.3 Resolution of inflammation

In the early phase of inflammation, excessive amounts of PGE₂ are released. Cell-cell interactions eventually generate ‘stop signals’ that serve as endogenous anti-inflammatory mediators (148). Production of PGE₂ results in the downstream synthesis of other eicosanoids, resulting in formation of anti-inflammatory agents via lipid class switching (149, 150). Resolution is a critical component of the inflammatory response characterized by an active switch in enzyme activity from pro-inflammatory eicosanoids to pro-resolving lipid mediators. The signaling pathways that lead to PGE₂, in turn, actively switch on the transcription of enzymes necessary for the generation of other classes of eicosanoids such as resolvins and protectins. Failing to inactivate the immune system can be detrimental to the host. Glucocorticoids and other therapeutics that interfere with inflammatory pathways are very effective in blocking inflammation; however, their long-term use could result in a

dangerously immune-compromised state (14). The discovery of pro-resolution metabolites provides an alternative strategy in reducing inflammation by which EPA and DHA exert pro-resolving properties and reduce inflammatory complications.

Resolvins act by reducing neutrophil traffic, regulating cytokine and reactive oxygen species, and lowering the magnitude of inflammatory response (**Figure 2.4**). Inhibition of neutrophil infiltration is largely responsible for inducing resolution, and resolvins do so by inhibiting neutrophil chemotaxis, adhesion receptor expression, defensin release, and transepithelial migration (14). Protectins are recognized for their anti-inflammatory and protective actions in neural systems, liver, lung and the eye (151). Maresins are formed via macrophage LOXs and have properties that are similar to resolvins and protectins (152). Resolvins, protectins, and maresins can aid resolution by increasing the expression of chemokine receptor 5 on apoptotic neutrophils, thereby promoting chemokine clearance via macrophage engulfment and limiting additional neutrophil infiltration (15). Thus, macrophages also play a critical role in resolving inflammation in addition to initiating the inflammatory response. Other cell types, including dendritic cells, are likely involved, yet more research is needed.

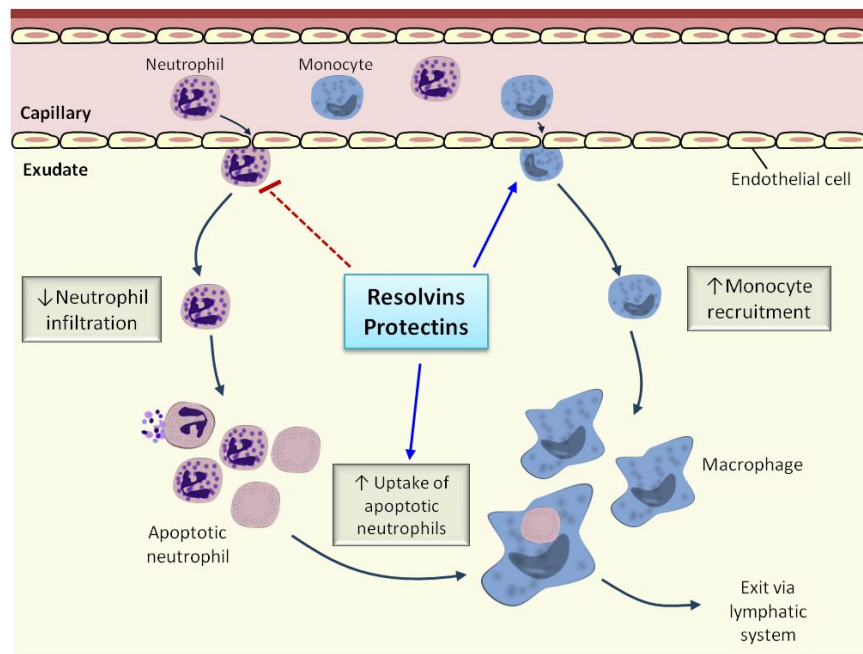


Figure 2.4 Acute inflammation temporal actions. The switch in predominant cell types is driven by a shift to the production of specialized pro-resolution lipid mediators including the resolvins and protectins. Adapted from Serhan, Chiang, and Van Dyke (153)

2.3.4 M2 macrophage activation

Nonphlogistic phagocytosis of apoptotic neutrophils is a critical step in resolving inflammation (153). Resolvins stimulate macrophage uptake of apoptotic neutrophils and promote active clearance of phagocytes from the site of inflammation, all while not increasing pro-inflammatory cytokine production (154). This switch in macrophage phenotype exemplifies the exceptional plasticity of macrophages and perhaps the potential role of EPA and DHA derived-metabolites in promoting a pro-resolving environment. Macrophages often are classified as two subtypes; although there likely are intermediate phenotypes depending on the local environment. Classically activated macrophages (M1) occur in a pro-inflammatory environment (e.g., TNF- α) and are often associated with obesity and insulin resistance, whereas alternatively activated macrophages (M2) occur in a more anti-inflammatory environment (e.g., IL-10) and more common in the lean state (**Figure 2.5**). M1 macrophages emerge during cell-mediated immune response, which induce inflammatory pathways (e.g., NF- κ B) to secrete pro-inflammatory cytokines and reactive oxygen species that antagonize insulin signaling (6). In contrast, M2 macrophages emerge during the later stages of an immune response to downregulate inflammatory activities, initiate resolution of inflammation, and protect against the negative effects of diet-induced obesity. M2 macrophages often are generated following exposure to IL-4 and IL-13 and generate high levels of anti-inflammatory cytokines (e.g., IL-10). Some evidence suggests that M2 macrophages directly increase insulin signaling in adipocytes, yet it may be that M2 macrophages indirectly contribute to adipocyte insulin sensitivity by inhibiting M1 macrophages (6). More recently, it has been postulated that M2 macrophages recruited to adipocytes take up FFAs and transport them to the liver, a process analogous to reverse cholesterol transport, rather than allowing FFAs to remain in circulation and promote insulin resistance (155).

Evidence has shown that oxidized metabolites of EPA and DHA promote the emergence of pro-resolving M2 macrophages (6). Resolvins, specifically resolvin E1, enhance clearance of apoptotic neutrophils via ChemR23, a G protein-coupled receptor (GPR) expressed on monocytes, macrophages, and dendritic cells (156, 157). Resolvin E1 binding to ChemR23 regulates phosphorylation of several signaling proteins, including Akt and ribosomal S6, resulting in nonphlogistic phagocytosis and clearance of apoptotic cells (**Figure 2.5**) (157).

Moreover, resolvin E1 has been shown to dampen LTB₄-induced NF-κB activation in neutrophils by binding to the LTB₄ receptor 1 as a partial agonist (158). Resolvin D1 also has been shown to enhance phagocytic and clearance functions of macrophages by binding to lipoxin A₄ receptor and GPR32 (159). Taken together, EPA and DHA derived resolvins promote resolution in part by interacting with GPRs to promote M2 macrophages. Identification of additional resolvin properties will likely expand as more cell surface binding sites are discovered.

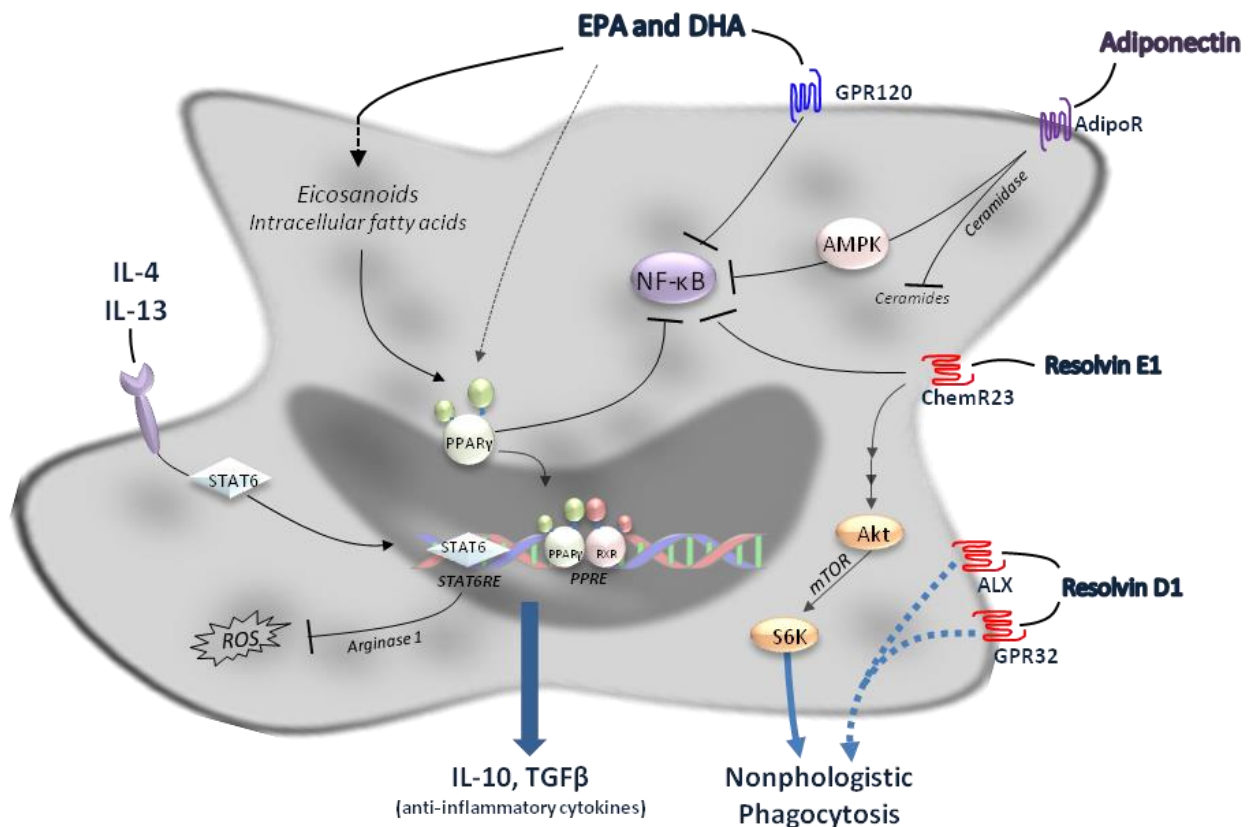


Figure 2.5 EPA and DHA promote alternatively activated macrophages. EPA incorporated into the cell membrane form eicosanoids with anti-inflammatory properties following an inflammatory stimulus. Several of these fatty acid metabolites serve as ligands for peroxisome proliferator-activated receptor gamma (PPAR γ), which directly regulate gene expression and inhibit NF-κB induced transcription. G-protein coupled receptor 120 (GPR120) functions as an EPA/DHA receptor/sensor on the cell surface of macrophages and adipocytes, mediating the insulin sensitizing effects by repressing macrophage-induced inflammation. Resolvin E1 binding to ChemR23 regulates phosphorylation of several signaling proteins, resulting in nonphlogistic phagocytosis and clearance of apoptotic cells. Resolvin D1 also enhances nonphlogistic phagocytosis and clearance by binding to lipoxin A₄ receptor (ALX) and GPR32.

2.3.5 Nuclear Receptors

Several transcription factors have been identified as EPA and DHA targets, including a number of nuclear receptors. Fatty acids can bind and control the activity of several nuclear receptors, including retinoid X receptor (RXR), liver X receptor (LXR), farnesoid X receptor (FXR), sterol regulatory element binding protein (SREBP), hepatic nuclear factor-4-alpha (HNF-4 α), and peroxisome proliferator activated receptors (PPAR) (160).

A key factor in regulating lipogenesis is the expression SREBP, which regulates the expression of fatty acid, cholesterol, and TG synthesizing enzymes (161). There are two isoforms, SREBP-1c and SREBP-2. The SREBP-2 isoform preferentially activates genes involved in cholesterol synthesis and uptake. Endoplasmic reticulum (ER) stress activates the SREBP-1c isoform, which promotes synthesis of lipogenic enzymes and induces lipotoxicity (162-164). EPA and/or DHA have been shown to suppress SREBP-1c expression, thereby reducing lipogenesis (165-167). The currently accepted mechanism is that EPA and DHA inhibit LXR α and RXR α heterodimer binding to the SREBP-1c gene promoter (165). The LXR/RXR heterodimer regulates expression of SREBP-1c gene by binding to the SREBP-1c promoter (168). Therefore, EPA/DHA inhibition of LXR/RXR binding would suppress SREBP-1c expression and decrease lipogenic enzyme activity. The reduction in SREBP-1c also could be secondary to EPA+DHA inhibition of SREBP-1c maturation, which leads to reduced SREBP-1c mRNA levels (169).

PPAR activation is another important mechanism by which EPA and DHA promote this switch in macrophage phenotype. Metabolic and immune functions share PPARs, nuclear receptors that when activated function as transcription factors, regulating genes involved in inflammation and metabolism. Odegaard and colleagues were the first to demonstrate that PPAR γ regulates M2 activation in adipose tissue (5). EPA and DHA, as well as their metabolites, can inhibit NF- κ B induced transcription via activation of PPAR γ . Moreover, activating PPAR γ enhances adipocyte differentiation, lipid storage, and insulin sensitivity. Multiple mechanisms have been proposed for these anti-inflammatory actions of PPARs, including NF- κ B interference as well as the breakdown of inflammatory eicosanoids via induction of peroxisomal β -oxidation (170).

Activation of PPAR γ increases plasma adiponectin levels, underscoring the importance of PPARs in insulin sensitivity. Dual activation of PPAR γ and PPAR α may further enhance insulin sensitivity by increasing both adiponectin and the expression of its receptors (171). Therefore, the potential insulin sensitizing effect of EPA+DHA can be explained in part by the ability of PPAR γ agonists to increase adiponectin activity. Adiponectin serves as an endogenous anti-inflammatory and insulin sensitizing molecule; increasing adiponectin levels inhibits the M1 and promotes the M2 phenotype (172, 173). More recently, adiponectin was shown to reduce ceramide levels by inducing ceramidase activity (174). Disruption of ceramide accumulation would be expected to improve insulin signaling. Therefore, PPAR γ agonists such as EPA and DHA could enhance insulin sensitivity in part by adiponectin-induced ceramide depletion. The recent development of PPAR agonists by several drug companies exemplifies the role of PPARs as a viable target against inflammatory-related complications. Of the three PPAR subtypes (β , α , and γ) that vary in function and ligand recognition, PPAR β (or PPAR δ) is the least understood (175). PPAR α regulates various genes involved in maintaining glucose and fatty acid homeostasis. Both saturated and unsaturated fatty acids serve as PPAR α ligands. In contrast, PPAR γ is preferential for PUFAs (175). Antidiabetic drugs, such as thiazolidinediones, fatty acids, and eicosanoids can serve as ligands for PPAR γ . Fatty acids are not the most efficient activators of PPAR γ , rather oxidized metabolites of PUFAs (e.g., 15-deoxy PGJ₂) appear to be more potent activators of PPAR γ .

PUFA also can bind to RXR, a transcription factor that heterodimers with PPARs; the strongest RXR activation has been observed for DHA and arachidonic acid (176). Moreover, PUFA have been shown to interfere with oxysterol activation by competing for LXR binding, thereby antagonizing oxysterol activation by LXR, which binds oxysterols and regulates expression of genes involved in bile acid synthesis(165). However, substantial cross-talk likely exists between SREBP, LXR, RXR, and PPAR signaling and, therefore, distinguishing between nuclear receptor signaling remains a challenge.

2.3.6 GPR120

Induction of PPAR γ by EPA and DHA suggests a potential involvement in M2 macrophage activation; however, the molecular mechanisms are not entirely clear. Research conducted in Olefsky's lab has demonstrated that G-protein coupled receptor 120 (GPR120)

functions as an EPA/DHA receptor/sensor on the cell surface of macrophages and adipocytes, mediating the insulin sensitizing effects by repressing macrophage-induced inflammation (124). Inflammatory cascades mediated by TLR4 and TNF- α receptors recruit TGF β -activated kinase 1 (TAK1) to phosphorylate I κ B, activate NF- κ B, and subsequent transcription of pro-inflammatory genes. (124). Upon EPA or DHA activation, GPR120 is internalized bound to β -arrestin, sequestering the binding protein necessary to activate TAK1 (177). Therefore, the GPR120 complex inhibits downstream pro-inflammatory pathways, suggesting that GPR120 agonists, including EPA and DHA, could be targeted to ameliorate inflammatory disease. As GPR120 agonists begin development, it will be important to explore their clinical effects as well as their potential use in obesity-induced insulin resistance.

2.3.7 Clinical evidence of n-3 PUFA anti-inflammatory effects

Despite a large body of evidence demonstrating anti-inflammatory properties of EPA+DHA, there is little clinical evidence demonstrating improvements in circulating inflammatory marker concentrations in healthy adults (**Table 2.4**).

The absence of a consistent EPA+DHA effect on circulating inflammatory markers could be due to a variety of reasons. The subject population, dosage, duration, and source (i.e., supplements or fish) of EPA+DHA contribute to the inconsistencies. Variations in fatty acid and macronutrient composition of the diet would also influence the effectiveness of EPA+DHA in reducing inflammation. For example, high fat diets likely reduce the effects of EPA+DHA supplementation (178). Thus, controlling macronutrient intake, especially fat intake, would produce a more consistent effect of EPA+DHA on inflammatory markers. Subject differences in underlying inflammation and weight status are also important considerations; discrepancies in study design (i.e., randomization, blinding, etc.) and methodology (i.e., biomarkers to assess inflammation) are additional factors. Consequently, large RCTs are needed taking into account these factors to establish the role and mechanisms of EPA+DHA in improving inflammation. Moreover, using standardized measures and accounting for individual variability trials will provide a more consistent body of evidence.

Table 2.4. Randomized controlled trials evaluating the effects of EPA+DHA on serum inflammatory biomarker concentrations in healthy adults¹

Study	Country	Design	Sample (n)	Population	Mean age (y)	EPA+DHA (mg/d)	Placebo	Duration (mth)	Serum Measures	EPA+DHA effect ²
Thies et al., 2001(179)	UK	P, DB, PC	48	Healthy adults	62	1000	Palm & Sunflower oil	3	ICAM, VCAM, IL-1B, TNF α ,	↓sICAM-1 ²
Ciubotaru et al., 2003(180)	USA	R, DB, PC	30	Postmenopausal women on HRT	60	2500	Safflower oil	1.2	IL-6, CRP	↓, IL-6, CRP
Madsen et al., 2003(181)	Denmark	R, DB, PC, CO	60	Healthy adults	38	6600; 2000	Olive oil	3	CRP	None
Vega-Lopez et al., 2004 (182)	USA	R, DB, PC	80	Healthy adults	29	1500	NR	3	CRP	None
Geelen et al., 2004 (183)	Netherlands	R, DB, PC	84	Healthy adults	50-70	1260	Sunflower oil	3	CRP	None
Fujioka et al., 2006 (184)	Japan	P, DB, PC	161	Healthy adults	38	860	Olive oil	3	CRP, TNF-receptors	None
Sanders et al., 2006 (185)	UK	R, DB, PC	79	Healthy adults	33	1500 (DHA only)	Linoleic acid	1	CRP	None
Yusof et al., 2008 (186)	UK	R, DB, PC	21	Healthy men	44	2100	Coconut oil	2	IL-6, CRP, ICAM, VCAM	↓sICAM-1
Pot et al., 2010 (187)	Netherlands	R, DB, PC	77	Healthy adults	59	1500	Sunflower oil	3	Cytokines, ICAM, VCAM	None
Thienprasert et al., 2009 (188)	Thailand	R, PC	180	Healthy children	11	1200	Soybean oil	6	IL-1B, IL-6, IL-10	None
Nieman et al., 2009 (189)	USA	P, DB, PC	23	Endurance Athletes	25	2400	Soybean & Canola oil	1.5	IL-1, IL-6, IL-8, CRP	None
Bloomer et al., 2009 (190)	USA	R, DB, PC, CO	15	Exercise-trained men	26	4432	Soybean oil	1.5	CRP, TNF α	↓ TNF α , CRP
Kiecolt-Glaser et al., 2011 (191)	USA	R, DB, PC	68	Healthy young adults	24	2500	Oil Mixture	3	IL-6, TNF α	↓TNF α (trend)
Dangardt et al., 2010 (192)	Sweden	R, DB, PC, CO	25	Obese adolescents	16	1220	MCT	3	IL-6, IL-1B, TNF α , CRP, ICAM1, VCAM,	↓TNF α , IL-6, IL-1B; ↑VCAM
Root et al., 2013 (193)	USA	R, DB, PC	57	Young adults	21	580	Safflower oil	1	IL-6, IL-10, TNF α , CRP	None

OVERALL: Little to no reduction in TNF α , IL-6, CRP (4 / 15 studies [27%] show benefit)

¹IL, interleukin, MCT, medium-chain triglycerides; R, randomized; DB, double-blind; HRT, hormone replacement therapy; ICAM, intercellular adhesion molecule; PC, placebo-controlled; CO, crossover; NR, not reported; VCAM, vascular cell adhesion protein,

²Omega-3 supplementation reduces sICAM-1 fairly consistently as reviewed by Yang et al. (194)

2.4 DIETARY RECOMMENDATIONS FOR EPA+DHA

Despite accumulating evidence that dietary EPA+DHA reduces risk of CVD (1, 67, 195), the optimal dose of n-3 PUFAs has yet to be resolved. In 2002, the Institute of Medicine (IOM) report on Dietary Reference Intakes (DRIs) for Energy, Carbohydrates, Fiber, Fat, Fatty Acids, Cholesterol, Protein, and Amino Acids, concluded that there was insufficient evidence to set DRIs for EPA and DHA (196). Since the release of the Report, the evidence base regarding EPA and DHA has grown dramatically which has led a number of organizations and expert groups around the world to have issued evidence-based recommendations for EPA and/or DHA. Here we review current recommendations for n-3 PUFA intakes set by various federal agencies and expert groups and the evidence that was used to set them.

Although chronic disease was addressed in the DRIs for vitamin C and calcium, DRIs are intended to meet the needs of healthy people, not individuals with disease. A challenge is defining the word “healthy.” Importantly, the intent is to offer dietary guidance for the promotion of health and the prevention of chronic diseases, which are rampant in the United States. Questions such as these make setting a DRI for the non-classical nutrients (such as EPA and DHA) important.

There currently is no DRI for EPA or DHA. There is an adequate intake (AI) for ALA (1.6 g/d for adult men and 1.1 g/d for adult women). This is based on the observed median intake in the United States at which no nutrient deficiency is present. In addition, an acceptable macronutrient distribution range (AMDR) for ALA has been set at 0.6-1.2% of energy (196), up to 10% of which can be provided by EPA and/or DHA. It is important to note that this is not an AMDR (or a DRI) for EPA and/or DHA; it simply indicates that consumption of between 0.06% and 0.12% of energy as EPA and/or DHA will “count” towards meeting the AMDR for ALA.

Many organizations and expert committees acknowledge the important role of EPA and DHA in human nutrition (Table 2.5). Dietary Guidelines for Americans (DGA) 2010 recommends consuming about 8 oz/wk of a variety of seafood to reduce cardiac deaths among individuals with or without pre-existing CVD (1). This would provide about 250 mg/d of EPA+DHA, which is sufficient to obtain cardioprotective effects for primary prevention of

CVD. This as a minimum intake was recently argued by Musa-Veloso et al. (197), who reported that intakes in excess of 250 mg/d elicit a 35.1% greater reduction in risk of sudden cardiac death compared to intakes <250 mg/d, in subjects free of known CHD. The American Heart Association (AHA) recommends that adults without CHD eat fish (particularly fatty fish) at least twice a week (195), which provides about 500 mg/d (59). Individuals with CHD are advised to consume 1 g/d of EPA+DHA for secondary prevention of CVD, preferably from seafood and in consultation with a physician (30). For TG lowering, 2-4 g/d of EPA+DHA are recommended as capsules under a physician's care. In terms of their effects on blood EPA+DHA levels, fish oil supplements provide similar benefits as seafood (198). Although concern has been raised about high doses causing excessive bleeding in some individuals, there is little evidence of any increased risk for clinically significant bleeding (199, 200). Nevertheless, individuals are advised to consult a physician before taking larger doses of n-3 PUFA (>2 g/d). Unlike fish oil supplements, seafood contains many nutrients such as high quality protein and vitamin D that may confer cardioprotective benefits beyond n-3 PUFAs.

Table 2.5 Recommended Intakes of EPA+DHA

Organization	Population	EPA+DHA Recommendation
Academy of Nutrition and Dietetics (201)	Adults	≥500 mg/d
	Adults without CHD	Fatty fish ≥2 times/wk (≈500 mg/d)*
American Heart Association (195)	Patients with CHD	≈1 g/d
	Patients with high TG	2-4 g/d
Dietary Guidelines for Americans 2010 (1)	Adults	≥250 mg/d
International Society for the Study of Fatty Acids and Lipids (202)	Adults	≥500 mg/d
	Pregnant/lactating women	≥500 mg/d (≥300 mg/d of DHA)
European Food Safety Agency (203)	Adults	≥250 mg/d
	Pregnant/lactating women	≥250 mg/d (+ 100-200 mg/d DHA)
World Health Organization (204)	Adults	≥250 mg/d

*Calculated to be approximately 500 mg/d (59)

Inadequate intake of DHA by pregnant women may impair neural development in the neonate (205-209). Sufficient DHA during pregnancy and lactation is crucial for proper brain development of infants. Thus, recommendations have been made for pregnant and lactating women. The International Society for the Study of Fatty Acids and Lipids recommends that

pregnant and/or lactating women consume ≥ 300 mg/d of DHA (202); European Food Safety Agency recommends women consume ≥ 250 mg/d of EPA+DHA plus an additional 100-200 mg/d of DHA (203). Children, pregnant women, and women who may become pregnant are advised to avoid eating fish with higher levels of mercury. Dietary Guidelines for Americans 2010 recommends eating a variety of seafood (two 4 oz servings per week) to reduce the amount of mercury consumed from any one seafood type (1).

2.4.1 Dietary sources of EPA+DHA

Seafood is the major source of EPA+DHA in the diet. Thus, the food-based strategy for increasing EPA+DHA intake is to consume more seafood. Species with higher EPA+DHA should be given greater emphasis. **Table 2.6** shows the variability of EPA and DHA content in seafood depending on the species, geographic region, and season of catch (210). Eating a single serving of wild Atlantic salmon (3.5 oz or 100 g) three times a week would provide approximately 789 mg/d of EPA+DHA. Therefore, recommendations can be met by consuming seafood; non-fish consumers have several alternative options.

Table 2.6 EPA and DHA content in selected seafood (100 g, cooked, dry heat) (211, 212)

Seafood	Energy kcal	EPA mg	DHA mg	EPA+DHA mg	Mercury ppm
Salmon, Atlantic, farmed	206	690	1457	2147	0.02
Salmon, Atlantic, wild	182	411	1429	1840	0.02
Salmon, coho, wild	139	401	658	1059	0.02
Salmon, sockeye	169	110	524	634	0.02
Makerel, Atlantic	262	504	699	1203	0.05
Herring, Pacific	250	1242	883	2125	0.08
Herring, Atlantic	203	909	1105	2014	0.08
Whitefish	172	406	1206	1612	0.09
Catfish, wild	105	100	137	237	0.03
Trout	150	468	520	988	0.07
Scallops ^a	111	72	104	176	0.01
Tilapia	128	5	130	135	0.01
Crab, blue ^a	83	101	67	168	0.07
Bass, sea	124	206	556	762	0.15
Tuna, Albacore, canned	128	230	630	860	0.35
Tuna, light, canned	116	50	220	270	0.13
Shrimp ^a	119	135	141	276	0.01
Cod, Atlantic	105	4	154	158	0.11
Cod, Pacific	85	42	118	160	0.11
Halibut	111	80	155	235	0.24
Swordfish	172	127	772	899	1.00
Grouper	118	35	213	248	0.45
Mackerel, king	134	174	227	401	0.73
Orange Roughy	105	6	25	31	0.57

Food fortification is another means for obtaining EPA+DHA. Efforts are being made to enrich various food products, including eggs, yogurt, milk, and spreads, with fish oil derived EPA+DHA. Fish oil contains 20-80% EPA+DHA by weight and this provides another option for consuming these fatty acids (2, 213). Products are being fortified with EPA+DHA by feeding animals n-3 PUFA and enriching their tissues with EPA+DHA, adding EPA+DHA oils directly to foods, or micro-encapsulating the oil prior to incorporation to maintain stability and avoid lipid oxidation (214). Biotechnology also has enabled the production of EPA and DHA oils from non-marine sources, including specific strains of algae, genetically modified soybean oil, and bioengineered yeast (215). It has been reported recently that global consumer spending on EPA+DHA fortified products will increase from \$25.4 billion in 2011 to \$34.7 billion in 2016 (216). North America accounts for 43% of these sales. **Table 2.7** displays a variety of foods that contain EPA and/or DHA. The rapid development of non-traditional foods fortified with EPA+DHA raises the question of food-nutrient interaction, particularly, how EPA+DHA fortification influences the composition and stability of the food products (217). Nonetheless, alternative dietary sources of EPA+DHA will be important to meet the growing demand for EPA+DHA among consumers.

Table 2.7 EPA and DHA content in selected foods (212, 214, 217)

Food	Serving Size	EPA+DHA mg
Non-marine sources		
Eggs	1 egg	20-40
Chicken, dark meat	3.5 oz	65-75
Turkey, dark meat	3.5 oz	15-20
Fortified sources		
Milk	8 oz	25-50
Soy milk	8 oz	32-50
Orange Juice	8 oz	50
Yogurt	4 oz	16-32
Eggs	1 egg	50-150
Margarine/spread	2 tbsp	60-100
Pork ^a	3.5 oz	50 - 800
Peanut Butter	2 tbsp	32
Wheat bread	1 slice	30-50

EPA+DHA content and source varies by brand; these ranges are based on nutrition labels and USDA National Nutrient Database for Standard Reference, Release 25.

^a Hogs fed diets rich in algal source of EPA+DHA (218)

2.4.2 Establishing a DRI for EPA+DHA

The available data for cardiac mortality provides a basis for establishing a DRI for EPA+DHA. Current intakes (≈ 100 mg/d) are not sufficient; setting a DRI for EPA+DHA is important for realizing health benefits, specifically CVD prevention. In the Harris et al. (59) analysis, 566 mg/d of EPA+DHA was the average intake associated with the greatest reduction (37%) in risk of CHD mortality. Based on this data, an AI of 566 mg/d could be considered for EPA+DHA intake. However, if we assume 566 mg/d was the median requirement, i.e., it reduced risk of CHD mortality in 50% of the population, then 566 mg/d would be considered as an estimated average requirement (EAR). The recommended daily allowance (RDA) could then be calculated using the standard deviation (224 mg/d). Two standard deviations above the EAR would be approximately 1 g/d, which would be the amount of EPA+DHA expected to meet the needs of nearly all healthy individuals (Figure 2.6). Of course, such requirements are speculative. Recommendations calling for >1 g/d of EPA+DHA are typically aimed at secondary prevention of CVD. To date, no UL for EPA+DHA has been set by any authoritative body. The FDA has stated that levels up to 3 g/d are Generally Recognized as Safe (219), although other authorities have reported no adverse effects at intake levels up to 5-6 g/d (200, 220).

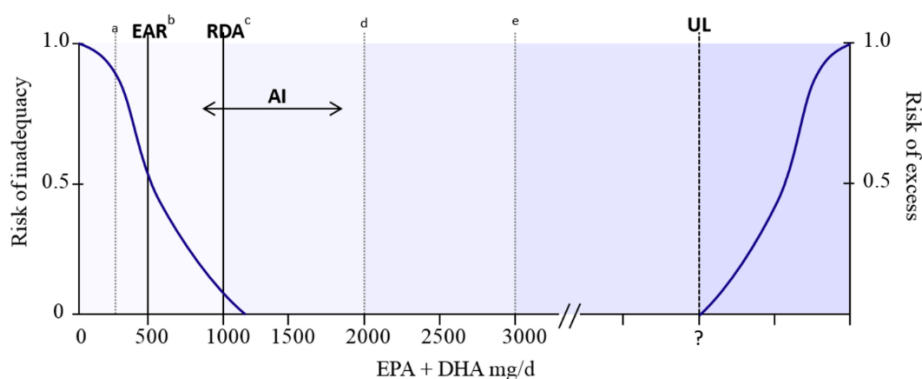


Figure 2.6 Potential dietary reference intakes for EPA+DHA based on cardiovascular benefit. The dashed lines highlight the uncertainty regarding the indicated RDA, suggesting additional research is still needed. Abbreviations: AI, adequate intake; EAR, estimated average requirement; RDA, recommended daily allowance; UL, tolerable upper limit

^a 250 mg/d recommended by Dietary Guidelines for Americans 2010, European Food Safety Agency, and World Health Organization

^b 500 mg/d recommended by Academy of Nutrition and Dietetics, International Society for the Study of Fatty Acids and Lipids, and American Heart Association (for those without CHD)

^c 1.0 g/d recommended by American Heart Association for secondary prevention of CHD)

^d 2.0 g/d recommended by American Heart Association for individuals with high triglyceride levels

^e ≤ 3.0 g/d Generally Recognized As Safe by Food and Drug Administration

2.5 CHALLENGES TO SETTING A DRI

There are many challenges in establishing a DRI for EPA and DHA. Yet, with these challenges also come opportunities to explore areas of research needed. Some of these challenges are:

1. EPA and DHA are individual nutrients. Thus, despite often being consumed in combination, it remains unclear whether there is a greater need for one versus the other, and whether a DRI for each one should be set independent of the other
2. Limitations in dietary intake assessment methods and nutrient databases for EPA+DHA present challenges in obtaining accurate, current intake data.
3. Lack of a consensus regarding the most appropriate and reliable biomarker for determining EPA+DHA status in humans.
4. The safety of markedly increased intakes of EPA+DHA needs to be defined.

2.5.1 EPA vs. DHA

Numerous studies have demonstrated beneficial effects of EPA+DHA; however, relatively little is known regarding the unique effects of EPA compared to DHA. Also, with increasing consumer awareness, many are beginning to question whether there is an appropriate form (TG or ethyl ester) or EPA to DHA ratio. As mentioned previously, additional research is needed to compare the specific effects of EPA versus DHA on health outcomes. It is not uncommon though to have DRIs for a mixture of nutrients, which is the case for macronutrient recommendations. Protein, fat, and carbohydrates, each containing mixtures of individual components with unique physiological effects, all have established AMDRs (196). In addition, both protein and carbohydrate have an RDA, while n-6 PUFA and ALA have an AI, as previously discussed. Thus, concerns over the ideal EPA to DHA ratio should not deter creating a DRI for these fatty acids.

Mozaffarian and Wu recently reviewed the evidence for shared or distinct cardiovascular effects of EPA and DHA (221). In animal and human studies, both EPA and DHA modulate inflammation, reduce platelet aggregation, lower TG levels, and may increase cardiac diastolic filling and arterial compliance. In contrast, observational and clinical studies

suggest that DHA may have a greater TG-lowering effect in addition to increasing the proportions of large LDL and HDL particles. The clinical relevance of DHA effects on lipoprotein particle size is unclear. Mozaffarian and Wu concluded that EPA and DHA have collective as well as complementary benefits; available evidence is insufficient to specify recommendations for their individual intakes or ratio of their intake. Both EPA and DHA provide cardiovascular benefits and therefore increasing consumption of either EPA or DHA would be advantageous (221). More research is needed to understand the distinct health effects of EPA and DHA as well as the effects of docosapentaenoic acid (DPA, 22:5, n-3), an elongated metabolite of EPA and intermediate between EPA and DHA; however, given their complementary effects and combined presence in fish and fish oil, it is appropriate to focus on their collective consumption in setting a DRI.

2.5.2 Dietary assessment methods

Reliable and valid dietary intake assessments are crucial in determining DRIs. This is a challenge for all nutrients, including EPA and DHA. Moreover, the quality of nutrient intake data can vary considerably among studies (196). The dosage, duration, source of EPA+DHA (i.e., supplements or fish), patient type, concomitant medications, other dietary and lifestyle factors also contribute to the inconsistencies among studies and complicate establishing DRIs. Vegans and vegetarians have limited food choices for EPA+DHA, and have low blood levels of EPA+DHA (222). Very few studies have examined whether vegetarians have greater ALA conversion efficiency compared to meat and fish eaters (223, 224). Further research focusing on the endogenous production of EPA and DHA in vegans and vegetarians is needed. Reddy et al. (225) found that infants born to vegetarians had lower DHA in their plasma and cord artery phospholipids compared with infants born to non-vegetarians, while docosapentaenoic acid (DPA, 22:5, n-6) was greater ($p < 0.001$). Whether the partial replacement of DHA with DPAn-6 has any physiological consequence requires further investigation. Research on the endogenous production of EPA and DHA in vegans and vegetarians is needed to set a DRI for this population.

Inter-individual variability in response to EPA+DHA also presents a significant challenge to defining DRIs, which is an issue IOM committees encounter when identifying standardized and consistent data on nutrient intakes. Differences in age, sex, weight, perhaps

race as well as overall health status complicate the discussion of optimal EPA+DHA intake. The impact of genetics on EPA+DHA levels (synthesis, absorption, metabolism) also is a likely determinant of response. A recent study in Framingham reported that after controlling for dietary intake of EPA+DHA (and age, sex, smoking), heredity accounted for over one-third of the variability in the O3I (226). There is increasing evidence to suggest that specific genotypes (e.g., apolipoprotein E, fatty acid desaturases, and peroxisome proliferator-activated receptors) modulate the response to EPA+DHA intake (227). Although the literature on genetic determinants of the response to increased EPA+DHA is very limited, with further research, genotyping may become a useful tool to determine optimal intakes for certain populations.

Maintaining and updating food composition databases (i.e., USDA National Nutrient Database for Standard Reference (212)) are important in assessing n-3 PUFA intake. For example, NHANES, the major source of current dietary intake data for the U.S. population, relies on the USDA database to accurately calculate nutritional values. The current version, Release 25, issued in September 2012, expanded the n-3 PUFA composition data for foods and dietary supplements. Although this improved accuracy of n-3 PUFA intake estimates, challenges remain in accurately determining intake. A limitation of food records is that individuals frequently underestimate their intake. For that reason, a reliable and validated biomarker of n-3 PUFA status is necessary to validate dietary intake data.

2.5.3 Biomarkers of EPA+DHA status

In the previous DRI Report, the IOM acknowledged the biological potency of EPA and DHA, yet indicated that there was a lack of accepted biomarkers of intake for EPA and DHA (196). Significant progress has been made since the DRI Report and several markers of EPA+DHA intake are now available, including plasma, erythrocyte, and adipose tissue levels. The use of blood markers of fatty acid intake has made it possible to evaluate outcome measures related to disease. Plasma fatty acids reflect intake over the past few days, whereas adipose tissue fatty acids are more reflective of long-term fatty acid intake (228, 229). The erythrocyte content of EPA and DHA, i.e., the Omega-3 Index, is a useful biomarker of n-3 PUFA status as well. It reflects tissue levels and correlates with intake. The use of the Omega-3 Index has made it possible to evaluate EPA+DHA status relative to diseases. Harris and von Schacky proposed that the Omega-3 Index be considered a risk factor for CHD death (7). A

low Omega-3 Index is associated with increased risk for non-fatal acute coronary syndromes (230). The Omega-3 Index is highly correlated with cardiac membrane EPA+DHA levels ($r=0.81$, $p<0.0001$) (27). Measuring EPA+DHA content of RBC membranes (as opposed to whole plasma or plasma phospholipids) provides insight into longer term intake versus short term intake (231, 232). Utilizing a standardized assessment method, such as the Omega-3 Index, is important for assessing n-3 PUFA status.

2.5.4 Safety of increased EPA+DHA intake

There are some concerns over excessive bleeding at higher doses of n-3 PUFAs (≥ 3 g/d), despite several studies have examining this question, and uniformly reporting no increased risk for clinically significant bleeding (199). In order to obtain ≥ 3 g/d of n-3 PUFAs, one would have to consume multiple servings of seafood each day, an achievable yet uncommon practice in the United States. However, as food fortification continues to rise, concerns over high n-3 PUFA intakes will become a more relevant topic for discussion. In 2011, the Norwegian Scientific Committee for Food Safety conducted a safety review of EPA+DHA and found no adverse effect on bleeding time up to levels as high as 6.9 g/d (220). More recently, the European Food Safety Authority concluded that intakes up to ≈ 5 g/d of n-3 PUFA do not appear to increase risk of bleeding complications (200). While additional research will continue to clarify the effect of EPA and DHA supplementation on bleeding time, current evidence indicates that n-3 PUFAs do not increase risk of adverse bleeding episodes.

Consumption of fish raises the issue of human exposure to methylmercury, a toxic form of mercury found in long-lived fish and top-level predators, such as king mackerel, swordfish, shark, tilefish, and albacore tuna. In 2004, the FDA issued an advisory for women who may become pregnant, pregnant women, nursing mothers, and young children to avoid certain types of fish high in mercury (233). A recent analysis of prospective cohort studies examined the combined effects of methylmercury and n-3 PUFA in middle-age men, reporting that eating fish high in n-3 PUFAs and low in methylmercury was associated with reduced risk of MI (234). Risk-benefit analyses indicate that lowering fish consumption would have serious public health consequences (2, 234-236). Moreover, fish oil supplements contain little to no mercury (237).

2.6 HUMAN MODEL OF ENDOTOXEMIA

Inflammation is initiated by the immune system in response to injury, irritation or infection; however, prolonged or chronic inflammation is involved in the etiology of numerous acute and chronic diseases. Studies of inflammation are often confounded by the absence of well-defined onset time of inflammation, differences in the duration of disease, and inter-individual differences in the sensitivity and immune response to various types of stimuli (238). Human models of systemic inflammation have been developed in order to study the molecular and physiological effects of inflammation in a controlled setting. Endotoxin, or bacterial lipopolysaccharide (LPS) from *Escherichia coli*, is a potent stimulator of the innate immune response. LPS features a surface pattern not found in human tissue (i.e., pathogen-associated molecular pattern), serving as ligands for the pattern recognition receptor expressed on immune cells called toll-like receptor-4 (TLR4)(239). Administration of LPS, even at a low dose (0.6 ng/kg body weight) elevates circulating concentrations of inflammatory cytokines via activation of TLR-4 pathway (**Figure 2.7**) (238, 240). This model mimics inflammatory responses observed in initial host responses to infection.

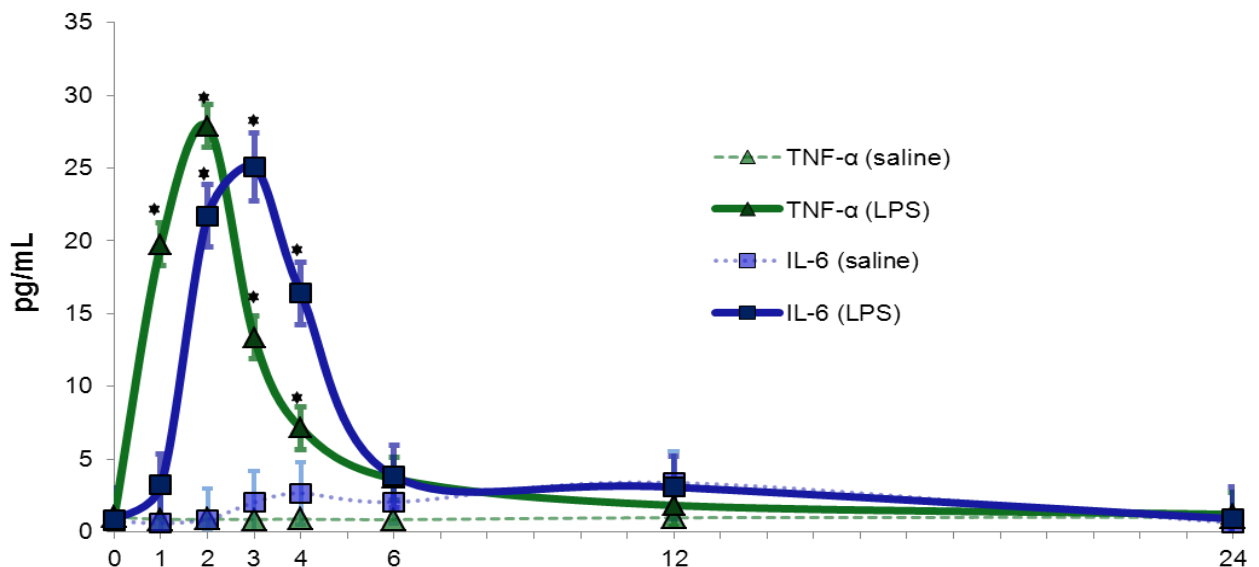


Figure 2.7. Low dose intravenous LPS administration (0.6 ng/kg) in healthy males (n=6) produces significant increases in pro-inflammatory cytokines (TNF- α and IL-6) compared to saline control. Data obtained from pilot study (Flock et al., Experimental Biology 2012).

Acute changes observed during experimental endotoxin challenges resemble those observed chronically in obesity, insulin resistance, and atherosclerosis (241-243). Mehta et al. (241), demonstrated that a LPS challenge (3 ng/kg body weight) in twenty healthy adults induced acute inflammation as well as systemic insulin resistance following modulation of adipose tissue inflammatory and insulin signaling pathways. A follow up study using a lower dose (0.6 ng/kg) provided further evidence of human insulin resistance induction during low-grade inflammation *in vivo* (243). Therefore, endotoxemia models are useful in furthering our understanding of the human response to inflammation as well as identifying therapeutic agents that may ameliorate inflammatory conditions.

The endotoxemia model has been proved to be safe and efficacious for evaluating anti-inflammatory compounds (240, 244-246). Michaeli et al. (246) found that supplementing fifteen healthy men with fish oil (700 mg EPA+DHA/d) for 3-4 weeks blunted fever responses following an *in vivo* LPS challenge (2 ng/kg); however, no impact on cytokine production was observed (measured for 6 hours after challenge). Potential limitations include dose and duration of supplementation as it may not have been sufficient for significant changes in cell membrane EPA+DHA content. Nonetheless, the endotoxemia model serves as a robust means of assessing the anti-inflammatory as well as pro-resolving effects of different levels of RBC EPA and DHA.

2.7 RATIONALE FOR CURRENT RESEARCH

Despite evidence indicating cardioprotective as well as anti-inflammatory effects of long-chain n-3 PUFA, there remain limitations for making dietary recommendations. Differences in subject population, dose, and duration explain some of the inconsistencies reported in clinical trials. Moreover, individual variability in response to n-3 PUFA consumption has made it difficult to determine appropriate intake recommendations. Therefore, the primary objective of the first study is to examine the dose-response effects of consuming EPA+DHA within dietary ranges on RBC content of EPA+DHA (i.e., Omega-3 Index [O3I]), a biomarker of EPA+DHA intake and predictor of CVD risk (7). The secondary objective is to identify individual characteristics that modify the response to EPA+DHA intake. We hypothesize that EPA+DHA intake will dose-dependently increase O3I following 5 months of supplementation and that baseline O3I and body weight will

predict the response. These results will have significant implications for making EPA+DHA recommendations to achieve a target OSI for CVD risk reduction on an individual basis.

Long-chain n-3 PUFA have been reported to have anti-inflammatory effects that may help reduce the risk of many chronic diseases (247). However, questions remain regarding the role of n-3 PUFA in the etiology and treatment of inflammatory conditions. Nutritionally-achievable doses of n-3 PUFA typically have not been shown to affect circulating inflammatory markers in healthy adults (184, 248); however, the effect of long-term n-3 PUFA intake across a range of nutritionally-achievable doses has not been defined. Moreover, the relationship between RBC fatty acids and inflammatory markers in healthy adults has not been well-documented. Therefore, the objective of the second study is to evaluate the dose-response effects of nutritionally-achievable doses of EPA+DHA on circulating inflammatory marker concentrations in healthy adults, and secondly, to explore the relationship between baseline and changes in RBC content of n-3 and n-6 PUFA with circulating inflammatory marker concentrations. We hypothesize that no dose-response effect of EPA+DHA intake will be observed given the non-inflamed healthy population as well as use of dietary doses.

Clinical studies examining the anti-inflammatory effects of n-3 PUFA are typically confounded by the absence of well-defined onset time of inflammation, differences in the duration of inflammation, as well as inter-individual variability in the sensitivity and immune response to various types of stimuli (238). Low-dose intravenous administration of purified lipopolysaccharide (LPS) serves as a well-controlled and standardized model to study the anti-inflammatory effects of n-3 PUFA *in vivo*. Therefore, the objective of the third study is to explore the relationship between RBC content of n-3 and n-6 PUFA with changes in peripheral blood mononuclear cell gene expression following an *in vivo* LPS challenge in healthy adult men. We hypothesize that RBC content of EPA and DHA will be associated with a lower peak pro-inflammatory gene expression following the LPS challenge.

Collectively, this research will provide an in depth analysis of how n-3 PUFA intake alters cell membrane composition as well as immune function in healthy adults. Clinical evidence has been inconsistent, therefore, examining the metabolism and immunomodulatory effects of n-3 PUFA in healthy adults will help clarify the role of n-3 PUFA consumption in disease prevention.

CHAPTER III:

DETERMINANTS OF ERYTHROCYTE OMEGA-3 FATTY ACID CONTENT IN RESPONSE TO FISH OIL SUPPLEMENTATION: A DOUBLE-BLIND RANDOMIZED CONTROLLED TRIAL

3.1 ABSTRACT:

Background: Erythrocyte membrane content of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which constitutes the Omega-3 Index (O3I), predicts cardiovascular disease mortality. The amount of EPA+DHA needed to achieve a target O3I is poorly defined as are the determinants of the O3I response to a change in EPA+DHA intake.

Objective: The objective of this study was to develop a predictive model of the O3I response to EPA+DHA supplementation in healthy adults, specifically identifying factors that determine the response.

Design: A randomized, placebo-controlled, double blind, parallel-group study was conducted in 125 healthy men and women. One of five doses (0, 300, 600, 900, 1,800 mg) of EPA+DHA was given daily as fish oil supplements for approximately 5 months. The O3I was measured at baseline and end of study.

Results: There were no significant differences between groups in clinical characteristics at baseline. The O3I increased in a dose-dependent manner ($p < 0.0001$), with the dose of EPA+DHA alone accounting for 68% (quadratic, $p < 0.0001$) of the variability in the O3I response. Dose adjusted per unit body weight (g/kg) accounted for 70% (linear, $p < 0.0001$) of the change in O3I. Additional factors that improved prediction of treatment response were baseline O3I, age, sex, and physical activity. Collectively, these explained 78% of the response variability ($p < 0.0001$).

Conclusion: Our findings validate the O3I as a biomarker of EPA+DHA consumption, and identify additional factors, particularly body weight, that can be used to tailor EPA+DHA recommendations to achieve a target O3I.

3.2 INTRODUCTION

The long-chain omega-3 (n-3) polyunsaturated fatty acids (PUFA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), are recommended for reducing the risk of cardiovascular disease (CVD), especially sudden cardiac death (1, 30, 202, 203). However, a Dietary Reference Intake for EPA+DHA has not been established. Use of biomarker-based approaches has made it possible to study the association of different blood or tissues levels of EPA+DHA on important health benefits or outcomes, such as risk of CVD events. The Omega-3 Index (O3I), which is the sum of EPA+DHA content in red blood cell (RBC) membranes, is a biomarker of n-3 PUFA status (7, 249) that is highly correlated with myocardial EPA+DHA content (250, 251). An O3I of $\geq 8\%$ has been recommended as a cardioprotective level (7) based on associations with reduced risk of primary cardiac arrest (28), sudden cardiac death (8), coronary atherosclerosis (9), and acute coronary syndrome (230, 252). In studies of Americans not taking n-3 FA supplements, mean O3I values range from of 4% to 5% (253-256). In two larger observational studies of US adults that did not exclude supplement users, O3I values were somewhat higher, averaging 5.3% (257) and 5.6% (258).

Due to limitations in the current evidence, dietary recommendations for achieving a target O3I cannot be made. Observational studies confirm that dietary or supplemental intake of EPA+DHA is associated with higher levels of the O3I (230, 253, 259, 260), and additional factors, such as body weight and health status, modify this relationship (230, 253, 258-261). However, these studies lack precision and accuracy due to use of food frequency questionnaires and other dietary recall methods (262).

Similarly, results from past supplementation studies have limitations that restrict their usefulness for making O3I-based recommendations. Specifically, trials have been too short in duration (254, 263-265), have not examined a range of dietary doses (254, 263, 266), had too few participants (254, 266, 267), reported high variability in measurement results (268), and/or reported higher than expected baseline values (268). Importantly, no prior supplementation studies analyzed the contribution of demographic and clinical factors to the O3I response to supplemental EPA+DHA.

Therefore, the objective of the present study was to model the O3I response to supplemental EPA+DHA intakes within dietary ranges, and identify factors that modify this response. We hypothesized that modeling the body-weight adjusted dose of EPA+DHA as a predictor would yield a more precise estimation of response to treatment than dose alone, and that additional factors also would influence the O3I response to supplementation. This information is important for making EPA+DHA recommendations to achieve a target O3I for CVD risk reduction on an individual basis.

3.3 METHODS

3.3.1 Participant recruitment and screening

Healthy, young males and females (20-45 years of age) who reported low or no habitual consumption of oily fish (e.g. salmon, tuna, and herring; <4 servings per month) and not taking n-3 PUFA supplements or consuming n-3 PUFA supplemented foods were recruited. All race and ethnic groups were eligible. Exclusion criteria included serious medical conditions, history of diabetes, or smoking; chronic anti-inflammatory medications; consumption of n-3 PUFA supplements and n-3 PUFA-supplemented foods in the past 3 months; pregnant, nursing, or planning a pregnancy; planning to change dietary habits; and body mass index (BMI) <20 or >30 kg/m².

Potential subjects were screened initially via telephone interview to determine if they met the following criteria: age, self-determined health status, weight, fish intake, and willingness to participate in the study and adhere to all aspects of the study protocol. Potential subjects who met the telephone screening criteria were scheduled for additional screening at the Penn State Clinical Research Center. After written informed consent was obtained, study participants were comprehensively evaluated in an examination that included anthropometric measurements, biochemical assessment for traditional CVD risk factors, complete blood count and standard chemistry panel to rule out the presence of serious illness, medical history, and physical examination. The medical history included questions regarding the participants' self-reported exercise and physical activity habits, which were used to assign participants to one of five physical activity levels (None = little to no exercise; Light = 1-3 d/wk; Moderate = 3-5 d/wk; Heavy = 6-7 d/wk; Very heavy = twice per day). The Harris-Benedict equation was used to

estimate individual daily calorie requirements based on body weight, height, age, and reported physical activity. The study protocol was approved by the Institutional Review Board at the Pennsylvania State University and registered on ClinicalTrials.gov (NCT01078909). All procedures followed were in accordance with the ethical standards of the Helsinki Declaration of 1975, revised in 2000.

3.3.2 Study Design

This was a randomized, placebo-controlled, double-blind, parallel-group study. Participants (n=125) were randomized to one of five doses (0, 300, 600, 900, 1,800 mg) of EPA+DHA given daily as fish oil supplements (Nordic Naturals, Watsonville, CA) for 5 months, the approximate time it takes for membrane fatty acid composition to reach a new steady state (267). A computer-generated randomization scheme was developed in advance, stratified by sex and age to ensure even distribution among treatment groups. Using this randomization scheme, eligible participants were assigned to blinded treatments at the baseline visit. The treatments were matched to a coded alpha identifier with a sealed envelope containing the code break for treatment; bottles were labeled A, B, C, D, or E by the manufacturer corresponding to different treatments. All researchers, clinicians, and participants were blinded to treatment assignment. The head nurse of the Clinical Research Center kept the envelope sealed until completion of the study. Researchers became unblinded after preliminary analyses were performed. The intervention was designed to provide doses of EPA+DHA that could be achievable by consumption of oily fish. For example, a single serving (100 g) of light canned tuna provides approximately 270 mg of EPA+DHA, whereas the same serving size of wild Atlantic salmon provides approximately 1,840 mg of EPA+DHA (212).

All participants were instructed to consume (with food) six identical capsules per day containing either placebo or fish oil that collectively delivered the target dose of EPA+DHA in triglyceride form. The study clinician was provided with a sealed envelope containing the code break for treatment assignment that was opened following study completion. Analysis of the fish oil capsules verified that they contained 20% EPA (20:5, n-3), 13% DHA (22:6, n-3), 17% palmitic acid (16:0), 14% oleic acid (18:1, n-9), 8% palmitoleic acid (16:1, n-7), 8% myristic acid (14:0), 4% stearic acid (18:0), 4% eicosadienoic acid (20:2, n-6), and small amounts of

other fatty acids. The soybean-oil placebo capsules contained 53% linoleic acid (18:2, n-6 [LA]), 23% oleic acid, 10% palmitic acid, 6% alpha-linolenic acid (18:3, n-3 [ALA]), 4% stearic acid (18:0), and small amounts of other fatty acids. The total amount of fatty acid provided per day by the capsules in each regimen is presented in **Table 3.1**.

All participants were instructed to maintain their weight and activity level and their usual (limited) consumption of fatty fish as well as their non-consumption of off-study fish oil capsules during the course of the study. The participants were supplied with log sheets and contacted monthly to ensure compliance and discuss any difficulties with taking the capsules. Also, participants reported back to the Clinical Research Center after 8 weeks to return log sheets, remaining containers, and to receive new supplies.

Table 3.1. Fatty acid composition (mg/daily dose) of the study interventions ¹

Fatty Acid	EPA+DHA				
	0 mg/d	300 mg/d	600 mg/d	900 mg/d	1800 mg/d
Myristic, 14:0	11	79	147	215	420
Palmitic, 16:0	563	629	694	760	957
Stearic, 18:0	238	242	246	251	264
Palmitoleic, 16:1 n-7	8	83	159	235	461
Oleic, 18:1 n-9	1269	1187	1104	1021	773
Linoleic, 18:2 n-6	2918	2454	1991	1527	136
Linoelaidic, 18:2 n-6 <i>trans</i>	33	44	55	66	99
Eicosadienoic, 20:2 n-6	3	35	68	100	196
α -linolenic, 18:3 n-3	351	300	249	198	46
Arachidonic, 20:4 n-6	2	11	20	29	55
Eicosapentaenoic (EPA), 20:5 n-3	9	191	374	556	1103
Docosapentaenoic, 22:5 n-3	1	20	40	59	118
Docosahexaenoic (DHA), 22:6 n-3	6	121	237	352	698
Total EPA+DHA	15	312	610	908	1801

¹Values were calculated from independent analysis of fatty acid composition (only fatty acids detected $\geq 1\%$ of total fatty acids for either activate treatment or placebo capsules are shown).

3.3.3 Blood sample collection

Whole blood samples were collected by venipuncture in the fasting state (12 h with nothing but water, 48 h without alcohol, and 2 h without vigorous exercise) before and after the intervention. A general chemistry profile was obtained as was a complete blood count using

fresh blood samples (Chem 24 panel; Quest Diagnostics, Pittsburgh, PA). Whole blood was centrifuged at 1500 x g for 15 min at 4 °C. Except for endpoints that required unfrozen specimens, samples were stored at -80 °C until they were analyzed.

Serum parameters

Total cholesterol (TC) and triglycerides (TG) were measured by enzymatic analysis (Quest Diagnostics, Pittsburgh, PA; CV < 2% for both). HDL cholesterol (HDL-C) was estimated according to the modified heparin-manganese procedure (CV < 2%). The Friedewald equation (269) was used to calculate LDL cholesterol (LDL-C = TC - [HDL + TG/5]).

Liver enzymes were measured as part of a general chemistry battery of blood tests (Chem 24 panel; Quest Diagnostics). Serum high-sensitivity C-reactive protein (hs-CRP) was measured by latex-enhanced immunonephelometry (Quest Diagnostics; assay CV < 8%).

RBC fatty acid analysis

Red blood cells were isolated from blood samples drawn into heparin-containing tubes. RBC fatty acid composition was analyzed by gas chromatography with flame ionization detection as previously described (7). Briefly, unwashed, packed RBCs were directly methylated with boron trifluoride and hexane at 100 °C for 10 min. The fatty acid methyl esters thus generated were analyzed using a GC2010 Gas Chromatograph (Shimadzu Corporation, Columbia, MD) equipped with an SP2560, fused silica capillary column (Supelco, Bellefonte, PA). Fatty acids were identified by comparison with a standard mixture of fatty acids characteristic of RBC (GLC 727, NuCheck Prep, Elysian, MN) which also was used to determine individual fatty acid response factors. Fatty acid composition was expressed as a percent of total identified fatty acid (CV < 3.7%). High and low O3I controls were included in every analytical run.

3.3.4 Statistical analysis

All statistical analyses were performed using Minitab (version 16.2, Minitab, State College, PA). Differences between treatments groups were tested by analysis of variance (ANOVA) using a general linear model. Baseline values were included as a covariate. Tukey-adjusted P values were used for *post-hoc* comparisons among the 5 groups. Adjusted P values

of < 0.05 were considered significant. Continuous data are reported as the mean \pm SEM. For descriptive purposes, categorical data are presented as frequencies and percentages. Fit statistics were assessed for continuous variables to identify any outliers (± 3 SD) and for normality. Non-normally-distributed data are reported as median and interquartile range (IQR).

Regression modeling was performed using the Assistant menu-based tool for regression. Univariate regression models were used to determine the effects of each subject characteristic on the O3I. The Best Subsets Regression procedure was used to compare all possible models and identify the best-fitting models when including other subject characteristic predictors beyond dose (e.g. sex, age, body weight, physical activity, race, baseline O3I). Multivariable regression models were used to determine the effect of dose on O3I in conjunction with these additional predictors. Interactions terms between treatment and participant characteristics (i.e., sex, age, body weight, and physical activity) also were tested to identify inter-individual differences in O3I response to treatment. Final models were selected based on optimized fit statistics (smallest Mallows' C_p) and explanatory power (largest adjusted R^2). Graphic representations were generated in Minitab as scatter plots for outcome vs. predictor with regression lines. Change scores were calculated as the end of treatment value minus baseline value. Residual vs. fit plots were examined to ensure homoscedasticity.

3.4 RESULTS

The study design and flow of participants are shown in **Figure 3.1**. A total of 495 individuals were screened between September 2011 and March 2012; 125 participants who met the inclusion criteria were randomly assigned to a treatment group. All baseline measurements were completed between October 2011 and April 2012. Nine subjects withdrew from the study between baseline and the final time point, which left 116 participants who completed the study. The reasons for withdrawing were: inability to comply with the intervention (5 subjects), no longer interested (2 subjects), moved (1 subject), or no reason given (1 subject). Returned capsule counts and log sheets were used to assess compliance.

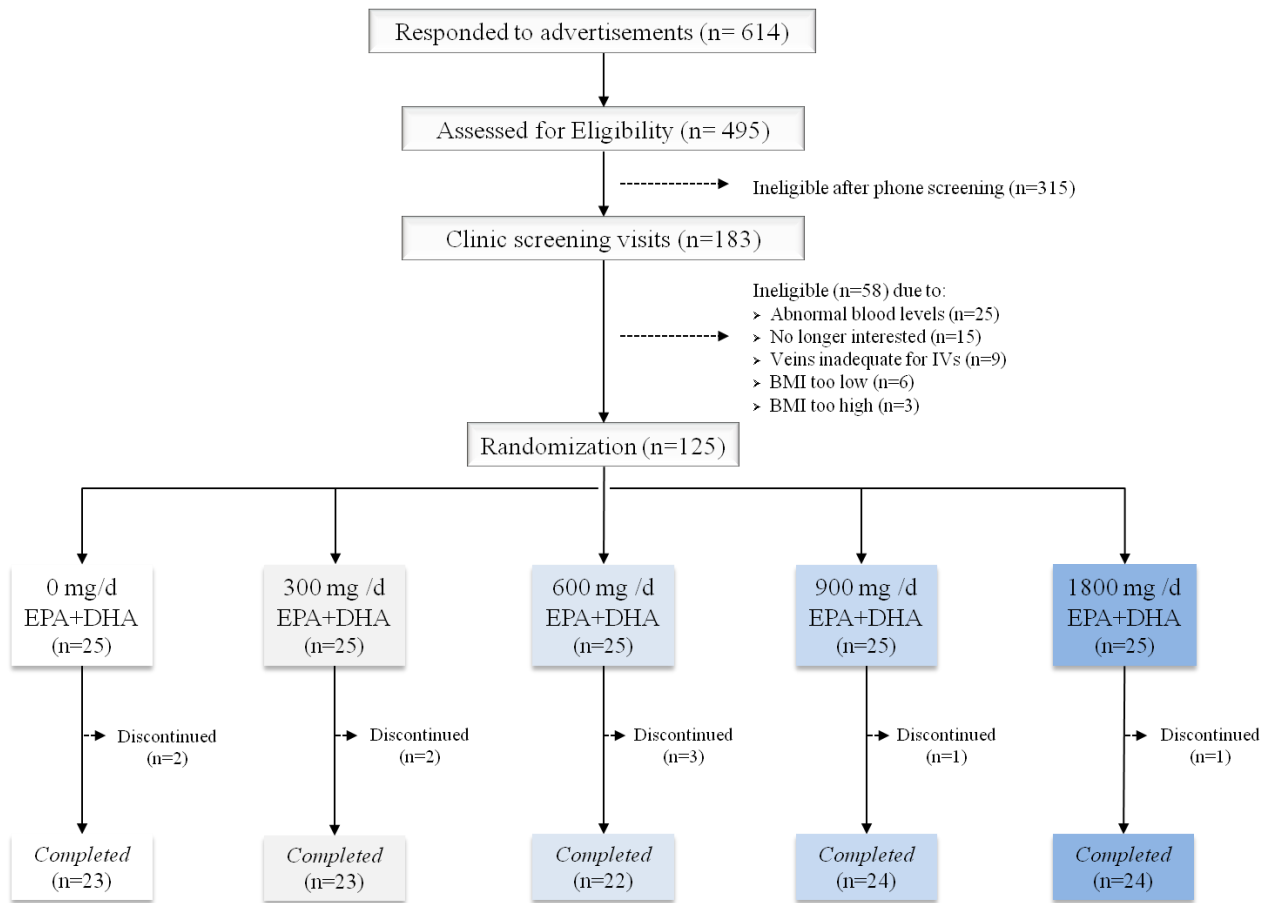


FIGURE 3.1. Flow diagram of the enrollment, screening, random assignment, withdrawals, and follow-up of the study participants.

The final study population was young, healthy, normal weight adults with a low O3I status. Study participants were predominantly white and non-Hispanic. Among study completers, compliance was high (mean: 97%, range: 85-100%). One participant with a very high O3I at baseline (i.e., 8.3%) was identified as an outlier and excluded from the analysis to ensure the sample was representative of individuals who consumed low n-3 PUFA, our target study population. Baseline characteristics of the 115 participants are shown in **Table 3.2**.

Table 3.2 Baseline characteristics of the participants who completed the study (n=115)¹

	EPA+DHA				
	0 mg/d	300 mg/d	600 mg/d	900 mg/d	1800 mg/d
N	23	23	21	24	24
Age (y)	25.7 ± 1.4 ²	25.8 ± 1.5	27.1 ± 1.6	25.8 ± 1.3	26.0 ± 1.2
Male, n (%)	11 (48)	12 (55)	11 (48)	13 (54)	13 (54)
Race, n (%)					
White	19 (83)	18 (82)	17 (81)	17 (71)	22 (92)
Black	1 (4)	0 (0)	0 (0)	0 (0)	0 (0)
Asian	2 (9)	4 (18)	4 (19)	3 (12)	2 (8)
Hispanic	1 (4)	0 (0)	0 (0)	4 (17)	0 (0)
Body mass index (kg/m ³)	24.6 ± 0.6	23.4 ± 0.5	24.5 ± 0.6	24.0 ± 0.4	25.4 ± 0.6
Blood pressure (mm Hg)					
Systolic	111 ± 3	111 ± 2	114 ± 2	112 ± 2	116 ± 2
Diastolic	75 ± 2	72 ± 2	76 ± 2	74 ± 1	76 ± 1
Lipids and Lipoproteins (mg/dL)					
Total cholesterol	171.8 ± 5.7	172.9 ± 8.1	163.0 ± 6.0	165.0 ± 6.3	171.0 ± 6.6
LDL-C	100.1 ± 5.4	104.6 ± 6.9	92.6 ± 5.1	93.1 ± 5.3	97.0 ± 5.7
HDL-C	55.5 ± 2.1	49.3 ± 2.6	53.3 ± 2.8	53.9 ± 2.9	55.0 ± 3.2
Triglyceride	81.7 ± 6.6	97.3 ± 5.6	84.2 ± 7.7	89.9 ± 5.7	94.6 ± 7.7
Glucose (mg/dL)	90.1 ± 0.8	88.9 ± 1.4	87.9 ± 1.2	86.8 ± 1.0	89.7 ± 1.2
CRP (mg/L) ³	0.50 (0.2-2.0)	0.70 (0.2-1.4)	0.60 (0.2-0.7)	0.75 (0.3-2.7)	0.80 (0.2-1.5)
Erythrocyte n-3 fatty acid content (% by weight)					
EPA	0.49 ± 0.04	0.42 ± 0.04	0.40 ± 0.02	0.51 ± 0.06	0.46 ± 0.03
DHA	3.88 ± 0.16	3.87 ± 0.22	3.85 ± 0.18	3.80 ± 0.22	3.82 ± 0.20
Omega-3 Index	4.37 ± 0.18	4.29 ± 0.24	4.28 ± 0.19	4.31 ± 0.27	4.28 ± 0.22

¹ Groups were not significantly different for any of these metrics at baseline (p for group effect <0.05). LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein-cholesterol; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid.

² Mean +/- SEM (all such values).

³ Median; IQR in parentheses.

There were no significant differences between groups at baseline with respect to participant characteristics as well as lipids and lipoproteins, liver enzymes, glucose, or hs-CRP (Table 3.2). Erythrocyte fatty acid content was similar between groups. The mean O3I at study entry (±SEM) was 4.3 ± 0.1%, with a range of 2.3 to 6.8% (Figure 3.2). On average, women had a higher O3I than men (p<0.001). Body weight, BMI, blood pressure, and heart rate did not change significantly during the study (not shown).

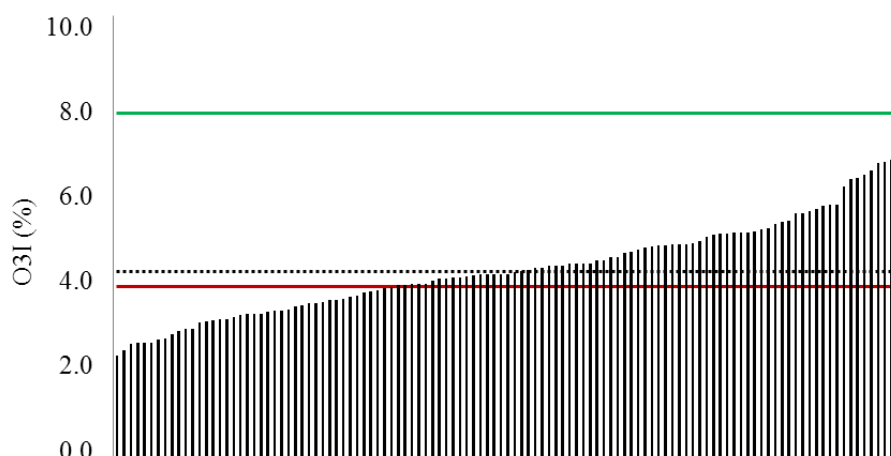


FIGURE 3.2. Distribution of the percentage of red blood cell (RBC) EPA+DHA values (Omega-3 Index [O3I]) in the study population at baseline (n=115). Lines at 8% and 4% indicate proposed low- and high-risk horizons, respectively, and the dotted line at 4.3% is the population average

3.4.1 Serum parameters

Total cholesterol, LDL-C, HDL-C, TG, glucose, liver enzymes, CRP, and measures of health status, remained unchanged for all treatment groups (**Supplementary Table 1**).

3.4.2 Erythrocyte fatty acids

EPA+DHA supplementation increased the O3I in a dose-dependent manner (**Table 3.3, Figure 3.3**). The increase in both EPA and DHA resulted in a significant increase in O3I of 121% (from 4.3% to 9.5%) for the 1,800 mg/d dose, 75% for 900 mg/d dose, 59% for the 600 mg/d dose, and 44% for the 300 mg/d dose (all $p < 0.0001$ vs. placebo). This effect was accompanied by a significant decrease in total n-6 PUFA (**Supplementary Table 2**). No change in O3I was observed for the placebo group from baseline. Participants taking 300 mg/d achieved a median O3I of 6.1% (IQR: 5.8% - 7.1%); however, no participant in our study assigned to 600 mg/d or less achieved an O3I of 8% (**Figure 3.3**). Participants taking 900 mg/d achieved a median O3I of 7.6% (IQR: 6.6% - 8.3%), whereas the 1,800 mg/d group achieved a median O3I of 9.9% (IQR: 8.9% - 10.5%).

Table 3.3. Effects of EPA+DHA supplementation on Omega-3 Index (n=115)¹

Omega-3 Index	0 mg/d	300 mg/d	600 mg/d	900 mg/d	1800 mg/d	P value
Baseline	4.37 ± 0.22	4.29 ± 0.22	4.28 ± 0.23	4.31 ± 0.22	4.28 ± 0.22	0.998
Post ²	4.39 ± 0.27 ^a	6.18 ± 0.27 ^b	6.80 ± 0.28 ^{b,c}	7.53 ± 0.26 ^c	9.48 ± 0.26 ^d	<0.0001
Change ²	0.02 ± 0.24 ^a	1.89 ± 0.24 ^b	2.52 ± 0.26 ^{b,c}	3.22 ± 0.24 ^c	5.20 ± 0.24 ^d	<0.0001

¹ Data shown represent least square mean ± standard error of the mean. Values with different superscript letters are significantly different, P < 0.05 (Tukey-adjusted values from post-hoc tests). P values are for the main effect of treatment

² Baseline values included as a covariate

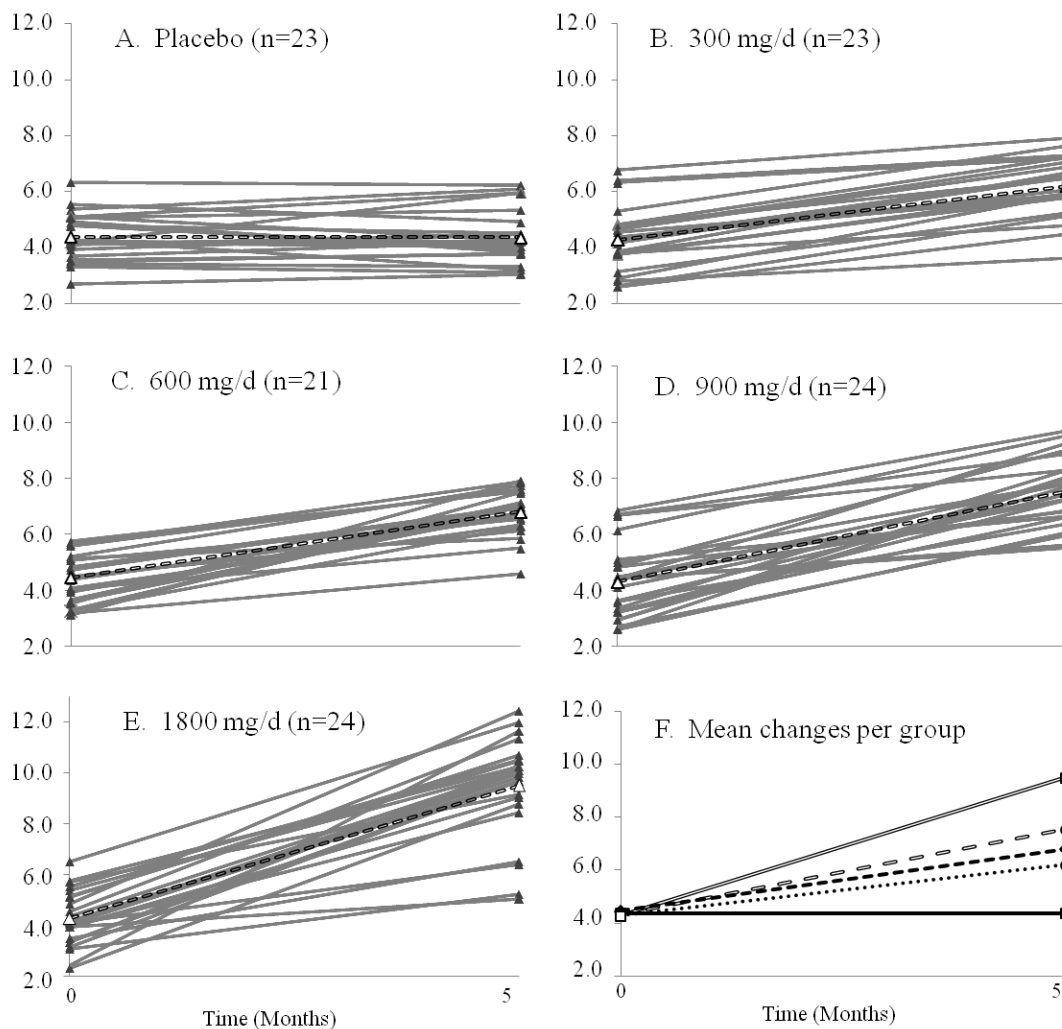


FIGURE 3.3. Changes in the Omega-3 Index (O3I) before and after healthy adults were supplemented for 5 months with either 0 (A; n = 23), 300 (B; n = 23), 600 (C; n = 21), 900 (D; n = 24), or 1800 (E; n = 24) mg/d of EPA+DHA. Values obtained using the regression procedure (Minitab 16.2, State College, PA). In Figs. 3.3 A-E, each participant is denoted by a solid line with a darkened triangle symbol (▲). The mean change per group is denoted as a dashed line with a white triangle symbol (Δ). Fig. 3.3 displays the mean changes for each supplement group: 0 (■), 300 (●), 600 (◆), 900 (○), and 1800 (□) mg/d of EPA+DHA.

3.4.3 Regression modeling

Univariate models of O3I response to treatment

Regression modeling was used to assess the relationship between supplemental EPA+DHA intake and O3I. A linear regression model demonstrated that the change in O3I was largely determined by the dose of EPA+DHA administered ($r^2 = 65.4\%$, $p < 0.0001$; **Table 3.4**). However, the Minitab's Assistant tool selected a quadratic fit to model the relationship between treatment dose and change in O3I ($r^2 = 67.7\%$, $p < 0.0001$; **Figure 3.4**). Baseline O3I and % DHA in RBCs also independently predicted the change in O3I ($r^2 = 4.9\%$, $p = 0.02$; $r^2 = 4.5\%$, $p = 0.02$, respectively); thus, individuals with a low baseline O3I status experienced a greater percent rise in O3I as a result of the intervention. However, no other measured participant characteristics (i.e., race, blood pressure, blood lipid levels, alcohol intake, meal frequency, or compliance) were significant independent predictors of the change in O3I in univariate models.

Table 3.4 Regression models predicting change in Omega-3 Index (n=115)¹

Predictor	Coefficient ± SE	P value	r^2 (adjusted r^2)
<i>Univariate - Linear</i>			
Intercept	0.0065 ± 0.0018	<0.0001	0.654
Treatment Dose (g)	0.0266 ± 0.0018	<0.0001	(0.651)
<i>Univariate - Quadratic</i>			
Intercept	0.0024 ± 0.0022	0.274	0.677
Treatment Dose (g)	0.0435 ± 0.0063	<0.0001	(0.671)
Treatment Dose-squared	-0.0090 ± 0.0032	0.006	
<i>Univariate- Body-weight adjusted</i>			
Intercept	0.0056 ± 0.0017	0.001	0.698
g/kg	2.0000 ± 0.0124	<0.0001	(0.695)
<i>Multivariable Model 1</i>			
Intercept	0.0255 ± 0.0042	<0.0001	
g/kg	2.0092 ± 0.1122	<0.0001	0.754
Baseline O3I	-0.4653 ± 0.0923	<0.0001	(0.750)
<i>Multivariable Model 2²</i>			
Intercept	0.0437 ± 0.0054	<0.0001	
g/kg	2.0042 ± 0.1089	<0.0001	
Baseline O3I	-0.5796 ± 0.1008	<0.0001	0.779
Age	0.0003 ± 0.0001	0.023	(0.766)
Sex	-0.0035 ± 0.0020	0.084	
PA	0.0005 ± 0.0011	0.675	
PA x dose (g/kg)	0.3236 ± 0.1284	0.013	

¹ Values were obtained using the regression procedure (Minitab 16.2, State College, PA). PA, physical activity.

² Interaction terms centered prior to fitting regression model.

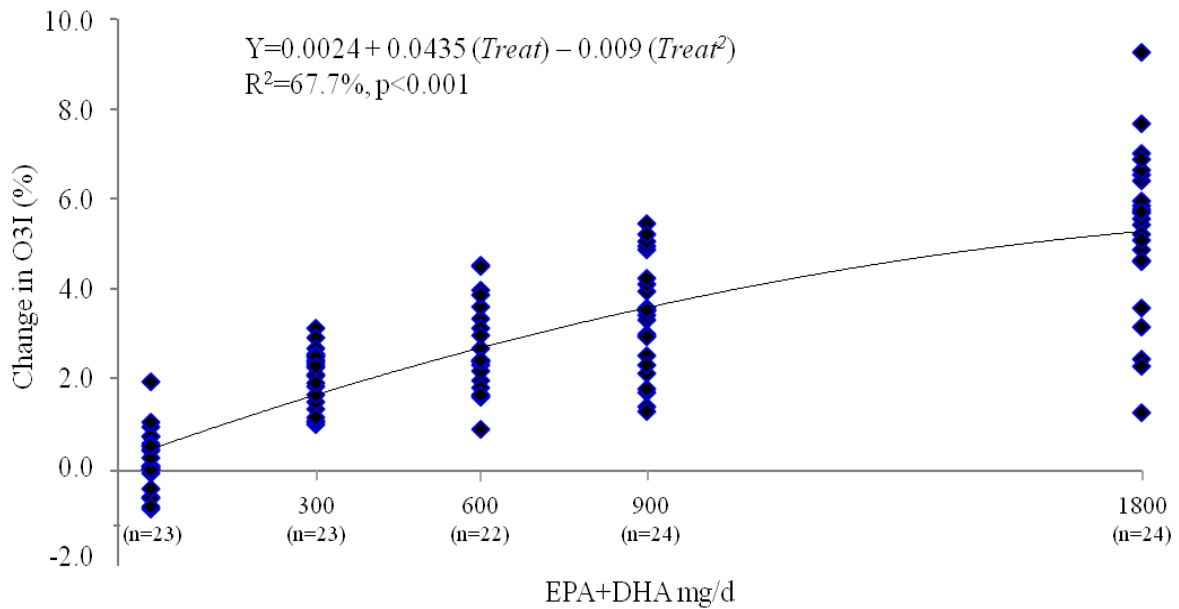


FIGURE 3.4. Treatment dose significantly predicts changes in Omega-3 index (O3I; n=115). Values obtained using the regression procedure (Minitab 16.2, State College, PA). EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid.

The dose of EPA+DHA adjusted per unit body weight (g/kg) also was a strong univariate predictor of change in O3I ($r^2=69.8\%$, $p<0.0001$; **Figure 3.5**). Increasing the dose of EPA+DHA per unit body weight (g EPA+DHA/kg body weight) resulted in a greater O3I response; individuals with lower body weight and on higher doses experienced the greatest increase in O3I.

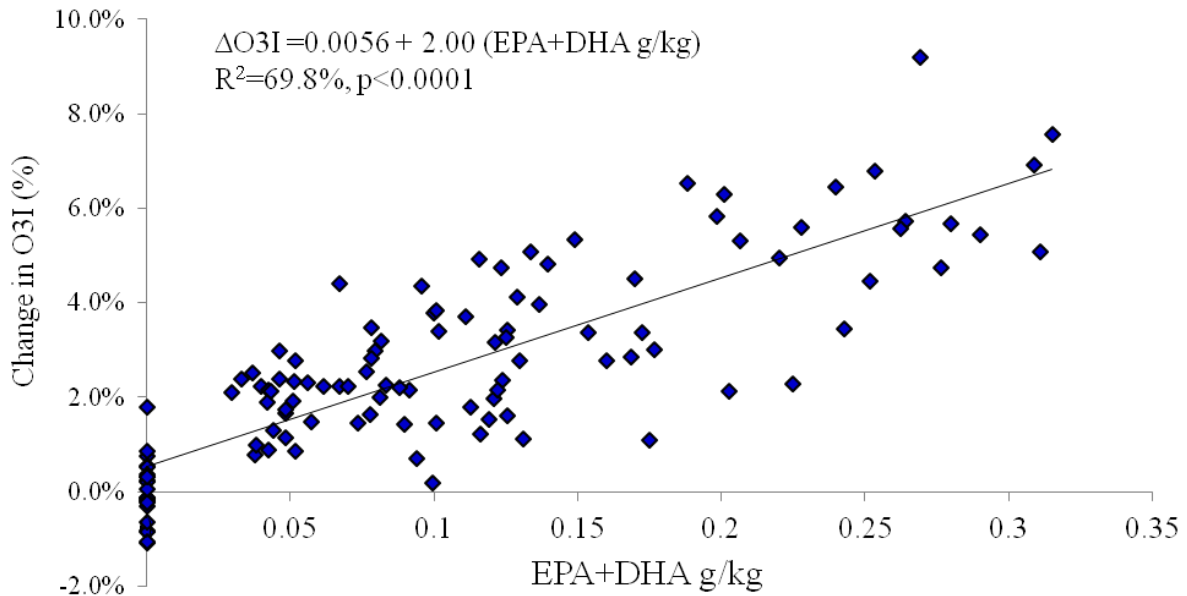


FIGURE 3.5. The gram amount of EPA+DHA consumed per kilogram of body weight significantly predicts changes in Omega-3 index (O3I; n = 115). Values obtained using the regression procedure (Minitab 16.2, State College, PA). EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid.

Multivariable models predicting the change in the O3I

Various statistical models with increasing complexity were identified to model changes in the O3I. Adding baseline O3I as a predictor to the body-weight adjusted model improved prediction of the O3I response ($r^2 = 75.4\%$, $p < 0.0001$; **Table 3.4**). Additional factors, including age, sex, and physical activity also predicted change in the O3I ($r^2 = 77.9\%$, $p < 0.0001$; **Table 3.5**). Lower O3I status ($p < 0.0001$) and older age ($p = 0.02$) each predicted greater increases in O3I. The level of physical activity interacted with the effect of dose per unit of body on the O3I response; increased physical activity level predicted greater increases in O3I when included in the model of dose per body weight ($p = 0.01$). Female sex tended to predict greater increases in O3I, although this was not statistically significant ($p = 0.08$). Including these factors in the model, relative to the univariate model of dose per unit body weight, explained an additional 8.1% variability in change in O3I.

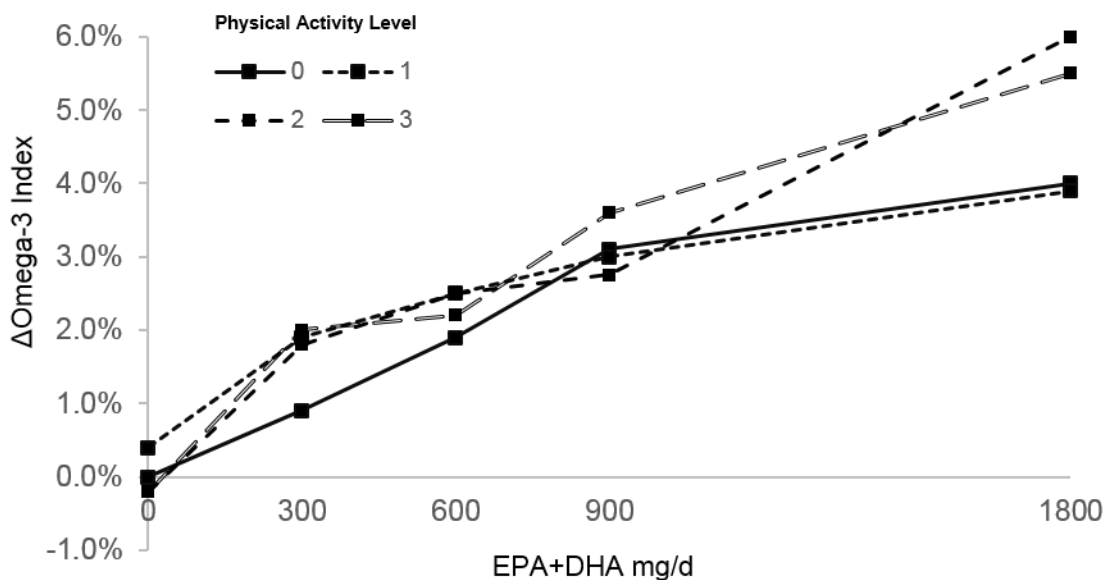


Figure 3.6 The level of physical activity interacted with the effect of dose on the O3I response; increased physical activity level predicted greater increases in O3I as dosed increased.

3.5 DISCUSSION

The present study modeled the effect of EPA+DHA supplementation and participant characteristics on the O3I response. We found that variations in dose explained 68% of the variability in the response; including body weight, baseline O3I, age, physical activity, and sex in the model explained an additional 10% of the variability in response (**Table 3.4**).

It is estimated that Americans consume <100 mg/d of EPA+DHA (270), well below the current recommendations of 250-500 mg/d for healthy adults (1, 30, 202, 203). In the present study, participants had an average baseline O3I of 4.3%, which is consistent with previous studies of adults reporting low habitual fish intake (257, 263). Our results suggest that a healthy, normal weight adult with low fish intake who increased their dietary intake by 250-500 mg/d of EPA+DHA would experience an increase in O3I values of about 1-2 percentage points (5.3% - 6.3%). Thus, our results demonstrate that increasing consumption of EPA+DHA to current recommended dietary intakes in people who rarely consume oily fish would result in increased O3I levels associated with reduced acute coronary syndrome (230) and CVD mortality (8, 28).

Additional EPA+DHA intake beyond current recommendations are needed to achieve the higher target O3I values associated with the greatest reduction in CVD risk (8, 28, 230). Based on our findings, we estimate that an average healthy adult with a low O3I (i.e., 4.3%) would require at least 1 g/d of EPA+DHA for 5 months to achieve an O3I of 8% (**Table 3.4**). This dose has been shown to reduce all-cause mortality, cardiac death, and sudden death in post-MI patients (4) and approximates the average intake in Japan where CHD death rates are reduced relative to the rate in the U.S. (271). Equations developed using data in our study also could be used to estimate EPA+DHA intakes in research studies with greater accuracy and sensitivity than questionnaire-based dietary assessment methods.

The response to supplementation that we observed for the highest dose agrees with prior studies that administered EPA+DHA for up to 12 months (254, 267, 268). On average, O3I increased from 4.3% to 9.5% (5.2 percentage point increase from baseline) with 1.8 g/d of EPA+DHA (60% EPA, 40% DHA) over a period of 5 months. In a recent study by Browning et al. (268), a similar increase (+5 percentage points) was observed in older healthy adults supplemented with 1.9 g/d of EPA+DHA (46 % EPA, 54% DHA) for 12 months. In comparison, Katan et al. (267) reported healthy men supplemented with ≈ 2 g/d of EPA+DHA (85% EPA, 15% DHA) for 12 months increased the O3I by 4 percentage points, suggesting that, in addition to duration and dose, the relative proportions of EPA and DHA may influence O3I response. Katan et al (267) used a supplement that was almost completely EPA, which may explain the lower O3I response considering turnover of DHA in RBC membranes is slower than that of EPA (267, 272, 273). We used EPA+DHA supplements containing the same ratio of EPA to DHA contained in most over the counter fish oil supplements (274). Von Schacky et al. (9) also used this ratio in a randomized controlled trial of coronary heart disease patients consuming 3 g/d of EPA+DHA (62% EPA, 38% DHA) for 3 months followed by 1.5 g/d for 21 months. Patients in the fish oil group increased the O3I by 5.5 percentage points (from 3.4% to 8.9%), had less progression and more regression of coronary artery disease as measured by changes on coronary angiography compared to the placebo group (9). Further research is needed to differentiate the specific effects of EPA and DHA on cardiovascular health before dietary recommendations can be made for EPA and DHA individually (or their ratio) (221).

Body weight explained additional variability in O3I response to EPA+DHA supplementation. Individuals with lower (vs. higher) body weight tended to have a greater response to a given EPA+DHA intake. This suggests that EPA+DHA recommendations to achieve a target O3I may be most appropriately made on a body weight basis, similar to current dietary protein requirements (196). Using the body-weight adjusted values (**Table 3.4**, **Figure 3.5**), it can be estimated that an individual weighing 75 kg would require about 1.2 g/d of EPA+DHA to increase their O3I from 4.3% to 8%; however, the requirement would only be 0.9 g/d if the same individual weighed 55 kg, or in contrast, 1.5 g/d for an individual weighing 95 kg, a range representing 3-5 typical fish oil capsules per day. Thus, accounting for individual differences in body weight could potentially improve precision for EPA+DHA recommendations.

Individuals with a higher baseline O3I experienced a lower O3I response to treatment. This finding is consistent with previous evidence demonstrating that individuals with higher EPA+DHA concentrations incorporate additional EPA+DHA at a slower rate than those with lower baseline concentrations (254, 265). We also found that incorporation of EPA+DHA into RBC membranes increased in a dose-dependent, and potentially saturable manner (**Figure 3.4**), suggesting that RBC membrane EPA+DHA concentrations are regulated to some degree and at some point reach a point of saturation.

Our multivariable model, which included body-weight adjusted dose, baseline O3I, age, sex, and physical activity, accounted for over three-fourths of the variability in O3I response to supplementation (**Table 3.4**). Smoking status has been shown previously to be inversely associated with O3I (259, 260, 275); we excluded smokers from our study and therefore were unable to assess the effect of smoking on O3I response.

Age was a predictor of the change in O3I in the multivariable model. Older individuals experienced a greater increase in their O3I as a result of the intervention, although the effect size was small, and individuals in our study were relatively young overall (aged 20-45 years). Nonetheless, aging may cause alterations in n-3 PUFA metabolism. Vandel et al. (276) reported that elderly adults (average 74 years old) given 1 g/d of EPA + DHA for 3 weeks experienced a 42% higher DHA incorporation into plasma lipids than young adults (average 24 years old); EPA incorporation was similar for both groups. Reasons for these age-related differences in n-3 PUFA metabolism are not well understood, although the emerging link

between low DHA status and cognitive decline in the elderly has encouraged clinical trials to examine the role of n-3 PUFA in older populations (277). Epidemiological evidence has frequently reported an association between age and O3I (230, 253, 257, 258, 260, 278-280); however, more clinical studies are needed to understand the mechanisms as well as relevance of age-related differences in response to EPA+DHA supplementation.

We also found that women on average had a higher O3I than men at study entry, with a strong trend for sex ($p < 0.10$) to be a predictor of O3I responses in the multivariable model. The relationship between sex and O3I has been reported previously, although it is inconsistent and not well understood (230, 258-260, 279, 280). Body weight might be responsible in part for the variability in sex, since women tend to weigh less than men (i.e., body weight and sex were collinear); however, this factor was accounted for in the model by adjusting the dose per unit body weight.

We were surprised to find that a physical activity and treatment interaction significantly predicted O3I response to treatment. Participants who were more physically active tended to experience greater increases in O3I as dose increased. This suggests that exercise may enhance incorporation of EPA+DHA in RBC membranes in subjects taking fish oil supplements, although a mechanism to explain this relationship is not immediately obvious. It is possible that improvements in blood flow as a result of increased physical activity enhances EPA+DHA incorporation into RBC membranes. However, increasing RBC content of EPA+DHA also improves membrane fluidity and permeability, which could theoretically improve oxygen diffusion through the membrane to exercising muscles. Therefore, consistent moderate physical activity may preferentially incorporate EPA+DHA into the RBC membrane for optimal oxygen delivery. Such a hypothesis needs to be clinically tested.

The TG-lowering effects of supplemental n-3 PUFA also have been well-demonstrated, particularly in individuals with elevated TG (281), and an inverse relationship between O3I and serum TG has been previously reported (230). However, no changes in serum TG, LDL-C, or HDL-C concentrations were observed in present study. The lack of TG-lowering effect can be explained by the normotriglyceridemic study population as well as the use of < 2 g/d doses of EPA+DHA (195).

3.5.1 Strengths and limitations

Among the strengths of this study were the placebo-controlled, double-blind study design that compared five doses of EPA+DHA; a relatively large sample size; a low dropout rate (7%); an adequate duration of supplementation (≈ 5 months); and the use of validated analytical methods to determine biomarker response to treatment. Moreover, our statistical approach involved unbiased selection procedures to identify variables for optimized modeling of O3I responses to EPA+DHA supplementation.

Limitations include the predominantly Caucasian, young, healthy population studied as well as the absence of background dietary and other demographic, behavioral, physiologic, or genetic data that might have allowed us to predict with greater power the changes in the O3I. A recent Framingham cross-sectional analysis found that pedigree (i.e., ancestry) explained 24% of the variability in O3I (258), while dietary EPA+DHA intake and fish oil supplement use explained 40% of the variability in O3I (dose or duration of supplement use was not considered). We did not examine genetic differences in the present study; however, because of our dose-response, randomized design and number of participants, this study design is not optimal for discovering genetic predictors of the O3I response to supplementation. Nonetheless, genome-wide association studies in large populations given a fixed dose of EPA+DHA may help identify genetic loci responsible for additional variability in the O3I response (258, 282).

The lack of information on levels of EPA+DHA in participants' background diets, as well as other nutrients in the habitual diet could have affected the O3I response to supplemental EPA+DHA via effects on metabolism as well as uptake into cell membranes through effects on enzymatic and non-enzymatic oxidation (283, 284). For example, variations in choline intake can affect the rise in RBC n-3 PUFA levels with supplementation (285). Therefore, obtaining dietary data prior to and during the intervention could be useful for future research. Body composition data also would be worthwhile to collect. Adipose tissue serves as a storage site for fatty acid in the form of TG; therefore, additional research is needed to determine how the amount and location of body fat (i.e. subcutaneous versus visceral adiposity) may impact the O3I response to EPA+DHA intake.

3.5.2. Conclusions

Marine derived n-3 PUFA supplementation explained two-thirds of the variability in response of RBC EPA+DHA content and several factors beyond dose (i.e., body weight, baseline O3I, age, physical activity, and sex) add more precision to the predictive model. These results can be used to estimate an individual's required supplemental intake for achieving a target O3I to make biomarker-based dietary recommendations for EPA+DHA, and conversely, to estimate dietary intake based on levels of O3I (with or without including additional predictive factors identified herein). However, future studies are needed to assess how EPA and DHA individually or in different ratios affect O3I responses. Additional research also is needed to evaluate whether background diet and nutrients beyond EPA+DHA affect biomarker responses. Finally, more research is needed to clarify the association between changes in the O3I and different disease states and health outcomes. In conclusion, our study has provided models that quantitatively demonstrate the relationship between dietary supplemental doses of EPA+DHA and biomarker responses, providing a useful tool for future research studies of marine n-3 PUFA that, in aggregate, can inform evidence-based dietary recommendations.

3.6. SUPPLEMENTARY MATERIAL

Table 3.5. (Supplementary Table 1) Effects of EPA + DHA supplementation on general health profile (n=115)¹

	EPA+DHA					P value for treatment effect ²
	0 mg/d	300 mg/d	600 mg/d	900 mg/d	1800 mg/d	
Sodium (mmol/L)	139.5.1 ± 0.3	139.6 ± 0.3	139.4 ± 0.3	139.3 ± 3	139.4 ± 0.3	0.95
Potassium (mmol/L)	4.0 ± 0.1	4.1 ± 0.1	4.0 ± 0.1	4.1 ± 0.1	4.0 ± 0.1	0.20
Chloride (mmol/L)	105.1 ± 0.4	105.0 ± 0.4	105.4 ± 0.4	104.9 ± 0.4	105.3 ± 0.4	0.90
Uric Acid (mg/dL)	5.2 ± 0.1	5.1 ± 0.1	5.1 ± 0.1	5.0 ± 0.1	5.0 ± 0.1	0.81
Phosphorus (mg/dL)	3.4 ± 0.1	3.7 ± 0.1	3.7 ± 0.1	3.7 ± 0.1	3.6 ± 0.1	0.12
Calcium (mg/dL)	9.3 ± 0.1	9.4 ± 0.1	9.3 ± 0.1	9.4 ± 0.1	9.4 ± 0.1	0.94
AST (U/L)	18.7 ± 1.6	21.6 ± 1.6	20.5 ± 1.7	19.9 ± 1.6	18.9 ± 1.6	0.72
ALT (U/L)	18.4 ± 1.7	16.6 ± 1.7	18.5 ± 1.7	17.8 ± 1.6	18.0 ± 1.7	0.94
ALP (U/L)	58.5 ± 1.6 ^a	55.7 ± 1.6	55.6 ± 1.7	56.8 ± 1.6	51.0 ± 1.6 ^b	0.02
LD (U/L)	147.6 ± 7.6	159.6 ± 7.6	144.4 ± 7.9	155.0 ± 7.4	142.2 ± 7.6	0.45
Bilirubin, Total (mg/dL)	0.8 ± 0.1	0.8 ± 0.1	0.9 ± 0.1	0.7 ± 0.1	0.7 ± 0.1	0.53
Bilirubin, Direct (mg/dL)	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	1.5 ± 0.1	0.76
Glucose (mg/dL)	87.4 ± 1.2	88.2 ± 1.2	89.9 ± 1.2	89.9 ± 1.2	88.6 ± 1.2	0.67
BUN (mg/dL)	14.4 ± 1.0	13.7 ± 1.0	13.9 ± 1.0	13.8 ± 0.9	15.4 ± 0.9	0.72
Creatinine (mg/dL)	0.8 ± 1.9	0.8 ± 1.9	4.4 ± 2.0	3.4 ± 1.8	0.9 ± 1.9	0.50
BUN/Creatinine Ratio	17.0 ± 0.7	14.9 ± 0.8	15.9 ± 0.8	16.3 ± 0.7	16.7 ± 0.7	0.35
Protein, Total (g/dL)	6.8 ± 0.1	6.8 ± 0.1	6.6 ± 0.1	6.8 ± 0.1	6.8 ± 0.1	0.65
Albumin (g/dL)	4.4 ± 0.1	4.5 ± 0.1	4.3 ± 0.1	4.4 ± 0.1	4.4 ± 0.1	0.52
Globulin (g/dL)	2.3 ± 0.1	2.3 ± 0.1	2.3 ± 0.1	2.4 ± 0.1	2.4 ± 0.1	0.65
A/G Ratio	1.9 ± 0.1	2.0 ± 0.1	1.9 ± 0.1	1.9 ± 0.1	1.9 ± 0.1	0.22
TC (mg/dL)	165.2 ± 4.3	172.0 ± 4.3	169.4 ± 4.5	167.6 ± 4.2	166.9 ± 4.2	0.84
LDL-C (mg/dL)	96.0 ± 3.6	100.7 ± 3.6	100.7 ± 3.7	95.4 ± 3.5	98.9 ± 3.5	0.74
HDL-C (mg/dL)	51.6 ± 1.7	55.5 ± 1.7	50.7 ± 1.8	55.5 ± 1.7	51.9 ± 1.7	0.14
Triglycerides (mg/dL)	84.4 ± 5.8	80.7 ± 5.8	90.5 ± 6.0	81.8 ± 5.6	81.0 ± 5.7	0.76
TC/HDL-C	3.4 ± 0.1	3.2 ± 0.1	3.5 ± 0.1	3.2 ± 0.1	3.4 ± 0.1	0.06
hs-CRP (mg/L) ³	0.40 (0.2-1.3)	0.50 (0.2-1.2)	0.50 (0.3-1.1)	0.60 (0.4-1.7)	0.60 (0.2-1.2)	0.87
Iron, Total (mcg/dL)	121.8 ± 9.6	119.5 ± 9.6	124.3 ± 10.0	107.6 ± 9.5	98.8 ± 9.6	0.29

¹ Least-square mean +/- SEM (all such values). AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; BUN, blood urea nitrogen; LD, lactate dehydrogenase. Values with different superscript letters are significantly different, P < 0.05 (Tukey-adjusted values from post-hoc tests).

² P values are for the main effect of treatment. Baseline values included as a covariate. Significance set at P < 0.002 to account for multiple testing.

³ Median; IQR in parentheses.

Table 3.6 (Supplementary Table 2) Effects of EPA + DHA supplementation on RBC fatty acid profile (n=115)¹

Fatty Acid	Common Name	EPA+DHA					P value for treatment effect ²
		0 mg/d	300 mg/d	600 mg/d	900 mg/d	1800 mg/d	
<i>Saturated fatty acids</i>							
C14:0	Myristic	0.37± 0.02	0.37 ± 0.02	0.37 ± 0.02	0.34 ± 0.02	0.39 ± 0.02	0.53
C16:0	Palmitic	21.82 ± 0.20	21.85 ± 0.20	22.13 ± 0.20	21.97 ± 0.20	22.08 ± 0.20	0.76
C18:0	Stearic	17.19 ± 0.16	17.37 ± 0.16	17.23 ± 0.17	17.36 ± 0.16	17.43 ± 0.16	0.82
C20:0	Arachidic	0.19 ± 0.01	0.17 ± 0.01	0.18 ± 0.01	0.18 ± 0.01	0.17 ± 0.01	0.71
C22:0	Behenic	0.15 ± 0.01	0.16 ± 0.01	0.15 ± 0.01	0.16 ± 0.01	0.16 ± 0.01	0.92
C24:0	Lignoceric	0.32 ± 0.03	0.36 ± 0.03	0.34 ± 0.03	0.38 ± 0.03	0.39 ± 0.04	0.59
<i>Monounsaturated fatty acids</i>							
C16:1n7	Palmitoleic	0.33 ± 0.02	0.28 ± 0.02	0.27 ± 0.02	0.27 ± 0.02	0.25 ± 0.02	0.11
C18:1n9	Oleic	13.52 ± 0.15	13.32 ± 0.15	13.51 ± 0.16	13.21 ± 0.15	13.03 ± 0.15	0.12
C20:1n9	Gadoleic	0.25 ± 0.01 ^a	0.23 ± 0.01	0.23 ± 0.01	0.21 ± 0.01 ^b	0.21 ± 0.01 ^b	<0.001
C24:1n9	Nervonic	0.32 ± 0.03	0.36 ± 0.03	0.34 ± 0.04	0.37 ± 0.03	0.39 ± 0.03	0.65
<i>Trans fatty acids</i>							
C16:1n7t	Palmitelaidic	0.15 ± 0.01	0.15 ± 0.01	0.16 ± 0.01	0.15 ± 0.01	0.16 ± 0.01	0.14
C18:1t	Elaidic	1.02 ± 0.04	0.95 ± 0.04	0.95 ± 0.04	0.96 ± 0.04	0.95 ± 0.04	0.56
C18:2n6t	Linoelaidic	0.33 ± 0.01	0.31 ± 0.01	0.29 ± 0.01	0.31 ± 0.01	0.29 ± 0.01	0.38
<i>n-6 Polyunsaturated fatty acids</i>							
C18:2	Linoleic (LA)	13.68 ± 0.28 ^a	13.02 ± 0.28	13.33 ± 0.29 ^a	12.81 ± 0.27	12.05 ± 0.27 ^b	0.001
C18:3	γ-linolenic (GLA)	0.16 ± 0.01 ^a	0.14 ± 0.01	0.14 ± 0.01	0.14 ± 0.01	0.12 ± 0.01 ^b	<0.001
C20:2	Eicosadienoic	0.38 ± 0.01 ^a	0.35 ± 0.01 ^{ab}	0.33 ± 0.01 ^{bc}	0.33 ± 0.01 ^{bc}	0.31 ± 0.01 ^c	<0.0001
C20:3	Dihomo-γ-linolenic (DGLA)	2.00 ± 0.05 ^a	1.82 ± 0.05 ^{ab}	1.73 ± 0.05 ^b	1.72 ± 0.05 ^{bc}	1.56 ± 0.05 ^c	<0.0001
C20:4	Arachidonic (AA)	16.10 ± 0.24 ^a	15.21 ± 0.24 ^{ab}	14.54 ± 0.25 ^b	14.43 ± 0.24 ^{bc}	13.58 ± 0.24 ^c	<0.0001
C22:4	Docosatetraenoic	4.10 ± 0.10 ^a	3.45 ± 0.10 ^b	3.05 ± 0.11 ^{bc}	2.90 ± 0.10 ^{cd}	2.58 ± 0.10 ^d	<0.0001
C22:5	Docosapentaenoic	0.76 ± 0.03 ^a	0.58 ± 0.03 ^b	0.52 ± 0.03 ^{bc}	0.50 ± 0.02 ^{cd}	0.42 ± 0.02 ^{cd}	<0.0001
<i>n-3 Polyunsaturated fatty acids</i>							
C18:3	α-linolenic (ALA)	0.20 ± 0.01 ^a	0.18 ± 0.01	0.17 ± 0.01	0.17 ± 0.01	0.15 ± 0.01 ^b	0.001
C20:5	Eicosapentaenoic (EPA)	0.47 ± 0.09 ^a	0.91 ± 0.09 ^b	1.23 ± 0.10 ^{bc}	1.44 ± 0.09 ^c	2.46 ± 0.09 ^d	<0.0001
C22:5	Docosapentaenoic	2.42 ± 0.09 ^a	3.13 ± 0.09 ^b	3.24 ± 0.09 ^{bc}	3.54 ± 0.08 ^c	3.90 ± 0.08 ^d	<0.0001
C22:6	Docosahexaenoic (DHA)	3.87 ± 0.16 ^a	5.30 ± 0.16 ^b	5.60 ± 0.17 ^{bc}	6.06 ± 0.16 ^c	7.03 ± 0.16 ^d	<0.0001

¹ Least-square mean +/- SEM (all such values). Values indicate erythrocyte content by % weight. Different superscript letters are significantly different, P < 0.05 (Tukey-adjusted values for post-hoc tests)² P values are for the main effect of treatment. Baseline values included as a covariate. P<0.002 considered significant after accounting for multiple comparisons.

CHAPTER IV:

DOSE REPOSE EFFECTS OF LONG-CHAIN OMEGA-3 FATTY ACIDS ON CIRCULATING INFLAMMATORY MARKER CONCENTRATIONS IN HEALTHY ADULTS: A RANDOMIZED CONTROLLED TRIAL OF FISH OIL SUPPLEMENTATION

4.1. ABSTRACT

Background: Long-chain omega-3 (n-3) PUFA, namely EPA and DHA, have potential anti-inflammatory effects. Results of interventional studies, however, have been inconsistent.

Objective: The primary objective of this study was to determine the dose-response effects of supplemental EPA+DHA intake on circulating TNF- α , IL-6, and C-reactive protein (CRP) concentrations in healthy adults, and secondly, to evaluate the associations between RBC membrane content of n-3 and omega-6 (n-6) PUFA and inflammatory markers.

Design: A randomized, placebo-controlled, double blind, parallel study was conducted in healthy men and women. One of five doses (0, 300, 600, 900, or 1,800 mg) of EPA+DHA was given daily for approximately 5 months. Fasting blood samples collected before and after supplementation.

Results: There were no significant effects of EPA+DHA intake on IL-6 or CRP; however, a marginally significant treatment effect was observed for TNF- α ($p < 0.08$). Higher quartiles of RBC DHA content were associated with lower TNF- α at baseline ($p = 0.001$), whereas the lowest quartile of DPA acid had significantly higher CRP ($p < 0.001$). The highest quartile of AA content had significantly higher TNF- α and IL-6 ($p < 0.05$), whereas the highest quartile of LA content had significantly lower IL-6 compared to the lowest quartile ($p < 0.05$). There were no significant associations between changes in RBC content of n-3 or n-6 PUFA and changes in inflammatory markers.

Conclusion: Our findings indicate that EPA+DHA intake, across a range of nutritionally-achievable doses, has no dose-response effect on circulating TNF- α , IL-6, or CRP in healthy adults after 5 months of supplementation. However, despite no relationship between changes in RBC PUFA content and inflammatory markers, observed baseline associations warrant further investigation.

4.2. INTRODUCTION

Diet plays a key role in modulating inflammation and thereby reducing the burden of chronic disease, including cardiovascular disease (CVD). Elevated blood concentrations of pro-inflammatory cytokines such as IL-6 and TNF- α , as well as acute phase reactants such as C-reactive protein (CRP), are used to evaluate disease status and serve as risk factors for the development of CVD (286, 287). Long-chain omega-3 (n-3) PUFA, specifically EPA and DHA, have been reported to have anti-inflammatory potential that may help reduce the risk of many chronic diseases (247). The n-3 PUFA docosapentaenoic acid (DPA), which is also present in fish and animal products, may possess similar anti-inflammatory potential (57), whereas omega-6 (n-6) PUFA are typically considered to be more pro-inflammatory (288).

Long-chain n-3 PUFA have become one of the most commonly used supplements in the United States, often for anti-inflammatory purposes (216, 289). However, questions remain regarding the role of n-3 PUFA in the etiology and treatment of inflammatory conditions. Although population studies have generally linked diets higher in EPA+DHA to lower levels of inflammation (118, 290-292), randomized controlled trials with n-3 PUFA have yielded mixed results for the effects of supplementation on both inflammatory markers and CVD outcomes (11, 182, 248, 263, 293-298). Differences in subject population, dose, duration, and background diet composition may explain some of the inconsistencies reported in clinical trials. Furthermore, there is a lack of studies examining a range of nutritionally-achievable doses. Thus, there remains a need to clarify the potential anti-inflammatory effects of longer-term supplemental n-3 PUFA intake across a range of nutritionally-achievable doses in healthy adults.

Measurement of blood markers of fatty acid intake also offers an opportunity to study the relationships between chronic intake of n-3 and n-6 PUFA and inflammation (299). The EPA+DHA content of the RBC membrane (termed the Omega-3 Index [O3I]) is a useful biomarker of dietary n-3 PUFA intake as it reflects intake averaged over the course of the RBC lifespan (\approx 120 d) and correlates with tissues and cells throughout the body (231, 251, 300). Associations between varying levels of RBC fatty acids and inflammatory markers have been examined in non-healthy populations (301-303); however, the relationship between RBC fatty acids and inflammatory markers in healthy adults has not been well-documented. Therefore,

examining the relationship between n-3 and n-6 PUFA content in RBC membranes and inflammatory marker concentrations may help elucidate the inflammatory effects associated with n-3 and n-6 PUFA intake.

Previously, we reported the effects of five doses of EPA+DHA on RBC fatty acids (304). The objective of the present study was to evaluate the dose-response effects of EPA+DHA on circulating markers of inflammation (TNF- α , IL-6, and CRP) in healthy adults, and secondly, to explore the relations between both the baseline and the post-supplementation changes in RBC content of n-3 and n-6 PUFA with circulating inflammatory marker concentrations. We also measured circulating white blood cell populations given *in vitro* and *ex vivo* evidence for the potential of n-3 PUFA to alter immune cell proliferation (305).

4.3. METHODS

4.3.1 Subject recruitment and screening

Healthy young adults (n = 125) between the ages of 20-45 y and with BMI between 20-30 kg/m² reporting no or low habitual oily fish consumption (<4 servings per month) and not taking n-3 PUFA supplements were recruited. Details of the study have been previously reported (304). The study was approved annually by the Pennsylvania State University Institutional Review Board.

4.3.2. Study design

Each participant was randomly assigned to take one of five doses (0, 300, 600, 900, 1,800 mg) of EPA+DHA daily as soybean oil placebo or fish oil supplements (Nordic Naturals, Watsonville, CA) for approximately 5 months (**Figure 4.1**). The fatty acids provided by the supplements in all regimens have been described in detail previously (304). The fish oil capsules contained approximately 20% EPA, 2% DPA, and 13% DHA. Participants agreed to maintain their weight, activity level, usual (limited) fish consumption, and not take any other n-3 PUFA supplements during the study. Daily log sheets and monthly check-ins were completed to verify compliance. Participants returned to the Pennsylvania State University Clinical Research Center every 8 weeks to receive new supplies, return empty containers, and provide completed log sheets.

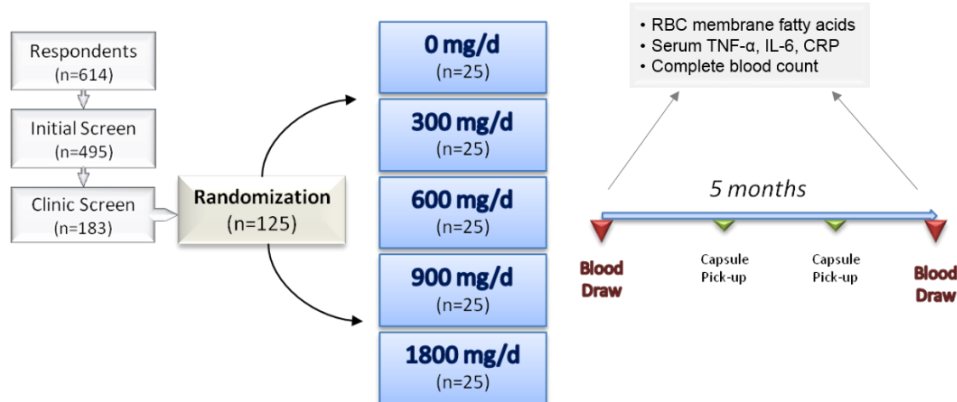


Figure 4.1. Study design and flow of subjects.

4.3.3 Blood sample collection

At the beginning of the study and after the treatment period, participants reported to the Clinical Research Center after a 12 hour overnight fast to provide a blood sample by venipuncture. Whole blood was centrifuged at 1500 x g for 15 min at 4 °C. Serum was collected and stored at -80 °C until they were analyzed, except for the complete blood count (CBC), which was measured from EDTA anticoagulated whole blood samples (Quest Diagnostics, Pittsburgh, PA). Red blood cells (RBC) were collected following separation from plasma by centrifugation and frozen at -80 °C until analyzed. Fatty acid analysis was performed as previously described (304).

4.3.4. Inflammatory marker concentrations

Serum concentrations of TNF- α and IL-6 were measured with high-sensitivity ELISA kits (R&D Systems, Minneapolis, MN) in duplicate (CV <10%). Serum high-sensitivity CRP was measured by latex-enhanced immunonephelometry (Quest Diagnostics; assay CV < 8%).

4.3.5. Statistical analysis

Statistical analyses were performed using Minitab (version 16.2, Minitab, State College, PA). Analytes that were assayed in duplicate (i.e., TNF- α and IL-6) were averaged before analysis. Fit statistics were assessed for each variable to identify any outliers and for normality. To ensure a healthy non-inflamed population, baseline and endpoint TNF- α and IL-6 values >3.0 ng/L as well as CRP values >3.0 mg/L were identified as outliers and removed from the

analysis (306, 307). A natural log transformation was applied to baseline and endpoint values of IL-6 and CRP because of non-normal distribution (skew > 1), and further analysis of associations and treatment effects were performed on the transformed values.

Independent two-sample t tests were used to assess sex differences in baseline inflammatory marker concentrations. Associations between baseline inflammatory marker concentrations and BMI, body weight, blood pressure, and age were assessed using Pearson correlation tests. The general linear model was used to test the effects of treatment on inflammatory marker concentrations and CBC measures. Baseline values were included as covariates. We present 3 models for the effect of supplementation on TNF- α , IL-6, and CRP concentrations: adjusted for baseline value (model 1), adjusted for baseline value, sex, and age (model 2), and adjusted for baseline value, sex, age, blood pressure, and body weight (model 3). Tukey-adjusted p-values were used for *post-hoc* comparisons between treatment groups, with adjusted $p < 0.05$ considered significant.

Baseline RBC membrane content of n-3 PUFA (alpha-linolenic acid [ALA], EPA, DPA, DHA) and n-6 PUFA (linoleic acid [LA], arachidonic acid [AA]) were assessed as quartiles and compared with circulating inflammatory marker concentrations using ANOVA. Tukey-adjusted p values were used for *post hoc* comparisons among quartiles. Changes in RBC membrane content were compared with changes in inflammatory marker concentrations using Pearson correlation tests. Change scores were calculated as the end of supplementation value minus baseline value. Scatterplots were generated to illustrate exploratory analyses of continuous relationships between baseline RBC PUFA content and inflammatory marker concentrations with Pearson correlation coefficients and unadjusted p-values reported for each comparison.

4.4. RESULTS

4.4.1 Participant characteristics

The study design and flow of participants have been reported previously (304) and summarized in **Figure 4.1**. Of the 125 participants, nine subjects withdrew from the study, leaving 116 participants who completed the study (mean compliance: 97%, range 85-100%). Two additional participants were removed from the analysis due to either having very high O3I at study entry (>8%) or underlying health condition (leukopenia). Elevated baseline TNF-

α (n=4), IL-6 (n=2), or CRP (n=12) data were excluded from the analysis in order to limit our analyses to healthy adults and remove statistical outliers. Remaining subjects with elevated endpoint TNF- α (n=0), IL-6 (n=3), or CRP (n=4) were excluded from the treatment effect and change score analyses.

There were no significant differences between the treatment groups at baseline with respect to n-3 and n-6 PUFA content of RBC membranes (304), CBC measures, and inflammatory marker concentrations (Table 4.1). The mean O3I at study entry (\pm SEM) was $4.3 \pm 0.1\%$, which is representative of Americans not taking n-3 PUFA supplements (253, 254, 308). Men had higher baseline TNF- α concentrations compared with women (1.5 ± 0.1 ng/L vs. 1.2 ± 0.1 ng/L, $p < 0.001$), whereas women had higher CRP than men (1.1 ± 0.1 mg/L vs. 0.6 ± 0.1 mg/L, $p = 0.001$). Individuals with higher baseline TNF- α also had higher body weight, diastolic blood pressure, and systolic blood pressure ($p < 0.001$ for all), whereas CRP was associated with higher BMI ($p = 0.009$) (Supplementary Table 1). There were no significant associations between age and baseline inflammatory marker concentrations.

Table 4.1. Baseline blood characteristics (n=114)¹

	EPA+DHA				
	0 mg/d (n=23)	300 mg/d (n=23)	600 mg/d (n=21)	900 mg/d (n=24)	1800 mg/d (n=23)
White blood cells, <i>cells/μL</i>					
Neutrophils	3080 \pm 230	3130 \pm 150	3380 \pm 270	3230 \pm 160	3240 \pm 170
Lymphocytes	1800 \pm 90	1910 \pm 120	1870 \pm 80	1940 \pm 100	1880 \pm 100
Monocytes	395 \pm 25	421 \pm 19	480 \pm 29	429 \pm 23	454 \pm 27
Eosinophils	133 \pm 16	154 \pm 26	168 \pm 29	168 \pm 30	158 \pm 26
Basophils	22.2 \pm 2.0	23.0 \pm 2.7	28.1 \pm 3.8	29.6 \pm 3.3	20.4 \pm 2.4
RBC count, $10^{12}/L$					
Hemoglobin, <i>g/L</i>	142 \pm 2.9	139 \pm 2.7	140 \pm 3.0	141 \pm 2.6	139 \pm 2.4
Hematocrit, %	42 \pm 0.8	41 \pm 0.8	41 \pm 0.9	42 \pm 0.7	41 \pm 0.7
MCV, <i>fL</i>	89 \pm 0.7	91 \pm 0.4	89 \pm 0.8	91 \pm 0.7	90 \pm 0.8
MCH, <i>pg/cell</i>	31 \pm 0.3	31 \pm 0.2	30 \pm 0.3	31 \pm 0.2	31 \pm 0.3
MCHC, <i>g/L</i>	342 \pm 1.2	342 \pm 1.2	339 \pm 1.4	339 \pm 1.0	339 \pm 1.0
RDW, %	13 \pm 0.2	13 \pm 0.1	13 \pm 0.2	13 \pm 0.1	13 \pm 0.1
Platelet count, $10^9/L$					
	217 \pm 8.7	227 \pm 10.5	232 \pm 11.0	229 \pm 11.5	212 \pm 8.0
Markers of Inflammation					
TNF- α^2 , ng/L	1.4 (1.1, 1.6)	1.4 (1.3, 1.5)	1.3 (1.1, 1.5)	1.4 (1.2, 1.5)	1.5 (1.2, 1.6)
IL-6 ³ , ng/L	0.9 (0.7, 1.1)	0.9 (0.7, 1.1)	1.0 (0.8, 1.2)	0.9 (0.7, 1.1)	1.0 (0.8, 1.2)
CRP ⁴ , mg/L	0.5 (0.3, 0.8)	0.6 (0.4, 1.1)	0.5 (0.3, 0.6)	0.6 (0.4, 1.0)	0.6 (0.4, 0.9)

Values are means \pm SEM. Groups were not significantly different for any of these metrics at baseline (p for group effect < 0.05). MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; RDW, RBC distribution width.

² N=110, means (95% CI)

³ N=112, geometric means (95% CI)

⁴ N=102, geometric means (95% CI)

4.4.2. Effect of supplementation on circulating inflammatory markers

Following supplementation, there were no significant differences between treatment groups with respect to IL-6 or CRP concentrations (**Table 4.2**); however, a marginally significant treatment effect was observed for TNF- α ($p < 0.08$). The 1,800 mg/d group, on average, experienced a 10% reduction in TNF- α concentrations from baseline, although no group was significantly different from placebo.

Table 4.2. Effects of EPA+DHA supplementation on inflammatory markers concentrations¹

Inflammatory marker	0 mg/d	300 mg/d	600 mg/d	900 mg/d	1800 mg/d	P value
TNF- α , ng/L ²						
<i>Model 1</i>	1.38 (1.28, 1.47)	1.38 (1.29, 1.47)	1.43 (1.33, 1.52)	1.41 (1.32, 1.49)	1.26 (1.17, 1.35)	0.07
<i>Model 2</i>	1.38 (1.29, 1.47)	1.39 (1.30, 1.48)	1.42 (1.33, 1.51)	1.41 (1.32, 1.49)	1.26 (1.17, 1.35)	0.08
<i>Model 3</i>	1.39 (1.30, 1.47)	1.39 (1.30, 1.48)	1.42 (1.33, 1.52)	1.41 (1.33, 1.50)	1.24 (1.14, 1.34)	0.04
IL-6, ng/L ³						
<i>Model 1</i>	0.97 (0.81, 1.15)	0.80 (0.67, 0.96)	1.03 (0.86, 1.24)	1.06 (0.89, 1.26)	0.98 (0.83, 1.17)	0.18
<i>Model 2</i>	0.97 (0.81, 1.15)	0.81 (0.67, 0.97)	1.03 (0.86, 1.23)	1.06 (0.89, 1.26)	0.98 (0.83, 1.17)	0.20
<i>Model 3</i>	0.98 (0.82, 1.16)	0.82 (0.68, 0.98)	1.03 (0.85, 1.23)	1.07 (0.90, 1.28)	0.95 (0.80, 1.13)	0.27
CRP, mg/L ⁴						
<i>Model 1</i>	0.52 (0.40, 0.67)	0.41 (0.31, 0.53)	0.54 (0.41, 0.70)	0.45 (0.33, 0.60)	0.42 (0.31, 0.55)	0.33
<i>Model 2</i>	0.52 (0.40, 0.68)	0.41 (0.31, 0.54)	0.54 (0.41, 0.71)	0.45 (0.33, 0.60)	0.41 (0.31, 0.54)	0.42
<i>Model 3</i>	0.52 (0.40, 0.68)	0.44 (0.33, 0.58)	0.54 (0.41, 0.70)	0.47 (0.35, 0.64)	0.36 (0.27, 0.48)	0.30

¹ Model 1 includes treatment adjusted for baseline value. Model 2 includes treatment adjusted for baseline value, sex, and age; Model 3 includes treatment adjusted for baseline value, sex, age, blood pressure, and body weight. P values are for the main effect of treatment. CRP, C-reactive protein.

² N=110, least squares mean (95% CI).

³ N=109, geometric means (95% CI).

⁴ N=98, geometric means (95% CI).

There were no significant treatment effects on CBC measures with the exception of lymphocyte concentrations (**Supplementary Table 2**). The 1,800 mg/d group had, on average, 17% higher lymphocyte concentrations following supplementation compared to the placebo (Tukey-adjusted $p = 0.02$; **Figure 4.2**). Both the 900 mg/d and 1,800 mg/d groups significantly increased lymphocyte concentrations from baseline ($p < 0.01$).

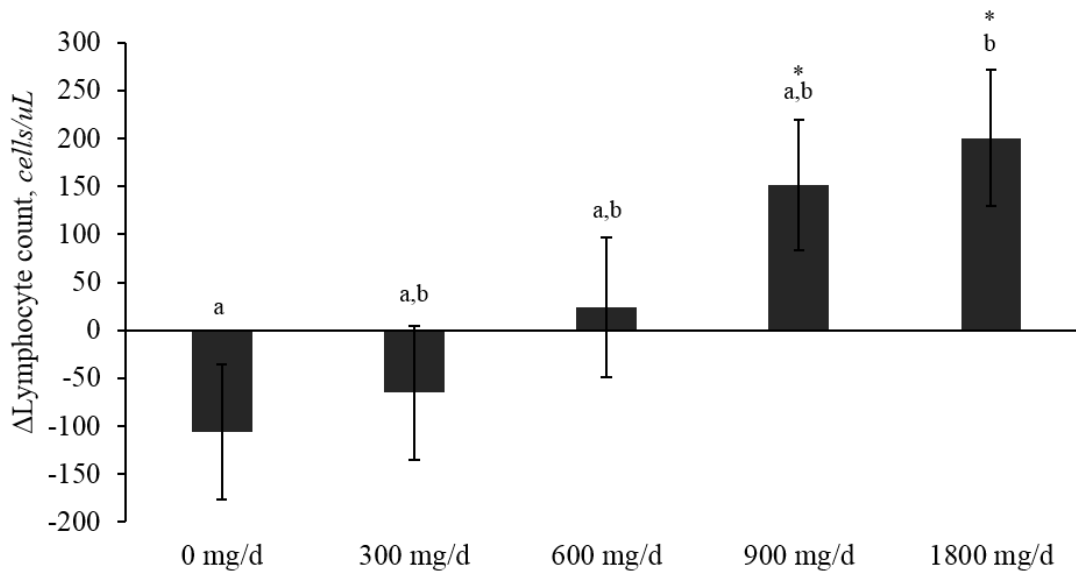


Figure 4.2. Changes in lymphocyte concentrations following 5 months of EPA+DHA supplementation. Values are means \pm SEM (n=114). Values with different letters are significantly different, $p < 0.05$ (Tukey-adjusted values for post hoc tests). Asterisk indicates group is significantly different from baseline ($p < 0.01$).

4.4.3. Associations between RBC content of PUFA and circulating inflammatory markers

Baseline inflammatory marker concentrations were compared between quartiles of n-3 and n-6 PUFA content in RBC membranes (Table 4.3). There were no significant differences in any inflammatory marker concentrations across quartiles of ALA or EPA content. Higher quartiles of DHA content were associated with lower TNF- α concentrations ($p=0.001$), whereas higher quartiles of AA content were associated with higher TNF- α concentrations ($p=0.005$) (Table 4.3; Figure 4.3). Participants in the highest quartile of AA content (quartile 4) also had, on average, 29% higher IL-6 concentrations compared with participants in the lowest quartile of AA content (quartile 1) (Table 4.3; Figure 4.4). In contrast, participants in the highest quartile of LA content had, on average, 29% lower IL-6 concentrations compared with participants in the lowest quartile of LA content (Table 4.3; Figure 4.4). Furthermore, participants in the lowest quartile of DPA content had significantly higher CRP concentrations compared with participants in all other quartiles of DPA content ($p < 0.001$).

Table 4.3 Inflammatory marker concentrations according to quartiles of n-3 and n-6 PUFA content in RBC membranes¹

	Quartiles of RBC fatty acid content				P value
	Q1	Q2	Q3	Q4	
ALA, 18:3 n-3					
TNF- α ² , ng/L	1.54 (1.37, 1.70)	1.38 (1.22, 1.55)	1.29 (1.12, 1.45)	1.33 (1.16, 1.49)	0.14
IL-6 ³ , ng/L	1.01 (0.84, 1.22)	0.99 (0.86, 1.13)	0.83 (0.67, 1.04)	0.94 (0.78, 1.12)	0.74
CRP ⁴ , mg/L	0.43 (0.31, 0.60)	0.52 (0.39, 0.70)	0.60 (0.42, 0.87)	0.73 (0.50, 1.07)	0.15
EPA, 20:5 n-3					
TNF- α , ng/L	1.47 (1.31, 1.63)	1.32 (1.20, 1.44)	1.46 (1.30, 1.62)	1.27 (1.15, 1.39)	0.21
IL-6, ng/L	0.97 (0.79, 1.19)	0.94 (0.79, 1.12)	0.92 (0.77, 1.10)	0.93 (0.77, 1.11)	0.98
CRP, mg/L	0.66 (0.44, 0.99)	0.46 (0.35, 0.61)	0.67 (0.48, 0.93)	0.49 (0.34, 0.69)	0.26
DPA, 22:5 n-3					
TNF- α , ng/L	1.37 (1.20, 1.54)	1.32 (1.16, 1.49)	1.36 (1.20, 1.53)	1.47 (1.30, 1.64)	0.60
IL-6, ng/L	1.03 (0.83, 1.28)	0.85 (0.71, 1.01)	0.95 (0.80, 1.13)	0.94 (0.79, 1.11)	0.50
CRP, mg/L	1.05 (0.73, 1.53) ^a	0.40 (0.29, 0.55) ^b	0.43 (0.33, 0.57) ^b	0.53 (0.41, 0.71) ^b	<0.001
DHA, 22:6 n-3					
TNF- α , ng/L	1.55 (1.39, 1.71) ^a	1.48 (1.33, 1.63) ^a	1.36 (1.21, 1.51) ^{ab}	1.13 (0.97, 1.29) ^b	0.001
IL-6, ng/L	0.91 (0.75, 1.09)	1.05 (0.89, 1.23)	0.97 (0.80, 1.18)	0.84 (0.69, 1.02)	0.34
CRP, mg/L	0.55 (0.40, 0.75)	0.57 (0.40, 0.81)	0.54 (0.38, 0.75)	0.59 (0.39, 0.89)	0.98
Omega-3 index (EPA+DHA)					
TNF- α , ng/L	1.56 (1.40, 1.72) ^a	1.49 (1.34, 1.65) ^a	1.33 (1.18, 1.49) ^{ab}	1.13 (0.98, 1.29) ^b	0.001
IL-6, ng/L	0.92 (0.76, 1.10)	0.99 (0.82, 1.19)	1.02 (0.84, 1.22)	0.84 (0.70, 1.01)	0.46
CRP, mg/L	0.52 (0.37, 0.74)	0.68 (0.48, 0.96)	0.55 (0.39, 0.77)	0.51 (0.36, 0.72)	0.62
Linoleic acid, 18:2 n-6					
TNF- α , ng/L	1.46 (1.29, 1.64)	1.45 (1.32, 1.58)	1.41 (1.27, 1.56)	1.20 (1.09, 1.32)	0.08
IL-6, ng/L	1.03 (0.86, 1.24) ^a	1.01 (0.84, 1.22) ^a	1.02 (0.85, 1.23) ^a	0.73 (0.63, 0.84) ^b	0.014
CRP, mg/L	0.55 (0.38, 0.80)	0.67 (0.47, 0.94)	0.55 (0.39, 0.78)	0.48 (0.35, 0.67)	0.60
Arachidonic acid, 20:4 n-6					
TNF- α , ng/L	1.18 (1.00, 1.33) ^a	1.36 (1.20, 1.52) ^{ab}	1.39 (1.25, 1.53) ^{ab}	1.59 (1.41, 1.77) ^b	0.005
IL-6, ng/L	0.79 (0.66, 0.94) ^a	0.97 (0.80, 1.17) ^{ab}	0.91 (0.75, 1.10) ^{ab}	1.11 (0.95, 1.31) ^b	0.06
CRP, mg/L	0.55 (0.39, 0.78)	0.57 (0.40, 0.81)	0.56 (0.40, 0.79)	0.56 (0.38, 0.81)	0.99

¹ Quartiles are specific for the content of each fatty acid in RBC membranes. P values are for trend across quartiles calculated using ANOVA. Values with different superscript letters are significantly different, $p < 0.05$ (Tukey-adjusted values for post hoc tests). ALA, alpha-linolenic acid; CRP, C-reactive protein; DPA, docosapentaenoic acid; Q, quartile.

² n=110, least squares means (95% CI).

³ n=112, geometric means (95% CI).

⁴ n=102, geometric means (95% CI).

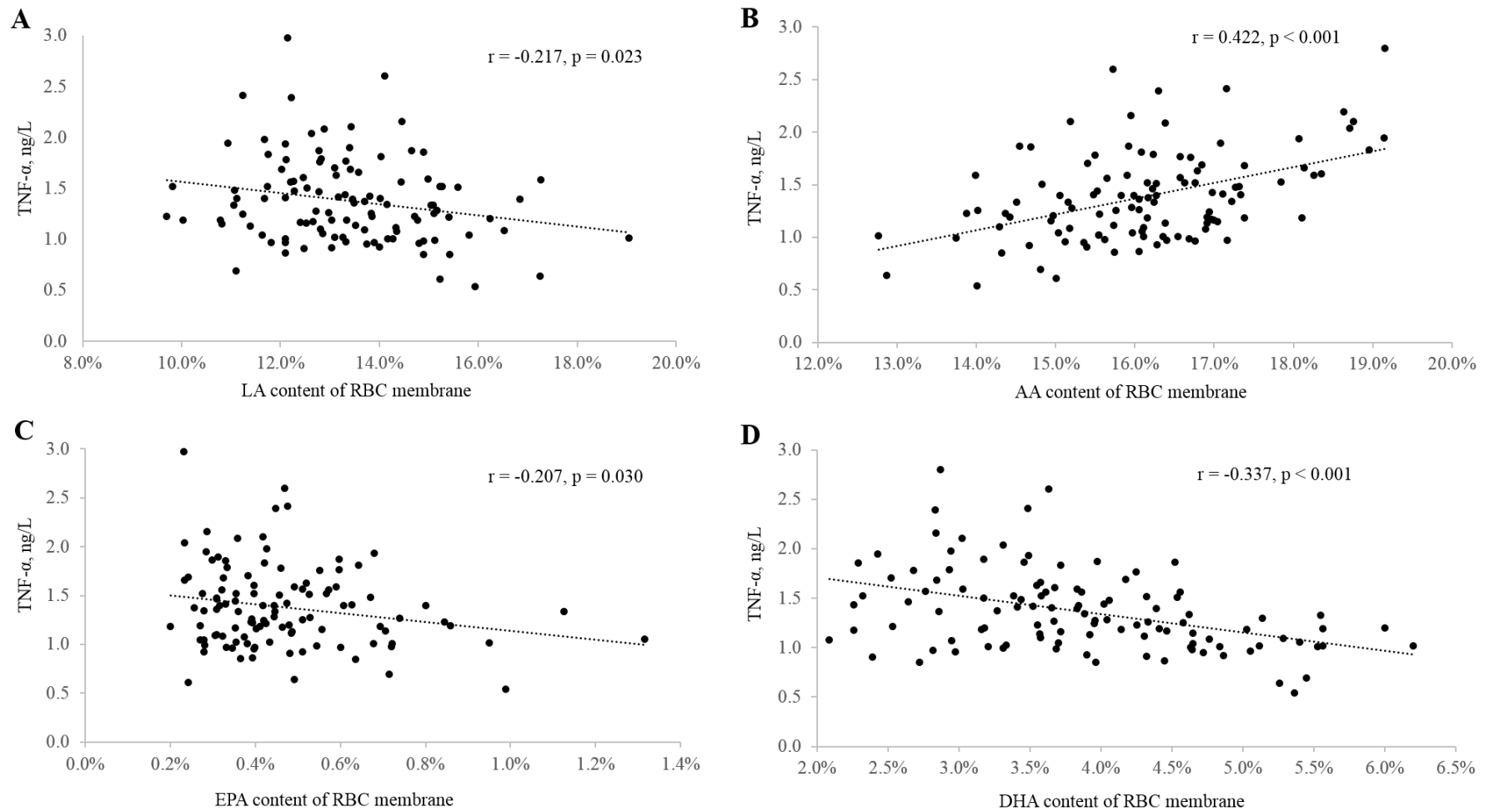


Figure 4.3. Scatterplots for baseline RBC membrane content of LA (*A*), AA (*B*), EPA (*C*), and DHA (*D*) versus circulating TNF- α concentrations in healthy adults (n=110). Pearson correlation coefficients and unadjusted p-values are reported for each comparison. AA, arachidonic acid; LA, linoleic acid.

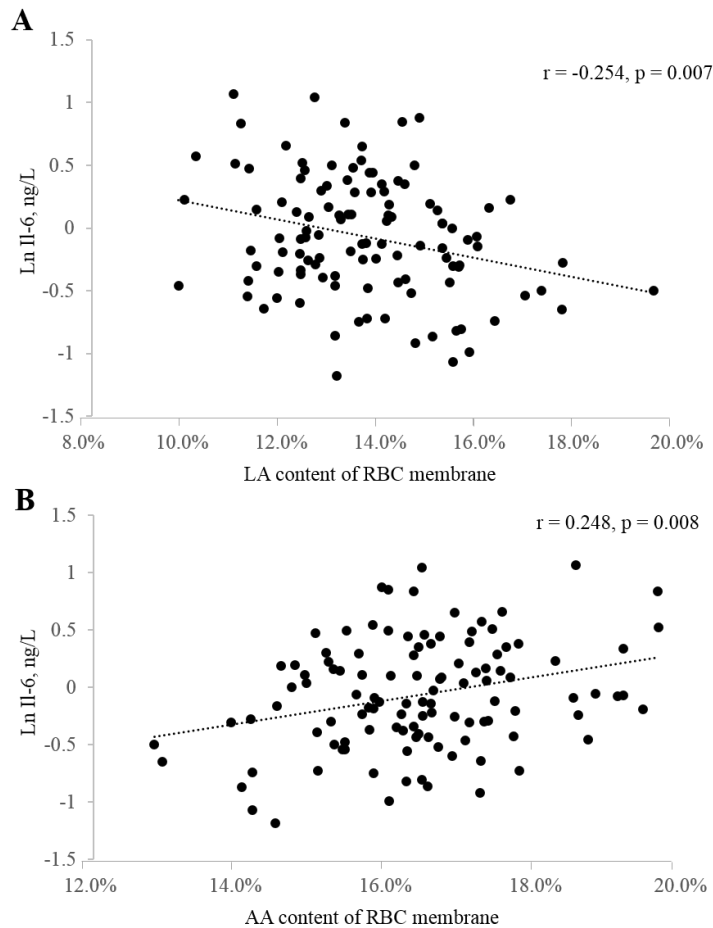


Figure 4.4. Scatterplots for baseline RBC membrane content of LA (*A*) and AA (*B*) versus circulating IL-6 concentrations in healthy adults (n=112). Pearson correlation coefficients and unadjusted p-values are reported for each comparison. AA, arachidonic acid; LA, linoleic acid.

Changes in inflammatory marker concentrations were compared with changes in n-3 and n-6 PUFA content in RBC membranes following supplementation (**Table 4.4**). No significant associations between the change in inflammatory marker concentrations versus change in n-3 or n-6 PUFA content were observed. However, changes in lymphocyte concentrations were positively associated with changes in EPA and DPA content following supplementation (p=0.001 and p=0.008, respectively; **Supplementary Figure 1**).

Table 4.4. Pearson correlations of change in RBC PUFA content with change in inflammatory marker¹

Fatty Acid	Δ TNF ²		Δ IL6 ³		Δ CRP ⁴	
	r	p value	r	p value	r	p value
Δ ALA, 18:3 n-3	0.14	0.15	-0.02	0.84	0.02	0.85
Δ EPA, 20:5 n-3	-0.12	0.23	-0.01	0.96	-0.05	0.62
Δ DPA, 22:5 n-3	-0.10	0.29	-0.03	0.74	-0.08	0.44
Δ DHA, 22:6 n-3	-0.11	0.24	-0.07	0.47	0.00	0.99
Δ Omega-3 Index	-0.12	0.21	-0.05	0.61	-0.02	0.85
Δ LA, 18:2 n-6	0.00	0.97	0.01	0.93	-0.04	0.70
Δ AA, 20:4 n-6	0.11	0.27	0.04	0.72	0.03	0.80

¹Pearson correlation coefficients with unadjusted p-values. AA, arachidonic acid; ALA, alpha-linolenic acid; CRP, C-reactive protein; DPA, docosapentaenoic acid; LA, linoleic acid.

²N = 110

³N = 109

⁴N = 98

4.5. DISCUSSION

The primary objective of this study was to determine the effect of supplemental EPA+DHA intake (from fish oil supplements), across a range of nutritionally-achievable doses, on circulating inflammatory marker concentrations in healthy adults after 5 months of supplementation. There were no significant effects of EPA+DHA intake on IL-6 or CRP concentrations; however, a marginally significant treatment effects was observed for TNF- α . Prior clinical research has been inconsistent, although a few studies utilizing high doses of EPA+DHA (> 2 g/d) have shown limited potential for anti-inflammatory effects in non-healthy populations (180, 191, 302, 309-313). Fish oil supplementation studies of healthy adults rarely find beneficial effects of EPA+DHA intake on inflammatory marker concentrations (182, 296-298), potentially due in part to low baseline inflammatory marker concentrations and absence of modifiable elevations in inflammation. Our results suggest that even in healthy adults, 1,800 mg/d EPA+DHA may exert a modest anti-inflammatory effect over the course of 5 months supplementation as evident by a marginally significant decrease in TNF- α concentrations. Further evidence in support of this modest effect is provided by the associations between RBC content of n-3 PUFA content and circulating TNF- α concentrations at baseline.

The anti-inflammatory effects of n-3 PUFA can be attributed, in part, to the incorporation of n-3 PUFA into membrane phospholipids and subsequent alterations in cell

signaling and lipid mediator production (305). Thus, we examined the relationship between RBC content of n-3 and n-6 PUFA and inflammatory marker concentrations before and after supplementation. The change in n-3 and n-6 PUFA content of RBC was not related to changes in TNF- α , IL-6, or CRP concentrations following supplementation. This analysis is limited by negligible treatment effects on inflammatory markers following 5 months supplementation. However, it may suggest that RBC fatty acids, although an important biomarker, may not be the direct pool for inflammatory regulation (i.e. RBC fatty acids are not serving as substrate pools for bioactive lipid production and/or directly modulating inflammatory gene expression).

There were, however, several baseline associations between RBC content of n-3 PUFA and inflammatory markers that support the modest anti-inflammatory effect of increased dietary n-3 PUFA intake over years, especially in people with very low habitual n-3 intake. We found that higher RBC content of DHA was associated with lower TNF- α at baseline, while individuals with the lowest content of DPA had the highest CRP at baseline. Whether low DHA and/or DPA content is a cause or effect of elevated inflammatory marker concentrations remains uncertain.

Dietary sources of DPA include seafood and meat products (primarily from ruminant animals) (314, 315); however, DPA is predominately derived from endogenous elongation of EPA and/or ALA (57, 221, 316). In humans, DPA can be retroconverted to EPA, suggesting that DPA serves as a storage pool for EPA (57, 317). Therefore, it is possible that the low DPA content association with slightly higher CRP may be due to increased EPA utilization, thereby promoting DPA to EPA conversion in order to replenish EPA stores. A case-control study by Sun et al. (57) reported that plasma DPA, but not EPA or DHA, was inversely associated with IL-6 concentrations in adult females (mean age 60 years); neither EPA, DPA, nor DHA was associated with CRP concentrations (57). In contrast, Reinders et al. (318) found that serum DPA and DHA, but not EPA, were each associated with lower CRP in healthy Finnish men (mean age 52 years). The authors noted that concentrations of DPA in serum are lower compared to EPA or DHA and likely contributed to the large regression coefficient with CRP (318). In contrast, RBC content of DPA is typically greater than EPA and less than DHA content (254, 258, 263, 304). We found that RBC content of DPA, but not EPA or DHA, was inversely associated with CRP concentrations in healthy adults (mean age 26 years), whereas neither EPA, DPA, nor DHA was associated with IL-6 concentrations.

Differences in n-3 PUFA measurements (plasma vs. RBC fatty acids) as well as subject population (e.g., age, sex, BMI, etc.) explain in part the contrasting associations reported for DPA and inflammatory marker concentrations. Race-specific genetic differences also may explain inconsistencies across studies. A recent prospective multiethnic study of U.S. adults (mean age 61.5 years) found that plasma phospholipid content of DPA was inversely associated with CVD incidence in whites and Chinese, but not in African-Americans or Hispanics (319); EPA and DHA concentrations were each inversely associated with CVD in whites, but not in Chinese, African-Americans, or Hispanics in the fully adjusted model (319). Therefore, the relationship between DPA and inflammation also may be influenced by genetic differences in n-3 PUFA metabolism.

The association between n-6 PUFA and inflammatory marker concentrations also varied depending upon the specific fatty acid. Individuals with the highest RBC content of AA had higher TNF- α and IL-6 concentrations at baseline compared to those with lower AA content. This may reflect the ability of AA to generate eicosanoids that can promote pro-inflammatory cytokine production (288). In contrast, individuals with the highest LA content had lower IL-6 concentrations compared to those with the lowest LA content. Upon further investigation, we found that baseline LA and AA content were significantly inversely correlated (**Supplementary Figure 2**). This is particularly interesting since it has been proposed that dietary intake of LA is pro-inflammatory, and thus increases CVD risk, largely based on LA being a precursor of AA (320, 321). The ability of AA to modulate inflammation via the generation of eicosanoids has been well-documented (288), however, the potential for LA to influence inflammation is less certain (322). These observations underscore limitations with simply focusing on ratios of total n-6 PUFA vs. total n-3 PUFA since we report unique relationships between individual fatty acids that do not support equivalent effects (e.g. RBC LA content was inversely associated with plasma IL-6). Additional studies examining differences between LA and AA in modulating inflammation are needed.

There were several interesting relationships between baseline inflammatory marker concentrations and participant characteristics. Men had higher TNF- α concentrations compared with women, whereas women had higher CRP than men. These findings are in agreement with previous publications (323-329). Women tend to weigh less than men; however, women on average still had lower TNF- α and higher CRP concentrations than men

after accounting for body weight or BMI (data not shown). Sex-specific differences in adiposity and body fat distribution likely account for these contrasting results (326, 330). Women generally have a higher body fat percentage than men and store more fat in the femoral-gluteal region (lower body), whereas men generally store fat in the abdominal region as part of the visceral depot (326, 331). Sex hormones, particularly estrogen, also may account for these sex differences (326, 332, 333). We did not include measures of body composition or sex hormone concentrations in the present study, although we did find that increased body weight was associated with higher TNF- α and increased BMI was associated with higher CRP. Therefore, sex-specific differences in TNF- α and CRP concentrations may be due to a combination of adiposity, body fat distribution, and sex hormones.

We also found that increased diastolic and systolic blood pressures were both associated with higher TNF- α . Several studies have demonstrated an association between elevated TNF- α and hypertension (334-339). TNF- α has been shown to alter kidney hemodynamics and mediate both increases and decreases in blood pressure, as reviewed by Ramseyer et al. (340). The mechanism by which TNF- α influences hypertension, however, is uncertain and the question remains as to whether increased TNF- α is a cause or effect of hypertension.

Unexpectedly, our study found that 1,800 mg/d EPA+DHA increased circulating lymphocyte concentrations in healthy adults relative to the placebo group. We also found that increasing n-3 PUFA content in the RBC membrane was associated with increasing lymphocyte concentrations following supplementation. Weaver et al. (341) also observed an increase in the number of circulating lymphocytes in healthy adults (n=27) after 4 weeks of supplementation with fish oil (860 mg/d EPA+DHA) and borage oil (831 mg/d gamma-LA). The mechanisms for this effect of EPA+DHA supplementation are unclear. Peripheral blood lymphocytes pretreated with n-3 PUFA have been shown to dose-dependently reduce the cell surface expression of L-selectin, which facilitates lymphocyte adhesion to endothelial cells as well as migration of lymphocytes to lymphoid tissues and sites of inflammation (342). Moreover, clinical evidence indicate that EPA+DHA supplementation selectively reduces plasma soluble intercellular adhesion molecule-1 concentrations in healthy adults ≤ 55 years (194). Therefore, the observed increase in circulating lymphocyte concentrations at increasing

EPA+DHA doses may be explained in part by reduced expression of specific cellular adhesion molecules, and thus a greater proportion of lymphocytes freely circulating than adhered to the endothelium. Another possible explanation for the increase in lymphocyte concentrations could be the effects of lipid mediators formed from n-3 PUFA (e.g., prostaglandins and leukotrienes), which have been shown to influence hematopoietic stem cell or myeloid progenitor cell differentiation (343). We did not differentiate between types of lymphocytes and thus are unable to determine whether the observed increase in circulating lymphocytes can be attributed to a particular cell type (i.e. T cell, B cell, NK cell). More clinical research is needed to assess the effects of supplemental n-3 PUFA intake on circulating white blood cell and cellular adhesion molecule concentrations.

4.5.1. Strengths and limitations

The present study had a number of strengths, including the placebo-controlled, double-blind study design comparing five treatment groups, large sample size, well-characterized study cohort, relatively longer duration of supplementation (5 months), and use of a validated biomarker of cell membrane fatty acid content. The study population had a baseline O3I representative of the average American not taking n-3 PUFA supplements. We instructed participants to reschedule visits if they were experiencing or recovering from any acute illness or injury and secondarily excluded from analysis elevated inflammatory markers. This analytical approach eliminated highly influential outliers from our analysis and increased the sensitivity of models to detect effects in our target population of healthy adults. Moreover, the study also had a dropout rate of 7%, which is relatively low considering the duration of the study intervention.

The predominantly Caucasian, young, healthy participants as well as the absence of dietary assessment are limitations. Population subgroups may have unique responses to supplemental EPA+DHA intake, thus, future studies should consider identifying racial differences as well as genetic determinants of n-3 PUFA metabolism. Although participants were instructed to limit their intake of n-3 PUFA from food, specifically fatty fish, and continue their habitual diets throughout the duration of the intervention, we did not have data on the dietary habits and thus intake of fatty acids from food, as well as non-fatty acid components, could have confounded our findings.

Lastly, TNF- α , IL-6, and CRP are well-established circulating inflammatory markers, yet provide little information regarding the origin of the underlying inflammation. Consequently, we are unable to determine whether the relationship between n-3 and n-6 PUFA content of RBC membranes and these inflammatory markers reflect events originating in certain tissues (e.g. liver, adipose tissue, etc.). Omega-3 PUFA supplementation also may affect serum cytokine concentrations not measured in the present study (e.g., IL-2, IL-8, IL-10). Measuring tissue-specific inflammatory markers (e.g. resistin) as well as serum markers beyond TNF- α , IL-6, and CRP would provide greater insight into the potential role of n-3 and n-6 PUFA in regulating inflammation.

4.5.2. Conclusions

Nutritionally-achievable doses of EPA+DHA over a period of 5 months had no effect on circulating IL-6 or CRP concentrations in healthy adults; however, 1,800 mg/d EPA+DHA may exert a modest anti-inflammatory effect as evident by a marginally significant reduction in TNF- α . Additional markers of inflammation, including cellular adhesion molecules, need to be examined to more fully understand the clinical effects of supplemental EPA+DHA intake on inflammation as well as to clarify the observed increase in lymphocyte concentrations following 1,800 mg/d EPA+DHA. We also found that increased RBC content of DHA was associated with lower TNF- α at baseline, whereas low DPA content was associated with higher CRP. In contrast, changes in RBC membrane content of n-3 or n-6 PUFA were not associated with changes in inflammatory marker concentrations following EPA+DHA supplementation, indicating a need for longer term studies and exploration of other biomarkers of fatty acid content (e.g., white blood cells) that may be more directly involved in inflammatory regulation. Collectively, our findings indicate that EPA+DHA intake, across a range of nutritionally-achievable doses, has no dose-response effect on circulating TNF- α , IL-6, or CRP in healthy adults after 5 months of supplementation. However, despite no relationship between changes in RBC PUFA content and inflammatory markers, observed baseline associations warrant further investigation.

4.6. SUPPLEMENTARY MATERIAL

Table 4.5 (Supplementary Table 1) Pearson correlation coefficients of participant characteristics with baseline inflammatory marker concentrations ¹

Variable	TNF- α ²		IL-6 ³		CRP ⁴	
	r	p value	r	p value	r	p value
Age	-0.09	0.35	0.16	0.10	0.18	0.07
BMI	0.17	0.08	0.16	0.09	0.26	0.009
Body Weight	0.34	<0.001	0.10	0.27	0.01	0.90
SBP	0.34	<0.001	-0.05	0.59	-0.04	0.68
DBP	0.33	0.001	-0.01	0.89	-0.03	0.74

¹Pearson correlation coefficients with unadjusted p-values.

²N = 110

³N=112, log transformed

⁴N=102, log transformed

Table 4.6 (Supplementary Table 2). Effect of EPA+DHA supplementation on complete blood count measures (n=114)¹

	EPA+DHA					p value
	0 mg/d	300 mg/d	600 mg/d	900 mg/d	1800 mg/d	
White blood cells, <i>cells/uL</i>						
Neutrophils	2820 ± 160	2760 ± 160	2780 ± 170	3060 ± 160	2900 ± 170	0.68
Lymphocytes	1780 ± 70 ^a	1820 ± 70 ^{ab}	1910 ± 73 ^{ab}	2040 ± 70 ^{ab,3}	2080 ± 100 ^{b,2}	0.009
Monocytes	368 ± 18	384 ± 18	399 ± 19	374 ± 17	410 ± 18	0.47
Eosinophils	138 ± 13	169 ± 13	140 ± 13	125 ± 13	134 ± 13	0.16
Basophils	24.8 ± 2.3	26.3 ± 2.3	23.5 ± 2.4	28.2 ± 2.2	26.6 ± 2.3	0.66
RBC Count, $10^{12}/L$	4.5 ± 0.1	4.5 ± 0.1	4.5 ± 0.1	4.5 ± 0.1	4.3 ± 0.1	0.34
Hemoglobin, <i>g/L</i>	138 ± 1.4	138 ± 1.4	139 ± 1.5	138 ± 1.4	137 ± 1.4	0.85
Hematocrit, %	41 ± 0.5	41 ± 0.5	41 ± 0.5	41 ± 0.4	40 ± 0.5	0.58
MCV, <i>fL</i>	91 ± 0.3	91 ± 0.3	91 ± 0.4	91 ± 0.3	90 ± 0.3	0.21
MCH, <i>pg/cell</i>	31 ± 0.1	31 ± 0.1	31 ± 0.1	31 ± 0.1	31 ± 0.1	0.61
MCHC, <i>g/L</i>	341 ± 2.2	339 ± 2.3	336 ± 2.2	339 ± 2.2	341 ± 2.3	0.52
RDW, %	13 ± 0.1	13 ± 0.1	13 ± 0.2	13 ± 0.1	13 ± 0.1	0.74
Platelet Count, $10^9/L$	218 ± 6.8	220 ± 6.6	207 ± 6.9	213 ± 6.5	222 ± 6.8	0.57

¹All values are least-square mean +/- SEM. P values are for the main effect of treatment. Baseline values included as a covariate. Values with different subscript letters are significantly different, p<0.05 (Tukey-adjusted values for post hoc tests). MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; RDW, red blood cell width.

²Significantly different compared to the baseline value (Bonferroni-adjusted, p<0.01).

4.6. SUPPLEMENTARY MATERIAL

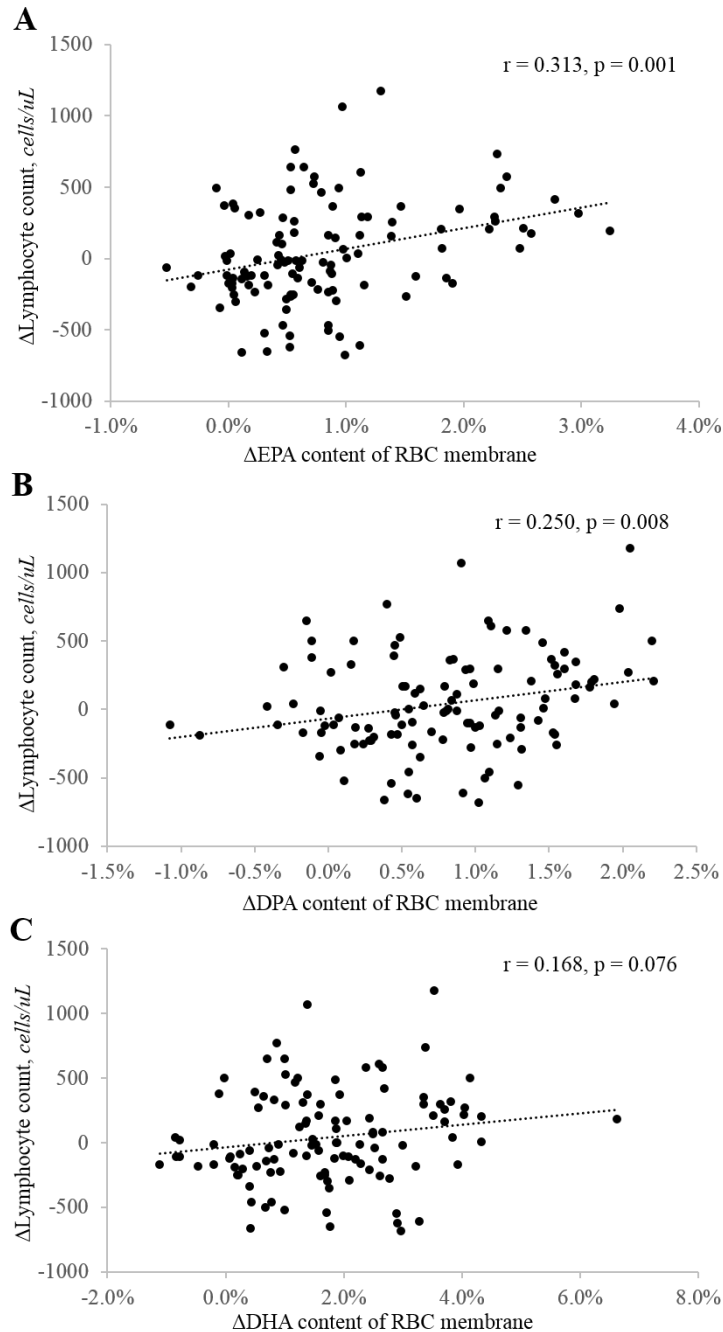


Figure 4.5 (Supplementary Figure 1). Scatterplots for change RBC membrane content of EPA (A), DPA(B), and DHA (C) versus change in lymphocyte concentrations following fish oil supplementation in healthy adults ($n=114$). Pearson correlation coefficients and unadjusted p-values are reported for each comparison. DPA, docosapentaenoic acid.

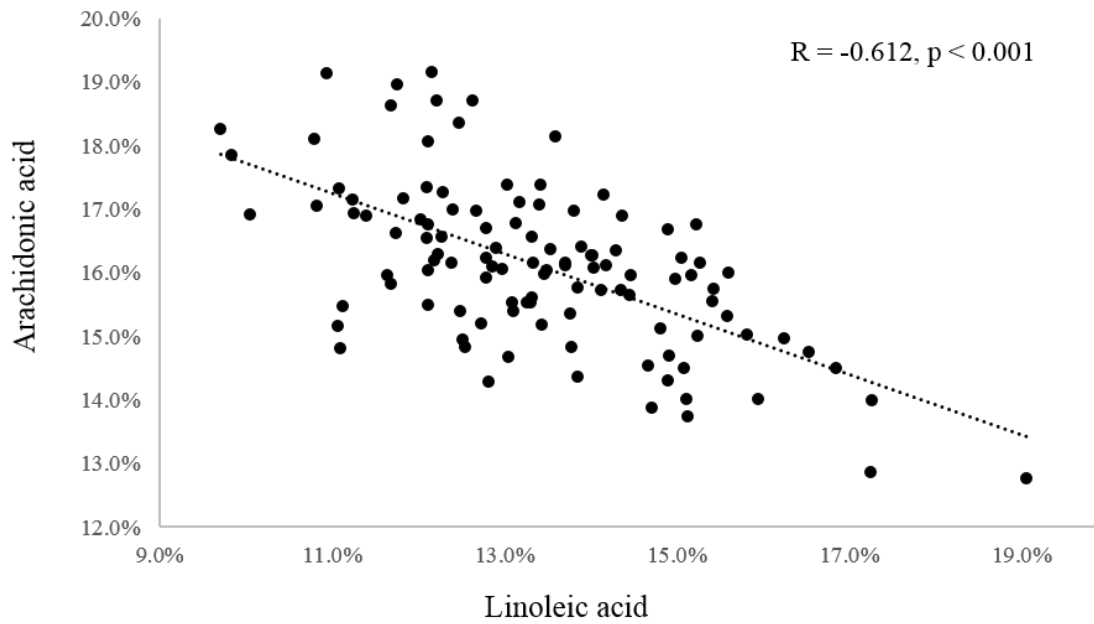


Figure 4.6 (Supplementary Figure 2). Baseline linoleic acid (18:2, omega-6) content in RBC membranes inversely correlates with baseline arachidonic acid (20:4, omega-6) content.

CHAPTER V:

CHANGES IN PERIPHERAL BLOOD MONONUCLEAR CELL GENE EXPRESSION FOLLOWING *IN VIVO* ENDOTOXIN CHALLENGE IN HEALTHY ADULT MALES

5.1 ABSTRACT:

Background: Administration of lipopolysaccharide (LPS) in humans results in the activation of peripheral blood mononuclear cells (PBMC) via toll-like receptor 4 (TLR4) signaling, initiating the release of inflammatory mediators in systemic circulation. Long-chain omega-3 (n-3) polyunsaturated fatty acids (PUFA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), have potent anti-inflammatory and pro-resolving effects.

Objective: To evaluate changes in gene expression following a low-dose *in vivo* LPS challenge in healthy adult men, and secondly, to explore the relationship between subject characteristics, red blood cell (RBC) PUFA content, and gene expression.

Design: Eight healthy adult males between the ages of 20-45 years with different RBC membrane content of EPA and DHA were injected with sterile protein-free LPS (0.6 ng/kg body weight). Total RNA was extracted from PBMC samples collected at 0, 2, 4, and 24 hours post-LPS injection. Real-time qPCR analysis was performed to assess changes in 14 target genes involved in the TLR4 pathway.

Results: Low-dose LPS challenge stimulated an inflammatory response in healthy adult men as indicated by increased PBMC expression of IL-1 β , TNF- α , and IL-6 at 2 hours (p<0.05). Expression of anti-inflammatory genes, IL-10 and PPAR γ , increased at 4 hours (p<0.05). Baseline serum albumin/globulin (A/G) was inversely associated with pro-inflammatory gene expression at 2 hours (p<0.01). RBC content of docosapentaenoic acid (DPA, 22:5 n-3), but not EPA or DHA, was associated with increased IL-1 β expression at 4 hours as well as larger reductions in IL-1 β expression between the 4 and 24 hour time points (p<0.01 for both).

Conclusion: These data offer insight into the progression of the inflammatory response in addition to identifying factors that may influence innate immune responses.

5.2 INTRODUCTION

Inflammation is initiated by the immune system in response to injury, irritation or infection; however, prolonged or chronic inflammation is involved in the etiology of numerous acute and chronic diseases. Studies of inflammation are often confounded by the absence of well-defined onset time of inflammation as well as differences in the duration of disease and inter-individual differences in the sensitivity and immune response to various types of stimuli (238). Intravenous administration of purified lipopolysaccharide (LPS) from *Escherichia coli* (gram-negative bacteria) even at a low dose (0.6 ng/kg body weight) elevates circulating concentrations of inflammatory cytokines via activation of toll-like receptor (TLR)-4 pathway and mimics inflammation that is observed in initial host responses to infection (**Figure 5.1**) (238, 240). Moreover, acute changes observed during experimental endotoxin challenges resemble those observed chronically in obesity, insulin resistance, and atherosclerosis (241-243). Therefore, the endotoxemia model is useful in furthering our understanding of the human response to inflammation as well as identifying therapeutic agents that may ameliorate inflammatory conditions.

Accumulating evidence indicates that long-chain omega-3 polyunsaturated fatty acids (n-3 PUFA), namely eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), have potent biological activities, including anti-inflammatory and pro-resolving effects (139). Incorporation of EPA and DHA into the membrane phospholipids of various cells can modulate signaling events and alter a variety of metabolic activities (139). Increasing cell membrane content of EPA and DHA reduces the production of arachidonic acid (AA)-derived eicosanoids in favor of synthesizing eicosanoids with more anti-inflammatory properties (15). Serhan et al. (149) demonstrated that increasing cellular uptake of EPA and DHA enhances the production of resolvins and protectins, which are novel lipid mediators proposed to reduce and resolve inflammatory responses (139). EPA and DHA also have been shown to interfere with LPS induced cyclooxygenase-2 (COX-2) expression by altering the composition of lipid rafts and thereby modulating TLR-mediated signaling (140, 141).

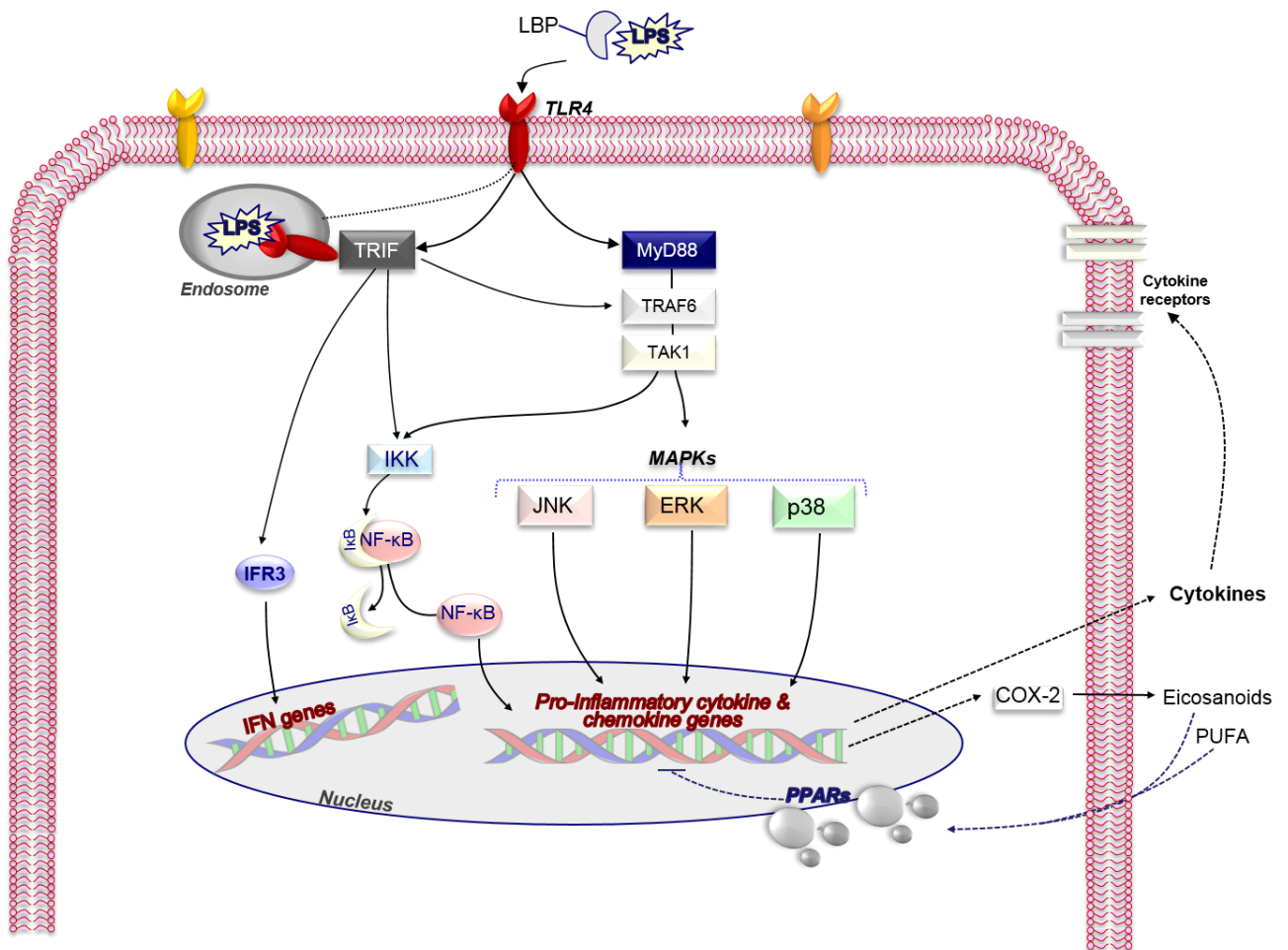


Figure 5.1 Stimulation of Toll-like receptor 4 (TLR4) signaling pathway by lipopolysaccharide (LPS). LPS recognition by immune cells (e.g., macrophages) is facilitated by LPS-binding protein (LBP). LPS/TLR4 signaling can be separated into myeloid differentiation primary response gene (MyD88)-dependent and MyD88-independent pathways. Upon initial LPS stimulation, MyD88 recruits and activates adaptor proteins, including TNF receptor associated factor 6 (TRAF6), which activates transforming growth factor- β -activated kinase 1 (TAK1). TAK1 then activates downstream mitogen-activated protein kinase (MAPK) pathways as well as inhibitor of nuclear factor kappa-B (I κ B) kinase (IKK). Activation of MAPK pathways leads to induction of transcription factors responsible for increasing expression of pro-inflammatory cytokines. Activated IKK phosphorylates I κ B, leading to degradation of I κ B and translocation of NF- κ B to the nucleus where it also increases expression of pro-inflammatory cytokines. Increased pro-inflammatory cytokine production can then stimulate additional inflammatory pathways via cytokine receptors in a feed-forward manner. The LPS/TLR4 complex is eventually internalized and retained in the endosome, where TLR adaptor molecule, toll/IL-1R domain containing adaptor inducing interferon (TRIF) facilitates MyD88-independent signaling, thereby activating interferon regulatory factor 3 (IRF3) and late-phase NF- κ B for the induction of interferons (IFN) and additional cytokine genes. LPS-induced TLR4 signaling also induces expression and activity of Cyclooxygenase-2 (COX-2), thereby promoting synthesis of eicosanoids and lipid mediators. Certain eicosanoids and long-chain polyunsaturated fatty acids (PUFA) act as ligands for peroxisome proliferator-activated receptors (PPARs), a group of nuclear receptors that upon stimulation can direct differentiation of immune cells towards anti-inflammatory phenotypes. Adapted from Lu et al. (344) and Kawai et al. (345).

The mechanisms by which n-3 PUFA affect gene expression are multifaceted. These include changes in enzyme activity, activating or suppressing signaling molecules, interacting directly with DNA, as well as interacting with proteins involved in the processing of transcription factors (13). Several genome-wide gene expression profile studies have previously demonstrated the upregulation and downregulation of thousands of genes following an intravenous endotoxin challenge (346-349). Similarly, a number of recent studies have investigated changes in gene expression in response to n-3 PUFA supplementation (11, 341, 350, 351). Here we evaluate changes in the expression of TLR-4 pathway genes following a low-dose endotoxin challenge in healthy adult men and explore the relationship between subject characteristics, RBC membrane content of n-3 and n-6 PUFA, and inflammatory gene expression. We hypothesized that inflammatory response genes (e.g., TLR4, COX2, TNF- α , IL-6, IL-1 β) will be upregulated in response to a low-dose LPS challenge and higher RBC content of n-3 PUFA, namely EPA and DHA, will be associated with a lower peak response.

5.3 METHODS

5.3.1 Subject Population

Eight healthy adult males between the ages of 20-45 years and BMI between 20-30 kg/m² were included in the study following the completion of a fish oil supplementation study as previously reported and is summarized in **Figure 5.2** (304). Participants received 0, 300, 600, 900, or 1,800 mg/d of EPA+DHA for approximately 5 months. Exclusion criteria included serious medical conditions, history of diabetes, smoking, and chronic anti-inflammatory medications, habitual oily fish consumption (<4 servings per month), and use of n-3 PUFA supplements.

5.3.2 Endotoxin Challenge

Following the completion of the supplementation study, subjects participated in a low dose intravenous endotoxin challenge (**Figure 5.2**). Subjects reported to the Clinical Research Center (CRC) at 7:00 AM on the day of testing; vital signs were measured and an intravenous catheter was inserted into an antecubital forearm vein. A baseline blood sample was collected

and a normal saline solution was injected into the catheter. A sterile solution of protein-free endotoxin (reference endotoxin, *E.coli* O113:H10:K:neg, manufactured under GMP, provided by NIH) was injected at a dose of 0.6 ng/kg body weight. Blood samples were collected from the venous catheter at 0, 2, 4, and 24 hours post-injection. Subjects were monitored continuously by trained CRC staff for blood pressure and body temperature. Subjects remained at the CRC until the 8 hour after initial injection and returned in the morning for the 24 hour sample. All meals were provided and controlled for 24 hours.

The study protocol was approved by the Institutional Review Board at the Pennsylvania State University approved and registered on ClinicalTrials.gov (NCT01078909). All procedures followed were in accordance with the ethical standards of the Helsinki Declaration of 1975, revised in 2000.

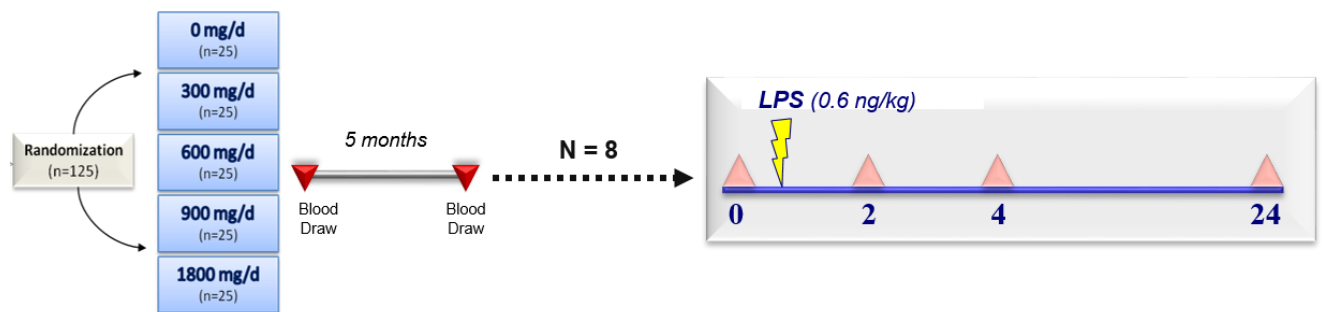


Figure 5.2. Study design and endotoxin challenge time course.

5.3.3 Inflammatory marker concentrations

Serum concentrations of TNF- α and IL-6 were measured at baseline and 2 hours post-LPS injection with high-sensitivity ELISA kits (R&D Systems, Minneapolis, MN) in duplicate (CV <10%). Serum high-sensitivity CRP was measured by latex-enhanced immunoephelometry (Quest Diagnostics; assay CV < 8%).

5.3.4 Mononuclear cell collection

Peripheral blood mononuclear cells (PBMC) were collected at 0, 2, 4, and 24 hours post-LPS injection. We selected these time points based on previous studies using the endotoxemia model indicating that the greatest changes in PBMC gene expression occurred within 6 hours and resolved by 24 hours (347, 348, 352). Whole blood was collected directly into a cell preparation tubes containing sodium citrate and Ficoll Hypaque density fluid (BD Vacutainer CPT) and immediately centrifuged for 30 minutes (1,800 g, 15° C for 30 min). The plasma layer was removed and stored at -80C. The PBMC layer was removed, washed with PBS, and centrifuged (900 g, 15° C for 15 min). After two washes in PBS, the cells were resuspended in 100 uL of RNALater, and then stored in -80 C.

5.3.5 Total RNA extraction

Total RNA was extracted as per mirVana miRNA Isolation protocol (Ambion) following removal of RNALater. RNA was isolated from the PBMC samples of 8 healthy Caucasian males. The quality and quantity of the isolated RNA was assessed by NanoDrop (NanoDrop spectrophotometer Wilmington, DE, USA).

5.3.6 Real-time PCR

Real-time qPCR analysis was performed by the Genomics Core Facility of the Pennsylvania State University by reverse-transcribing DNase-treated RNA using the High Capacity cDNA Reverse Transcription kit and the protocol provided with the kit (Life Technologies, Carlsbad, CA). Quantification was performed by adding 10 or 20 ng of cDNA in a reaction with 2X TaqMan Universal PCR Master Mix and TaqMan Gene Expression assays in a final volume of 20 uLs. **Table 5.1** describes the 14 target genes and **Figure 5.1** displays their involvement in the TLR4 pathway. The amplification protocol consisted of 10 min at 95°C, followed by 40 cycles of 15 sec at 95°C and one min at 60°C in the 7300 Real-Time PCR System. Cycle threshold (Ct) values of the genes and reference genes (cyclophilin and beta-2-microglobulin) were used with the $\Delta\Delta C_t$ method to determine relative levels of gene expression. Final results are expressed as fold change, using beta-2-microglobulin as the endogenous control and comparing 2, 4, and 24 h to the 0 hour time point.

Table 5.1 Description and function of targeted genes involved in the TLR-4 pathway

Gene Symbol	Gene Description	Function(s)
TLR4	Toll-like receptor-4	Pathogen recognition activator of innate response
TICAM1	Toll-like receptor adaptor molecule-1 (or TRIF)	Mediates protein-protein interactions between TLRs and signal-transduction components; MyD88 independent NF- κ B and IRF activation
PTGS2	Cyclooxygenase-2 (COX-2)	Key enzyme in eicosanoid synthesis.
<i>Cytokines</i>		
IL1B	Interleukin 1-beta	Pro-inflammatory cytokine
TNF	Tumor-necrosis factor -alpha	Pro-inflammatory cytokine
IL6	Interleukin 6	Pro-inflammatory cytokine
IL10	Interleukin 10	Anti-inflammatory cytokine
<i>Protein Kinases</i>		
IKKBK	Inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase beta	Phosphorylates the inhibitor in the inhibitor/NF- κ B complex, leading to the activation of NF- κ B
MAPK1	Mitogen-activated protein kinase 1 (ERK2)	<i>ERK pathway</i> ; activated by stress, phosphorylates a number of transcription factors
MAPK8	Mitogen-activated protein kinase 8 (JNK1)	<i>JNK pathway</i> ; activated by stress and pro-inflammatory cytokines; phosphorylates a number of transcription factors
MAPK14	Mitogen-activated protein kinase 14 (p38 α)	<i>p38 pathway</i> ; activated by stress, pro-inflammatory cytokines, and LPS; phosphorylates a number of transcription factors.
<i>Nuclear Receptors</i>		
PPARA	Peroxisome proliferator-activated receptor alpha	Lipid homeostasis; activation promotes catabolism of fatty acids.
PPARG	Peroxisome proliferator-activated receptor gamma	Adipocyte differentiation; activation promotes fatty acid uptake, adipogenesis, and insulin sensitization.

5.3.7 Statistical analyses

Statistical analyses were performed using Minitab (version 16.2, Minitab, State College, PA). Continuous data are reported as the mean \pm SD or SEM. For descriptive purposes, categorical data are presented as frequencies and percentages. Fit statistics were assessed for continuous variables to identify any outliers (± 3 SD) and for normality. Gene expression data are presented as relative abundance normalized to the 0 hour time point. Relative Expression Software Tool (REST) was used to correct for exact PCR efficiencies and the mean Ct (REST

2009, Qiagen, Hilden, Germany). Paired t-tests were used to assess differences between timepoints. Pearson correlations were performed in Minitab to determine the relationships between subject characteristics, serum parameters, RBC fatty acids, and changes in gene expression. Correlation coefficients and unadjusted p-values <0.025 are reported.

5.4 RESULTS

LPS (0.6 ng/kg) was administered to healthy Caucasian male volunteers (n=8), and blood samples were taken for analysis. **Table 5.2** displays the baseline characteristics of the included subjects; Omega-3 Index (O3I) ranged from 3.06% to 9.98%. All subjects developed a mild fever 2-6 hours post-LPS injection (**Figure 5.3**). Additional symptoms included mild headache (n=5), fatigue (n=4), malaise (n=3), nausea (n=3), and myalgia (n=3). Serum TNF- α and IL-6 concentrations significantly increased 2 hours post-LPS injection (**Supplementary Figure 1**).

Table 5.2. Baseline characteristics of healthy, Caucasian participants (n=8)¹

Variables	Overall Mean \pm SD
Age, yr	26.5 \pm 6.9
O3I, % (range)	5.96 (3.06 - 9.98)
BMI, mg/k ²	24.9 \pm 3.4
SBP, mm Hg	120 \pm 4.8
DBP, mm Hg	76 \pm 9.2
Glucose, mg/dL	92 \pm 5.3
Albumin, g/dL	4.3 \pm 0.2
Globulin, g/dL	2.1 \pm 0.1
Albumin/Globulin	2.1 \pm 0.1
Lipids and Lipoproteins, mg/dL	
TC	157 \pm 37
LDL-C	98 \pm 32
HDL-C	43 \pm 12
TC_HDL ratio	3.9 \pm 1.5
TG mg/dL	84 \pm 54
Inflammatory Markers	
TNF α (pg/mL)	1.6 \pm 0.3
IL6 (pg/mL)	1.3 \pm 0.5
CRP (mg/L)	0.5 \pm 0.3

¹Data are means \pm SD

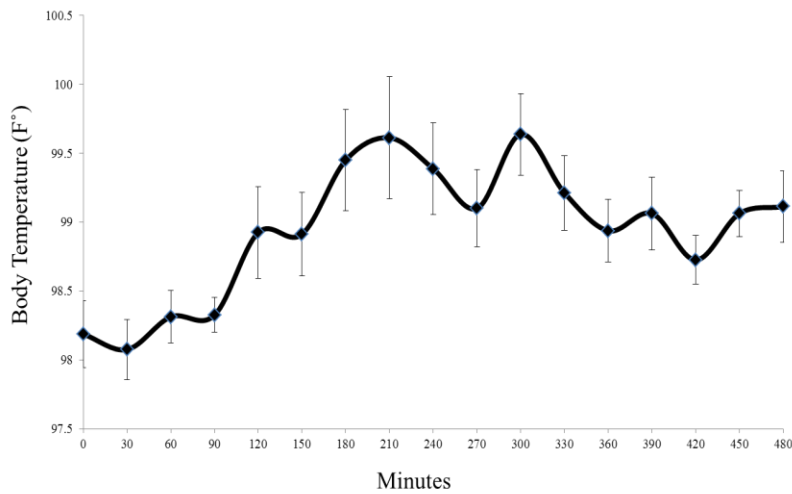


Figure 5.3. Change in body temperature after intravenous LPS administration in 8 healthy adult males. Data reported as mean \pm SEM. Body temperature increased 2 hours post-LPS injection and remained elevated.

5.4.1 Changes in gene expression

RNA obtained from the 8 male subjects demonstrated changes in gene expression within the first 2-4 hours of LPS administration. The mean changes in relative abundance are presented in **Table 5.3**. As expected, variability in both timing and magnitude of individual gene expression was evident.

Table 5.3 Mean PBMC gene expressions before and after LPS stimulation (n=8)¹

	0 hr	2 hr	4 hr	24 hr
TLR4	1.0 \pm 0.0	2.3 \pm 0.9	3.1 \pm 0.8	1.1 \pm 0.2
COX2	1.0 \pm 0.0	32.4 \pm 24.0	14.5 \pm 10.0	3.2 \pm 1.9
IKBKB	1.0 \pm 0.0	2.4 \pm 0.5	2.0 \pm 0.4	1.5 \pm 0.4
TICAM1	1.0 \pm 0.0	2.4 \pm 0.5	2.6 \pm 0.5	1.7 \pm 0.4
MAPK1	1.0 \pm 0.0	1.4 \pm 0.6	0.9 \pm 0.2	1.3 \pm 0.2
MAPK8	1.0 \pm 0.0	1.4 \pm 0.2	0.9 \pm 0.1	1.0 \pm 0.2
MAPK14	1.0 \pm 0.0	3.6 \pm 1.0	4.6 \pm 1.1	1.2 \pm 0.3
TNF	1.0 \pm 0.0	2.0 \pm 0.4	1.3 \pm 0.2	0.9 \pm 0.1
IL6	1.0 \pm 0.0	2.5 \pm 0.5	4.8 \pm 3.5	1.0 \pm 0.3
IL1B	1.0 \pm 0.0	45.2 \pm 21.6	21.2 \pm 8.6	1.8 \pm 0.6
IL10	1.0 \pm 0.0	1.0 \pm 0.4	10.7 \pm 4.3	0.9 \pm 0.3
PPARA	1.0 \pm 0.0	1.4 \pm 0.2	1.2 \pm 0.1	1.3 \pm 0.3
PPARG	1.1 \pm 0.1	1.1 \pm 0.3	14.6 \pm 5.9	1.6 \pm 0.3

¹ Means \pm SEM. Relative abundance; normalized to reference gene (beta-2-microglobulin) and 0 hour time point.

5.4.1.1 TLR4

The LPS cell receptor, TLR4, increased 2-4 hours post-LPS injection in all but one subject (**Figure 5.4A**). TLR4 expression at 4 hours was significantly greater compared to the baseline 0 hour time point ($p=0.03$). The TLR4 adaptor molecule, TICAM1, increased 2-4 hours post-LPS injection for all subjects and was highly correlated with TLR4 at 2, 4, and 24 hour time points ($r=0.959$, $p<0.001$; $r=0.873$, $p=0.005$; $r=0.926$, $p=0.001$, respectively; **Figure 5.4B**). TICAM1 expression was significantly greater at 2 and 4 hour time points compared to baseline ($p=0.03$ and $p=0.01$, respectively).

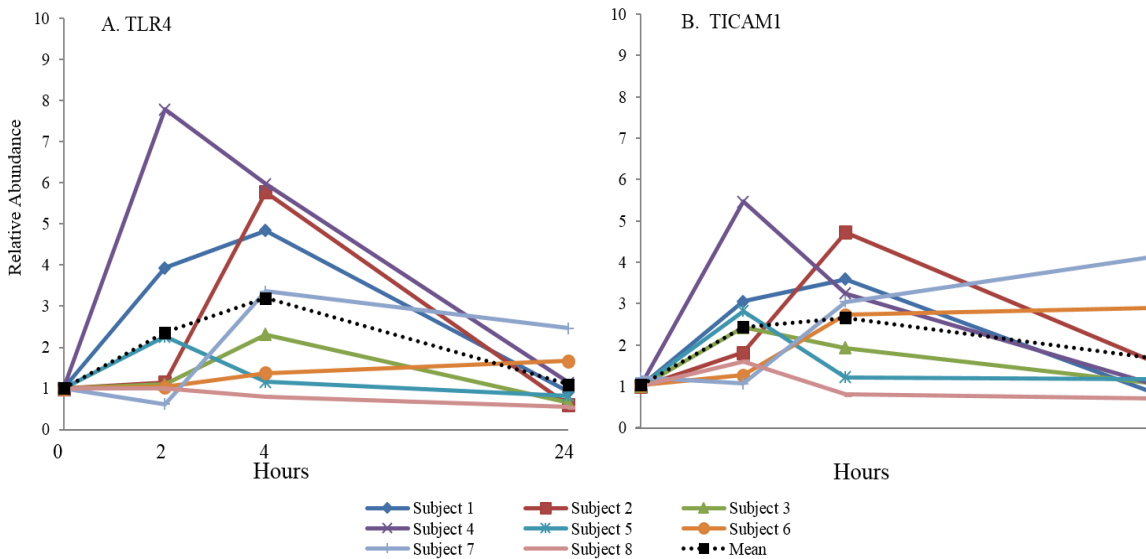


Figure 5.4. Changes in TLR4 (A) and TICAM1 (B) expression after intravenous LPS administration in 8 healthy adult males. Each participant is plotted as a different colored line. The mean expression per time point is denoted as a black dashed line.

5.4.1.2 Kinases

Expression of kinases involved in the inflammatory response also tended to increase 2-4 hours post-LPS injection, particularly IKBKB and MAPK14 (**Figure 5.5**). IKBKB and MAPK14 expression at 2 hours was significantly greater compared to baseline ($p=0.02$ and $p=0.03$, respectively). At 2 hours, there were 3- and 4-fold increases in IKBKB expression over

baseline in subjects 3 and 4, respectively, as well as 7-, 4-, and 8-fold increases of MAPK14 expression in subjects 1, 2, and 4, respectively. At 4 hours, there were 3-fold increases in IKBKB expression over baseline in subjects 1 and 2 as well as 7-, 10-, 6-, 5-, and 5-fold increases in MAPK14 expression in subjects 1, 2, 4, 6, and 7, respectively. By 24 hours, IKBKB and MAPK14 expression returned to near baseline levels in all but two individuals (subject 6 and 7) whose peak expression level was more delayed. Changes in MAPK1 were minimal with the exception of subject 4; at 2 hours, there was a six-fold increase in MAPK1 expression. Otherwise, no major changes (i.e., >two-fold change) in MAPK1 expression were observed (Figure 5.5).

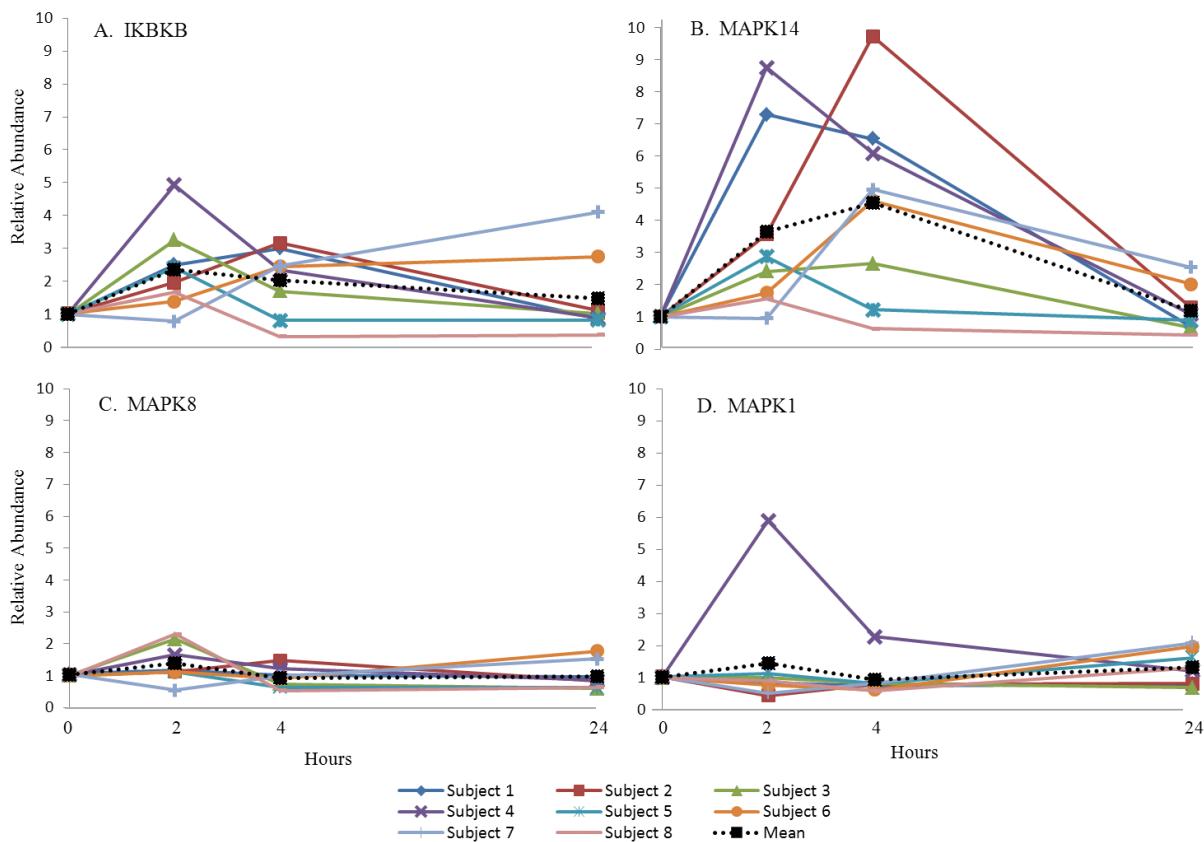


Figure 5.5. Changes in kinase expression after intravenous LPS administration in 8 healthy adult males. In Figs. 5.5A-5D, each participant is plotted with a different colored line and symbol. The mean expression per time point is denoted as a black dashed line.

5.4.1.3. COX-2

COX-2 expression changed considerably depending on the specific subject. All but two individuals (subjects 3 and 8) increased COX-2 expression post-LPS injection. At 2 hours, there were 197-, 31-, and 20-fold increases of COX-2 over baseline in subjects 1, 4, and 5, respectively. At 4 hours, there were 19-, 83-, and five-fold increases of COX-2 over baseline in subjects 1, 2, and 4, respectively. By 24 hours, expression returned to baseline levels except for subject 7, which showed a 16-fold increase over baseline. Therefore, maximum COX-2 expression is reached at 2 hours post-LPS injection for most subjects.

5.4.1.4. Cytokines

Several pro-inflammatory cytokines (i.e., TNF- α , IL-1 β , IL-6,) were upregulated within the first 2 hours, especially IL-1 β (**Figure 5.6**). TNF- α , IL-1 β , and IL-6 expression were each greater at 2 hours compared to baseline ($p=0.05$, $p=0.08$, and $p=0.02$, respectively). At 2 hours, there were 175-, 12-, 6-, 98-, 12-, and 13-fold increases in IL-1 β over baseline in subjects 1, 2, 3, 4, 5, 6, and 7, respectively. At 4 hours, subjects 2 and 7 further increased IL-1 β expression (67- and 32-fold, respectively); by 24 hours, expression returned to near baseline levels for all subjects. Therefore, 2 hours appears to be where maximum expression is reached in IL-1 β for most subjects, which is similar for TNF- α and IL-1 β . Despite subject 2 reaching a maximum IL-6 expression at 4 hours (29-fold increase), the majority of subjects achieved a maximum expression at 2 hours (although all less than a fivefold increase).

Expression of the anti-inflammatory cytokine, IL-10, remained unchanged until the 4 hour time point. IL-10 expression was significantly greater at 4 hours compared to baseline ($p=0.05$). At 4 hours, there were 9-, 15-, 39-, 8-, and 6-fold increases in IL-10 expression over baseline in subjects 1, 2, 4, 6, and 7, respectively. All subjects achieved maximum IL-10 expression at 4 hours and returned to baseline levels by 24 hours.

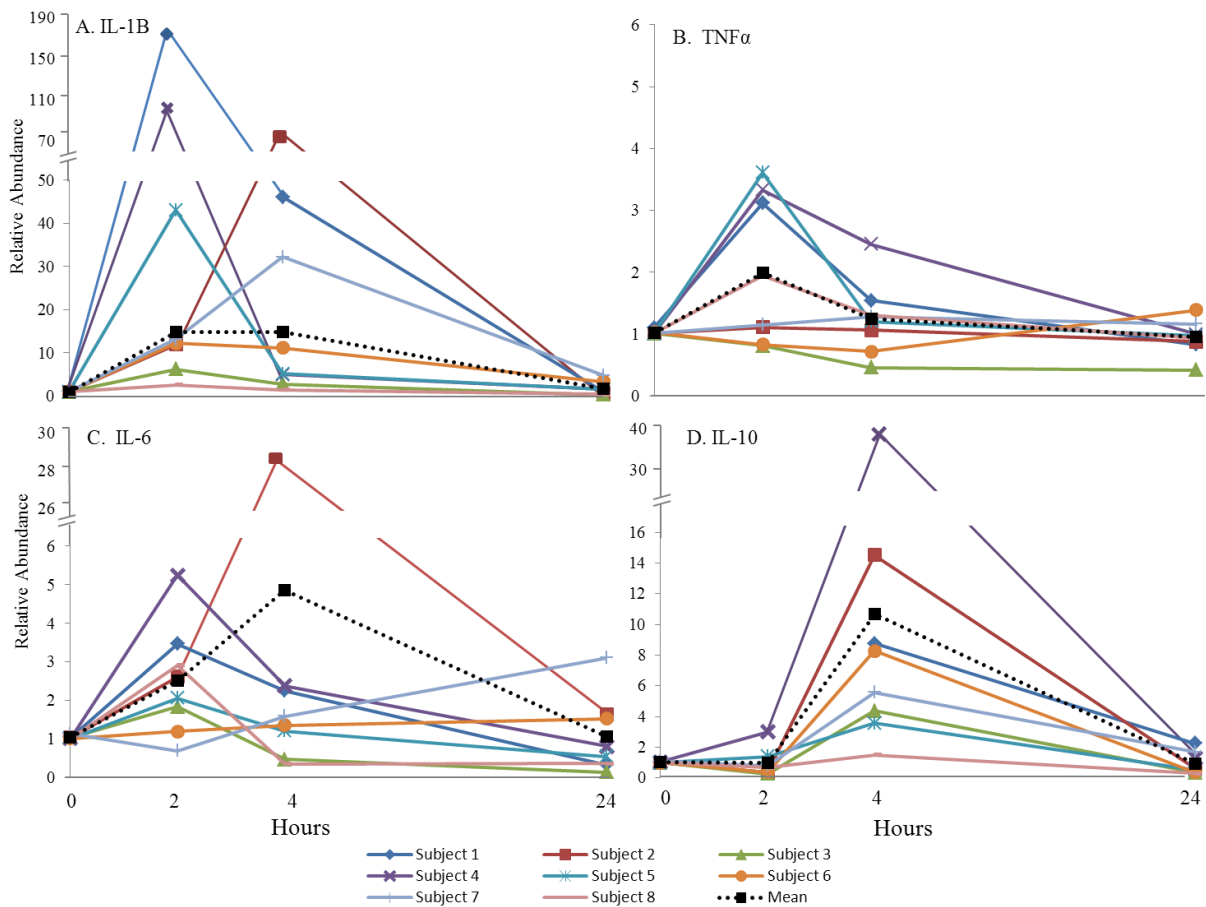


Figure 5.6. Changes in cytokine expression after intravenous LPS administration in 8 healthy adult males. In Figs. 5.7A-D, each participant is plotted with a different colored line and symbol. The mean expression per time point is denoted as a black dashed line.

5.4.1.5 PPARs

Minimal changes in PPAR α were observed; however, similar to IL-10, PPAR γ expression increased in all subjects at the 4 hour time point (**Figure 5.7**). PPAR γ expression at 4 hours was significantly greater compared to baseline ($p=0.05$). At 4 hours, there were 29-, 12-, 50-, 7-, and 11-fold increases in PPAR γ expression over baseline in subjects 1, 3, 4, 6, and 7, respectively. By 24 hours, expression returned to baseline levels. The timing and maximum expression of PPAR γ at 4 hours was consistent for all subjects.

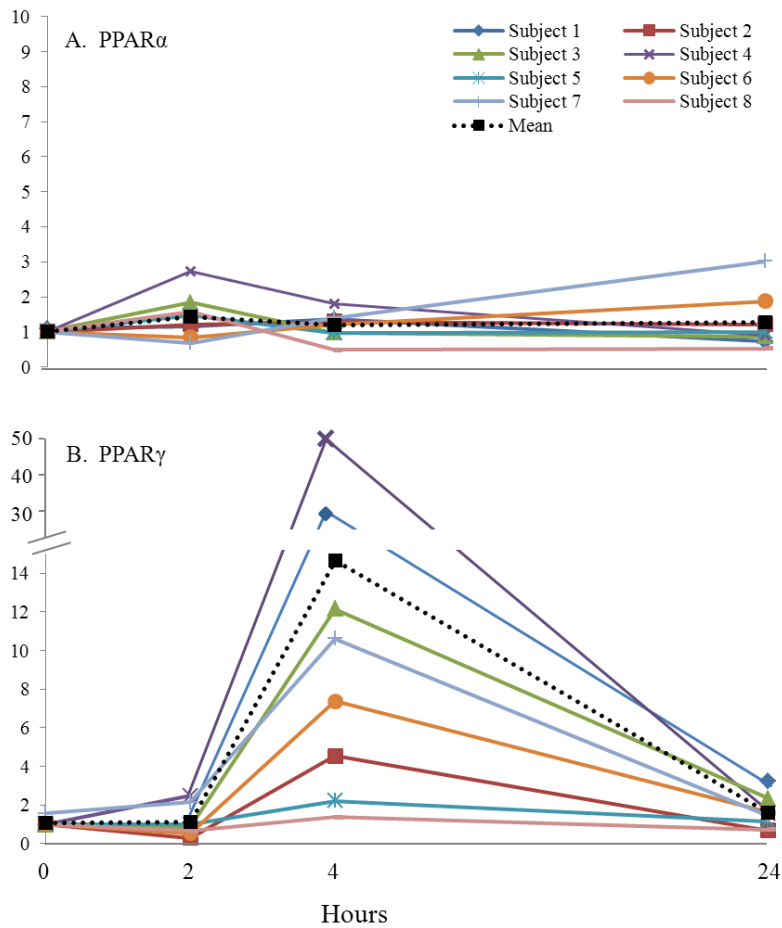


Figure 5.7. Changes in PPAR α (A) and PPAR γ (B) expression after intravenous LPS administration in 8 healthy adult males. Each participant is plotted with a different colored line and symbol. The mean expression per time point is denoted as a black dashed line.

5.4.2 Correlations explaining divergence in gene expression

Pearson correlations were conducted to explore the relationship between gene expression and subject characteristics, serum parameters, and RBC membrane content of n-3 and n-6 PUFA. Subject 2 was an outlier for IL-6 expression and, therefore, excluded from IL-6 expression correlation analyses.

5.4.2.1 Subject characteristics

BMI was positively correlated with IL-6 and PPAR γ expression at 4 hours ($r=0.866$, $p=0.012$; $r=0.823$, $p=0.012$, respectively). Higher SBP at baseline was associated with lowered IL-6 expression at 2 hours ($r=-0.844$, $p=0.008$).

5.4.2.2 Serum parameters

Baseline serum CRP did not correlate with any of the genes measured. Moreover, serum TNF α and IL-6 concentrations did not correlate with the expression of TNF- α and IL-6, despite both measures displaying clear elevations at 2 hours (**Table 5.4**). However, serum IL-6 concentrations at 2 hours were positively correlated with TLR4 expression at 2 hours ($r=0.807$, $p=0.016$) and PPAR γ expression at 4 hours ($r=0.945$, $p<0.001$); serum TNF- α at 2 hours was correlated with PPAR γ expression at 24 hours ($r=0.903$, $p=0.002$).

Table 5.4. Correlations coefficients comparing serum TNF- α and IL-6 concentrations with TNF- α and IL-6 gene expression levels (n=8)

Gene	Serum TNF- α		Serum IL-6	
	0 hour	2 hour	0 hour	2 hour
<i>TNFα</i>				
0 hour	-0.093	0.632	-0.194	0.464
2 hour	-0.064	0.041	0.048	0.216
4 hour	-0.593	0.135	0.205	0.585
24 hour	-0.423	-0.392	0.756*	-0.064
<i>IL-6</i>				
0 hour	0.262	0.566	-0.678	0.203
2 hour	-0.379	0.305	0.106	0.611
4 hour	0.104	-0.124	0.060	-0.224
24 hour	-0.225	-0.214	0.214	-0.135

Pearson correlation coefficients.

* Unadjusted $p<0.05$

Baseline low-density lipoprotein-cholesterol (LDL-C) was inversely correlated with IL-1 β expression at 4 hours ($r=-0.772$, $p=0.025$); however, no other correlations between baseline blood lipids levels and gene expression were observed.

Most notably, albumin/globulin (A/G) ratio was inversely correlated with TLR4, IKBKB, MAPK14, TICAM1, IL-6, and PPAR α expression at 2 hours ($r=-0.814$, $p=0.014$; $r=-0.865$, $p=0.006$; $r=-0.836$, $p=0.01$; $r=-0.899$, $p=0.002$; $r=-0.906$, $p=0.002$; $r=-0.833$, $p=0.01$, respectively). Albumin values were within the reference range (3.6–5.1 g/dL), whereas globulin values were towards the lower end of the reference range (2.1–3.7 g/dL; **Table 5.2**).

5.4.2.3 RBC content of n-3 and n-6 PUFA

Baseline RBC membrane content of EPA or DHA did not correlate with the expression of any genes measured. However, the intermediate fatty acid, docosapentaenoic acid (DPA, 22:5 n-3), was positively correlated with IL-1 β expression at 4 hours ($r=0.848$, $p=0.008$) as well as a larger reduction in IL-1 β expression between the 4 and 24 hour time points ($r=-0.854$, $p=0.007$). This is consistent with our previous observation that DPA, but not EPA or DHA, predicted changes in serum CRP concentrations following a low-dose LPS challenge in healthy men and women (data not yet published).

RBC membrane content of linoleic acid (LA, 18:2 n-6) and alpha-linolenic acid (ALA, 18:3 n-3) were each positively correlated with PPAR α expression at 2 hours ($r=0.881$, $p=0.004$; $r=0.856$, $p=0.007$, respectively). No associations between gene expression and arachidonic acid (AA, 20:4 n-6) were observed.

5.5. DISCUSSION

Intravenous endotoxin administration in healthy adult males produced changes in PBMC gene expression within 2-4 hours. Exposure to protein-free sterile LPS induced genes associated with pattern recognition, signal transduction, transcription factors, and cell proliferation. However, by 24 hours, expression levels had been resolved. This is in contrast to chronic inflammatory conditions in which continued exposure to an inflammatory stimulus promotes a sustained inflammatory response (348, 353). LPS is cleared from the blood within

30 minutes of intravenous LPS administration (354); therefore, secondary mediators amplified by the initial host response are responsible for many of the downstream effects, including changes in genes associated with the innate immunity (348, 355).

Our findings indicate that the TLR4-mediated pathway was upregulated in PBMCs following *in vivo* LPS stimulation as evident by increased expression of several kinases and pro-inflammatory cytokines involved in the innate immune response. Major increases in the expression of MAPK14 and IKBKB occurred 2-4 hours after LPS administration. Talwer et al. (15) also observed an induction of MAPK14 in PBMCs 6 hours after intravenous endotoxin challenge (4.0 ng/kg). MAPK14 encodes for p38 α MAPK, which is activated by stress and/or inflammatory cytokines and regulates cell proliferation, differentiation, and survival (356). IKBKB encodes for IKK- β , a protein that phosphorylates the inhibitor in the inhibitor/NF- κ B complex, thereby causing dissociation of the inhibitor and translocation of NF- κ B to the nucleus where it activates transcription of various genes, including cytokines. Increased MAPK14 and IKBKB expression in response to LPS administration highlights the activation of separate signaling pathways in stimulating the innate immune response, although these signaling pathways often interact with each other via biochemical crosstalk. For example, Craig et al. (357) showed that MAPK kinase 6, which phosphorylates and activates p38 MAPK, also augmented IKK- β activity in rat cardiac myocytes. Thus, optimal induction of cytokines in response to an inflammatory stimulus likely involves multiple signaling pathways. Exploring the crosstalk among these pathways would provide greater insight into the inflammatory response.

Increased TICAM1 expression in coordination with increased TLR4 expression also indicates stimulation of the MyD88-independent TLR4 signaling pathway. The LPS/TLR4 complex is internalized and retained in the endosome, where it is known to trigger recruitment of TICAM1 to facilitate the activation of interferon regulatory factor 3 (IRF3) as well as the late-phase activation of NF- κ B (**Figure 5.1**) (345). Induction of interferons and interferon-inducible genes, although not measured in the present study, are important for anti-viral and anti-bacterial responses (344). Further exploration of TICAM1 activation may therefore have significant implications in vaccine development as well as in treating inflammation.

Induction of COX-2 expression was observed in all but two subjects. This increase in COX-2 expression would be expected to cause an increase in the synthesis of eicosanoids, fatty acid derived lipid mediators with potent pro- and/or anti-inflammatory properties. The paradoxical role of COX-2 to promote inflammation and its resolution reflects the temporal nature of COX-2 expression and the changing cell populations that create different microenvironments (358). Increasing cell membrane content of EPA and DHA has been shown to promote the synthesis of eicosanoids with more anti-inflammatory properties in addition to the production of novel lipid mediators proposed to reduce and resolve inflammatory responses (15, 139). In the present study, there was no association between EPA, DHA, or the combination of EPA and DHA content in RBC membranes with COX-2 expression. Small sample size and interindividual variability are clearly limiting factors in assessing this relationship. Nonetheless, clinical evidence assessing the effect of n-3 PUFA on COX-2 expression as well as activity is needed.

Previous analyses of serum cytokine kinetics using the endotoxemia model indicate that peak TNF- α and IL-1 β concentrations occur approximately 1.5-2 hours after LPS administration followed by a rise in IL-6 concentrations and subsequently by IL-10 (354, 359-361). We observed peak TNF- α and IL-1 β expression at 2 hours; IL-6 expression also reached maximum expression for most subjects at 2 hours, although two subjects had peak IL-6 expression at 4 hours. These findings are similar to those reported by Prabhakar et al. (352) who found that TNF- α , IL-1 β , and IL-6 expression increased within 1 hour of LPS treatment (3.0 ng/kg) in six healthy adults and persisted at the 3 hour time point. By 24 hours, TNF- α and IL-1 β expression had returned to near baseline levels whereas IL-6 was slightly below the baseline expression level (352). We also observed slightly lower IL-6 expression at 24 hours for some subjects relative to baseline (**Figure 5.6C**). This is likely a result of negative feedback and the increase in anti-inflammatory mediators between 4 and 24 hours (i.e., IL-10).

Interestingly, serum concentrations of TNF- α and IL-6 at 2 hours did not correlate with either TNF- α or IL-6 expression at 2 or 4 hours. Individual differences in response to an inflammatory stimulus may explain this null finding as well as other cells and inflammatory mediators not measured here. For example, circulating cytokines could be derived from

cytokine-producing cells other than PBMC, such as neutrophils. Moreover, the proportion of specific white blood cells present in blood changes dramatically following an *in vivo* LPS challenge. Talwar et al. (53) observed a rapid increase in neutrophils, whereas PBMCs decreased at 6 hours following an *in vivo* LPS challenge (4 ng/kg) in 8 healthy adults. The proportion of lymphocytes in PBMC population fell by almost 50%, whereas the percentage of monocytes increased by threefold. Furthermore, the proportion of T cells and natural killer cells within the lymphocyte population remained the same at 6 hours, while the proportion of B cells increased (53). Therefore, LPS induced changes in the proportion of white blood cells in circulation, including different subtypes of PBMCs, may explain some of the observed changes in gene expression following the LPS challenge. Additional research examining specific white blood cell gene expression and circulating cytokines levels following an *in vivo* LPS challenge would help explain functional differences among white blood cells in inflammatory regulation.

IL-10 and PPAR γ were among the most consistent LPS-induced genes. In contrast to pro-inflammatory cytokines (i.e., TNF- α , IL-1 β , IL-6), there was a delay in increased mRNA abundance of IL-10 (Figure 5.6D). Similarly, PPAR γ expression increased 4 hours after endotoxin challenge, 2 hours following the maximum expression of notable pro-inflammatory genes. Collectively, these data offer insight into the progression of the inflammatory response. The initial acute pro-inflammatory phase is followed by counterregulation (i.e., increased IL-10 and PPAR γ expression), concluding with full recovery and a return to homeostasis. However, the interrelationship between IL-10 and PPAR γ is not clearly understood. Omega-3 PUFA and their oxidized metabolites are natural ligands of PPAR γ (362), a nuclear transcription factor that, when activated, inhibits NF- κ B and thereby suppresses subsequent inflammatory processes. Lytle et al. (363) showed that administration of PPAR γ agonists had an anti-inflammatory effect independent of IL-10 in an animal model of inflammatory bowel disease. In contrast, Kim et al. (364) found that administration of PPAR γ agonists improved asthmatic features in mice via regulation of IL-10 expression and IL-10 receptor activation. Differences in the disease model and study conditions may be responsible for the discrepancy (364).

Nonetheless, our results illustrate a significant and consistent increase in both IL-10 and PPAR γ expression 4 hours following the LPS challenge in healthy adult men.

Obesity is typically accompanied by an elevated inflammatory state (365, 366). We found that higher BMI was associated with higher IL-6 and PPAR γ expression at 4 hours post-LPS administration. Serum IL-6 concentrations as well as IL-6 expression at 2 hours were both positively correlated with PPAR γ expression at 4 hours and therefore the observed association between BMI and PPAR γ may have been due to higher IL-6 expression. Nonetheless, body weight gain by PPAR γ agonists has been reported in rodent (367-370) and human studies (371, 372). PPAR γ activation reportedly increases food intake (373), suggesting that the central nervous system might be a site for PPAR γ action. PPAR γ is a well-known regulator of adipocyte differentiation (374); however, additional research is clearly needed to better understand PPAR γ signaling and its effects on body weight.

Elevated LDL-C is associated with increased CVD risk; however, LDL also binds and neutralizes circulating LPS, a mechanism by which increased serum LDL protects against the lethal effects of LPS (375-377). We found that higher serum LDL-C levels were associated with lower IL-1 β expression, suggesting that elevated LDL-C levels may reduce LPS-induced inflammation. Therefore, it could be theorized that in familial hypercholesterolemia, elevated serum LDL-C is due to a genetic mutation in the LDL-receptor gene, and may be an evolutionary adaptation to protect against threats of infection; the adverse effects of hypercholesterolemia (i.e., atherosclerosis) normally do not become life threatening until after reproductive years. Adequately powered clinical studies are needed to evaluate the role of lipoproteins in the inflammatory response and to more fully understand the functional aspects of lipoproteins beyond lipid transport.

Surprisingly, baseline A/G was a better predictor of inflammatory response than serum TNF- α , IL-6, or CRP. We found that lower baseline A/G was associated increased expression of TLR4, IKBKB, MAPK14, TICAM1, IL-6, and PPAR α at 2 hours following the LPS administration. Low A/G can attributed to reduced hepatic production of albumin and/or increased hepatic or lymphocyte production of globulins, antibodies often elevated in inflammatory conditions (378-380). Subjects in the present study had globulin levels within the

normal range; although, it is possible that having higher globulin levels prior to a LPS challenge may enhance the immune system's ability to more quickly and effectively identify and neutralize LPS. Serum CRP is currently the standard clinical measure to assess inflammatory status; however, our data suggests looking into A/G as a systemic index that could help predict individual response to acute inflammation.

Strategies to strengthen counter-regulatory mechanisms and accelerate recovery have significant implications in the prevention and treatment of inflammatory conditions. Omega-3 PUFA, specifically EPA and DHA, have been shown to possess anti-inflammatory and pro-resolving properties (11, 13, 14); however, neither EPA, DHA, nor the combination of EPA and DHA content in RBC membranes were related to inflammatory gene expression following the LPS challenge. Unexpectedly, greater DPA content was associated with increased IL-1 β expression at 4 hours as well as a larger reduction in IL-1 β expression between the 4 and 24 hour time points. The mechanisms behind these associations are not clear. DPA can be retroconverted to EPA, suggesting that DPA serves as a storage pool for EPA (57, 317); therefore, changes in EPA utilization for eicosanoid synthesis would alter DPA content and explain the association with IL-1 β expression. However, it is also plausible that DPA and/or DPA metabolites promote resolution similar to oxidized metabolites of EPA and DHA (139).

Sources of only DPA are not available in foods or in supplement form, and thus clinical evidence about its effects are absent. Cell culture work has provided some insight regarding the effects of DPA in inflammation. Akiba et al. (381) evaluated the effects of DPA compared with EPA and DHA on platelet aggregation and AA metabolism in rabbit platelets. The results showed that DPA was a more potent inhibitor of collagen- and AA-induced platelet aggregation than either EPA or DHA, suggesting that DPA may be interfering with the COX pathway. Kanayasu-Toyoda et al., (382) found that pretreatment of endothelial cells with DPA resulted in a dose-dependent increase in migration, which was achieved at one-tenth the concentration required for maximal stimulation. Tsuji et al. (383) showed that migrating activity of endothelial cells stimulated with vascular endothelial growth factor was suppressed by DPA pretreatment. More specifically, treatment of endothelial cells with DPA caused suppression of vascular endothelial growth factor-receptor 2 expression in both plastic dish and

collagen gel cultures (383). Collectively, these data indicate that DPA has an inhibitory effect on angiogenesis in part through the suppression of vascular endothelial growth factor-receptor 2 expression. Endothelial cell migration is an important process in blood vessel repair. Similarly, alternative macrophage activation is an important step in resolving inflammation; DPA may have implications in both processes although the mechanisms require further investigation. Discerning between the *in vivo* effects of specific n-3 PUFA also can be difficult due to interconversion. Nonetheless, our observations indicate a need to examine the function and biological role of individual n-3 PUFA, including DPA, in the context of inflammation.

We also found that higher RBC content of LA and ALA was associated with greater PPAR α expression at 2 hours. PPAR α is a nuclear receptor that mediates the effects of dietary fatty acids on gene expression, including genes involved in fatty acid oxidation (384). Long-chain PUFA, including LA and ALA, can bind and activate PPAR α , thereby inducing expression of transport proteins and key enzymes involved in fatty acid oxidation (385). Metabolites of LA or ALA also bind PPAR α and therefore may be responsible in part for the observed association (386). However, it is unclear as to why only LA and ALA, and no other long-chain PUFA with PPAR α binding affinity were associated with PPAR α expression.

5.5.1 Strengths and Limitations

A key strength as well as novelty of the present study is the use of a well-controlled *in vivo* LPS challenge to evaluate changes in inflammatory genes expression. We also collected PBMCs using specialized cell-preparation tubes, which reduce the risk of contamination. The 2, 4, and 24 hour time points measured were based on prior endotoxin challenge studies and provided information on both the initiation as well as resolution of inflammation (347, 348, 352); however, some peak responses may have been missed and therefore additional time points, especially within the first 6 hours, would have provided a more precise time point determination of maximum gene expression following the LPS challenge.

Limitations include small sample size, lack of a saline control, and the inclusion of only male Caucasian subjects. Financial constraints restricted the number of subjects analyzed whereas only male Caucasian subjects were included to focus the analysis on a homogenous population with diverse RBC fatty acid content. Another limitation of the study includes the

use of non-separated PBMCs. Changes in gene expression may be caused in part by changes in the proportion of certain blood cells. As previously discussed, Talwar et al. (348) observed a decrease in the percentage of lymphocytes whereas the proportion of monocytes increased 6 hours after LPS administration (4 ng/kg) in healthy adults. Changes in the number and proportion of PBMCs would alter the contribution of RNA associated with each cell type. Almost double the amount of RNA is present in activated T cells compared to resting T cells (387); our mixed PBMC sample analyses do not account for variations in RNA content per cell. Therefore, examining changes in cell-specific gene expression following an *in vivo* LPS challenge would be more accurate.

RBC membrane fatty acids, although an important biomarker, may not be the ideal biomarker to examine inflammatory regulation. Measuring the fatty acid content of PBMCs might be a more appropriate method. The amount of these cell types in circulation vary depending on the environment and therefore present additional challenges. Nonetheless, examining the fatty acid content of specific immune cells involved in the inflammatory response would provide greater insight into the role of PUFA in inflammatory regulation.

Lastly, a significant number of correlations were conducted exploring the linear relationship between gene expression and additional study measures. P-values were reported without adjustment, therefore, increasing the risk of Type I errors. Moreover, nonlinear relationships were not assessed and should be examined considering the individual variability.

5.5.2. Conclusion

Low dose *in vivo* LPS administration in healthy adult Caucasian males activated an acute phase host response as indicated by increased expression of genes involved in the TLR4-mediated pathway. The inflammatory response is characterized by a rapid amplification of pro-inflammatory gene expression (e.g., TNF α , IL-1 β , IL-6) followed by a subsequent rise in anti-inflammatory gene expression (e.g., IL-10) and return to homeostasis within 24 hours. RBC content of EPA and/or DHA was unrelated to changes in gene expression, although DPA content was associated with IL-1 β expression. Taken together, our data provides novel insight into the changes in gene expression following an acute inflammatory challenge that may be further explored to identify targets for therapeutic intervention.

5.6. SUPPLEMENTARY MATERIAL

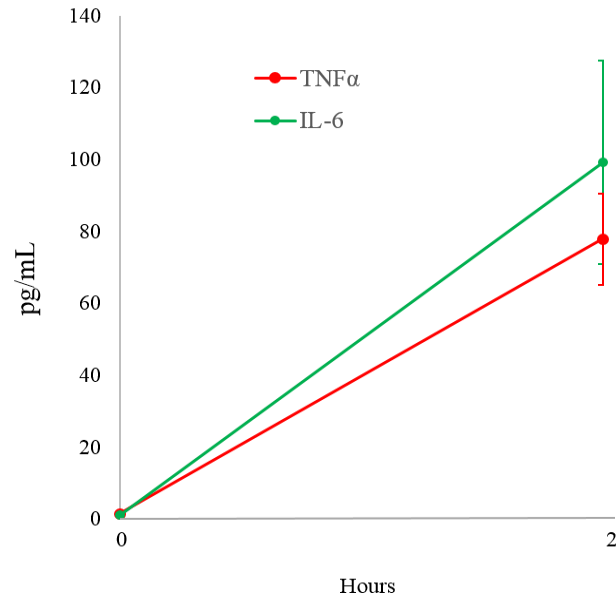


Figure 5.8. (Supplementary Figure 1) Change in serum TNF- α and IL-6 concentrations 2 hours after intravenous LPS administration in 8 healthy adult males. Data reported as mean \pm SEM. Both serum TNF- α and IL-6 significantly increased 2 hours post-LPS injection.

CHAPTER VI:

RESEARCH SUMMARY AND FUTURE DIRECTIONS

The studies in this dissertation were designed to explore the immunometabolic effects of long-chain n-3 PUFA in modifying cell membrane composition and inflammatory processes. Specifically, these studies investigated (1) the dose-response effects of n-3 PUFA consumption on RBC membrane composition and factors that influence fatty acid incorporation, (2) the effects of n-3 PUFA consumption on circulating inflammatory markers as well as the relationship between RBC membrane fatty acid content and inflammatory marker concentrations, and (3) the effects of RBC membrane n-3 PUFA on changes in gene expression following an endotoxin challenge in humans. The purpose of this chapter is to briefly summarize the main findings and identify additional areas for research.

6.1 Incorporation of dietary n-3 PUFA into cell membranes

Increased cell membrane content of n-3 PUFA, specifically EPA and DHA, is associated with a reduced risk of various CVD related outcomes (8, 9, 28, 230, 252). Therefore, understanding the effectiveness of dietary EPA+DHA intake as well as other clinical characteristics in altering cell membrane fatty acid composition provides valuable information for making n-3 PUFA recommendations. In the first study, we found that EPA+DHA supplementation in the form of fish oil explained two-thirds of the variability in response of RBC membrane EPA+DHA content. Moreover, several factors beyond dose (i.e., body weight, age, physical activity, and sex) added more precision to the predictive model. These results can be used to make dietary recommendations to achieve a target cell membrane composition of EPA+DHA for CVD risk reduction on an individual basis.

This data in addition to a large body of evidence supporting a cardioprotective benefit of increased EPA+DHA intake provide strong justification for setting a DRI for EPA and DHA. However, much remains to be done to establish DRIs for EPA and DHA. Areas of research that would help further characterize and define optimal EPA+DHA intake include:

1. We need a better understanding of what the requirements are for the individual long chain n-3 PUFA, and the factors that affect their conversion and biological function.
2. The effect of background diet, including both macronutrient and micronutrient intake, on how n-3 PUFA is processed and incorporated into cell membranes needs to be further examined. For example, how do the incorporation rates vary if an individual is consuming a low-fat or high-fat diet? Would certain micronutrient, phytonutrient, or antioxidant levels in the diet influence n-3 PUFA metabolism?
3. Further examining the effect of adiposity and variations in body composition on the metabolism and effectiveness of n-3 PUFA is warranted. We found that body weight had a small effect on the incorporation of EPA+DHA into RBC membranes, indicating a need to explore potential differences in n-3 PUFA metabolism based on the amount as well as location of body fat.
4. The appropriate endpoints to use for establishing a DRI for n-3 PUFA need to be reviewed. We used an established biomarker that has been associated with CVD risk (i.e., Omega-3 Index). Nonetheless, biomarkers beyond the Omega-3 Index should be explored in the context of influencing health outcomes.
5. More intervention trials are needed for EPA+DHA in the primary prevention of CVD in the general population as well as in the prevention and/or treatment of inflammatory conditions.
6. Individual differences among n-3 PUFAs (e.g., ALA, EPA, DPA, DHA) with respect to health outcomes, conversion efficiency, and metabolism require further investigation. For example, would consuming isolated EPA or DHA be as effective as EPA+DHA? Is there an ideal ratio of EPA to DHA? Conversely, would isolated sources of other n-3 PUFA provide similar benefits?
7. There is a need to expand the evidence base about the impact of EPA+DHA intake on other health outcomes beyond CVD, such as cancer and cognitive health.
8. Further information is needed about the effects of genetic variation and epigenetic regulation of EPA+DHA on health outcomes. Such information would further explain individual variability in response to EPA+DHA intake.

9. The safety issues related to different doses of EPA+DHA intakes need further characterization in total and in at-risk populations. Very high doses (e.g., 3 g/d EPA+DHA) have been regarded as safe by the FDA, yet no upper limit has been defined. Also, long-chain PUFA, including EPA and DHA, are susceptible to peroxidation and may cause damage in certain conditions if antioxidant protection against oxidative stress is insufficient. Therefore, researchers and practitioners should remain cautious in advising that increasing EPA+DHA intake is beneficial for all populations.

A minimum daily intake of 250 – 500 mg of EPA+DHA appears to be the general consensus among the scientific community for healthy adults; however, until DRIs are established, consumers and health professionals will continue to be uncertain as to how to interpret various recommendations and decide on appropriate n-3 PUFA dietary recommendations. Addressing the current challenges outlined above will help clarify the role of n-3 PUFA consumption in disease prevention and ultimately guide development of DRIs.

6.2 Role of n-3 PUFA in inflammation

Our findings indicate that long-chain n-3 PUFA, either consumed or contained in cell membranes, may help attenuate inflammation. In the second study, despite no differences between treatments, we found that ingestion of 1,800 mg/d of EPA+DHA over a period of 5 months reduced serum TNF- α concentrations in healthy adults. Surprisingly, both 900 mg/d and 1,800 mg/d EPA+DHA increased circulating lymphocyte concentrations. We did not differentiate between the types of lymphocytes (e.g., T cell, B cell, etc.) and thus unable to determine whether the observed increase can be attributed to a particular cell type. However, we speculate that this observed increase in circulating lymphocyte concentrations at increasing EPA+DHA doses may be explained in part by reduced expression of specific cellular adhesion molecules, resulting in a greater amount of lymphocytes freely circulating than adhered to the endothelium. Nonetheless, this finding clearly highlights a need to further assess the clinical effects of EPA+DHA intake on circulating immune cell populations as well as cellular adhesion molecule concentrations.

Many mechanisms have been proposed regarding the anti-inflammatory properties of n-3 PUFA. However, human and animal studies have not yielded consistent beneficial effects. In the second study, RBC content of DHA was associated with lower TNF- α , while AA content was associated with higher TNF- α and IL-6. This seems to support the notation that n-3 PUFA are ‘anti-inflammatory’ and n-6 PUFA are ‘pro-inflammatory’; however, higher LA content in RBC membranes was associated with lower IL-6. The main source of n-6 PUFA in the diet is LA and, therefore, labeling n-6 PUFA as ‘pro-inflammatory’ needs to be re-examined. Furthermore, DPA has received little to no attention relative to EPA and DHA. We found that low DPA content in RBC membranes, but not EPA or DHA, was associated with higher CRP. Whether low DPA content is a cause or effect of higher CRP is unclear. We speculate that DPA serves as a storage pool for EPA and, therefore, low DPA in an inflamed environment may be due to increased EPA utilization. In the third study, neither EPA, DHA, nor the combination of EPA and DHA content in RBC membranes were related to inflammatory gene expression following the low-dose LPS challenge; however, greater DPA content was associated with increased IL-1 β expression. Although the mechanism for this observation is unclear, it is possible that changes in the microenvironment could alter DPA to EPA conversion rates and explain, in part, the observed associations between DPA and inflammatory status. Nonetheless, our findings indicate that DPA may be just as biologically relevant as EPA and DHA and, therefore, included when exploring the role of n-3 PUFA in inflammation. Future studies should examine individual differences of n-3 PUFA in modulating inflammatory regulation and how variations in membrane n-3 PUFA content influence inflammatory response.

Genetic variation in n-3 PUFA metabolism also exists, which could modulate the physiological response to n-3 PUFA and help elucidate 22% of the variability unexplained in our first study as well as the variability in anti-inflammatory benefits associated with n-3 PUFA intake. The subject population (e.g., body weight, inflammatory status, age) is an important factor when evaluating the anti-inflammatory effects of n-3 PUFA intake. Furthermore, fatty acid desaturase genotypes have emerged as important modulators of n-3 PUFA bioconversion, therefore, interactions of TNF- α , PPAR, and COX genotypes with n-3 PUFA have significant

implications pertaining to inflammatory outcomes (227). More research is needed to explore these genetic determinants of inter-individual variation in response to n-3 PUFA as well as interactions between gene polymorphisms and n-3 PUFA in the context of inflammation.

RBC membrane fatty acids, although an important and well-established biomarker, may not be the ideal biomarker to examine inflammatory regulation (i.e. RBC fatty acid changes are not directly related to changes in inflammation). We found that changes in RBC membrane n-3 PUFA content did not correlate with changes in inflammatory marker concentrations. The study population consisted of healthy non-inflamed adults and, therefore, changes in circulating inflammatory markers were minuscule. However, there also were no associations between EPA and/or DHA content in RBC membranes and the changes in PBMC gene expression following a low-dose LPS challenge in healthy men. Although the small sample size was a clear limitation in this study, a more appropriate marker to examine the immunomodulatory effects of n-3 PUFA in humans would be PBMCs, immune cells more directly involved in the inflammatory response. However, using PBMC fatty acid content as a biomarker presents an additional challenge of cell-specificity as PBMCs consist of a variety of cell types, including T cells, B cells, and monocytes. Furthermore, the proportion of these cell types in circulation often vary depending on the changing environment. An excellent experiment would be to determine the fatty acid content of each cell type as well as the amount of each cell type present in circulation before and after an *in vivo* inflammatory challenge. Such information would provide valuable insight on the role of fatty acids, including n-3 PUFA, in inflammatory regulation.

A significant challenge moving forward will be accounting for the varying degrees of adiposity and inflammation within individuals and between tissues. Here we studied n-3 PUFA and inflammation in healthy adults; however, a large percentage of Americans are obese, which is known to cause low-grade inflammation and subsequent insulin resistance. The impact of n-3 PUFA on inflammatory regulation may be completely different in an obese state and thus the role of adiposity in modulating n-3 PUFA needs to be explored. Also, we typically use circulating TNF- α , IL-6, and CRP to assess inflammatory status; however, it may not be an accurate assessment of inflammation within a specific tissue. For example, skeletal

muscle production of IL-6 during exercise may have unique immunological effects, that differ from that of liver and/or adipose-derived IL-6, which is commonly implicated in the development of insulin resistance and chronic disease (388). Therefore, researchers should be cautious in generalizing circulating markers and consider tissue/cell specificity. Measuring tissue-specific inflammatory markers (e.g. resistin) as well as serum markers beyond TNF- α , IL-6, and CRP would provide greater insight into the potential role of n-3 PUFA in regulating inflammation.

Although not presented herein, we examined correlation patterns among genes measured as well as the expression of several microRNAs before and after the *in vivo* LPS challenge. Increased DPA content was associated with lower microRNA155 expression at 24 hours post-LPS injection ($r=-0.856$, $p=0.007$). MicroRNA155 is a non-coding RNA induced by pro-inflammatory stimuli, including LPS, that post-transcriptionally inhibits various target genes and recently shown to promote cardiac inflammation (389). MicroRNAs are a relatively new discovery and therefore additional research is still needed to understand the effects of altering microRNA expression in the context of inflammation. Nonetheless, exploring compounds that can post-transcriptionally regulate gene expression could have significant implications in identifying therapeutic strategies for the prevention and/or treatment of inflammation. Again, our data suggests that DPA may be an important fatty acid involved in inflammatory regulation that warrants further investigation.

Lastly, advances in fatty acid biochemistry and molecular techniques will continue to be instrumental in understanding the interrelationship between immune function and n-3 PUFA. Recently discovered pro-resolving lipid mediators derived from EPA and DHA appear to be promising targets for controlling inflammation. Additional receptors, pathways, and cellular interactions await discovery that will likely expand our understanding of the dynamics and kinetics of the resolution program. Moreover, the next generation of tools and technology will be critical in developing selective strategies to target specific cellular processes. Specialized drug delivery systems and improved imaging techniques will prove to be valuable procedures. Most anti-inflammatory options to date have very broad activities, often inhibiting a variety of immune functions. Developing therapeutic options that target specific receptors,

proteins, and/or cells would reduce unwanted side effects and avoid interfering with all immune functions. Therefore, a major objective for future research of immunometabolism should be to identify immunomodulatory agents, such as n-3 PUFA, that promote immune and metabolic systems back to homeostasis. Although much work remains to be done, there are certainly exciting possibilities for the clinical application of n-3 PUFA in managing and resolving inflammation. Inflammation will continue to be a normal and necessary bodily response, yet identifying strategies that can reduce the severity of inflammation as well as support recovery have significant implications in health and disease.

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PUBLICATIONS

Flock MR, Skulas-Ray AC, Harris WH, Gaugler TL, Fleming JA, Kris-Etherton PM. Effects of supplemental long-chain omega-3 fatty acids and erythrocyte membrane fatty acid content on circulating inflammatory markers in healthy adults. *In submission*.

Flock MR, Skulas-Ray AC, Harris WH, Etherton TD, Fleming JA, Kris-Etherton PM. Determinants of erythrocyte omega-3 fatty acid content in response to fish oil supplementation: a dose-response randomized controlled trial. *J Am Heart Assoc*. 2013; 2(6):e000513.

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Flock MR, Rogers CJ, Prabhu KS, Kris-Etherton PM. Immunometabolic role of omega-3 fatty acids in obesity-induced inflammation. *Diabetes Metab Res Rev*. 2013 Sept; 29(6): 431-445

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Flock MR, Green MH, Kris-Etherton PM. Effects of adiposity on the plasma lipid response to dietary saturated fatty acids and cholesterol. *Adv Nutr*. 2011 May; 2(3): 261-274.

AWARDS

Kligman Graduate Fellowship, Pennsylvania State University 2013-14
Thomas H. Smouse Memorial Fellowship, American Oil Chemists' Society 2012-14
Clinical Emerging Leader Award, American Society for Nutrition 2013
Office of Dietary Supplements Travel Award, National Institutes of Health 2013
Ruth Pike Scholarship, Pennsylvania State University 2012-13
Wrigley Science Institute Predoctoral Fellowship, American Society for Nutrition 2012
Graduate Research Exhibition 2nd place Award, Pennsylvania State University 2012
Graham Fellowship Award, Pennsylvania State University 2010-11