AN ABSTRACT OF THE DISSERTATION OF

Jane V. Higdon for the degree of Doctor of Philosophy in Nutrition and Food Management presented on November 23, 1999. Title: The Effect of Oleate, Linoleate, and EPA/DHA Supplementation of Postmenopausal Women on In Vivo Lipid Peroxidation and LDL Susceptibility to Ex Vivo Oxidation.

Abstract approved: ____________________________

Rosemary C. Wander

While replacement of dietary saturated fat with unsaturated fat has been advocated to reduce cardiovascular disease risk, diets high in polyunsaturated fatty acids (PUFA) could increase low density lipoprotein (LDL) susceptibility to oxidation, potentially contributing to the pathology of atherosclerosis. To assess in vivo lipid peroxidation and susceptibility of LDL surface and core lipids to ex vivo oxidation, in women consuming increased amounts of specific unsaturated fatty acids, 15 postmenopausal women took daily supplements of sunflower oil providing 12.3 g/day of oleate, safflower oil providing 10.5 g/day of linoleate, and fish oil providing 2.0 g/day of eicosapentaenoate (EPA) and 1.4 g/day of docosahexaenoate (DHA) during a crossover trial. Plasma F2-isoprostanes (F2-isoP), malondialdehyde (MDA), and thiobarbituric acid reacting substances (TBARS) were measured to assess lipid peroxidation in vivo. Ex vivo oxidation of LDL was monitored by measuring the formation of phosphatidylcholine hydroperoxides.
(PCOOH) and cholesteryl linoleate hydroperoxides (CE18:2OOH) during copper-mediated oxidation. Plasma free F2-isoP and MDA concentrations were lower after EPA/DHA supplementation than after oleate (P = 0.001, F2-isoP and 0.02, MDA) and linoleate supplementation (P = 0.04 for both F2-isoP and MDA). However, plasma TBARS concentrations were higher after EPA/DHA than after oleate (P = 0.001) and linoleate supplementation (P = 0.0004). During LDL oxidation, the lag phase for PCOOH formation was shorter in EPA/DHA- than oleate- (P = 0.0001) and linoleate-enriched LDL (P = 0.002), while the lag phase for CE18:2OOH was shorter in EPA/DHA- than oleate- (P = 0.01) but not linoleate-enriched LDL. The maximal rate of PCOOH formation was lower in EPA/DHA- than linoleate- (P = 0.007) but not oleate-enriched LDL, while the maximal rate of CE18:2OOH formation was lower in EPA/DHA- than oleate- (P = 0.03) and linoleate-enriched LDL (P ≤ 0.0001). The maximal concentrations of PCOOH and CE18:2OOH were lower in EPA/DHA- than oleate- (P ≤ 0.05) and linoleate-enriched LDL (P ≤ 0.01). Oleate-enrichment generally decreased the oxidative susceptibility of LDL surface and core lipids, while EPA/DHA-enrichment did not increase LDL oxidative susceptibility compared to linoleate-enrichment. This study emphasizes the need for more than one relevant assay of in vivo lipid peroxidation.
The Effect of Oleate, Linoleate, and EPA/DHA Supplementation of Postmenopausal Women on In Vivo Lipid Peroxidation and LDL Susceptibility to Ex Vivo Oxidation

by

Jane V. Higdon

A DISSERTATION

submitted to

Oregon State University

in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

Presented November 23, 1999
Commencement June 2000
Doctor of Philosophy dissertation of Jane V. Higdon presented on
November 23, 1999

APPROVED:

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I understand that my dissertation will become part of the permanent collection of
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Jane V. Higdon, Author
CONTRIBUTION OF AUTHORS

Dr. Rosemary C. Wander was involved in the design, analysis, and writing of each manuscript. Shi-Hua Du assisted in the data collection for each manuscript. Drs. Jason D. Morrow, Jiankang Liu, and Bruce N. Ames assisted in the data collection for and the writing of the first manuscript (Chapter 3). Dr. Ye-Sun Lee and Tianying Wu assisted in the data collection for the second manuscript (Chapter 4).
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CHAPTER 1

INTRODUCTION

By definition aerobic organisms cannot survive without oxygen. Because O₂ is potentially toxic, as well as mutagenic, aerobic organisms cannot survive without antioxidant defense systems. The potential for O₂ toxicity is derived from its propensity to form a number of reactive oxygen species (ROS), which are capable of reacting with and damaging vital molecules, including proteins, DNA, and lipids. Ideally the production of ROS should be balanced by an organism’s antioxidant defenses. The term, “oxidative stress,” was first introduced by Sies in 1985. Oxidative stress occurs when there is an imbalance between pro- and antioxidants, leading to potential oxidative damage (Halliwell & Gutteridge, 1999). Thus, oxidative stress can result from conditions that increase the generation of ROS or conditions that diminish the antioxidant defenses in some manner.

Oxidative damage is thought to contribute to the pathology of a number of diseases, including atherosclerosis and cancer (McCall & Frei, 1999). The balance between pro- and antioxidants in an organism can be disrupted by an increased presence of ROS, either extrinsic or intrinsic to the organism. For example toxins may be ROS themselves, or they may be metabolized by an organism in a manner
that generates ROS. Additionally, physiologic responses, such as the activation of the immune system, may result in excessive production of ROS as in tissue injury or chronic inflammatory disease (Halliwell, Gutteridge, & Cross, 1992).

Oxidative damage may also result if the capacity of an organism’s antioxidant systems is exceeded by the generation of ROS. Antioxidant systems include: 1) enzymes that catalytically inactivate ROS, 2) proteins that sequester pro-oxidants such as transition metal ions, 3) low-molecular weight scavengers of ROS which can be synthesized or obtained in the diet, and 4) mechanisms for the repair of oxidative damage to DNA, lipids, and proteins (Halliwell & Gutteridge, 1999).

Because of the role oxidative stress appears to play in a number of chronic diseases, as well as the potential for nutritional and pharmacological intervention, the assessment of oxidative stress in humans has received a considerable amount of attention from researchers. The reaction of ROS with cellular macromolecules can lead to a number of different products, which are subject to decomposition or metabolism (de Zwart, Meerman, Commandeur, & Vermeulen, 1999). Therefore, the potential mechanism for and likely targets of oxidative damage must be considered in the selection of assays of oxidative stress with respect to a particular disease entity.

Lipids in cell membranes as well as those present in lipoproteins may be oxidized by ROS, resulting in a chain reaction of lipid peroxidation capable of damaging cellular membrane proteins and ion channels (Richter, 1987), as well as
modifying LDL (Steinberg, Parthasarathy, Carew, Khoo, & Witztum, 1989). LDL, modified by oxidation, has been shown to stimulate changes on a cellular level that may account for the development or at least the acceleration of atherosclerosis (Steinberg, 1997). For these reasons, biomarkers of lipid peroxidation have been utilized to measure oxidative stress in living organisms.

Lipids vary in their susceptibility to peroxidation in that not all fatty acids are equally susceptible to abstraction of a hydrogen atom by ROS (Cosgrove, Church, & Pryor, 1987). Bis-allylic hydrogens are most easily abstracted from unsaturated fatty acids, because the weakest carbon-hydrogen bonds are those at the bis-allylic methylene positions (between 2 double bonds) (Gardner, 1989). In studies of homogeneous solutions of purified fatty acids, the number of bis-allylic methylene positions and the susceptibility of fatty acids to oxidation correlate positively (Cosgrove et al., 1987). Based on this research it has been suggested that highly unsaturated fatty acids, i.e., fatty acids with more double bonds, may be more susceptible to oxidation than less saturated fatty acids in vivo also.

Increased dietary intake of specific unsaturated fatty acids results in increased plasma and tissue concentrations of those same fatty acids (Katan, Deslypere, van Birgelen, Penders, & Zegwaard, 1997). If fatty acids oxidized in vivo are similar in their relative oxidative susceptibilities to fatty acids oxidized in homogeneous systems in vitro, individuals who consume more dietary unsaturated fatty acids might be at greater risk of oxidative stress with possible pathological consequences. Even in models only slightly more complex than homogeneous
solutions, e.g., in vitro oxidation of aqueous micelles, it is not clear that a direct correlation between the oxidative susceptibility of lipids and their degree of unsaturation is maintained (Yazu, Yamamoto, Ukegawa, & Niki, 1996). In vivo, the relative oxidative susceptibilities of unsaturated fatty acids are complicated further by the effects of highly unsaturated fatty acids on membrane structure and function, as well as on the function of an individual's antioxidant and detoxification systems.

Concern regarding the susceptibility of unsaturated dietary fatty acids to oxidation in vivo is relevant at a time when dietary recommendations aimed at reducing morbidity and mortality from cardiovascular disease promote the substitution of unsaturated fatty acids for saturated fatty acids (LaRosa et al., 1990). The effect of dietary unsaturated fatty acids on the oxidative susceptibility of LDL must also be considered given the large body of research supporting the theory that the oxidation of LDL plays a role in the development of atherosclerosis (Steinberg, 1997). Epidemiological studies have found that diets rich in the monounsaturated fatty acid (MUFA), oleic acid (18:1n-9), are associated with decreased mortality from cardiovascular disease compared to modern western diets that are high in the omega-6 (n-6) polyunsaturated fatty acid (PUFA), linoleic acid (18:2n-6) (Helsing, 1995; Keys et al., 1986). In contrast, diets rich in the highly unsaturated n-3 PUFA, eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3) due to the consumption of fish have also been associated with significant decreases in mortality from cardiovascular disease (Daviglus et al., 1997;
Kromhout, Bosschieter, & de Lezenne Coulander, 1985; Zhang, Sasaki, Amano, & Kesteloot, 1999). If the relative oxidative susceptibilities of oleate, linoleate, and EPA/DHA demonstrated in homogeneous systems in vitro were maintained when incorporated into tissue and lipoproteins in vivo, oxidative stress would be highest when the diet was rich in EPA/DHA and lowest when the diet was rich in oleate.

These issues are of particular relevance to postmenopausal women for whom cardiovascular disease is the major cause of mortality in the United States (Manolio et al., 1993). Until quite recently women have been underrepresented in studies of cardiovascular disease risk, despite evidence that findings regarding cardiovascular disease in men cannot always be extrapolated to women (Thaul & Hotra, 1993).

The purpose of the research described in this dissertation was to determine the effects of increased consumption of oleate, linoleate, and EPA/DHA by postmenopausal women on oxidative stress in vivo, as well as the susceptibility of LDL to ex vivo oxidation.
The Oxidative-modification Hypothesis of Atherosclerosis

The development and progression of atherosclerosis is a complex process which progresses through a series of stages beginning with increased endothelial permeability, resulting in influx of LDL and recruitment of monocytes (Ross, 1993). Early atherosclerotic lesions can be identified in childhood (Berenson, Srinivasan, & Nicklas, 1998). These fatty streaks are composed mainly of foam cells, which are essentially lipid-engorged monocyte/macrophages. Proliferation and migration of intimal smooth muscle results in an elevated intimal lesion, known as atherosclerotic plaque. Further lipid accumulation (predominantly LDL) and cell death possibly related to the cytotoxic effects of oxidized lipids result in plaque that is vulnerable to disruption or rupture. Plaque disruption is generally the initial event in coronary thrombus formation, the critical factor in the mortality and morbidity from coronary heart disease (Dalager-Pedersen, Ravn, & Falk, 1998).

Over the past 20 years, a large body of evidence has accumulated in support of the hypothesis that oxidatively modified LDL plays an important role in the pathogenesis of atherosclerosis (Tribble, 1999). According to this hypothesis LDL accumulate in the arterial subendothelial space and become mildly oxidized by resident vascular cells (Diaz, Frei, Vita, & Keaney, 1997). So called “minimally modified” LDL stimulate the resident vascular cells to produce factors that attract
monocytes (monocyte chemotactic protein 1; MCP1) and stimulate their differentiation to macrophages (macrophage colony stimulating factor; MCSF). Accumulating monocytes and macrophages can promote further oxidation of LDL, resulting in oxidative modification to apolipoprotein B-100 (apo-B). The resultant changes in apo-B, such as increased negative charge, allow the more fully oxidized form of LDL to be recognized and taken up by macrophage scavenger receptors (Navab et al., 1996). Unlike the LDL receptor, the scavenger receptor is not down-regulated when cellular cholesterol content increases. Thus oxidized LDL may account for the formation of the lipid-laden foam cells found in fatty streaks (Steinberg, 1997). Other proatherogenic properties of oxidized LDL include but are not limited to 1) cytotoxicity toward cultured endothelial cells (Hessler, Morel, Lewis, & Chisolm, 1983), 2) inhibition of nitric oxide (NO) induced vasodilation (Chin, Azhar, & Hoffman, 1992), and 3) stimulation of intimal smooth muscle cell proliferation (Chatterjee, 1998).

Although the majority of support for the oxidative modification hypothesis of atherosclerosis is based on investigations performed in vitro there also exists evidence that LDL oxidation is related to the presence and severity of atherosclerosis in vivo. Oxidized LDL can be detected in the lipoprotein fraction extracted from human atherosclerotic lesions but not from plasma (Yla-Herttuala et al., 1989). Antibodies generated against oxidized LDL react with antigens in atherosclerotic lesions but not with those in normal arterial segments (Palinski et al., 1989). Higher plasma concentrations of immunoreactive oxidized LDL were
demonstrated in patients having acute myocardial infarctions than in control subjects (Holvoet et al., 1995). Additionally, higher levels of autoantibodies that react with oxidized LDL have been found in patients with carotid atherosclerosis (Salonen et al., 1992) and in patients with angiographically demonstrated coronary artery disease (Lehtimaki et al., 1999) than in control subjects. However, it is unclear from these studies whether oxidized LDL plays a critical role in the etiology of atherosclerosis or whether it results from the inflammatory process accompanying atherosclerosis.

**LDL Oxidation**

Human LDL are large spherical particles with diameters of 19-25 nm and molecular weights between 1.8 and 2.8 million. According to Esterbauer et al (1992), one average LDL particle with a molecular weight of 2.5 million would contain approximately 1600 molecules of cholesteryl ester and 170 molecules of triacylglycerol in its central lipophilic core. The core would be surrounded by a monolayer of 700 phospholipid molecules (~64% phosphatidylcholine, 7% lysophosphatidylcholine, and 26% sphingomyelin) and 600 free cholesterol molecules. The phospholipids are oriented with their polar heads toward the surface of the LDL particle, allowing it to travel in an aqueous solution (plasma). Embedded in the outer layer, but in contact with the core, is a large glycosylated protein (550 kDa), apolipoprotein B (apoB). The fatty acid content of LDL may vary considerably due to the dietary content of individual fatty acids, but as much as half of LDL fatty acids are unsaturated and therefore susceptible to oxidation.
LDL contain a number of lipophilic antioxidants, including α-tocopherol, present in the largest quantities (~6 molecules per LDL particle in unsupplemented individuals), as well as γ-tocopherol, carotenoids, and ubiquinol 10, in much smaller concentration, e.g., ~50% of LDL particles contain ubiquinol 10 (Halliwell & Gutteridge, 1999).

It is unclear exactly how LDL oxidation takes place in the vessel wall, but 2 distinctly different models of LDL oxidation in vitro have been proposed. The first model will be referred to as the classical model, which was developed by Esterbauer and colleagues (Esterbauer, Striegl, Puhl, & Rotheneder, 1989). This model was first developed for copper-mediated LDL oxidation, but has been applied to a number of other LDL oxidation systems. Monitoring the kinetics of LDL oxidation mediated by a relatively high concentration of Cu$^{2+}$ (10 mol Cu$^{2+}$/mol LDL), resulting in a relatively high radical flux reveals 3 consecutive time phases: 1) the lag phase, 2) the propagation phase, and 3) the decomposition phase. During the lag phase LDL are gradually depleted of antioxidants, while only minimal lipid peroxidation occurs. Once LDL antioxidants have been depleted, lipid peroxidation accelerates to a rapid rate in the propagation phase. Ultimately, the rate of lipid hydroperoxide decomposition matches and then overtakes the rate of formation, resulting in a plateau and a decomposition phase.
Figure 2.1. Classical LDL oxidation kinetics. The formation of cholesteryl ester hydroperoxides during copper-mediated LDL oxidation is plotted over time. Lag, propagation, and decomposition phases are illustrated with broken lines.

The chemistry of lipid peroxidation thought to take place in the classical model of LDL oxidation is based on studies of the oxidation of purified fatty acids in homogeneous systems. As depicted in figure 2.2, the oxidation of unsaturated fatty acids is a free radical-mediated process consisting of 3 events: (1) initiation, (2 & 3) propagation, and (4) termination (Wagner, Buettner, & Burns, 1994).

\[
\begin{align*}
L-H + X^\bullet & \rightarrow L^\bullet + X-H \\
L^\bullet + O_2 & \rightarrow LOO^\bullet \\
LOO^\bullet + L-H & \rightarrow L^\bullet + LOOH \\
LOO^\bullet + LOO^\bullet/L^\bullet & \rightarrow \text{Non-radical product (NRP)}
\end{align*}
\]

Figure 2.2. Theoretical model of lipid peroxidation by a free radical process. (1) A sufficiently energetic radical oxidant, X\^\bullet, abstracts a hydrogen atom from a lipid, L\^\bullet, resulting in a carbon-centered radical, L\^\bullet. L\^\bullet reacts rapidly with O\_2 to produce a lipid peroxyl radical, LOO\^\bullet (2), which is the chain carrying radical of the propagation cycle (3). The propagation cycle continues until (4) termination results in the formation of non-radical product (NRP) (Wagner et al., 1994).
In the classical model of LDL oxidation, also derived from studies of homogeneous systems, α-tocopherol acts as an antioxidant, inhibiting lipid peroxidation until depleted from LDL (Upston, Terentis, & Stocker, 1999).

\[
\begin{align*}
\alpha\text{-TOH} + X^* & \rightarrow \alpha\text{-TO}^* + XH \quad (1) \\
\alpha\text{-TOH} + \text{LOO}^* & \rightarrow \alpha\text{-TO}^* + \text{LOOH} \quad (2) \\
\alpha\text{-TO}^* + \text{LOO}^*/X^* & \rightarrow \text{Non-radical product (NRP)} \quad (3)
\end{align*}
\]

**Figure 2.3.** Theoretical model of α-tocopherol antioxidant action in the classical model of LDL oxidation. (1) Inhibition of lipid peroxidation by the reaction of α-tocopherol (α-TOH) with radical oxidant (X*) to form α-tocopheroxyl radical (α-TO•) and inactive oxidant (XH). (2) Inhibition of lipid peroxidation by the reaction of α-TOH with a lipid peroxyl radical (LOO*) to form α-TO• and lipid hydroperoxide (LOOH). (3) Reaction of α-TO• with LOO* to form non-radical product (NRP) (Upston et al., 1999).

The mechanism for initiation of lipid peroxidation in Cu\(^{2+}\)-mediated LDL oxidation remains somewhat unclear. Originally it was assumed that Cu\(^{2+}\) ions act by decomposing preexisting peroxides in LDL to the chain propagating peroxyl and alkoxy radicals: 1) \(\text{LOOH} + \text{Cu}^{2+} \rightarrow \text{LOO}^* + \text{Cu}^+ + \text{H}^+\) and 2) \(\text{LOOH} + \text{Cu}^+ \rightarrow \text{LO}^* + \text{Cu}^{2+} + \text{OH}^-\) (Halliwell & Gutteridge, 1999). However, methods for isolating LDL from plasma, which yield LDL preparations that have a full complement of antioxidants and no detectable lipid hydroperoxides (level of detection = 1 mol CEOOH/ 200 LDL particles) have been developed (Shwaery, Mowri, Keaney, & Frei, 1999), and Cu\(^{2+}\) readily induces oxidation in LDL prepared in this manner (Frei & Gaziano, 1993). The mechanism by which Cu\(^{2+}\)
initiates oxidation in the absence of preformed lipid hydroperoxides has been only partially defined, but Cu\(^{2+}\) binding to LDL (primarily apolipoprotein B) and reduction to Cu\(^{+}\) are required (Retsky, Chen, Zeind, & Frei, 1997). LDL associated \(\alpha\)-tocopherol has been proposed as a potential Cu\(^{2+}\) reductant, but no correlation has been found between LDL \(\alpha\)-tocopherol content and Cu\(^{2+}\) reduction (Proudfoot, Croft, Puddey, & Beilin, 1997).

**Tocopherol-mediated Peroxidation**

In vitro, under conditions of relatively low radical flux (in the case of copper-mediated oxidation < 3 mol Cu\(^{2+}\)/mol LDL), \(\alpha\)-tocopherol appears capable of acting as a prooxidant. Investigators at The Heart Research Institute (Sydney, Australia) have developed a model for tocopherol-mediated peroxidation (TMP), which takes into account the heterogeneous nature of the LDL particle, i.e., the polar surface and lipophilic core (Neuzil, Thomas, & Stocker, 1997). TMP is initiated when an aqueous radical oxidant is scavenged by \(\alpha\)-tocopherol (Figure 2.4; reaction 1), a reaction that is thermodynamically favored over abstraction of bis-allylic hydrogens from LDL core or surface lipids. Under conditions of high radical flux the probability is high that a second \(X^*\) will enter the LDL particle and react with \(\alpha\)-TO\(^*\), resulting in termination of lipid peroxidation through the formation of NRP (Figure 2.4; reaction 2). Thus, conditions of high radical flux would give rise to the lag phase observed in the model of classical lipid peroxidation. \(\alpha\)-TO\(^*\) is less polar than \(\alpha\)-TOH, allowing it to diffuse to the lipophilic core of the LDL particle. Under conditions of low radical flux \(\alpha\)-TO\(^*\) may
diffuse to the core, react with LH in the presence of O\textsubscript{2}, and produce LOO• + \(\alpha\)-TOH (Fig 2.4; reaction 3). Reaction of LOO• with \(\alpha\)-TOH gives rise LOOH + \(\alpha\)-TOO• (Figure 2.4; reaction 4). In this manner \(\alpha\)-tocopherol becomes the lipid peroxidation chain-carrying molecule. Such a model explains why under conditions of low radical flux, LDL lipids may be oxidized in the presence of \(\alpha\)-tocopherol. TMP may be prevented if coantioxidants such as ubiquinol-10 or ascorbate are able to transfer the radical character back to the aqueous environment (Upston et al., 1999).

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Equation</th>
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<tbody>
<tr>
<td>(\alpha)-TOH + X•</td>
<td>(\alpha)-TO• + XH</td>
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<tr>
<td>(\alpha)-TO• + X•</td>
<td>NRP</td>
</tr>
<tr>
<td>(\alpha)-TO• + LH</td>
<td>L•</td>
</tr>
<tr>
<td>L• + O\textsubscript{2}</td>
<td>LOO•</td>
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<tr>
<td>(\alpha)-TOH + LOO•</td>
<td>(\alpha)-TO• + LOOH</td>
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**Figure 2.4.** Reactions occurring in LDL during tocopherol-mediated peroxidation (TMP). Initiation of TMP occurs when an aqueous radical oxidant (X•) is scavenged by \(\alpha\)-tocopherol (\(\alpha\)-TOH), and an \(\alpha\)-tocopheroxyl radical (\(\alpha\)-TO•) is formed (1). Bimolecular termination reaction occurs under conditions of high radical flux (2). Under conditions of low radical flux \(\alpha\)-TO• diffuses to the LDL core where it reacts with bisallylic hydrogens in LH to form a peroxyl radical (LOO•) (3 & 4). Reaction of LOO• with another \(\alpha\)-TOH gives rise to lipid hydroperoxide (LOOH) and \(\alpha\)-TOO• (5). The cyclic nature of reactions 3, 4, and 5 allows the formation of many LOOH with little \(\alpha\)-TOH consumption (Upston et al., 1999).

While neither model (classical or TMP) has been proven to occur in vivo, several aspects of the TMP model are relevant to what is known regarding
oxidation conditions in the arterial wall. Potential oxidants in the arterial wall include myeloperoxidase, lipoygenase, metal ions, and reactive nitrogen species, all of which have been demonstrated to cause TMP in vitro. Although the rate of radical formation in the artery wall is unknown, it is not unreasonable to assume that it is low, allowing LDL oxidation in the presence of α-tocopherol (Upston et al., 1999). There is some evidence that intimal lipid peroxidation occurs in the presence of vitamin E. Evaluation of human atherosclerotic plaque reveals α-tocopherol concentration comparable to that of plasma, despite the presence of oxidized lipids (Suarna, Dean, May, & Stocker, 1995). Free radical-induced oxidation of linoleate generates 4 stereoisomers (2 cis,trans and 2 trans, trans). The presence of lipophilic hydrogen donors such as α-tocopherol influences the relative distribution of those isomers. In the presence of α-tocopherol cis, trans (Z,E) hydroperoxide isomers are primarily formed, while in the absence of α-tocopherol more stable trans, trans (E,E) isomers are preferentially formed (Upston et al., 1999). Therefore, recent findings of a relative abundance of the cis, trans (ZE) isomers of cholesteryl linoleate hydroperoxide in human carotid atherosclerotic lesions suggest oxidation in the presence of α-tocopherol (Upston, Neuzil, & Stocker, 1996).

The Relative Oxidizability of Specific PUFA

Generally, the more double bonds present in an unsaturated fatty acid, the more readily it is assumed to oxidize in biological systems. These assumptions are based on the results of investigations into the autoxidation of unsaturated fatty
acids in homogeneous systems in vitro. In 1947 Holman and Elmer measured autooxidation of neat methyl and ethyl esters, without added initiator, by measuring O₂ absorption. They found linoleate to be 40 times more reactive than oleate, linolenate 2.4 times more reactive than linoleate, and arachidonate (20:4n-6) 2 times more reactive than linolenate (Holman & Elmer, 1947). Forty years later investigators determined relative oxidizability of a series of PUFAs in homogeneous chlorobenzene solution by examining the kinetics of oxidation initiated by thermal decomposition of azo initiators (diazo compounds which decompose to produce 2 carbon centered radicals at a known constant rate). A measure of the ease with which a fatty acid underwent autooxidation, referred to as oxidizability, was calculated as the ratio of 2 rate constants: \( k_p/(2k_t)^{1/2} \)

where \( k_p \) represented the rate constant for the propagation reaction:

\[
\text{LOO}^\cdot + \text{LH} \quad \longrightarrow \quad \text{LOOH} + \text{L}^\cdot
\]

and \( k_t \) represented the rate constant for the termination reaction:

\[
2 \text{LOO}^\cdot \quad \longrightarrow \quad \text{NRP}.
\]

They found oxidizability to be linearly related to the number of bis-allylic methylene positions present in PUFA, e.g. the oxidizability of DHA (22:6n-3) was 5 times greater than that of linoleate (18:2n-6) (Cosgrove et al., 1987). For lipid peroxidation, the rate of propagation is determined by the various carbon-hydrogen bond dissociation energies along the fatty acid chain. The weakest carbon-hydrogen bonds are those between 2 double bonds, i.e., at the bis-allylic methylene positions (Gardner, 1989). Therefore it is not surprising that oxidative
susceptibility in homogeneous systems increased with the number of double bonds present in the PUFA.

Studies of multiphase in vitro systems, which may be more similar to LDL than homogeneous systems, suggest oxidative susceptibility may not be directly related to the degree of unsaturation of a fatty acid. A study of the photooxidation of emulsions of PUFA in aqueous solution found that oxidation of EPA and DHA, with 5 and 6 double bonds respectively, resulted in lower yields of malondialdehyde than arachidonic acid (AA) which possesses only 4 double bonds (Bruna, Petit, Beljean-Leymarie, Huynh, & Nouvelot, 1989).

Yazu et al. (1996) demonstrated that methyl esters of EPA (5 double bonds) are oxidized less rapidly by azo-initiators, in aqueous micelles than are linoleate esters (2 double bonds). Measurement of O₂ uptake during oxidation indicated that EPA reacted with twice as much O₂ as linoleate. This would suggest that they formed bicycloendoperoxides and hydroperoxides, respectively. EPA-derived bicycloendoperoxides, containing 2 molecules of O₂, would be more polar than linoleate hydroperoxides, containing one molecule of O₂. Thus, EPA-derived peroxyl radicals would be likely to localize at the surface of the micelle, rather than in the hydrophobic core, enhancing the rate of termination and reducing the rate of propagation since there is less oxidizable EPA at the surface than in the core (Yazu et al., 1996).

The effect of 3 different antioxidants, with different site reactivities in aqueous micelles, on the oxidizability of methyl linoleate and methyl EPA supports
the idea that peroxyl radicals derived from EPA tend to localize at the surface of
the micelle rather than in the core. The antioxidant di-tert-butyl-4-methyl phenol
(BHT) localizes preferentially in the micelle core, 2,2,5,7,8-pentamethyl-6-
chromanol (PMC) localizes at the micelle surface, and carboxy-2,5,7,8-tetra-
methyl-6-chromanol (Trolox) tends to remain in the aqueous phase. In methyl
linoleate micelles containing these antioxidants the length of the induction phase, a
measure of the efficacy of an antioxidant, decreased in the order: BHT > PMC >
Trolox. However, BHT did not inhibit the oxidation of methyl EPA efficiently in
micelles indicating that EPA-derived peroxyl radicals preferentially localized at the
micelle surface (Yazu et al., 1998).

The presence of EPA and linoleate in a 1:1 ratio in aqueous micelles
reduced the oxidation of total substrate (EPA and linoleate) 5-fold compared with
linoleate alone. The above data indicate that in the presence of linoleate, the
increased polarity of EPA-derived-peroxyl radicals enhances their diffusion to the
micelle surface, increasing the termination reaction rate, decreasing the propagation
rate, and ultimately decreasing the rate of total substrate oxidation in the
EPA/linoleate micelles (Yazu et al., 1996). The LDL particle consists of an
amphipathic surface monolayer and a hydrophobic core, suggesting it is more
likely to behave similarly to the multiphase system of the aqueous micelle than to
homogeneous solutions of fatty esters.

The effect of specific PUFA on oxidative susceptibility in biological
systems has also been studied in cell culture. In one set of studies the content of
specific PUFA was increased in L1210 murine leukemia cells by adding it to the culture media for 48 hours prior to inducing oxidation. During oxidation O₂ uptake was measured and lipid-derived free radical generation was monitored using electron paramagnetic resonance spin trapping. The rate of oxygen uptake and lipid radical generation increased exponentially with the number of bis-allylic hydrogen positions in the cellular lipids (Wagner et al., 1994). Oxidation of the same line of cells after they had been supplemented with specific PUFA and α-tocopherol resulted in a rate of α-tocopherol loss that was 10 fold greater in DHA-enriched cells than oleate-enriched cells (Kelley, Buettner, & Burns, 1995). The results of a study, in which human umbilical vein endothelial cells were incubated for 7 days with different PUFA and subsequently oxidized using Cu²⁺, were less definitive. The loss of PUFA from cellular phospholipids during oxidation was positively and directly related to the number of bis-allylic methylene positions, but conjugated diene formation was greater in linoleate than EPA or DHA incubated cells (Vossen, van Dam-Mieras, Hornstra, & Zwaal, 1995). Although these data could suggest increased oxidative susceptibility in linoleate-enriched endothelial cells, compared to EPA- and DHA-enriched cells, the conflicting results for the loss of PUFA from phospholipids make drawing a clear conclusion difficult.

**Specific PUFA and the Measurement of Oxidative Susceptibility**

Because lipid peroxidation is a complex, multistage process, many techniques have been developed for measuring it. However, no single method yet developed can be considered an accurate index of overall lipid peroxidation. Each
assay measures a different aspect of the lipid peroxidation process. Therefore the choice of methods should reflect the mechanism of interest, and more than one assay of lipid peroxidation should be applied whenever possible (Halliwell & Chirico, 1993).

Because lipid peroxidation results in the loss of unsaturated fatty acid side chains, one way to follow the oxidation of specific PUFA is to measure the loss of individual fatty acids. In order to measure individual fatty acids lipids must be extracted from the system under study, and the lipids must be hydrolyzed to release fatty acids which can then be measured using HPLC or gas chromatography (GC). As with almost all assays of lipid peroxidation, careful technique is required to avoid artifactual peroxidation during the assay (Halliwell & Gutteridge, 1999).

When a PUFA molecule is oxidized, a hydrogen atom is most likely to be abstracted from a carbon atom situated between 2 double bonds. The resultant carbon-centered radical is stabilized by molecular rearrangement creating a conjugated diene which absorbs UV light at 234 nm, and is the basis for the conjugated diene assay. In the presence of O₂, this conjugated dienoic carbon-centered radical (L•) forms a lipid hydroperoxyl radical (LOO•) and is capable of abstracting a hydrogen atom from another PUFA to form a lipid hydroperoxide (LOOH) and another L•. Thus continuous monitoring of conjugated diene formation reflects events occurring early in the lipid peroxidation process (Halliwell & Chirico, 1993). While conjugated dienes reflect the formation of lipid hydroperoxides, not all conjugated dienes are lipid hydroperoxides, and not all lipid
hydroperoxides are conjugated dienes, e.g. the hydroperoxide formed from the MUFA, oleate. Although continuous monitoring of conjugated diene formation can be effectively utilized in monitoring the oxidation of pure lipids, membrane fractions, and isolated lipoproteins, application of this assay to body fluids has been problematic due to the presence of other molecules with significant absorbance at 234 nm (Halliwell & Gutteridge, 1999). With respect to the measure of oxidative susceptibility in the presence of specific fatty acids, hydroperoxides of MUFA would not be measured. Moreover, differing rates of decomposition of specific LOOH could result in differences in net rate and maxima, which could not be distinguished from differing rates of formation (Frankel, 1998).

The assay of lipid hydroperoxides is a more specific approach to the measurement of the early events of lipid peroxidation. The application of high performance liquid chromatography (HPLC) with postcolumn chemiluminescence detection has resulted in sensitive and specific assays of hydroperoxides of the major lipid classes (phospholipids, cholesteryl esters, free cholesterol, and triglycerides) (Frei, Yamamoto, Niclas, & Ames, 1988). Unlike the conjugated diene assay, these assays measure LOOH directly. The lower level of detection of cholesteryl ester hydroperoxides (CEOOH), using this technique is approximately 10 pmol/ mg LDL protein (~ 1 molecule of CEOOH per LDL particle) and 0.1 nM in plasma. While this chemiluminescence technique is very useful for monitoring ex vivo oxidation, CEOOH, which are the most abundant LOOH detectable in LDL and plasma (Sattler, Mohr, & Stocker, 1994), are not generally detected in the
plasma of healthy individuals (Polidori et al., 1998), nor in carefully isolated LDL preparations (Shwaery et al., 1999). Thus, the use of these techniques to detect lipid peroxidation in vivo, while varying consumption of specific PUFA, is likely to be limited to those individuals already experiencing considerable oxidative stress. With respect to monitoring ex vivo oxidation, e.g. in LDL, modification of the HPLC separation for the LOOH assays described above offers the possibility of following the formation of hydroperoxides of specific PUFA using postcolumn chemiluminescence detection.

Because primary oxidation products such as lipid hydroperoxides are generally unstable under physiological conditions, practical assays of in vivo oxidation generally measure more stable secondary oxidation products (de Zwart et al., 1999). Specific secondary oxidation products do not result from the breakdown of all unsaturated fatty acids, and a number of different breakdown pathways are possible for a given unsaturated fatty acid. Consequently, examining the effect of specific dietary fatty acids on the formation of these decomposition products can be problematic.

One of the most frequently used assays of lipid peroxidation in biological samples and foods is the thiobarbituric acid (TBA) assay. Malondialdehyde (MDA) is an aldehyde formed during the decomposition of hydroperoxides of fatty acids with 3 or more double bonds (Frankel, 1998). In the TBA assay, MDA reacts to form a TBA adduct which absorbs light at 532 nm and fluoresces at 553 nm (Halliwell & Gutteridge, 1999). However, despite its widespread use, the TBA
assay has several drawbacks that make it especially unsuitable for use in vivo. Most of the thiobarbituric acid reacting substances (TBARS) detected by the assay are generated during the acid heating step of the assay. A number of substances found in biological samples absorb or fluoresce at the same wavelengths used to quantify the TBA-MDA adduct. Additionally, the conditions under which the assay is performed (the presence of metal ions or antioxidants) can greatly affect the color generation of the assay (Janero, 1990). These conditions tend to vary between laboratories, and care must be taken in the interpretation of the results of TBA assays performed under different conditions.

Recently a more sensitive and specific assay of MDA using gas chromatography/ negative chemical ion mass spectrometry (GC/MS) has been developed, which avoids some of the pitfalls of the TBA assay. This technique combines the specificity of mass spectrometry with relatively mild sample preparation, thus avoiding heat-generated artifacts encountered with the TBA assay (Yeo, Liu, Helbock, & Ames, 1999). In a study of MDA formation during in vitro oxidation of specific PUFA, varying in carbon chain length and number of double bonds, the GC/MS method was found to be 2-6 times more sensitive in detecting MDA than the TBA assay. However, in biological samples the TBA assay detected TBARS at concentrations 2-6 times higher than the GC/MS assay for MDA, likely due to the lack of specificity of the TBA assay, as well as artifactual production of MDA during the acid heating step (Liu, Yeo, Doniger, & Ames, 1997).
Because MDA is thought to result mainly from the oxidation of fatty acids with 3 or more double bonds, assays of MDA would be expected to measure the oxidation of arachidonate, EPA, and DHA, but not the oxidation of MUFA or PUFA with only 2 double bonds (oleate or linoleate). Thus, increased MDA concentrations may only reflect increased concentrations of PUFA with more than 2 double bonds. A number of different aldehydes are formed during the decomposition of LOOH. Some form mainly during the decomposition of n-3 PUFA hydroperoxides (propanal), while others are more likely to form during decomposition of n-6 PUFA (hexanal). Differential production of these aldehydes has already been explored using a rapid headspace gas chromatography method (Frankel et al., 1994). Additionally, the sensitive and specific GC/MS method applied to MDA has been applied to other aldehydes derived from lipid peroxidation, and holds promise for the assessment of in vivo lipid peroxidation of specific PUFA.

F₂-isoprostanes are prostaglandin-like products of nonenzymatic peroxidation of arachidonate. They are a well-established biomarker for in vivo oxidative stress, and have been shown to correlate with conditions of increased lipid peroxidation in animals and humans (Morrow & Roberts, 1999). In humans, elevated plasma F₂-isoprostanes have been demonstrated in conditions associated with enhanced oxidative stress such as chronic cigarette smoking (Morrow et al., 1995), hepatorenal syndrome, and systemic sclerosis (Morrow & Roberts, 1997). Increased concentrations of F₂-isoprostanes have been found in human
atherosclerotic lesions (Gniwotta, Morrow, Roberts, & Kuhn, 1997). Additionally, plasma F$_2$-isoprostane concentrations have been positively associated with plasma total homocyst(e)ine levels, an independent risk factor for cardiovascular disease (Voutilainen & et al., 1999).

Recently it has been demonstrated that the nonenzymatic oxidation of EPA (20:5n-3) in vitro results in a number of F$_3$-isoprostanes one of which (8-epi PGF$_{3\alpha}$) can be found in small amounts in plasma (Nourooz-Zadeh, Halliwell, & Anggard, 1997). Moreover, F$_4$-isoprostanes resulting from the nonenzymatic oxidation of DHA (22:6n-3) have been isolated in vitro and in vivo, and found to be elevated in the spinal fluid of humans with Alzheimer's disease (Roberts et al., 1998). In the future, the assessment of plasma concentrations of these 3 families of isoprostanes could provide insight into the relative contribution of individual PUFA to in vivo oxidative stress.

**Dietary Unsaturated Fatty Acids and LDL Oxidation**

The consistent positive association between dietary saturated fat content and plasma cholesterol concentrations (Mensink & Katan, 1992) has led a number of organizations to recommend that individuals reduce their consumption of saturated fatty acids (SFA) (LaRosa et al., 1990; National Cholesterol Education Program (NCEP), 1993). In addition to lowering total fat consumption, increasing the proportion of dietary fat derived from monounsaturated (MUFA) and polyunsaturated fatty acids (PUFA) has been advocated because such diets have
resulted in more favorable lipoprotein profiles, with respect to cardiovascular
disease risk (Berry et al., 1991; Gardner & Kraemer, 1995).

Substitution of n-6 PUFA for SFA has resulted in decreases of plasma LDL
cholesterol (LDL-C) concentrations, but has also been associated with less
desirable decreases in HDL cholesterol (HDL-C) (Berry et al., 1991; Gardner &
Kraemer, 1995). In some studies, substitution of MUFA for SFA resulted in
decreases in plasma LDL-C comparable to n-6 PUFA without the concomitant
decrease in plasma HDL-C (Mattson & Grundy, 1985). The traditional
Mediterranean diet, rich in the MUFA, oleic acid (18:1n-9), has been associated
with reduced mortality from cardiovascular disease (Helsing, 1995; Katan, Zock, &
Mensink, 1995).

Diets rich in fish have also been associated with decreased mortality from
cardiovascular disease (Daviglus et al., 1997; Kromhout et al., 1985; Zhang et al.,
1999). Although the mechanisms for this cardioprotective effect have yet to be
clarified, the highly unsaturated n-3 PUFA EPA and DHA may play a role.
Increased consumption of EPA and DHA consistently results in decreased plasma
triacylglycerol concentrations (Harris, 1989). Moreover, increased consumption of
EPA and DHA has been demonstrated to modulate inflammatory and thrombotic
responses as well as to decrease susceptibility to cardiac arrhythmias, all of which
may contribute to the cardioprotective effects of fish consumption (Simopoulos,
1996).
Because the oxidative modification of LDL appears to play a role in the pathology of atherosclerosis, understanding the factors affecting the oxidative susceptibility of LDL is critical to the investigation of therapeutic modalities for the prevention and treatment of atherosclerosis. The specific PUFA content of the LDL particle should be an important factor in determining its oxidative susceptibility because lipid appears to be the initial and most plentiful substrate for LDL oxidation (Reaven & Witztum, 1996).

A number of studies have demonstrated that, in humans, diets enriched in oleate compared to linoleate (the most common PUFA in the modern western diet) lead to LDL that are enriched in oleate and are less susceptible to ex vivo oxidation. The majority of these studies assessed LDL oxidative susceptibility by monitoring conjugated diene formation during copper-mediated oxidation. In several studies, oleate-enrichment of LDL resulted in decreased rate of formation as well as decreased maximal concentrations of conjugated dienes, over several hours of oxidation, when compared to linoleate-enrichment (Abbey, Belling, Noakes, Hirata, & Nestel, 1993; Reaven et al., 1991; Tsimikas et al., 1999). Other investigators have demonstrated lengthened lag phases in oleate-compared to linoleate-enriched LDL (Mata et al., 1996; Mata et al., 1997; Reaven et al., 1991). All of these findings indicate decreased oxidative susceptibility in oleate-enriched LDL. However, the use of the conjugated diene assay to compare oxidative susceptibilities of oleate and linoleate-enriched LDL should be questioned because oleate has only one double bond and unlike linoleate does not form a conjugated
diene when oxidized. Thus, diminished conjugated diene formation might only reflect decreased linoleate content rather than decreased oxidation. One study found increased maximal conjugated diene concentrations in oleate-compared to linoleate-enriched LDL (Carmena et al., 1996). However, the linoleate-rich supplement as well as the linoleate-enriched LDL was found to have higher antioxidant concentrations than its oleate-rich counterpart. Bonanome et al (1992) measured O2 consumption during oxidation of LDL with 2,2'-azobis(2-amidinopropane) hydrochloride (AAPH), a water-soluble diazo compound that generates free radicals by thermal decomposition. They found a lower rate of O2 consumption in oleate- compared to linoleate-enriched LDL, but no difference in lag phase.

The use of the TBA assay to assess MDA concentrations in LDL has met with contradictory results. Several studies showed decreased thiobarbituric acid reacting substances (TBARS) in oleate- compared to linoleate-enriched LDL (Berry et al., 1991; Mata et al., 1996; Mata et al., 1997), while one study showed no difference in native LDL, but increased formation of TBARS during copper-mediated oxidation of oleate-compared to linoleate-enriched LDL (Reaven et al., 1991). Several precursors for MDA have been proposed, including mono and bicycloendoperoxides, which result from the oxidation of PUFA containing 3 or more double bonds. Consequently the TBA assay is less sensitive when used to assay the oxidation of lipids containing mainly oleate and linoleate (Frankel, 1998). Viewed together most of the current research suggests that oleate-enrichment of
LDL decreases its oxidative susceptibility. However, the research has been limited by some of the methods that have been used to measure oxidation in the LDL particle.

The effect of increased consumption of n-3 PUFA on LDL oxidative susceptibility in humans is less clear. A number of studies have evaluated LDL oxidative susceptibility by continuous monitoring of conjugated diene formation during copper mediated oxidation. While several investigators demonstrated shorter lag phases in EPA/DHA-enriched LDL, suggesting increased oxidative susceptibility, they also found slower rates of diene formation, suggesting decreased oxidative susceptibility (Sorensen, Marckmann, Hoy, van Duyvenvoorde, & Princen, 1998; Suzukawa, Abbey, Howe, & Nestel, 1995; Wander, Du, Ketchum, & Rowe, 1996). One study of EPA/DHA supplementation that demonstrated shorter lag phase and slower rate of conjugated diene formation found that LDL linoleate content was the primary predictor of indices of oxidative susceptibility by multiple regression analysis, despite enrichment of LDL with EPA/DHA (Wander, Du, & Thomas, 1998).

Other investigators found that increasing EPA/DHA consumption provided no evidence of increased susceptibility of LDL to ex vivo oxidation. Bonanome et al (1996) monitored conjugated diene formation during AAPH-mediated LDL oxidation, and found no change in lag phase, rate, or maximum diene concentration after EPA/DHA supplementation of hypertriacylglycerolemic men. Brude et al (1997) found no difference in LDL oxidation whether it was copper-mediated or
cell-mediated in male smokers supplemented with EPA/DHA. Moreover, they found a decreased rate of conjugated diene formation during AAPH-induced oxidation after EPA/DHA supplementation. Nenseter et al (1992) supplemented men and women with EPA/DHA and found no difference in maximum conjugated diene concentration during copper-mediated oxidation, nor in the degradation of oxidized LDL by cultured macrophages. A study that examined the formation of volatile oxidation products of n-3 PUFA (propanal) and n-6 PUFA (hexanal) during copper mediated oxidation of LDL found increased propanal formation in the LDL of individuals fed fish oil high n-3 PUFA and increased hexanal formation in the LDL of individuals fed corn oil high in n-6 PUFA. However, the total volatile oxidation products did not differ between the two groups, suggesting increased n-3 PUFA consumption did not increase LDL oxidative susceptibility (Frankel et al., 1994).

In a porcine atherosclerosis model, Whitman et al (1994) found that increased EPA/DHA consumption led to LDL that demonstrated shorter lag times but slower rates of conjugated diene formation during copper-mediated oxidation. However, EPA/DHA supplementation resulted in no difference in atherosclerotic lesion development in all major blood vessels. In the same animal model, EPA/DHA supplementation of animals on a diet designed to enhance the regression of atherosclerotic lesions experienced significant atherosclerotic lesion regression in all major vessels despite the finding of shortened lag phase in EPA/DHA-enriched LDL compared to control (Barbeau, Klemp, Guyton, & Rogers, 1997).
A number of investigators have attempted to measure in vivo lipid peroxidation in individuals consuming increased EPA/DHA. Again, the results are contradictory. Early studies employed the plasma TBA assay, which lacks specificity and is prone to artifactual oxidation (Yeo et al., 1999; Janero, 1990). Several studies have demonstrated increased plasma and urinary concentrations of TBARS in individuals consuming increased EPA/DHA, and concluded that a diet rich in highly unsaturated n-3 PUFA might increase in vivo lipid peroxidation (Harats et al., 1991; Meydani et al., 1991; Nelson, Morris, Schmidt, & Levander, 1993). Because MDA is formed mainly from oxidation of PUFA with 3 or more double bonds, increased plasma concentrations of TBARS may only indicate increased plasma concentrations of highly unsaturated n-3 PUFA rather than increased lipid peroxidation in vivo. Other studies of EPA/DHA supplementation using different assays of in vivo oxidation have failed to find evidence of increased oxidative stress despite the presence of elevated plasma (Allard, Kurian, Aghdassi, Muggli, & Royall, 1997) or urinary TBARS concentrations (Wander, Du, Ketchum, & Rowe, 1996).

Thus, while enrichment of the LDL particle with specific unsaturated fatty acids is likely to affect its susceptibility to oxidation, investigation of this possibility has been limited by the measurement techniques previously available. To accurately assess the effects of increased consumption of specific fatty acids on oxidative stress, assays that are sensitive to and able to distinguish oxidation products of different fatty acids will be required. The following two manuscripts
represent an attempt to apply more sensitive and specific measurement techniques to questions regarding the effect of dietary fatty acids on in vivo and ex vivo oxidative susceptibility.
CHAPTER 3

SUPPLEMENTATION OF POSTMENOPAUSAL WOMEN WITH EPA/DHA-RICH FISH OIL DOES NOT INCREASE IN VIVO LIPID PEROXIDATION COMPARED TO OLEATE- AND LINOLEATE-RICH OILS AS ASSESSED BY PLASMA CONCENTRATIONS OF MALONDIALDEHYDE AND F₂-ISOPROSTANES

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Submitted to *The American Journal of Clinical Nutrition*
The American Society for Clinical Nutrition, Inc., Bethesda, MD, September, 1999
Abstract
While replacement of dietary saturated fat with unsaturated fat has been advocated for the reduction of cardiovascular disease risk, diets high in polyunsaturated fatty acids (PUFA) could increase oxidative stress, potentially contributing to the pathology of atherosclerosis. The purpose of this study was to examine several indices of in vivo lipid peroxidation, including F2-isoprostanes, malondialdehyde (MDA), and thiobarbituric acid reacting substances (TBARS) in the plasma of postmenopausal women taking dietary oil supplements rich in oleate, linoleate, and EPA/DHA. Fifteen postmenopausal women took daily supplements of sunflower oil providing 12.3 g/day of oleate, safflower oil providing 10.5 g/day of linoleate, and fish oil providing 2.0 g/day of EPA and 1.4 g/day of DHA in a 3-period, 3-treatment, crossover trial. Plasma free F2-isoprostane concentrations were lower after EPA/DHA supplementation than after oleate and linoleate supplementation (P = 0.001 and 0.04 respectively). However, when normalized to plasma arachidonate concentrations, F2-isoprostane values were higher after EPA/DHA supplementation than after linoleate supplementation (P = 0.04). Plasma MDA concentrations were lower after EPA/DHA supplementation than after oleate and linoleate supplementation (P = 0.02 and 0.04), but plasma TBARS concentrations were higher after EPA/DHA supplementation than after oleate or linoleate supplementation (P = 0.001 and 0.0004). Both plasma MDA and F2-isoprostane results suggested decreased lipid peroxidation in vivo when EPA/DHA consumption was increased, but when free F2-isoprostanes were normalized to plasma arachidonic acid concentrations this observation was modestly reversed.
Our findings do not support the contention that diets rich in PUFA increase lipid peroxidation in vivo.

**Introduction**

The consistent positive association between dietary saturated fat content and plasma cholesterol concentrations (1) has led a number of organizations to recommend that individuals reduce their consumption of saturated fatty acids (SFA) (2, 3). In addition to lowering total fat consumption, increasing the proportion of dietary fat derived from monounsaturated (MUFA) and polyunsaturated fatty acids (PUFA) has been advocated because such diets have resulted in more favorable lipoprotein profiles, with respect to cardiovascular risk (4, 5). More recently, dietary enrichment with MUFA, omega-6 (n-6) PUFA, and omega-3 (n-3) PUFA has been found to have somewhat different effects on risk factors for cardiovascular disease. Substitution of n-6 PUFA for SFA has resulted in favorable decreases of plasma LDL cholesterol (LDL-C) concentrations, but has also been associated with less desirable decreases in HDL cholesterol (HDL-C) (4). In some studies, substitution of MUFA for SFA resulted in decreases in plasma LDL-C comparable to n-6 PUFA without the concomitant decrease in plasma HDL-C (6). Diets rich in the MUFA, oleic acid (18:1n-9), have been associated with reduced risk of cardiovascular mortality (7). Diets high in the n-3 PUFA eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3) have resulted in decreases in plasma triacylglycerol concentrations (8). These highly unsaturated long-chain fatty acids are found in certain species of fish, and
diets rich in those same species of fish have been associated with decreased mortality from cardiovascular disease (9, 10).

Despite their favorable effects on lipid profiles, concern exists that diets relatively high in unsaturated fat could increase oxidative stress, a possible factor in the etiology of a number of chronic diseases including cardiovascular disease (11). In vitro studies of the oxidation of fatty acids in homogeneous solutions suggest that oxidative susceptibility increases with the number of double bonds in a fatty acid (12, 13), while studies of unsaturated fatty acids in multiphase systems suggest oxidative susceptibility may not be directly related to the degree of unsaturation (14). The results of studies examining the effects of increased proportions of highly unsaturated PUFA in the diet on indices of oxidative stress in vivo have been contradictory (15-17). However, much of the information regarding the effect of increased dietary PUFA on in vivo lipid peroxidation is based solely on the thiobarbituric acid (TBA) assay. Although widely used, the TBA assay has also been widely criticized due to its well-documented lack of specificity, and potential for artifactual oxidation (18).

Because primary oxidation products such as lipid hydroperoxides are unstable under physiological conditions, practical assays of in vivo oxidation generally measure more stable secondary oxidation products (19). A number of different breakdown pathways are possible, and specific secondary oxidation products do not result from the breakdown of all fatty acids. Consequently, examining the effect of specific dietary fatty acids on in vivo oxidative stress can
be problematic. For example, MDA is thought to result mainly from fatty acids with 3 or more double bonds, thus oxidation of MUFA or PUFA with only 2 double bonds, (e.g., linoleic acid) could not be detected by the TBA assay or more specific assays of MDA (20). An established assay of in vivo oxidative stress, F₂-isoprostanes, measures a product of the oxidation of arachidonate (AA; 20:4n-6), an n-6 PUFA (21). However, it is unclear whether the assessment of plasma F₂-isoprostanes would be useful in assessing in vivo oxidative stress related to changes in the proportions of specific dietary unsaturated fatty acids.

Because LDL oxidation appears to play an important role in the pathology of cardiovascular disease, it is important to assess the effect of increasing dietary unsaturated fatty acids on lipid peroxidation and oxidative stress in vivo. These issues are of particular relevance to postmenopausal women, for whom cardiovascular disease is the major cause of mortality in the United States (22). Until quite recently women have been underrepresented in studies of cardiovascular disease risk, despite evidence that findings regarding cardiovascular disease in men cannot always be extrapolated to women (23). For these reasons we assessed several indices of in vivo oxidative stress, including F₂-isoprostanes, MDA, and thiobarbituric acid reacting substances (TBARS) in the plasma of postmenopausal women taking daily supplements rich in oleate (n-9 MUFA) from sunflower oil, linoleate (n-6 PUFA) from safflower oil, and EPA/DHA (n-3 PUFA) from fish oil.
Subjects and Methods

SUBJECTS

Sixteen postmenopausal women, between 50 and 75 years of age, were recruited from the Oregon State University campus and the surrounding community using posters and newspaper advertisements. The study protocol was reviewed and approved by the institutional review board at Oregon State University and written consent was obtained from each participant prior to beginning the study.

Menopausal status was assessed on the basis of menstrual history (absence of normal menses for at least 12 months) and/or a history of use of hormone replacement therapy (HRT) for at least 12 months. A thorough medical history was obtained from each participant. Therapeutic agents or nutritional supplements known to have antioxidant or lipid-altering effects were specifically excluded. All participants were required to be normolipemic based on the results of plasma lipid profiles, analyzed by the Oregon State University Lipid Laboratory. Normal liver function, glucose status, and iron status were verified through medical history and the results of a fasting serum chemistry panel and a screening complete blood count CBC, analyzed by Good Samaritan Hospital Laboratory (Corvallis, OR). All prospective participants had their blood pressure screened prior to entering the study to ensure normal systolic and diastolic blood pressure. Height and weight were measured, and BMI calculated to select for participants who were not obese. Cigarette smoking was excluded by history. In order to participate in the study all participants agreed to refrain from taking any nutritional supplements other than
calcium or vitamin D, and to refrain from eating fish for the duration of the study. They were informed that the 15 g of oil would add 567 kJ (135 kcal) daily to their diets, and they agreed to maintain a stable body weight throughout the study, as well as to refrain from any significant change in physical activity.

Because one participant dropped out after the first period for reasons unrelated to the study, only the data obtained from the other 15 participants are included in the results. Occasional random loss of sample during laboratory analysis resulted in a further decrease in sample size for the statistical analysis of some of the assays reported.

**EXPERIMENTAL DESIGN**

In order to assess the effects of each of the 3 types of oil supplements on the individual participants, a 3-period, 3-treatment, blinded crossover trial was used. During each treatment period participants took 15 g of high oleate sunflower oil, high linoleate safflower oil, or fish oil rich in EPA/DHA. Each treatment period lasted 5 weeks and was followed by a 7-week washout interval, in order to minimize any carryover effect from the previous treatment. The initial 16 participants were randomly assigned to one of 6 treatment sequences, representing all possible sequences. The present crossover trial was also designed to avoid confounding of period and treatment effects by including each treatment in each period. This design allowed for the statistical assessment of carryover effects, described in the statistical analysis section (24). The total time of participation in the study was 27 weeks.
Because the laboratory was only able to process plasma samples from 4 participants at a time, treatment period starting dates were staggered so that only 4 participants started their treatment each of 4 consecutive weeks. After a 2-week run in period, measurements and blood samples were taken on Monday and Wednesday at the Oregon State University Metabolic Unit, prior to the start of the treatment and 5 weeks later, on Monday and Wednesday, during the last 3 days of the treatment period. At the start of the first 2 treatment periods, participants were given a 2-wk supply of supplements. After 2 weeks they returned to the metabolic unit to obtain their next 3-wk supply. At the start of the final treatment period, participants were given a 5-wk supply of supplements and returned only at the end of the treatment period for measurements and blood sampling. Thus, participants made a total of 14 visits to the Metabolic Unit during the course of the study. Compliance was assessed by counting leftover capsules, as well as by evaluating changes in specific fatty acid concentrations in plasma fatty acid profiles.

SUPPLEMENTS

The fish oil was obtained from the National Institute of Health’s Fish Oil Test Material Program (NIH-FOTMP) in sealed opaque containers, containing one hundred 1-g capsules. The high oleate sunflower oil (generously donated by Humpco, Memphis, TN) and the high linoleate safflower oil (Arista Industries, Darien, CT) were supplied in bulk and encapsulated after adjustment for antioxidant content (Professional Compounding Pharmacy, Corvallis, OR). The α-tocopherol concentration in the fish oil supplied by the NIH-FOTMP was assayed
by our laboratory and found to be 1.2 mg/g oil, while the \( \gamma \)-tocopherol concentration was 1.3 mg/g oil. The fish oil also contained tertiary butyl hydroquinone (TBHQ) added as an antioxidant to achieve the concentration of 0.17 mg/g oil. TBHQ is a food grade, oil-soluble antioxidant, which is metabolized within a few days of ingestion and excreted in the urine. Because it is not stored in tissues, it does not function as an antioxidant in vivo (25). After assaying the original \( \alpha \)- and \( \gamma \)-tocopherol concentrations in the sunflower and safflower oils, additional \( \alpha \)-tocopherol, \( \gamma \)-tocopherol and TBHQ (generously supplied by Eastman Chemical Company, Kingsport TN) were added in order to match the concentrations present in the fish oil, supplied by NIH-FOTMP. Thus, all 3 oil supplements supplied approximately 20 mg \( \alpha \)-TE in 15 g of oil consumed daily.

The potential for lipid peroxidation in the oil supplements was assessed by measuring peroxide values (26) and the \( p \)-anisidine value, a measure of the aldehyde content of fat (27). Assessment of lipid peroxidation was performed at the beginning of each treatment period on samples of each oil supplement, which had been kept refrigerated in opaque containers. Neither the \( p \)-anisidine value nor the peroxide value increased in any of the supplements by the final period of the study. Oil supplements were given to participants in opaque jars, with each day's dose contained in a resealing plastic bag. They were instructed to keep the opaque container in the refrigerator, except when removing the days oil supplement.
**DIET ANALYSIS**

In order to determine the nutritional content of her habitual diet, each participant was instructed in the technique of keeping a three-day (two weekdays and one weekend day) record of all foods and beverages consumed. Participants kept 3-day diet records during each of the 3 treatment periods. The nutritional content of each participant’s diet during each treatment period was analyzed using the computer software, Food Processor Plus (version 6.0; ESHA, Salem, OR).

**BLOOD COLLECTION**

Venous blood samples were collected into tubes containing Na₂EDTA (1g/L) after an overnight fast of approximately 12 hours. Samples were taken on two separate days at the beginning and end of each 5-week treatment period. Plasma samples were prepared within one hour of blood collection by centrifugation at 3000 rpm at 4°C for 15 minutes. Blood samples were kept in the dark and on ice until centrifugation. Plasma samples were immediately aliquotted and stored under argon at -70°C. Plasma samples were thawed only once, at the time of assay. Frozen plasma samples were shipped on dry ice overnight, from the Oregon State University Lipid Laboratory to Dr. Morrow’s laboratory at Vanderbilt University (Nashville, TN) as well as to Dr. Ames’ laboratory at the University of California (Berkeley, CA) approximately 3 months after the final blood draw. Plasma vitamin E, plasma fatty acid profiles, and plasma lipid and lipoprotein profiles were assayed at the OSU Lipid Laboratory at the end of each treatment
period, while plasma TBARS was assayed approximately 3 months after the final blood draw.

ASSAYS

Plasma total cholesterol concentrations were determined enzymatically, using a modification of the method of Allain et al (28). This cholesterol assay meets the National Cholesterol Education Program’s performance criteria for accuracy. Plasma triacylglycerol concentrations were measured using a modification of the method of McGowan et al (29). HDL cholesterol concentrations were measured enzymatically after precipitation of LDL and VLDL fractions with phosphotungstic acid and MgCl₂ (30). LDL cholesterol concentrations were calculated using the formula of Friedwald (31).

In order to obtain fatty acid profiles of plasma, as well as the supplemental oils, lipids were extracted with chloroform methanol (1:2, v/v), according to the method of Bligh and Dryer (32). After methylation, fatty acid methyl esters were measured by gas chromatography, using heptadecanoic acid (Nu-Chek Prep, Elysian, MN) as an internal standard, as described previously (33).

Because bis-allylic hydrogen atoms are most easily abstracted from fatty acids by reactive oxygen species, fatty acids with 2 or more double bonds are thought to be more susceptible to lipid peroxidation than SFA or MUFA (34). The peroxidation index multiplies the plasma concentration of specific PUFA by one less than the number of double bonds it possesses, and may be used as an index of
the overall susceptibility to lipid peroxidation, expressed by an individual's fatty acid profile.

Concentrations of α- and γ-tocopherol in the supplemental oils were measured by normal-phase HPLC (Shimadzu, Columbia, MD), using fluorometric detection (excitation λ 292; emission λ 330) based on a standardized method published by the International Union of Pure and Applied Chemistry (35). Oil samples were diluted in hexane and injected onto a silica column (Supelcosil 5μm; 250 x 4.6mm, Supelco Inc., Bellefonte, PA), using hexane: isopropanol (99:1 v/v) as a mobile phase, at a flow rate of 1 ml per minute.

Plasma α- and γ-tocopherol concentrations were measured by reversed phase HPLC with fluorometric detection, using a modification of the method of Arnaud et al (36). Tocopherols were extracted into hexane, evaporated under nitrogen, resuspended in methanol, and injected onto a C-18 column (Shim-pack CLS-ODS 5μ; 250x4.6mm, Shimadzu, Columbia, MD), using 100% methanol as a mobile phase, at a flow rate of 1.5 mL/min. Concentrations were determined using external standards. Recovery of added α-tocopherol to plasma samples averaged 92%. Application of our plasma assay to α-and γ-tocopherol standards (SRM 968b) obtained from the National Institute of Standards and Technology (NIST) Standards Reference Program (NIST; Gaithersburg, MD) yielded values within 6% of the NIST consensus values; intra-assay CV for α-tocopherol averaged 6.4% and inter-assay CV was 5.6%.
Free F₂-isoprostane concentrations in plasma were determined using gas chromatography (GC)/negative chemical ionization (NICI) mass spectrometry (MS) as described by Morrow and Roberts (37). Briefly, a deuterated prostaglandin F₂α internal standard was added to plasma, and F₂-isoprostanes were extracted with C-18 and silica mini-columns. The extracted F₂-isoprostanes were converted to pentafluorobenzyl ester trimethylsilyl ether derivatives and quantified using selected ion monitoring (m/z 569 for F₂-isoprostanes and m/z 573 for the internal standard) GC/NICI MS. This assay has been found to be highly accurate (96% accuracy) as well as highly sensitive; the lower limit of detection is in the low picogram range (38). Intra- and inter-assay CV for the assay in the present study were less than 10%.

Plasma malondialdehyde (MDA) concentrations were also measured using GC/MS in the negative chemical ionization mode as described by Yeo et al (39). A stable isotope internal standard, [²H₂] MDA, and an antioxidant, 2,6-tert-butyl-4-methylphenol (BHT; 67 μM), were added to the plasma samples. The samples were then incubated at room temperature in 6.6 N H₂SO₄ for 10 minutes to hydrolyze the aldehydes from the proteins. Plasma MDA was converted to a stable pentafluorophenyl hydrazine (PFPH) derivative at room temperature, and the derivative was quantified using the GC/MS in the negative chemical ionization mode (selective ion monitoring m/z 234 for MDA and m/z 236 for the internal standard). Intra- and inter-assay CV were 6.5%. This method combines the highly specific technique of GC/MS with mild sample preparation conditions, thus
avoiding cross reactivity and heat-generated artifacts characteristic of the thiobarbituric acid (TBA) method (40).

The TBA assay is a commonly used method of measuring MDA as an index of lipid peroxidation. Because of the lack of specificity of this assay, especially in biological systems, results are commonly expressed as thiobarbituric acid reacting substances (TBARS), rather than MDA. Plasma TBARS were measured in duplicate, using the method described by Yagi (41). To inhibit oxidation BHT (67 μM) was added to plasma samples before starting the assay, just as in the GC/MS assay for MDA. Plasma lipids were precipitated along with protein through the use of a phosphotungstic acid-sulfuric acid system. After centrifugation and removal of the supernatant, the precipitate was resuspended, and the TBA reagent (equal volumes of 0.67% TBA and glacial acetic acid) added. The mixture was heated for 60 minutes at 95° C. After cooling n-butanol was added, the mixture was vortexed and centrifuged, and the butanol layer was removed for fluorometric measurement at emission λ of 553 nm with excitation λ of 515 nm. External standards of 1,1,3,3-tetraethoxypropane were used to quantify TBARS in the plasma samples. Intra-assay CV averaged 8.4%, while inter-assay CV was 9.5%.

**STATISTICAL ANALYSIS**

The primary advantage of a crossover design is the increased precision in treatment comparisons, due to the removal of the between subject variation from the experimental error. A disadvantage of the crossover design is the possibility for carryover effects (i.e. a treatment given in one period may influence the treatment
response in the following period). A 7-week washout period was utilized after each treatment period in order to decrease the likelihood of carryover effects. Additionally, the random assignment of at least 2 subjects to all 6 possible treatment sequences allowed for the statistical assessment of carryover effects prior to inference regarding direct treatment effects.

Carryover effects were estimated and direct treatment effects analyzed, utilizing a mixed between-within subjects ANOVA procedure described by Kuehl (24). Briefly, the between subjects sources of variation consisted of 1) sequence of treatment and 2) subjects nested within sequence, while within subjects sources of variation consisted of 1) period, 2) treatment (direct), and 3) treatment (carryover). If treatment carryover effects were significant (P ≤ 0.05), estimates of differences among treatment means could be adjusted for the carryover effects. If carryover effects were not found to be statistically significant, the ANOVA was performed without the treatment (carryover) effect in the model. No significant treatment carryover effects were found for any of the data presented. Therefore, all least square means presented represent direct treatment effects which were not adjusted for carryover. Because no significant period-treatment interactions were encountered, the period-treatment interaction was not included in the final model. If the ANOVA demonstrated a significant treatment effect, pair-wise comparisons were performed using Fisher’s least significant difference test to determine significant differences among treatments. Results with P values ≤ 0.05 for a two-sided test were considered statistically significant. Analyses were accomplished

Prior to analysis data were screened graphically for normality, linearity, and homogeneity of variance. A modification of Levene’s test was used to ensure the assumption of equal within-group variance was not violated (42). Studentized residuals were used to screen for multivariate outliers. Observations with studentized residuals > 3, which did not resolve with transformation and demonstrated strong influence on the model were removed from the analysis. Data are expressed as least square means (LSM) ± standard error of the measurement (SEM), unless otherwise noted.

Results

SUBJECT CHARACTERISTICS

All participants were postmenopausal and taking HRT. Each participant continued her regimen of HRT, without alteration, for the duration of the study. Although the HRT regimens were not identical for each women, they were generally equivalent to 0.625 mg conjugated estrogens daily for hysterectomized women and 0.625 mg conjugated estrogens plus 2.5 mg medroxyprogesterone daily for women with intact uteruses. The participants did not take any chronic prescription medications other than HRT, nor did they take any nutritional supplements, other than calcium or vitamin D, for the duration of the study. Compliance with the supplement regimen was estimated to be 95% based on the
return of empty supplement containers and leftover supplement capsules. The results of the plasma fatty acid profiles for each participant during each treatment period were also consistent with a high level of compliance.

The initial characteristics of the subjects are presented in Table 3.1. Their mean age was 58 y (range 52-73 y). Although mean BMI was slightly greater than the desirable upper limit of 24.9, it was well under 30, the level associated with increased morbidity and mortality (43). All participants were considered normolipidemic on the basis of their lipid and lipoprotein profiles. Plasma total and LDL cholesterol concentrations were lower than the fiftieth percentile for this particular population of women (5.90-6.00 and 3.75-3.83 mmol/L, respectively), while HDL cholesterol concentrations were higher (1.40-1.45 mmol/L). Plasma triacylglycerol concentrations were well below 2.3 mmol/L, the upper limit of normal (3). Fasting blood glucose concentrations and iron status indicated by hemoglobin were within normal limits. The participants gained an average of 0.6 kg over the 9 months of the study.

Table 3.1. Initial characteristics of the subjects.* (n = 15)

<table>
<thead>
<tr>
<th>Age (y)</th>
<th>57.6 ± 5.8</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMI (kg/m²)</td>
<td>25.8 ± 3.5</td>
</tr>
<tr>
<td>Plasma cholesterol (mmol/L)</td>
<td>5.23 ± 0.55</td>
</tr>
<tr>
<td>Plasma triacylglycerol (mmol/L)</td>
<td>1.49 ± 0.47</td>
</tr>
<tr>
<td>HDL cholesterol (mmol/L)</td>
<td>1.57 ± 0.39</td>
</tr>
<tr>
<td>LDL cholesterol (mmol/L)</td>
<td>2.98 ± 0.61</td>
</tr>
<tr>
<td>Glucose (mmol/L)</td>
<td>4.93 ± 0.42</td>
</tr>
<tr>
<td>Hemoglobin (g/L)</td>
<td>132 ± 7</td>
</tr>
</tbody>
</table>

*Mean ± SD
DIETARY CONTENT OF SELECTED NUTRIENTS

Because the intake of nutrients given in Table 3.2 did not differ by supplement group, the values presented represent the average nutrient intake from three 3-day diet records. The participants consumed 29 ± 6% of energy as fat with the following distribution of energy between the 3 classes of fat: approximately 12% as saturated fat, 11% as monounsaturated fat, and 6% as polyunsaturated fat. Mean vitamin E intake was slightly below the recommended intake of 8 mg α-TE per day (44). Mean folic acid intake was below the recently revised recommended dietary allowance (RDA) of 400 μg per day, though well within the previous recommendation of 180 μg per day, while mean vitamin B12 and B6 intake were above the RDA of 2.4 μg and 1.5 mg respectively (45).
Table 3.2. Average daily intake of selected nutrients from three 3-day diet records.* (n = 15)

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>Recommended†</th>
<th>Intake</th>
</tr>
</thead>
<tbody>
<tr>
<td>Energy (kJ)</td>
<td>7959</td>
<td>7374 ± 1916</td>
</tr>
<tr>
<td>Protein (% of energy)</td>
<td>15</td>
<td>16 ± 3</td>
</tr>
<tr>
<td>Carbohydrate (% of energy)</td>
<td>-</td>
<td>55 ± 9</td>
</tr>
<tr>
<td>Total fat (% of energy)</td>
<td>30</td>
<td>29 ± 6</td>
</tr>
<tr>
<td>Saturated fat (g)</td>
<td>-</td>
<td>19 ± 8</td>
</tr>
<tr>
<td>MUFA (g)</td>
<td>-</td>
<td>18 ± 8</td>
</tr>
<tr>
<td>PUFA (g)</td>
<td>-</td>
<td>9 ± 5</td>
</tr>
<tr>
<td>Cholesterol (mg)</td>
<td>300</td>
<td>177 ± 90</td>
</tr>
<tr>
<td>Fiber (g)</td>
<td>20</td>
<td>20 ± 8</td>
</tr>
<tr>
<td>Vitamin E (mg α-TE)</td>
<td>8</td>
<td>6 ± 3</td>
</tr>
<tr>
<td>Vitamin C (mg)</td>
<td>60</td>
<td>138 ± 84</td>
</tr>
<tr>
<td>Selenium (μg)</td>
<td>55</td>
<td>57 ± 31</td>
</tr>
<tr>
<td>Folic Acid (μg)</td>
<td>400</td>
<td>265 ± 126</td>
</tr>
<tr>
<td>Vitamin B6 (mg)</td>
<td>1.5</td>
<td>1.6 ± 0.7</td>
</tr>
<tr>
<td>Vitamin B12 (μg)</td>
<td>2.4</td>
<td>3.6 ± 2.8</td>
</tr>
</tbody>
</table>

*mean ± SD
†Recommended value represents the 1998 recommended dietary allowances (RDA) for folic acid and vitamin B12 (45) and the 1989 RDA for all other nutrients (44).

**FATTY ACID INTAKE AND PLASMA FATTY ACID CONCENTRATIONS**

The dietary and the supplement content of 7 fatty acids of interest are presented in Table 3.3. The dietary content of those fatty acids did not differ significantly between the supplement groups. By design, each oil supplement supplied high concentrations of specific fatty acids. Fifteen g per day of the sunflower oil provided 12.3 g/day of oleic acid (18:1n-9), while 15 g of the safflower oil provided 10.5 g/day of linoleic acid (18:2n-6). The same quantity of fish oil provided 2.0 g/day of eicosapentaenoic acid (EPA; 20:5n-3) and 1.4 g/day of docosahexaenoic acid (DHA; 22:6n-3).
As a result of the oil supplement, the total dietary intake of specific fatty acids differed significantly between supplement groups. During the oleate supplement, total 18:1n-9 intake was 79% higher than that of the linoleate (P<0.0001) and 61% higher than that of the EPA/DHA supplement (P<0.0001). During the linoleate supplement, 18:2n-6 intake was 146% higher than that of the oleate (P<0.001) and 119% higher than that of the EPA/DHA supplement (P<0.0001). Because the participants were asked to exclude fish from their diet throughout the study, the EPA/DHA supplement greatly increased (approximately 50 to 100 fold) the 20:5n-3 and 22:6n-3 content of the diet when compared to that of the oleate and the linoleate supplements (P<0.0001 for all 4 comparisons). Although total intake of the n-6 polyunsaturated fatty acid, arachidonic acid (20:4n-6), was relatively low during all 3 supplements, it was significantly higher during the EPA/DHA supplement (P<0.0001) due to the higher 20:4n-6 content of the fish oil.
Table 3.3. Selected fatty acids supplied by diet, supplement, and total consumption, by supplement group.* (n = 15)

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Source</th>
<th>Diet (g/day)</th>
<th>Supplement (g/day)</th>
<th>Total (g/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>16:0</td>
<td>18:0</td>
<td>18:1 (n-9)</td>
</tr>
<tr>
<td>Sunflower oil (oleate)</td>
<td></td>
<td>7.21 ± 0.57</td>
<td>0.49</td>
<td>7.70 ± 0.57</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.46 ± 0.33</td>
<td>0.53</td>
<td>3.99 ± 0.33</td>
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<tr>
<td></td>
<td></td>
<td>13.47 ± 1.20</td>
<td>12.27</td>
<td>25.74 ± 1.20a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6.21 ± 0.90</td>
<td>0.58</td>
<td>6.79 ± 0.90b</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.05 ± 0.01</td>
<td>ND</td>
<td>0.05 ± 0.01a</td>
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<tr>
<td></td>
<td></td>
<td>0.01 ± 0.01</td>
<td>ND</td>
<td>0.01 ± 0.01a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.01 ± 0.01</td>
<td>ND</td>
<td>0.01 ± 0.01a</td>
</tr>
<tr>
<td>Safflower oil (linoleate)</td>
<td></td>
<td>7.28 ± 1.25</td>
<td>1.04</td>
<td>8.32 ± 1.25</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.31 ± 0.54</td>
<td>0.36</td>
<td>3.67 ± 0.54</td>
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<tr>
<td></td>
<td></td>
<td>12.51 ± 2.00</td>
<td>1.84</td>
<td>14.35 ± 2.00b</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6.24 ± 1.06</td>
<td>10.46</td>
<td>16.70 ± 1.06b</td>
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<tr>
<td></td>
<td></td>
<td>0.07 ± 0.01</td>
<td>ND</td>
<td>0.07 ± 0.01a</td>
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<td>0.00 ± 0.01</td>
<td>0.02</td>
<td>0.03 ± 0.02b</td>
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<td>0.03 ± 0.02</td>
<td>ND</td>
<td>0.03 ± 0.02a</td>
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<td>Fish oil (EPA/DHA)</td>
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<td>7.49 ± 1.03</td>
<td>2.16</td>
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<td>3.29 ± 0.47</td>
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<td>15.27 ± 2.03</td>
<td>0.76</td>
<td>16.03 ± 2.03b</td>
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<td>7.62 ± 1.37a</td>
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<td>0.02 ± 0.01</td>
<td>1.44</td>
<td>1.46 ± 0.01b</td>
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*mean ± SEM. Different letters represent significant differences between total (dietary + supplemental) fatty acid intake (P < 0.01). Dietary intake alone did not differ between supplement groups.

ND = not detectable

At the end of the supplement periods, changes in the plasma concentrations of fatty acids reflected the differences in dietary fatty acid content (Figure 3.1).
Mean plasma 18:1n-9 concentrations were 41% higher at the end of the oleate supplement, than the linoleate (P<0.0001), and 61% higher than the EPA/DHA supplement (P<0.0001). At the end of the linoleate supplement, plasma 18:2n-6 was 14% higher compared to the oleate (P = 0.02), and 34% higher compared to EPA/DHA supplement (P< 0.0001). Plasma 18:2n-6 concentrations also differed significantly between the oleate and the EPA/DHA supplements with 18:2n-6 concentrations 18% higher at the end of the oleate supplement (P = 0.01). Plasma concentrations of the n-3 fatty acids, 20:5n-3 and 22:6n-3, were significantly increased after the EPA/DHA supplement (P<0.0001). Plasma 20:5n-3 concentrations were approximately 10 times higher, while plasma 22:6n-3 concentrations were approximately 2.5 times higher after EPA/DHA supplementation than after the oleate and linoleate supplements. Plasma 20:4n-6, which can be synthesized from 18:2n-6, was 23% lower after the EPA/DHA supplement than after the oleate and linoleate supplements (P<0.001), despite the fact that total 20:4n-6 intake was highest during the EPA/DHA supplement (Total, Table 3.3).

Although total intake of saturated fatty acids (SFA) did not differ significantly between the 3 supplement groups, total plasma saturated fatty acid concentrations (ΣSFA) were significantly higher at the end of the oleate supplement compared to the linoleate (13%; P = 0.03) and the EPA/DHA supplements (18%; P = 0.004). As expected, plasma concentrations of all monounsaturated fatty acids (ΣMUFA) were higher at the end of the oleate
supplement than at the end of the linoleate (35%; \( P = 0.0002 \)) or the EPA/DHA supplements (50%; \( P < 0.0001 \)). Total plasma concentrations of polyunsaturated fatty acids (\( \Sigma \)PUFA) did not differ significantly. However, the ratio of plasma n-6 PUFA concentration to n-3 PUFA concentration differed significantly between the 3 supplement groups. The mean n-6/n-9 ratio was more than 3 times lower at the end of the EPA/DHA supplement (3.0) compared to the oleate (12.6; \( P<0.0001 \)) and more than 4 times lower when compared to the linoleate supplement (16.2; \( P<0.0001 \)). At the end of the oleate supplement the n-6/n-3 ratio was 28% lower when compared to the linoleate supplement (\( P = 0.01 \)).

**Figure 3.1.** Plasma concentrations of selected fatty acids after supplementation with 3 different oils. Each bar represents least square mean (LSM) ± SEM. Bars with diagonal lines represent plasma fatty acid concentrations after 5 weeks of the oleate supplement. Black bars represent plasma fatty acid concentrations after 5 weeks of the linoleate supplement and shaded bars after 5 weeks of the EPA/DHA supplement. Values with different letters are significantly different from each other (\( P \leq 0.05 \)).
At the end of the EPA/DHA supplement the mean peroxidation index was 28% higher compared to the linoleate and 31% higher compared to the oleate supplement (P<0.0001). The PI did not differ significantly between the linoleate and oleate supplement groups.

**PLASMA α- AND γ-TOCOPHEROL CONCENTRATIONS**

Each of the 3 oil supplements were matched for α- and γ-tocopherol content (20 mg α-TE/day). Additionally, mean dietary vitamin E (6 mg α-TE/day) did not differ significantly between the 3 supplement groups. However, as seen in Figure 3.2, mean plasma α-tocopherol concentrations were 13% lower at the end of EPA/DHA supplementation compared to oleate supplementation (P = 0.03). Plasma γ-tocopherol concentrations were also lower at the end of EPA/DHA supplementation: 46% lower than the mean plasma γ-tocopherol concentration at the end of oleate supplementation (P = 0.0004), and 33% lower than the end of linoleate supplementation (P = 0.008). If α- and γ-tocopherol concentrations are normalized to total plasma lipid content (plasma total cholesterol + plasma triacylglycerol) instead of plasma volume, the difference in α-tocopherol concentrations between supplement groups is eliminated, and for γ-tocopherol, only the significant difference between the EPA/DHA and the oleate supplements persist (P = 0.02).
**FIGURE 3.2.** Vitamin E concentrations normalized to plasma volume (Graph A) and plasma lipids (TC + TG, Graph B). Each oil supplement was matched for α- and γ-tocopherol concentration (1.2 mg/g α- and 1.3 mg/g γ-tocopherol). Bars represent least square mean (LSM) ± SEM. Bars with diagonal lines represent plasma tocopherol concentrations after 5 weeks of the oleate supplement. Black bars represent plasma tocopherol concentrations after 5 weeks of the linoleate supplement and shaded bars after 5 weeks of the EPA/DHA supplement. Values with different letters are significantly different from each other (P ≤ 0.05).

**ASSAYS OF IN VIVO OXIDATIVE STRESS**

Plasma free F2-isoprostanes, products of 20:4n-6 oxidation (Figure 3.3), were 15% lower after EPA/DHA supplementation when compared to oleate supplementation (P < 0.001) and 8% lower when compared to linoleate supplementation (P = 0.04). Free F2-isoprostanes did not differ significantly between oleate and linoleate supplementation. When normalized to plasma 20:4n-6 concentrations plasma free F2-isoprostanes values were 12% higher in the EPA/DHA supplemented group than the linoleate supplemented group (P = 0.04), while the value for oleate did not differ from either the EPA/DHA or the linoleate supplemented groups.
Plasma MDA concentrations (Figure 3.4), measured using the more specific method (GC/MS), show a similar trend to the F2-isoprostane concentrations. After EPA/DHA supplementation plasma MDA concentrations were 17% lower compared to oleate supplementation (P = 0.02), and 13% lower compared to linoleate supplementation (P = 0.04). Plasma MDA concentrations did not differ significantly between the oleate and the linoleate supplements.
Surprisingly, plasma TBARS concentrations (Figure 3.4), which are often referred to as an indicator of plasma MDA concentrations, showed the opposite trend. It is important to note that the TBARS concentrations are nearly 10 times higher than the MDA concentrations. After EPA/DHA supplementation, plasma TBARS were more than 21% higher when compared to oleate (P = 0.001) and 23% higher compared to linoleate supplementation (P = 0.0004). Plasma TBARS concentrations did not differ significantly between the oleate and the linoleate supplements.

**FIGURE 3.4.** Plasma concentrations of thiobarbituric acid reacting substances (TBARS) and malondialdehyde (MDA) at the end of supplementation with 3 different oils. Bars with diagonal lines represent plasma concentrations after 5 weeks of the oleate supplement. Black bars represent plasma concentrations after 5 weeks of the linoleate supplement and shaded bars after 5 weeks of the EPA/DHA supplement. Values with different letters are significantly different from each other (P ≤ 0.05). TBARS concentrations were nearly 10 times higher than the MDA concentrations.
Discussion

The results of the in vivo assays of oxidative stress did not uniformly support the idea that increased numbers of double bonds in dietary PUFA result in increased lipid peroxidation in vivo. In fact, the two assays purported to measure the same index of oxidative stress, malondialdehyde (the MDA and TBA assays), suggested opposite conclusions regarding the role of dietary n-3 fatty acids and in vivo oxidative stress. Plasma TBARS were approximately 21% and 23% higher after EPA/DHA supplementation compared to oleate and linoleate supplementation respectively, while the more sensitive and specific MDA assay resulted in MDA levels that were approximately 17% and 13% lower after EPA/DHA supplementation compared to oleate and linoleate. Liu et al. (13) previously demonstrated the increased sensitivity of the GC/MS assay over the TBARS assay in assessing MDA concentrations in unsaturated fatty acids oxidized in vitro. The fact that the TBARS concentrations in plasma were nearly 10 times higher than the MDA concentrations is likely due to the lack of specificity of the TBA assay for MDA, as well as artifactual production of MDA during the acid heating step. The GC/MS technique utilized in the present study combines the specificity of mass spectrometry with relatively mild sample preparation, thus avoiding heat-generated artifact encountered with the TBA assay (40).

Enzymatic and nonenzymatic oxidation of PUFA with more than 2 double bonds are thought to be sources of the aldehydic end product MDA. Thus, the oxidation of arachidonic acid as well as EPA and DHA are potential sources of
MDA. The conflicting results of the plasma MDA and TBA assays in the present study suggest the possibility of increased TBA-reactive substances other than MDA in plasma during fish oil supplementation, and/or the potential for increased MDA formation from EPA and DHA during the TBA assay, under harsh conditions not encountered during the MDA assay, nor in vivo.

F₂-isoprostanes are prostaglandin-like products of nonenzymatic peroxidation of 20:4n-6. They are an established biomarker for oxidative stress, and have been shown to correlate with conditions of increased lipid peroxidation in animals and humans (37). In humans, elevated plasma F₂-isoprostanes have been demonstrated in conditions associated with enhanced oxidative stress such as chronic cigarette smoking (46), hepatorenal syndrome, and systemic sclerosis (21). Additionally, increased concentrations of F₂-isoprostanes have been found in human atherosclerotic lesions (47) and recently, increased plasma F₂-isoprostane concentrations were associated with increased plasma total homocysteine levels, an independent risk factor for cardiovascular disease (48).

While plasma free F₂-isoprostane concentrations in the present study were 15% and 8% lower after EPA/DHA supplementation than after oleate and linoleate supplementation, when normalized to plasma 20:4n-6 concentrations free F₂-isoprostane values were 12% higher after EPA/DHA supplementation than after linoleate supplementation. Plasma 20:4n-6 concentrations were almost 20% lower after the EPA/DHA supplement than the linoleate and oleate supplements, despite the fact that total intake of 20:4n-6 was slightly though significantly higher during
the EPA/DHA supplement. Previous research in humans has demonstrated that
competition between n-3 and n-6 fatty acids can diminish the abundance of 20:4n-6
in plasma lipids through effects on synthesis and reesterification (49). The free F2-
isoprostane data from the present study suggest slightly increased oxidation of
20:4n-6, but provide no information regarding the oxidation of EPA or DHA.

Recently it has been demonstrated that the nonenzymatic oxidation of EPA
in vitro results in a number of F3-isoprostanes one of which (8-epi PGF3α) can be
found in small amounts in plasma (50). Moreover, F4-isoprostanes resulting from
the nonenzymatic oxidation of the DHA have been isolated in vitro and in vivo, and
found to be elevated in the spinal fluid of humans with Alzheimer’s disease (51).
In the future, the assessment of plasma concentrations of these 3 families of
isoprostanes could provide insight into the relative contribution of individual PUFA
to in vivo oxidative stress.

Several studies have suggested that increased consumption of n-3 fatty
acids may result in an increased potential for in vivo oxidative stress. These studies
are based primarily on the results of the TBA assay (15, 16). The results of the
present study and previous research at the Oregon State Lipid Laboratory (17) have
not demonstrated increased oxidative stress in vivo during fish oil supplementation
when more specific techniques than the TBA assay are used. For example, plasma
protein carbonyl concentrations, an assay of plasma protein oxidation, did not
increase during fish oil supplementation, despite a concomitant increase in plasma
TBARS (52). These findings support the use of more than one relevant assay of in
vivo oxidative stress in order to assess the effect of dietary fatty acids on lipid peroxidation.

Several investigators have recommended that increased dietary PUFA be accompanied by increased dietary vitamin E (53). In order to control for the effect of vitamin E on indices of oxidative stress in the present study, each of the 3 oil supplements was carefully matched for α- and γ-tocopherol content. Additionally, each participant’s dietary intake of vitamin E was calculated during each supplement period and did not differ among the 3 supplement periods. Despite this control of dietary intake some differences in plasma α- and γ-tocopherol concentrations were evident between supplement groups.

Plasma concentrations of α-tocopherol, considered the most potent antioxidant of the vitamin E isomers, were slightly (11%), though significantly lower after the EPA/DHA supplement than after the oleate supplement, but not the linoleate supplement. This finding might imply increased utilization of α-tocopherol as an antioxidant when the diet is rich in highly unsaturated n-3 fatty acids compared with a diet rich in monounsaturated fatty acids. However, plasma α-tocopherol concentrations during all 3 supplement periods were well within the range considered to be clinically normal (54), and the physiological significance of this small decrease remains unclear. Previous research at the Oregon State University Lipid Laboratory did not reveal a significant difference in plasma α-tocopherol concentrations with and without a similar level of EPA/DHA supplementation (17). The normalization of plasma α-tocopherol to plasma lipid
concentrations has been proposed as a valuable index of vitamin E status, when plasma lipids may be altered (55). When α-tocopherol concentrations were normalized to plasma total cholesterol + plasma triacylglycerol, the differences between supplement groups were eliminated, mainly due to the triacylglycerol-lowering effect of the fish oil.

Plasma γ-tocopherol concentrations were more markedly decreased than α-tocopherol (approximately 30%) during EPA/DHA supplementation than during oleate or linoleate supplementation. When plasma γ-tocopherol concentrations were normalized to plasma lipid concentrations, only the significant difference between the EPA/DHA and the oleate supplements persisted. Although most concern has focused on the antioxidant effects of α-tocopherol, some evidence suggests that γ-tocopherol may also play an important role in human health. γ-Tocopherol has been found to be more effective than α-tocopherol in inhibiting peroxynitrite-induced oxidation in vitro (56), but a more recent study did not confirm this result (57).

The results of the present study suggest that increased dietary n-3 PUFA may not increase susceptibility to oxidative stress in vivo, in the manner suggested by research in vitro. Increasing the content of n-3 PUFA has been found to alter the physical and functional properties of membranes (58). N-3 PUFA associated changes such as increased membrane fluidity may serve to protect the membrane lipids and proteins from oxidation rather than increase their oxidative susceptibility. Recently, increased dietary n-3 fatty acids have been found to reduce proton leak
through the inner mitochondrial membrane in mice. Proton leak is a term for the back flow of protons into the mitochondrial matrix without ADP phosphorylation, resulting in oxygen consumption that is uncoupled from ATP synthesis (59). Thus, mitochondrial membrane changes associated with increased dietary n-3 PUFA could result in decreased cellular oxidative stress. An individual’s susceptibility to oxidative stress is also influenced by the endogenous antioxidant defense system, comprised in part of antioxidant enzymes such as glutathione peroxidase (GSH-Px), superoxide dismutase (SOD), and catalase. Increased hepatic activity and m-RNA expression of GSH-Px, SOD, and catalase have been demonstrated in animals fed high EPA/DHA fish oil compared to those fed high linoleate corn oil (60, 61).

In the present study, the results of a more sophisticated assay for plasma MDA than the TBA assay suggests that oxidative stress is not increased by dietary supplementation with high EPA/DHA fish oil. However, when plasma free F₂-isoprostanes were normalized to plasma 20:4n-6 concentrations, the results suggested a slight increase in the oxidation of 20:4n-6. Based on these data, it is unclear whether the potentially beneficial effects of diets rich in n-3 PUFA are offset by an increased risk of lipid peroxidation in vivo. The conflicting results of the plasma MDA and TBA assays in the present study suggest that findings of increased oxidative stress during fish oil supplementation based solely on the results of the plasma TBA assay be reevaluated in light of more sensitive and specific assays of in vivo oxidative stress.
References


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CHAPTER 4

THE EFFECT OF OLEATE, LINOLEATE, AND EPA/DHA SUPPLEMENTATION OF POSTMENOPAUSAL WOMEN ON EX VIVO OXIDATION OF LDL PHOSPHOLIPIDS AND CHOLESTERYL ESTERS

Jane V. Higdon, Shi-Hua Du, Ye-Sun Lee, Tianying Wu, and Rosemary C. Wander

Submission planned to Journal of Lipid Research, Bethesda, MD
Abstract

While replacement of dietary saturated fat with monounsaturated and polyunsaturated fatty acids (MUFA and PUFA) has been advocated for the reduction of cardiovascular disease risk, diets high in PUFA could increase LDL susceptibility to oxidation, potentially contributing to the pathology of atherosclerosis. To investigate this possibility 15 postmenopausal women took daily supplements of sunflower oil (12.3 g/day of oleate), safflower oil (10.5 g/day of linoleate), and fish oil (2.0 g/day of EPA and 1.4 g/day of DHA) in a 3-period, 3-treatment, crossover trial. The formation of phosphatidylcholine hydroperoxides (PCOOH) and cholesteryl linoleate hydroperoxides (CE18:2OOH) during copper-mediated oxidation, as well as the loss of α-tocopherol and individual fatty acids from LDL phospholipids (PL) and cholesteryl esters (CE) were measured to assess the oxidative susceptibility of LDL surface and core lipids. The lag phase for PCOOH formation was shorter in EPA/DHA- than oleate- (P = 0.0001) and linoleate-enriched LDL (P = 0.002), while the lag phase for CE18:2OOH was shorter in EPA/DHA- than oleate- (P = 0.01) but not linoleate-enriched LDL. The maximal rate of PCOOH formation was lower in EPA/DHA- than linoleate- (P = 0.007) but not oleate-enriched LDL, while the maximal rate of CE18:2OOH formation was lower in EPA/DHA- than oleate- (P = 0.03) and linoleate-enriched LDL (P = 0.0001). The maximal concentrations of PCOOH and CE18:2OOH were lower in EPA/DHA- than oleate- (P < 0.05 for both) and linoleate-enriched LDL (P < 0.01 for both). In both the PL and CE fractions after 6 h of oxidation, loss of n-3
PUFA was greater and loss of n-6 PUFA less in EPA/DHA- than oleate and linoleate-enriched LDL (P < 0.05 for all). Loss of total PUFA from PL (~40%) and CE (~50%) was not significantly different between the 3 supplement groups. Oleate-enrichment generally decreased the susceptibility of LDL surface and core lipids to peroxidation suggesting that increased oleate consumption decreases LDL oxidative susceptibility. EPA/DHA-enrichment did not appear to increase LDL oxidative susceptibility, compared to linoleate-enrichment, suggesting that the health benefits related to increased fish consumption are not offset by an increase in LDL oxidative susceptibility.

Introduction

A large body of research supports the hypothesis that the oxidation of LDL in vessel walls plays a significant role in the development of atherosclerosis (1,2). For this reason factors that influence the oxidative susceptibility of LDL have been the subject of a number of investigations. Despite their favorable effects on lipid profiles (3,4), concern exists that diets relatively high in unsaturated fat could increase the oxidative susceptibility of LDL, thereby negating some of their cardioprotective effects.

Generally, the more double bonds present in an unsaturated fatty acid, the more readily it is assumed to oxidize in biological systems. These assumptions are based on the results of investigations into the autoxidation of unsaturated fatty acids in homogeneous systems in vitro (5). However, studies of multiphase systems in vitro (6,7), which appear to have more similarities to LDL than
homogeneous systems, suggest oxidative susceptibility may not be directly related to the degree of unsaturation of a fatty acid.

Studies that have examined the effect of consuming specific unsaturated fatty acids on the oxidative susceptibility of LDL have not resulted in clear support of the notion that the higher the degree of unsaturation of dietary PUFA, the greater the susceptibility of LDL to oxidation. While a number of studies have demonstrated that diets high in oleate (18:1n-9) result in LDL that are more resistant to ex vivo oxidation than diets high in linoleate (18:2n-6) (8-10), the effects of increasing consumption of the n-3 fatty acids, eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acids (DHA; 22:6n-3), on LDL oxidative susceptibility have been contradictory. Some studies have suggested that increased dietary intake of EPA and DHA increased LDL susceptibility to ex vivo oxidation (11-14), while others have found that increasing dietary EPA and DHA intake did not increase the oxidative susceptibility of LDL (15-19). The contradictory nature of these studies may be related to the assays of LDL oxidation as well as the criteria chosen to indicate LDL oxidative susceptibility. For example, shortened lag phase might be the criteria for increased oxidative susceptibility in one investigation, while increased rate or extent of oxidation might be used as the criteria in another investigation. Fish oil supplementation has been found to shorten lag time as well as paradoxically to decrease rate and extent of oxidation in a number of LDL oxidation studies (14,19,20).
Because LDL oxidation appears to play an important role in the pathobiology of atherosclerosis, it is important to assess the effect of increasing dietary unsaturated fatty acids on the oxidative susceptibility of LDL. This is of particular relevance to postmenopausal women, for whom cardiovascular disease is the major cause of mortality in the United States (21). Until quite recently women have been underrepresented in studies of cardiovascular disease risk, despite evidence that findings regarding cardiovascular disease in men cannot always be extrapolated to women (22). In order to evaluate the effects of specific dietary unsaturated fatty acids on LDL oxidative susceptibility, we isolated LDL from the plasma of postmenopausal women taking daily supplements rich in oleate from sunflower oil, linoleate from safflower oil, and EPA/DHA from fish oil. We measured phosphatidylcholine hydroperoxide (PCOOH) and cholesteryl ester hydroperoxide (CEOOH) formation during copper-mediated LDL oxidation to assess the oxidative susceptibility of LDL surface and core lipids respectively. Additionally, we assessed the loss of vitamin E as well as individual fatty acids from LDL during copper-mediated oxidation.

**Subjects and Methods**

**SUBJECTS**

Sixteen postmenopausal women, between 50 and 75 years of age, were recruited from the Oregon State University campus and the surrounding community using posters and newspaper advertisements. The study protocol was reviewed and
approved by the institutional review board at Oregon State University and written consent was obtained from each participant prior to beginning the study.

Menopausal status was assessed on the basis of menstrual history (absence of normal menses for at least 12 months) and/or a history of use of hormone replacement therapy (HRT) for at least 12 months. A thorough medical history was obtained from each participant. Therapeutic agents or supplements known to have antioxidant or lipid-altering effects were specifically excluded. All participants were required to be normolipidemic based on the results of plasma lipid profiles, analyzed by the Oregon State University Lipid Laboratory. Normal liver function, glucose status, and iron status were verified through medical history and the results of a fasting serum chemistry panel and a screening complete blood count CBC, analyzed by Good Samaritan Hospital Laboratory (Corvallis, OR). All prospective participants had their blood pressure screened prior to entering the study to ensure normal systolic and diastolic blood pressure. Height and weight were measured, and body mass index (BMI) calculated to select for participants who were not obese. Cigarette smoking was excluded by history. In order to participate in the study all participants agreed to refrain from taking nutritional supplements other than calcium or vitamin D, and to refrain from eating fish for the duration of the study. They were informed that the 15 g of oil would add 567 kJ (135 kcal) to daily to their diets, and they agreed to maintain a stable body weight throughout the study, as well as to refrain from any significant change in physical activity.
Because one participant dropped out after the first period for reasons unrelated to the study, only the data obtained from the other 15 participants are included in the results. Occasional random loss of sample during laboratory analysis resulted in a further decrease in sample size for the statistical analysis of some of the assays reported.

**EXPERIMENTAL DESIGN**

In order to assess the effects of each of the 3 types of oil supplements on the individual participants, a 3-period, 3-treatment, blinded crossover trial was used. During each treatment period participants took 15 g of high oleate sunflower oil, high linoleate safflower oil, or fish oil rich in EPA/DHA. Each treatment period lasted 5 weeks and was followed by a 7-week washout interval, in order to minimize any carryover effect from the previous treatment. The initial 16 participants were randomly assigned to one of 6 treatment sequences, representing all possible sequences. The crossover trial was also designed to avoid confounding of period and treatment effects by including each treatment in each period. This design allowed for the statistical assessment of carryover effects, described in the statistical analysis section (23). The total time of participation in the study was 27 weeks.

After a 2-week run in period, measurements and blood samples were taken on Monday and Wednesday at the Oregon State University Metabolic Unit, prior to the start of the treatment and 5 weeks later, on Monday and Wednesday, during the last 3 days of the treatment period. Participants made a total of 14 visits to the
Metabolic Unit during the course of the study. Compliance was assessed by
counting leftover capsules, as well as by evaluating changes in specific fatty acid
concentrations in plasma fatty acid profiles.

SUPPLEMENTS

The fish oil was obtained from the National Institute of Health’s Fish Oil
Test Material Program (NIH-FOTMP) in sealed opaque containers, containing one
hundred 1-g capsules. The high oleate sunflower oil (generously donated by
Humpco, Memphis, TN) and the high linoleate safflower oil (Arista Industries,
Darien, CT) were supplied in bulk and encapsulated after adjustment for
antioxidant content (Professional Compounding Pharmacy, Corvallis, OR). The α-
tocopherol concentration in the fish oil supplied by the NIH-FOTMP was assayed
by our laboratory and found to be 1.2 mg/g oil, while the γ-tocopherol
concentration was 1.3 mg/g oil. The fish oil also contained 0.17 mg tertiary butyl
hydroquinone (TBHQ)/g oil as an additional antioxidant. TBHQ is a food grade,
oil-soluble antioxidant, which is metabolized within a few days of ingestion and
excreted in the urine. Because it is not stored in tissues, it does not function as an
antioxidant in vivo (24). After assaying the original α- and γ-tocopherol
concentrations in the sunflower and safflower oils, additional α-tocopherol, γ-
tocopherol and TBHQ (generously supplied by Eastman Chemical Company,
Kingsport, TN) were added in order to match the concentrations present in the fish
oil. Thus, all 3 oil supplements supplied approximately 20 mg α-tocopherol
equivalents (α-TE) in 15 g of oil consumed daily.
The potential for lipid peroxidation in the oil supplements was assessed by measuring peroxide values (25) and the \( p \)-anisidine value, a measure of the aldehyde content of fat (26). Assessment of lipid peroxidation was performed at the beginning of each treatment period on samples of each oil supplement, which had been kept refrigerated in opaque containers. Neither the \( p \)-anisidine value nor the peroxide value increased in any of the supplements by the final period of the study. We have previously shown that storing the capsules under conditions similar to the way in which the participants stored them also did not increase peroxidation (19).

Concentrations of \( \alpha \)- and \( \gamma \)-tocopherol in the supplemental oils were measured by normal-phase HPLC, using fluorometric detection (excitation \( \lambda \) 292; emission \( \lambda \) 330) based on a standardized method published by the International Union of Pure and Applied Chemistry (27). Oil samples were diluted in hexane and injected onto a silica column (Supelcosil 5\( \mu \)m; 250 x 4.6mm, Supelco Inc., Bellefonte, PA), using hexane: isopropanol (99:1 v/v) as a mobile phase, at a flow rate of 1 ml per minute.

In order to obtain fatty acid profiles of the supplemental oils, lipids were extracted with chloroform methanol (1:2, v/v), according to the method of Bligh and Dryer (28). After methylation, fatty acid methyl esters were measured by gas chromatography, using heptadecanoic acid (Nu-Chek Prep, Elysian, MN) as an internal standard, as described previously (29).
DIET ANALYSIS FOR NUTRIENT CONTENT

Each participant was instructed in the technique of keeping a three-day (two weekdays and one weekend day) record of all foods and beverages consumed. Participants kept 3-day diet records during each of the 3 treatment periods. The nutritional content of each participant’s diet during each treatment period was analyzed using the computer software, Food Processor Plus (version 6.0; ESHA, Salem, OR).

BLOOD COLLECTION

Venous blood samples were collected into tubes containing Na$_2$EDTA (1g/L) after an overnight fast of approximately 12 hours. Plasma samples were prepared within one hour of blood collection by centrifugation (3000 rpm) at 4° C for 15 minutes, using a TJ-6 desktop centrifuge (Beckman; Palo Alto, CA). Blood samples were kept in the dark and on ice until centrifugation.

PLASMA LIPID AND LIPOPROTEIN PROFILE

Plasma total cholesterol concentrations were determined enzymatically, using a modification of the method of Allain et al (30). This cholesterol assay meets the National Cholesterol Education Program’s performance criteria for accuracy. Plasma triacylglycerol concentrations were measured using a modification of the method of McGowan et al (31). HDL cholesterol concentrations were measured enzymatically after precipitation of LDL and VLDL
fractions with phosphotungstic acid and MgCl₂ (32). LDL cholesterol concentrations were calculated using the formula of Friedewald (33).

**LDL ISOLATION AND PREPARATION**

Immediately after plasma was separated from red cells, LDL was isolated from plasma by single spin discontinuous density gradient ultracentrifugation as described by Chung et al (34), using a near vertical rotor. Briefly, plasma density was adjusted to 1.30 g/mL by adding solid KBr (0.4946 g/mL plasma). Four mL of density-adjusted plasma was carefully layered under 9 mL of 0.9% saline (density 1.006 g/mL) in a Quick-seal centrifuge tube (Cat. No. 34413, Beckman) using a 6-inch, 18 g needle and a 5 mL syringe. Sealed tubes were centrifuged in a near vertical rotor (NVT 65, Beckman) at 60,000 rpm for 120 minutes using an L5-75 ultracentrifuge (Beckman) set for slow acceleration at 7°C. Allowing for a slow deceleration period to prevent disruption of the gradients resulted in a total centrifugation time for LDL separation of 190 minutes. The LDL band was collected from the top of the centrifuge tube using a pasteur pipette. Immediately after isolation, samples for LDL composition were capped with argon and frozen at −70°C, while samples for LDL oxidation were dialyzed against phosphate-buffered saline (PBS; 0.15M NaCl, 0.01M NaH₂PO₄, pH 7.4) in the dark, at 4°C in order to remove EDTA. The PBS was degassed for 10 minutes, using a water aspirator, and purged with nitrogen for 5 minutes prior to use in order to remove oxygen from the solution. The buffer (LDL: PBS; 1:100 v/v) was changed 4 times during the 20-hour dialysis period.
The protein content of LDL was measured using the method of Lowry et al (35). After dialysis, each LDL sample was diluted to a concentration of 0.5 mg LDL protein/mL and oxidized using 4.69 μM CuSO₄ at 37°C. Aliquots removed just prior to the addition of CuSO₄ were designated as samples taken at 0 min of oxidation. Aliquots of LDL were removed at predetermined time points during oxidation for measurement of vitamin E, cholesteryl ester hydroperoxides (CEOOH), phosphatidylcholine hydroperoxides (PCOOH), and LDL fatty acid profile. Oxidation was terminated by the addition of EDTA at a concentration of 1.5 mg/mL and placing the samples on ice.

**LDL COMPOSITION**

LDL total cholesterol (TC) content was measured enzymatically using a modification of the method of Allain et al (30), while LDL triacylglycerol (TG) content was measured using a modification of the method of McGowan et al (31) (Sigma; St. Louis, MO). LDL free cholesterol (FC) and LDL phospholipid (PL) content were also measured using enzymatic methods (Wako; Richmond, VA). LDL phospholipids consist of 62%-66% phosphatidyl choline, 24%-28% sphingomyelin, 7%-7.4% lysophosphatidylcholine, and 2.3% other (2). The enzymatic assay for PL in LDL measured choline-containing phospholipids, accounting for 98% of the phospholipids in LDL. LDL cholesteryl ester (CE) content was calculated as (TC - FC) x 1.68 (36). Assays for LDL composition were performed at the end of each of the 3 treatment periods. The inter-assay CV
for TC, TG, FC, and PL between treatment periods were 3.0%, 5.7%, 9.4%, and 3.6% respectively.

**FATTY ACID PROFILES OF LDL PHOSPHOLIPIDS AND CHOLESTERYL ESTERS**

The fatty acid profiles of LDL lipids were analyzed at 0 and 360 minutes of CuSO₄-mediated oxidation. LDL lipids were extracted from 0.4 mL samples (0.5 mg LDL protein/mL) with chloroform methanol (1:2, v/v), using a modification of the method of Bligh and Dryer (28). Internal standards of diheptadecanoyl phosphatidylcholine (PC17:0) and cholesterol heptadecanoate (CE17:0) were added to each LDL sample prior to extraction. Lipids were separated using 20 x 20 cm Silica-Gel H thin-layer chromatography (TLC) plates (Alltech; Deerfield, IL). After washing the plates in chloroform/methanol (1:1, v/v), the lipid extract, dissolved in 50 μL chloroform, was applied as a streak 1 cm from the lower edge of the plate. Separation of the lipid classes was achieved by elution with diethylether/hexane (15:85, v/v). After drying with nitrogen, the plates were sprayed with an aqueous solution of 0.2% rhodamine 6G (Alltech). Cholesterol ester and phospholipid bands were identified by comparison with authentic standards applied to the same TLC plate as the samples (37). The uppermost band on the plate was identified as the cholesteryl ester fraction, while the phospholipid fraction did not migrate from the point of sample application at the base of the plate (Figure 4.1). Bands corresponding to cholesteryl ester and phospholipid fractions were scraped from the plate, methylated in the presence of heneicosanoic methyl ester (methyl 21:0) as
a standard, and analyzed using gas chromatography as described previously (29). Loss of individual LDL fatty acids was calculated by subtracting the concentration at 360 min of oxidation from the concentration at 0 min. The peroxidation index was calculated as follows: 

\[
\text{Peroxidation index} = (\Sigma PUFA \text{ with 2 double bonds} \times 1) + (\Sigma PUFA \text{ with 3 double bonds} \times 2) + (\Sigma PUFA \text{ with 4 double bonds} \times 3) + (\Sigma PUFA \text{ with 5 double bonds} \times 4) + (\Sigma PUFA \text{ with 6 double bonds} \times 5).
\]

**Figure 4.1.** Schematic TLC separation of simple lipids on a silica gel plate. With diethylether: hexane (15:85, v/v) as the solvent, cholesteryl esters (CE) migrate to the solvent front, followed by triacylglycerols (TG), free fatty acids (FFA), cholesterol (C), diacylglycerols (DG), monoacylglycerols (MG) and phospholipids (PL) (37).

**LDL VITAMIN E**

LDL vitamin E content was determined by reverse phase HPLC with fluorometric detection (excitation \(\lambda\) 295 nm, emission \(\lambda\) 330 nm), using a modification of the method of Arnaud et al (38). After precipitation of protein with 0.4 mL of ethanol, tocopherols from samples containing 100 \(\mu\)g of LDL protein at
0 min and 200 μg of LDL protein at 10 and 20 min of oxidation were extracted into 1 mL of hexane. Out of that 1 mL of hexane, 0.75 mL was removed and evaporated to dryness under nitrogen, resuspended in 0.25 mL of methanol, and 100 μL injected onto a C-18 column (Shim-pack CLS-ODS 5μ; 250x4.6mm, Shimadzu, Columbia, MD), using 100% methanol as a mobile phase, at a flow rate of 1.5 mL/min. Tocopherols (α and γ) were quantified in LDL using external standards of pure α- and γ-tocopherol (Sigma; St. Louis, MO) of known concentration. Recovery of added α-tocopherol to LDL samples averaged 92% and the interassay CV was 5.6%. LDL vitamin E was extracted and measured immediately after CuSO₄-mediated oxidation was terminated by the addition of EDTA. α- and γ-Tocopherol concentrations present in LDL were compared among the 3 supplement groups before oxidation and after 10 and 20 min of oxidation. The rate of α-tocopherol loss was assumed to be linear, and was estimated as the slope of the line of best fit between the 3 time points. The intercept of the same line with the time axis was used to estimate the time of vitamin E depletion. The rate of loss was not estimated for γ-tocopherol because the curves were clearly nonlinear. LDL α- and γ-tocopherol concentrations were normalized to LDL protein as well as to LDL lipid content (the sum of LDL total cholesterol, triacylglycerol, and phospholipid concentrations).
HYDROPEROXIDES IN OXIDIZED LDL

Phosphatidylcholine hydroperoxides (PCOOH) and cholesteryl ester hydroperoxides (CEOOH) were measured in LDL at 0, 20, 40, 60, 90, 120, 180, 240, 300, and 360 minutes of CuSCU-mediated oxidation using HPLC with postcolumn chemiluminescence detection, based on the methods of Sattler et al (39). These methods have been demonstrated to be highly sensitive and specific for PCOOH and CEOOH (40).

To remove traces of contaminating metals Chelex 100 resin (Bio-Rad, Richmond, CA) was added to all aqueous buffers (5 g/100mL), stirred for 1 hour and filtered to remove the Chelex. HPLC solvents were stored in dark bottles at 4°C over a 4 Å molecular sieve (∼100 g/L, Aldrich) to deplete them of hydroperoxides. Hexane was washed with water to remove trace amounts of hydroperoxides. One volume of water was added to 10 volumes of hexane in a brown bottle and stirred overnight. The water was allowed to settle for at least 4 h, and the upper hexane phase was removed for use in the assay (41).

Lipid hydroperoxides from 0.2 mL of unoxidized LDL and 0.1 mL (0.5 mg LDL protein/mL) of oxidized LDL were extracted into 1 mL of methanol (PCOOH) and 5 mL of washed hexane (CEOOH), yielding 2 phases (hexane and aqueous methanol). To measure PCOOH, the aqueous methanol extract was filtered and injected on to a NH2 column (LC-NH2, 5μ, 250x4.6mm, Supelco; Bellefonte, PA), using methanol/40mM NaH2PO4 (95:5, v/v) as a mobile phase, at a flow rate of 1.0 mL/min. Using a mixing tee, a postcolumn chemiluminescence
reagent consisting of 1:1 (v/v) methanol/100mM sodium borate buffer (pH 10) containing 1 mM isoluminol (6-amino-2,3,-dihydro-1,4-phthalazinedione; Sigma) and 3 mg/L microperoxidase (MP-11; Sigma), at a flow rate of 1.5 mL/min, was added to the eluent. The reaction of hydroperoxides and the postcolumn reagent results in the generation of light, which can be quantified using a chemiluminescence detector (S-3400, Soma Optics, Japan). See Figure 4.2 for a schematic diagram of the HPLC/chemiluminescence assay.

![Schematic diagram of HPLC/chemiluminescence assay](image)

**Figure 4.2.** Schematic diagram of HPLC/chemiluminescence assay (42).

The identification standard for PCOOH was prepared by oxidation of 20 mg of soybean phosphatidylcholine (Sigma) in 1 mL of methanol and 2 mM 2,2’-azobis(2-methylpropionitrile (AIBN; Aldrich, Milwaukee, WI) at 37°C for 6 h. 15(S)-hydroperoxyeicosatetraenoic acid (15(S)-HPETE; #44720, Cayman Chemicals, Ann Arbor, MI) was used as an external quantification standard. The
lower level of detection of PCOOH was approximately 50 pmol/mg LDL protein, and the interassay CV was 8.3%. PCOOH were extracted from LDL immediately after CuSO₄-mediated oxidation was terminated by the addition of EDTA. Extracted samples were capped with argon and immediately frozen at -80°C and were measured within 4 weeks of collection.

To measure CEOOH, 4 mL of the hexane extract was evaporated under nitrogen to dryness and resuspended in 0.25 mL of ethanol. Twenty to 100 μL were injected on to a C-18 column (Shim-pack CLS-ODS 5μ; 250x4.6mm, Shimadzu, Columbia, MD) using a mobile phase of methanol/tert-butanol (3:1, v/v) at a flow rate of 1 mL/min. The postcolumn chemiluminescence reagent was the same as that used in the PCOOH assay at a flow rate of 1.5 mL/min.

External standards for the identification of the hydroperoxides of CE18:1n-9 (CE18:1OOH), CE18:2n-6 (CE18:2OOH), CE20:4n-6 (CE20:4OOH), CE20:5n-3 (CE20:5OOH), and CE22:6n-3 (CE22:6OOH) were prepared separately by the oxidation of 20 mg of the pure cholesteryl ester (NuChek Prep, Elysian, MN) in 1 mL of toluene and 2 mM AIBN at 37°C for 6 h. After evaporation of the toluene under nitrogen the oxidized CE was resuspended in 1 mL of hexane and applied to a hexane-preconditioned Al₂O₃ solid phase extraction (SPE) column (Supelclean Alumina N; Supelco). The unoxidized CE was eluted with 15 mL of hexane and the CEOOH was eluted with 15 mL of tert-butylmethyl ether. Purified CEOOH standards were assayed individually using the HPLC/chemi-luminescence procedure described in the previous paragraph. Retention times for each peak in
each individual CEOOH standard were recorded. A mixture of all 5 CEOOH standards was also assayed in order to evaluate the separation of different species of CEOOH from another. The composition of the mobile phase of methanol/tert-butanol was varied incrementally from (1:1, v/v) to (5:1, v/v) in order to determine the best conditions for separation. Ultimately, the most useful separation of individual species of CEOOH was obtained using a mobile phase of methanol/tert-butanol (3:1, v/v).

The oxidation of PUFA may result in the formation of hydroperoxides with a number of positional isomers and various cyclic products. For example, 4 positional isomers of the hydroperoxide of 18:1n-9 have been identified, 2 of 18:2n-6, 6 of 20:4n-6, 8 of 20:5n-3, and 10 of 22:6n-3 hydroperoxide (43). The separation of the hydroperoxides obtained from specific cholesterol esters of LDL was problematic in that LDL contained a number of different cholesterol ester species. Not all of the peaks, found in standards made from oxidizing a single species of CE, could be identified. Using the HPLC conditions described above, CE20:5OOH could not be unequivocally identified in any of the LDL samples, as all its peaks were obscured by peaks of other CEOOH species. At least one peak representing the other 4 species of CEOOH could be identified in most of the LDL samples. 15(S)-HPETE was used as an external quantification standard for CEOOH. The lower level of detection for CE18:2OOH was approximately 10 pmol/mg LDL protein, and the interassay CV was 4.2%. CEOOH were extracted from LDL immediately after CuSO₄-mediated oxidation was terminated by the
addition of EDTA. Extracted samples were capped with argon and immediately frozen at -80°C and were measured within 6 days of collection.

Hydroperoxide concentrations were plotted over time of CuSO₄-mediated oxidation for CEOOH and PCOOH. Maximal rate was determined to be the slope of the line of best fit, using the method of least squares, through the 3 points that defined the steepest slope of the curve. The length of the lag phase was determined to be the time (value for X) at the intersection of the 2 straight lines of best fit as illustrated in Figure 4.3. The maximal concentration was taken to be the maximal concentration measured during 6 h of oxidation, and the time to one half of the maximal concentration was determined by drawing a perpendicular line from the oxidation curve at one half the value of the maximal concentration (y axis) to the time (x axis) as illustrated in Figure 4.3 (44).
Figure 4.3. Calculation of lag phase, maximal rate, maximal concentration, and time to half maximal concentration for CEOOH or PCOOH formation during ex vivo oxidation of LDL. Equations were obtained for the solid lines of best fit using the method of least squares. The duration of the lag phase was calculated by solving for X at the intersection of the two solid lines. The maximal concentration was taken to be the maximal concentration measured during 6 h of oxidation and the time to one half of the maximal concentration were determined by drawing a perpendicular line (broken lines) from the oxidation curve at one half the value of the maximal concentration (y axis) to the time (x axis).

STATISTICAL ANALYSIS

The primary advantage of a crossover design is the increased precision in treatment comparisons, due to the removal of the between subject variation from the experimental error. A disadvantage of the crossover design is the possibility for carryover effects, i.e., a treatment given in one period may influence the treatment response in the following period. A 7-week washout period was utilized after each treatment period to decrease the likelihood of carryover effects. Additionally, the random assignment of at least 2 subjects to all 6 possible treatment sequences
allowed for the statistical assessment of carryover effects prior to inference regarding direct treatment effects.

Carryover effects were estimated and direct treatment effects analyzed, utilizing a mixed between-within subjects ANOVA procedure described by Kuehl (23). Briefly, the between subjects sources of variation consisted of 1) sequence of treatment and 2) subjects nested within sequence, while within subjects sources of variation consisted of 1) period, 2) treatment (direct), and 3) treatment (carryover). If treatment carryover effects were significant (P ≤ 0.05), estimates of differences among treatment means could be adjusted for the carryover effects. If carryover effects were not found to be statistically significant, the ANOVA was performed without the treatment (carryover) effect in the model. No significant treatment carryover effects were found for any of the data presented. Therefore, all least square means presented represent direct treatment effects which were not adjusted for carryover. Because no significant period-treatment interactions were encountered, the period-treatment interaction was not included in the final model.

Pair-wise comparisons were performed using Fisher’s least significant difference test to determine significant differences among treatments. Results with P values ≤ 0.05 for a two-sided test were considered statistically significant. Analyses were accomplished using the SAS general linear model (GLM) procedure (version 6.12: SAS Institute Inc., Cary, NC).

Pearson product-moment correlation coefficients were calculated separately, for each of the 3 oil treatments. The correlation coefficient (r) was
considered statistically significant if its P value was $\leq 0.05$ for a two-sided test. Paired t tests (significance level $P \leq 0.05$) were used to compare 2 dependent variables of interest within a supplement group.

**Results**

**SUBJECT CHARACTERISTICS**

All participants were postmenopausal and taking HRT. Each participant continued her regimen of HRT, without alteration, for the duration of the study. Although the HRT regimens were not identical for each woman, they were generally equivalent to 0.625 mg conjugated estrogens daily for hysterectomized women and 0.625 mg conjugated estrogens plus 2.5 mg medroxyprogesterone daily for women with intact uteruses. The participants did not take any chronic prescription medications other than HRT, nor did they take any nutritional supplements, other than calcium or vitamin D, for the duration of the study. Compliance with the supplement regimen was estimated to be approximately 95% based on the return of empty supplement containers and leftover supplement capsules. The results of the plasma fatty acid profiles for each participant during each treatment period were also consistent with a high level of compliance.

The initial characteristics are presented in Table 4.1. The mean age of the participants was 58 y (range 52-73 y). Although the mean BMI of 25.8 was slightly greater than the desirable upper limit of 24.9, it was well under 30, the level at which morbidity and mortality associated with obesity has been found to
increase rapidly (45). The mean weight gain for the participants over the study
duration of 9 months of was 0.6 kg. All participants were assessed as
normolipidemic on the basis of their lipid and lipoprotein profiles. Plasma total
and LDL cholesterol for the participants were much lower than the fiftieth
percentile for this particular population of women (5.90-6.00 and 3.75-3.83
mmol/L, respectively), while HDL cholesterol concentrations were higher (1.40-
1.45 mmol/L). Plasma triacylglycerol concentrations were well below 2.3 mmol/L,
the upper limit of normal (46).

Table 4.1. Subject characteristics* (n = 15)

<table>
<thead>
<tr>
<th>Subject characteristic</th>
<th>Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>57.6 ± 5.8</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>25.8 ± 3.5</td>
</tr>
<tr>
<td>Plasma cholesterol (mmol/L)</td>
<td>5.23 ± 0.55</td>
</tr>
<tr>
<td>LDL cholesterol (mmol/L)</td>
<td>2.98 ± 0.61</td>
</tr>
<tr>
<td>HDL cholesterol (mmol/L)</td>
<td>1.57 ± 0.39</td>
</tr>
<tr>
<td>Plasma triacylglycerol (mmol/L)</td>
<td>1.49 ± 0.47</td>
</tr>
</tbody>
</table>

*Mean ± SD

DIETARY CONTENT OF SELECTED NUTRIENTS

The participants consumed an average of 29 ± 6% of their energy as fat
with approximately 12% of that energy as saturated fat, 11% as monounsaturated
fat, and 6% as polyunsaturated fat. Of the antioxidant nutrients, mean vitamin E
intake of 6 mg α-tocopherol equivalents (TE)/day was slightly below the
recommended intake of 8 mg α-TE/day. Mean vitamin C (138 mg/day) and
selenium (57 µg/day) intake met recommended levels of 60 mg/day and 55
µg/day, respectively (47) (Table 4.2).
Table 4.2. Average daily intake of selected nutrients from three 3-day diet records.* (n = 15)

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>Recommended†</th>
<th>Intake</th>
</tr>
</thead>
<tbody>
<tr>
<td>Energy (kJ)</td>
<td>7950</td>
<td>7374 ± 1916</td>
</tr>
<tr>
<td>Protein (g)</td>
<td>55</td>
<td>68 ± 19</td>
</tr>
<tr>
<td>Carbohydrate (% of energy)</td>
<td>-</td>
<td>55 ± 9</td>
</tr>
<tr>
<td>Total fat (% of energy)</td>
<td>≤30</td>
<td>29 ± 6</td>
</tr>
<tr>
<td>Saturated fat (g)</td>
<td>-</td>
<td>19 ± 8</td>
</tr>
<tr>
<td>MUFA (g)</td>
<td>-</td>
<td>18 ± 8</td>
</tr>
<tr>
<td>PUFA (g)</td>
<td>-</td>
<td>9 ± 5</td>
</tr>
<tr>
<td>Cholesterol (mg)</td>
<td>&lt;300</td>
<td>177 ± 90</td>
</tr>
<tr>
<td>Vitamin E (mg α-TE)</td>
<td>8</td>
<td>6 ± 3</td>
</tr>
<tr>
<td>Vitamin C (mg)</td>
<td>60</td>
<td>138 ± 84</td>
</tr>
<tr>
<td>Selenium (μg)</td>
<td>55</td>
<td>57 ± 31</td>
</tr>
</tbody>
</table>

* mean ± SD
†Recommended value represents the recommended dietary allowance (RDA) (47)

Fatty acid intake

The oil supplement and total dietary content of 7 fatty acids of interest are presented in Table 4.3. By design, each oil supplement supplied concentrations of specific fatty acids that were high but attainable through dietary manipulation. Fifteen g/day of the sunflower oil provided 12.3 g/day of oleate, while 15 g of the safflower oil provided 10.5 g/day of linoleate. The same quantity of fish oil provided 2.0 g/day of EPA and 1.4 g/day of DHA. As a result of the oil supplement, the total dietary intake of specific fatty acids differed significantly among supplement groups. During the oleate supplement, total 18:1n-9 intake was 79% higher than that of the linoleate (P<0.0001) and 61% higher than that of the EPA/DHA supplement (P<0.0001). During the linoleate supplement, 18:2n-6 intake was 146% higher than that of the oleate (P<0.001) and 119% higher than
that of the EPA/DHA supplement (P<0.0001). Because the participants were asked to exclude fish from their diet throughout the study, the EPA/DHA supplement greatly increased (approximately 50 to 100 fold) the 20:5n-3 and 22:6n-3 content of the diet when compared to that of the oleate and the linoleate supplements (P<0.0001 for all 4 comparisons). Although total intake of the n-6 polyunsaturated fatty acid, arachidonic acid (20:4n-6) was relatively low during all 3 supplements, it was significantly higher during the EPA/DHA supplement (P<0.0001) due to the higher 20:4n-6 content of the fish oil.

Table 4.3. Consumption of selected fatty acids (FA) derived from oil supplement (oil) and total consumption (oil supplement + dietary content) in g/d during each supplement period.*

<table>
<thead>
<tr>
<th>FA</th>
<th>oleate (sunflower oil)</th>
<th>linoleate (safflower oil)</th>
<th>EPA/DHA (fish oil)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>oil</td>
<td>diet + oil</td>
<td>oil</td>
</tr>
<tr>
<td>16:0</td>
<td>0.49</td>
<td>7.70 ± 0.57</td>
<td>1.04</td>
</tr>
<tr>
<td>18:0</td>
<td>0.53</td>
<td>3.99 ± 0.33</td>
<td>0.36</td>
</tr>
<tr>
<td>18:1 n-9</td>
<td>12.27</td>
<td>25.74 ± 1.20</td>
<td>1.84</td>
</tr>
<tr>
<td>18:2 n-6</td>
<td>0.58</td>
<td>6.79 ± 0.90</td>
<td>10.46</td>
</tr>
<tr>
<td>20:4 n-6</td>
<td>ND</td>
<td>0.05 ± 0.01</td>
<td>ND</td>
</tr>
<tr>
<td>20:5 n-3</td>
<td>ND</td>
<td>0.01 ± 0.01</td>
<td>0.02</td>
</tr>
<tr>
<td>22:6 n-3</td>
<td>ND</td>
<td>0.01 ± 0.01</td>
<td>ND</td>
</tr>
</tbody>
</table>

*Values for oil represent measurements made in duplicate; calculations based on 15 g oil/d. Values for diet + oil represent least square mean (LSM) ± SEM.
†Different letters represent significant differences in total fatty acid consumption between oil supplement groups (P ≤ 0.01).
‡ND = not detectable

**LDL COMPOSITION BY LIPID CLASS**

Molecules of cholesteryl ester and triacylglycerol form the central lipophilic core of the LDL particle. The core is surrounded by a monolayer of phospholipid molecules (~64% phosphatidylcholine, 7% lysophosphatidylcholine, and 26%
sphingomyelin) and free cholesterol (2). A large protein of 550 kDa, apolipoprotein B (apo B), is embedded in the outer layer though in contact with the core (2). The changes in LDL composition after oil supplementation are summarized in Figure 4.4. LDL triacylglycerol content after EPA/DHA supplementation was 31% lower than after oleate and 21% lower than after linoleate supplementation (P = 0.001 and 0.04, respectively), while triacylglycerol content did not differ significantly between oleate and linoleate supplementation. LDL phospholipid content was 5% higher after linoleate supplementation than after oleate and 6% higher than after EPA/DHA (P = 0.01 and 0.007, respectively), while phospholipid content did not differ between oleate and EPA/DHA supplementation. LDL cholesteryl ester and free cholesterol content did not differ significantly among the oil supplement groups.
Fig. 4.4. LDL composition by major lipid class at the end of supplementation with 3 different oils. Values are LSM ± SEM. Bars represent values at the end of 5 weeks of 15 g/d supplementation of oil high in oleate (diagonal lines), linoleate (solid), and EPA/DHA (shaded). TG represents triacylglycerols, FC free cholesterol, CE, cholesteryl esters, and PL phospholipids. Different letters represent significant differences among supplement groups (P < 0.05).

PUFA CONTENT OF LDL PHOSPHOLIPIDS PRIOR TO AND AFTER 6 HOURS OF OXIDATION

At the end of each supplement period, differences in the fatty acid content of LDL phospholipid prior to oxidation generally reflected the differences in dietary fatty acid content (Figure 4.5). After oleate supplementation LDL phospholipids contained 43% more 18:1n-9 than after linoleate and 52% more than after EPA/DHA supplementation (P = 0.0001), but did not differ between linoleate and EPA/DHA supplementation. After EPA/DHA supplementation LDL
phospholipid contained 25% less 18:2n-6 than after oleate supplementation and 34% less than after linoleate supplementation (P = 0.006 and 0.0004, respectively), but did not differ between oleate and linoleate supplementation. After EPA/DHA supplementation, LDL phospholipid content of 20:5n-3 increased more than 10 fold over oleate and linoleate supplementation (P < 0.0001), while LDL phospholipid content of 22:6n-3 more than doubled compared to oleate and linoleate supplementation (P < 0.0001). The oleate and linoleate supplement groups did not differ in LDL phospholipid 20:5n-3 and 22:6 n-3 content. After EPA/DHA supplementation, LDL phospholipid 20:4n-6 content was 25% lower than after oleate and 22% lower than after linoleate supplementation (P = 0.03), even though there were greater amounts in the diet, but did not differ significantly between oleate and linoleate supplement groups.

The peroxidation index (PI) is an estimate of the concentration of bisallylic hydrogens present in unsaturated fatty acids, and may therefore be viewed as an index of their susceptibility to oxidation. After EPA/DHA supplementation, the PI was 40% higher than after oleate or linoleate supplementation (P < 0.0001 for both comparisons), while the PI did not differ between oleate and linoleate supplement groups.

Losses of specific fatty acids from LDL phospholipids after 6 h of CuSO₄-mediated oxidation, determined to be the difference between the concentration of a given fatty acid prior to oxidation and the concentration after 6 h of oxidation, are also presented in Figure 4.5. The loss of 18:1n-9 from LDL phospholipids did not
differ among supplement groups. The reason for the small positive value is not clear. It may represent error in the assay (approximately 10%) or it may be the result of interferences in the oxidized phospholipid samples. The loss of 18:2n-6 from EPA/DHA-enriched LDL phospholipids was 36 nmol/mg LDL protein which was less than that lost from linoleate-enriched LDL (56 nmol/mg LDL protein; \( P = 0.05 \)) and oleate-enriched LDL (55 nmol/mg LDL protein; \( P = 0.06 \)). The loss of 18:2n-6 from LDL phospholipids was not significantly different between oleate- and linoleate-enriched LDL. In all 3 supplement groups, approximately one fourth of the 18:2n-6 present in LDL phospholipids was oxidized after 6 hours.

The loss of 20:5n-3 from EPA/DHA-enriched LDL phospholipids was 38 nmol/mg LDL protein compared to 5 nmol/mg LDL protein from oleate- and 3 nmol/mg LDL protein from linoleate-enriched LDL (\( P = 0.0001 \) for both comparisons). Loss of 22:6n-3 from EPA/DHA-enriched LDL phospholipids was 40 nmol/mg LDL protein compared to 18 nmol/mg LDL protein from oleate- and 16 nmol/mg LDL protein from linoleate-enriched LDL phospholipids (\( P = 0.0001 \) for both comparisons). Loss of 20:5n-3 and 22:6n-3 did not differ between oleate- and linoleate-enriched LDL phospholipids.

Although the loss of total PUFA from LDL phospholipid did not differ significantly among supplement groups, loss of n-6 PUFA from EPA/DHA-enriched LDL phospholipids was 82 nmol/mg LDL protein compared to 127 nmol/mg LDL protein from oleate- and 125 nmol/mg LDL protein from linoleate-enriched LDL (\( P = 0.02 \) for both comparisons). Loss of n-3 PUFA from
EPA/DHA-enriched LDL phospholipids was 89 nmol/mg LDL protein compared to 32 nmol/mg LDL protein from oleate- and 25 nmol/mg LDL protein from linoleate-enriched LDL phospholipids (P = 0.0001 for both comparisons). However, the fraction of n-6 PUFA lost after 6 h of oxidation was about one third for all 3 supplement groups, while the fraction of n-3 PUFA lost was closer to two thirds for all 3 supplement groups. After 6 h of oxidation the PI for EPA/DHA-enriched LDL phospholipids was 491 compared to 382 for oleate- (P = 0.004) and 400 for linoleate-enriched LDL phospholipids (P = 0.001). The PI for LDL phospholipids decreased by approximately 50% after 6 h of oxidation for each supplement group, resulting in a significantly greater absolute change in EPA/DHA supplement group when compared to oleate and linoleate supplement groups.
Fig. 4.5. Unsaturated fatty acid content of LDL phospholipids prior to and after 6 h of copper-mediated oxidation. Bars with different letters over them represent significant differences (P ≤ 0.05) in fatty acid content among supplement groups after 5 weeks of supplementation with oleate (diagonally striped bars), linoleate (solid bars), and EPA/DHA (shaded bars). Loss of individual fatty acids was calculated by subtracting the concentration at 6 h of oxidation from the concentration at 0 h. Bars with different numbers under them represent significant differences in fatty acid loss after 6 h of oxidation among supplement groups (P ≤ 0.05). PL PUFA represents the sum of all PUFA; n-6 and n-3 PUFA represent the sums of all n-6 and n-3 PUFA respectively in LDL phospholipids. PLPI represents the peroxidation index. The change in PLPI after 6 h of oxidation is presented in the far right section of the lower graph.
PUFA CONTENT OF LDL CHOLESTERYL ESTERS PRIOR TO AND AFTER 6 HOURS OF OXIDATION

Fatty acid content of LDL cholesteryl ester prior to oxidation also reflected the dietary fatty acid content at the end of the supplement period (Figure 4.6). LDL cholesteryl ester 18:1n-9 content was 28% higher after oleate supplementation than linoleate and 24% higher than after EPA/DHA supplementation (P = 0.0004 and 0.001, respectively), but did not differ between linoleate and EPA/DHA supplement groups. After linoleate supplementation LDL cholesteryl ester content of 18:2n-6 was 19% higher than after oleate and 23% higher than after EPA/DHA supplementation (P = 0.003 and 0.0006, respectively), but did not differ between oleate and EPA/DHA supplement groups. LDL cholesteryl ester 20:4n-6 content did not differ significantly among the 3 supplement groups. After EPA/DHA supplementation LDL cholesteryl ester 20:5n-3 content increased more than 10 fold over oleate and linoleate supplementation (P = 0.0001), while 22:6n-3 content more than doubled that of oleate and linoleate supplementation (P = 0.0001). Oleate and linoleate supplement groups did not differ in LDL cholesteryl ester 20:5n-3 or 22:6n-3 content. The PI for LDL cholesteryl esters was significantly higher after EPA/DHA supplementation compared to oleate and linoleate supplementation (P = 0.0002 and 0.008, respectively).

Losses of specific fatty acids from LDL cholesteryl esters are also presented in Figure 4.6. The loss of 18:2n-6 from linoleate-enriched LDL cholesteryl esters was 490 nmol/mg LDL protein, compared to only 321 nmol/mg LDL protein from EPA/DHA-enriched LDL cholesteryl esters (P = 0.02). Loss of 18:2n-6 from
oleate-enriched LDL cholesteryl esters was 452 nmol/mg LDL protein, compared to 321 nmol/mg LDL from EPA/DHA-enriched LDL, a difference which tended to be significant ($P = 0.07$). After 6 h of oxidation oleate-enriched LDL lost 48% of the 18:2n-6 originally present in its cholesteryl ester fraction, while EPA/DHA lost only 35% of its 18:2n-6 ($P = 0.03$). Linoleate-enriched LDL lost 44% of the 18:2n-6 from its cholesteryl ester fraction, which did not differ significantly from either oleate- or EPA/DHA-enriched LDL.

The loss of 20:5n-3 from EPA/DHA-enriched LDL cholestery esters was 99 nmol/mg LDL protein compared to 10 nmol/mg LDL protein from oleate- and 8 nmol/mg LDL protein from linoleate-enriched LDL ($P = 0.0001$ for both comparisons). Loss of 22:6n-3 from EPA/DHA-enriched LDL cholesteryl esters was 22 nmol/mg LDL protein compared to 11 nmol/mg LDL protein from oleate- ($P = 0.008$) and 8 nmol/mg LDL protein from linoleate-enriched LDL phospholipids ($P = 0.001$). Loss of 20:5n-3 and 22:6n-3 did not differ between oleate- and linoleate-enriched LDL cholesteryl esters.

Although the loss of total PUFA from LDL cholesteryl esters did not differ significantly among supplement groups, loss of n-6 PUFA from EPA/DHA-enriched LDL cholesteryl esters was 407 nmol/mg LDL protein compared to 579 nmol/mg LDL protein from oleate- ($P = 0.04$) and 602 nmol/mg LDL protein from linoleate-enriched LDL ($P = 0.02$). Loss of n-3 PUFA from EPA/DHA-enriched LDL cholesteryl esters was 138 nmol/mg LDL protein compared to 35 nmol/mg LDL protein from oleate- and 30 nmol/mg LDL protein from linoleate-enriched...
LDL cholesteryl esters (P = 0.0001 for both comparisons). As in LDL phospholipid, the fraction of n-3 PUFA (69-84%) lost after 6 h of oxidation was greater than that of n-6 PUFA (38-52%). After 6 h of oxidation the PI for EPA/DHA-enriched LDL cholesteryl esters was 955 compared to 635 for oleate- and 829 for linoleate-enriched LDL cholesteryl esters. None of the changes in the PI for LDL cholesteryl esters differed significantly among groups. The PI for LDL cholesteryl esters decreased by approximately 55% after 6 h of oxidation in each supplement group.
Figure 4.6. Unsaturated fatty acid content of LDL cholesteryl esters prior to and after 6 h of copper-mediated oxidation. Bars with different letters over them represent significant differences (P ≤ 0.05) in fatty acid content among supplement groups after 5 weeks of supplementation with oleate (diagonally striped bars), linoleate (solid bars), and EPA/DHA (shaded bars). Loss of individual fatty acids was calculated by subtracting the concentration at 6 h of oxidation from the concentration at 0 h. Bars with different numbers under them represent significant differences in fatty acid loss after 6 h of oxidation among supplement groups (P ≤ 0.05). CE PUFA represents the sum of all PUFA, n-6 and n-3 PUFA represent the sums of all n-6 and n-3 PUFA respectively in LDL cholesteryl esters. CE PI represents the peroxidation index. The change in CE PI after 6 h of oxidation is presented in the far right section of the lower graph.
LDL VITAMIN E CONTENT PRIOR TO AND DURING OXIDATION

The loss of vitamin E from LDL during CuSO₄-mediated oxidation is presented in Figure 4.7. When normalized to LDL protein (Figure 4.7A), LDL α-tocopherol concentration after EPA/DHA supplementation was significantly lower than after linoleate (19%; P = 0.04) and lower than after oleate supplementation (17%; P = 0.06). LDL α-tocopherol concentrations did not differ between oleate and linoleate supplement groups. After 10 minutes of CuSO₄-mediated oxidation EPA/DHA-enriched LDL contained less than half of the α-tocopherol found in oleate- and linoleate-enriched LDL (P = 0.0001), while linoleate-enriched LDL contained 27% less α-tocopherol than oleate-enriched LDL after 10 minutes of oxidation (P = 0.04). After 20 minutes of oxidation α-tocopherol concentration in EPA/DHA-enriched LDL was 10 times less than that of oleate-enriched LDL and 5 times less than that of linoleate-enriched LDL (P = 0.0001 and 0.04, respectively), while linoleate-enriched LDL contained less than half that of oleate-enriched LDL (P = 0.02). The rate of loss of α-tocopherol over 20 minutes of CuSO₄-mediated oxidation (Figure 4.7C) from oleate-enriched LDL was 32% slower than from linoleate-enriched LDL and 48% slower than from EPA/DHA-enriched LDL (P = 0.02 and 0.0007). The rate of α-tocopherol loss from linoleate- and EPA/DHA-enriched LDL did not differ significantly. The average estimated time of α-tocopherol depletion of LDL (Figure 4.7C) after EPA/DHA supplementation was 10 min less than after oleate and 4 min less than after linoleate supplementation.
(P = 0.0001 and 0.02, respectively). \( \alpha \)-Tocopherol depletion of LDL after linoleate was 6 minutes less than after oleate supplementation (P = 0.0006).

\( \alpha \)-Tocopherol concentrations, normalized to LDL lipid content, are shown in Figure 4.7B. When \( \alpha \)-tocopherol concentrations were so normalized, differences among supplement groups prior to oxidation became nonsignificant. The differences in \( \alpha \)-tocopherol concentrations at 10 and 20 minutes of oxidation, as well as the rate of loss of \( \alpha \)-tocopherol, remained significant despite normalization to LDL lipid content.
Figure 4.7. Loss of α-tocopherol from LDL after 10 and 20 min of copper-mediated oxidation. Graphs A and B illustrate mean LDL α-tocopherol concentrations at 0, 10 and 20 min of ex vivo oxidation. Squares represent the oleate supplementation group, circles linoleate, and triangles EPA/DHA. In graph A, α-tocopherol concentrations are normalized to LDL protein, while in graph B α-tocopherol concentrations are normalized to LDL lipid content. Graph C illustrates the differences in the mean rate of α-tocopherol loss from LDL (slope of the lines) during ex vivo oxidation, as well as the mean estimated time of α-tocopherol depletion of oxidizing LDL (x intercept). Different letters represent significant differences among supplement groups (P < 0.05).

When normalized to LDL protein (Figure 4.8A), LDL γ-tocopherol concentration after EPA/DHA supplementation was 29% lower than after oleate and 23% lower than after linoleate supplementation (P = 0.005 and 0.02, respectively) prior to oxidation. LDL γ-tocopherol concentrations prior to oxidation did not differ between oleate and linoleate supplement groups. After 10
minutes of oxidation EPA/DHA-enriched LDL contained 88% less \( \gamma \)-tocopherol than oleate- and 64% less than linoleate-enriched LDL \((P = 0.0001 \text{ and } 0.001, \text{ respectively})\), while linoleate-enriched LDL and oleate-enriched LDL did not differ. After 20 minutes of oxidation EPA/DHA-enriched LDL contained 4 times less \( \gamma \)-tocopherol than oleate-enriched LDL and 2 times less than that of linoleate-enriched LDL \((P = 0.0001 \text{ and } 0.002, \text{ respectively})\), while linoleate-enriched LDL contained 57% less \( \gamma \)-tocopherol than oleate-enriched LDL \((P = 0.01)\).

Normalization of \( \gamma \)-tocopherol to LDL lipid content did not alter any of the above differences as shown in Figure 4.8B.

**Figure 4.8.** \( \gamma \)-Tocopherol concentration of LDL prior to and after 10 and 20 min of copper-mediated oxidation. Squares represent \( \alpha \)-tocopherol concentrations after supplementation with oleate, circles linoleate, and triangles EPA/DHA. In graph A, \( \alpha \)-tocopherol concentrations are normalized to LDL protein, while in graph B concentrations are normalized to LDL lipid content. Different letters represent significant differences among supplementation groups \((P \leq 0.05)\).
PHOSPHATIDYLCHOLINE HYDROPEROXIDE FORMATION DURING LDL OXIDATION

The formation of oxidized products in LDL subjected to copper-mediated oxidation can be evaluated using several different measurements: 1) the length of the lag phase, 2) the maximal rate of hydroperoxide production during the propagation phase, 3) the maximal concentration of hydroperoxides, and 4) the time to one half maximal concentration of hydroperoxides. The oxidative susceptibility of LDL as determined by measuring the production of phosphatidylcholine hydroperoxides (PCOOH) and cholesteryl linoleate hydroperoxides (CE18:2OOH) are presented together in Figure 4.9. The duration of the lag phase in the production of PCOOH during CuSO₄-mediated oxidation was significantly shorter in EPA/DHA-enriched LDL than oleate- and linoleate-enriched LDL. Mean lag phase duration for EPA/DHA-enriched LDL was 27 minutes less than that of oleate-enriched LDL and nearly 18 minutes less than that of linoleate-enriched LDL (P = 0.0001 and 0.002, respectively), while the lag phase was 9 minutes shorter for linoleate-enriched LDL than oleate-enriched LDL (P = 0.08). The maximal rate of PCOOH formation during the propagation phase of LDL oxidation was 42% more rapid in linoleate-enriched LDL than in EPA/DHA-enriched LDL (P = 0.007) and 20% more rapid in linoleate-enriched LDL than in oleate-enriched LDL (P = 0.09). Maximal rates of PCOOH formation did not differ between oleate- and EPA/DHA-enriched LDL. The maximal concentration of PCOOH during CuSO₄-mediated LDL oxidation of EPA/DHA-enriched LDL was 28% lower than that of oleate-enriched LDL and 36% lower than that of linoleate-
enriched LDL (P = 0.04 and 0.01), while the maximal concentration of PCOOH did not differ between oleate- and linoleate-enriched LDL. The time required to reach half of the maximal concentration of PCOOH in oleate-enriched LDL was 28 min longer than in linoleate- and 46 min longer than in EPA/DHA-enriched LDL (P = 0.04 and 0.001, respectively), while it did not differ between linoleate and EPA/DHA-enriched LDL.
Figure 4.9. Susceptibility of LDL surface and core lipids to copper-mediated oxidation assessed by the formation of PCOOH and CE18:2OOH. Graph A depicts the duration of the lag phase, graph B the maximal rate of hydroperoxide formation, graph C the maximal concentration of hydroperoxides, and graph D the time it took to reach the maximal concentration of hydroperoxides. Bars with different letters over them represent significant differences among groups after 5 weeks of supplementation with 15 g/d of oil high in oleate (diagonally striped bars), linoleate (solid bars), and EPA/DHA (shaded bars) P ≤ 0.05.

**CHOLESTERYL LINOLEATE HYDROPEROXIDE FORMATION DURING LDL OXIDATION**

Even in LDL enriched with oleate or EPA/DHA the hydroperoxide of cholesteryl linoleate (CE18:2OOH) made up approximately 87% of the total CEOOH that could be quantified using the HPLC-chemiluminescence assay. For
this reason CE18:20OOH concentrations were the major determinant in the
measures of oxidative susceptibility of LDL cholesteryl esters, and are presented in
Figure 4.9. The duration of the lag phase in the production of CE18:20OOH during
CuSO₄-mediated oxidation was significantly longer in oleate-enriched LDL than
linoleate- and EPA/DHA-enriched LDL. Mean lag time for oleate-enriched LDL
was 13 minutes longer than that of linoleate-enriched LDL and nearly 18 minutes
longer than that of EPA/DHA-enriched LDL (P = 0.05 and 0.01, respectively),
while the lag time did not differ between linoleate-enriched LDL and EPA/DHA-
enriched LDL. The maximal rate of CE18:20OOH formation during the propagation
phase of LDL oxidation was 31% more rapid in linoleate- than in oleate-enriched
LDL, and 76% more rapid than in EPA/DHA-enriched LDL (P = 0.01 and 0.0001,
respectively). Additionally, maximal rate of CE18:20OOH formation was 34%
more rapid in oleate- than in EPA/DHA-enriched LDL (P = 0.03). The maximal
concentration of CE18:20OOH during CuSO₄-mediated LDL oxidation of
EPA/DHA-enriched LDL was 30% lower than that of oleate-enriched LDL and
49% lower than that of linoleate-enriched LDL (P = 0.01 and 0.0002, respectively),
while the maximal concentration of CE18:20OOH did not differ between oleate- and
linoleate-enriched LDL. The time required to reach half the maximal concentration
of CE18:20OOH was 23 min longer in oleate-enriched LDL than linoleate-enriched
LDL (p = 0.04) but did not differ from EPA/DHA-enriched LDL, nor did linoleate-
and EPA/DHA-enriched LDL differ significantly.
FORMATION OF HYDROPEROXIDES OF CHOLESTERYL ESTERS OF SPECIFIC FATTY ACIDS DURING LDL OXIDATION

Calculation of the parameters of LDL oxidative susceptibility discussed above using the sum of CE18:2OOH and CE22:6OOH, as well as using the sum of all CEOOH species measured (CE18:2OOH, CE20:4OOH, CE18:1OOH, and CE22:6OOH), did not result in any significant changes in the results obtained by using only the CE18:2OOH values.

The proportions of the maximal concentration of CEOOH, during 6 hours of LDL oxidation, contributed by the hydroperoxides of cholesteryl esters of specific fatty acids are presented in Table 4.4. Maximal CE18:1OOH concentrations were 23% higher in oxidized oleate-enriched LDL than linoleate-enriched LDL and 92% higher than in EPA/DHA-enriched LDL (P = 0.03 and 0.0001, respectively), while maximal CE18:1OOH concentrations were 56% higher in linoleate- than in EPA/DHA-enriched LDL (P = 0.02). Maximal CE18:2OOH concentrations were reviewed in the previous section (Figure 4.9). Maximal CE20:4OOH concentrations were 82% lower in oxidized EPA/DHA-enriched LDL than in oleate-enriched LDL and 121% lower than in linoleate-enriched LDL (P = 0.0003 and 0.0001). Maximal CE20:4OOH concentrations tended to be 21% lower in oxidized oleate-enriched LDL than linoleate-enriched LDL (P = 0.06). Maximal CE22:6OOH concentrations were more than 4.5 times higher in oxidized EPA/DHA-enriched LDL than in oleate- and linoleate-enriched LDL (P = 0.0001).
Table 4.4. Maximal concentrations of hydroperoxides of specific cholesteryl esters (nmol/mg LDL protein) measured during copper-mediated oxidation of LDL enriched with oleate, linoleate, and EPA/DHA.

<table>
<thead>
<tr>
<th></th>
<th>Oleate</th>
<th>Linoleate</th>
<th>EPA/DHA</th>
</tr>
</thead>
<tbody>
<tr>
<td>CE18:1OOH</td>
<td>5.8 ± 0.3\textsuperscript{a}</td>
<td>4.7 ± 0.6\textsuperscript{b}</td>
<td>3.0 ± 0.6\textsuperscript{c}</td>
</tr>
<tr>
<td>CE18:2OOH</td>
<td>66.8 ± 3.7\textsuperscript{a}</td>
<td>76.6 ± 4.6\textsuperscript{a}</td>
<td>51.4 ± 3.8\textsuperscript{b}</td>
</tr>
<tr>
<td>CE20:4OOH</td>
<td>4.7 ± 0.3\textsuperscript{a}</td>
<td>5.7 ± 0.6\textsuperscript{a}</td>
<td>2.6 ± 0.2\textsuperscript{b}</td>
</tr>
<tr>
<td>CE22:6OOH</td>
<td>0.7 ± 0.2\textsuperscript{a}</td>
<td>0.7 ± 0.1\textsuperscript{a}</td>
<td>3.8 ± 0.3\textsuperscript{b}</td>
</tr>
<tr>
<td>Sum CEOOH</td>
<td>77.0 ± 3.9\textsuperscript{a}</td>
<td>87.7 ± 5.4\textsuperscript{a}</td>
<td>60.8 ± 4.2\textsuperscript{b}</td>
</tr>
</tbody>
</table>

*Values are LSM ± SEM. Different letters represent significant differences among supplement groups (P ≤ 0.05).

Calculation of the time to one half maximal concentration allowed for comparison of the speed of oxidation of specific fatty acids esterified to cholesterol within a supplement group (Fig. 4-10). In EPA/DHA-enriched LDL the time to one half maximal concentration was 55 min shorter for CE22:6OOH than for CE18:2OOH (P = 0.0002) and 83 min shorter than for CE20:4OOH (P = 0.0007). This difference could not be analyzed in the other 2 supplement groups because CE22:6OOH could not be routinely detected in LDL that was not enriched with EPA/DHA. In oleate-enriched LDL the time to one half maximal concentration was 46 min longer for CE18:1OOH than for CE18:2OOH (P = 0.0001) and 26 min longer than for CE20:4OOH (P = 0.01). In linoleate-enriched LDL the time to half maximum was 20 minutes shorter for CE18:2OOH than for CE20:4OOH (P = 0.01).
Figure 4.10. Time to one half maximal concentration for CEOOH of specific fatty acids within oleate, linoleate, and EPA/DHA supplement groups. Each graph illustrates differences between specific CEOOH species after supplementation with oleate (graph A), linoleate (graph B), and EPA/DHA (graph C). CE22:6OOH was not routinely detected in the oleate or linoleate supplement groups. Different letters represent significant differences among different CEOOH within that supplement group (P ≤ 0.05).
Examination of the relationship between loss of α-tocopherol and formation of PCOOH in oxidizing LDL (Figure 4.11) revealed that, on average, significant PCOOH formation occurred before LDL was depleted of α-tocopherol in all three supplement groups. In EPA/DHA-enriched LDL α-tocopherol was lost more rapidly than in oleate- (P = 0.0001) and linoleate-enriched LDL (P = 0.02). No distinct lag phase could be identified in any of the PCOOH curves shown in Figure 4.11. However, unlike oleate- and linoleate-enriched LDL, in EPA/DHA-enriched LDL, the most rapid rate of PCOOH formation was observed between 0 and 20 minutes of oxidation. Once EPA/DHA-enriched LDL was depleted of α-tocopherol the rate of PCOOH formation slowed.

When CEOOH formation was examined with respect to LDL α-tocopherol depletion (Figure 4.12), a distinct lag phase, propagation phase and plateau, as described by Esterbauer et al (44), could be identified, and the maximal rate (propagation phase) began after LDL had been depleted of α-tocopherol in all 3 supplement groups. It should be noted that although α-tocopherol was lost most rapidly from EPA/DHA-enriched LDL, the rate of CE18:2OOH formation in EPA/DHA-enriched LDL was slowest (Figure 4.9).
Figure 4.11. The relationship between α-tocopherol concentrations and PCOOH concentrations in oxidizing LDL. Values are mean ± SEM. Loss of α-tocopherol is represented by solid circles, and formation of PCOOH is represented by open circles at the end of supplementation with 15 g/d of oil high in oleate (top graph), linoleate (middle graph), and EPA/DHA (bottom graph).
Figure 4.12. The relationship between α-tocopherol concentrations and CE18:2OOH concentrations in oxidizing LDL. Values are mean ± SEM. Loss of α-tocopherol is represented by solid circles, and formation of CE18:2OOH is represented by open circles at the end of supplementation with 15 g/d of oil high in oleate (top graph), linoleate (middle graph), and EPA/DHA (bottom graph).
Discussion

Because the outer monolayer of the LDL particle is composed mainly of phospholipids and free cholesterol, monitoring the formation of PCOOH during copper-mediated oxidation provides information regarding oxidation at the surface of the LDL particle. In the present study, oleate enrichment of LDL resulted in a significantly longer lag phase for the formation of PCOOH than EPA/DHA-enrichment and a longer lag phase than linoleate-enrichment that showed a trend toward significance. Moreover, the rate of formation of PCOOH was significantly slower in oleate-enriched than linoleate-enriched LDL. A number of studies have shown that diets rich in oleate result in LDL that are less susceptible to ex vivo oxidation than LDL resulting from diets rich in linoleate (9,10,48). The source of the decrease in oxidative susceptibility found in oleate-enriched LDL is unclear because in most studies the increase in LDL 18:1 n-9 content is accompanied by a decrease in LDL 18:2n-6 content (49). Because the 18:2n-6 content of LDL phospholipids did not differ significantly between the oleate- and linoleate-enriched LDL, the present study suggests that the decrease in oxidative susceptibility of oleate-enriched LDL was likely a result of the increased 18:1n-9 content of LDL phospholipids. Lee et al (49) obtained similar results by monitoring conjugated diene formation during AAPH-induced oxidation of liposomes made with 1,2 dioleoyl-sn-glycero-3-phosphatidylcholine and proposed that 18:1n-9 may act as a competitive inhibitor of the oxidation of PUFA.
In the present study, enrichment of LDL with the highly unsaturated n-3 PUFA, EPA and DHA, resulted in a mean maximal rate of formation of PCOOH that was comparable to that of oleate-enriched LDL and significantly lower than that of linoleate-enriched LDL. The maximal concentration of PCOOH measured over 6 h of copper-mediated oxidation was lower in EPA/DHA-enriched LDL than oleate- or linoleate-enriched LDL. The time to one half maximal concentration of PCOOH was shorter in EPA/DHA-enriched LDL than oleate-enriched LDL but not significantly shorter than linoleate-enriched LDL, suggesting that although maximum PCOOH concentrations were lower for EPA/DHA- compared to oleate-enriched LDL, maximal concentrations were attained more rapidly. Analysis of the major lipid classes in LDL indicated that the phospholipid content of EPA/DHA-enriched LDL was 6% lower than that of linoleate-enriched LDL. However, the rate of PCOOH formation was 33% slower and the maximal concentration 36% lower in EPA/DHA-enriched LDL, suggesting that the differences were not likely due simply to a difference in LDL phospholipid content.

Although the α- and γ-tocopherol content of EPA/DHA-enriched LDL were somewhat lower than that of linoleate and oleate-enriched LDL (5 vs. 6 mol α-tocopherol /mol LDL; 0.8 vs. 0.6 mol γ-tocopherol /mol LDL), the rate of loss of both tocopherols was significantly more rapid in EPA/DHA-enriched LDL during copper-mediated oxidation. Of the antioxidants naturally occurring in LDL, α-tocopherol is present in the greatest amounts while γ-tocopherol is generally present in only about half of LDL particles (2). During copper-mediated oxidation with
high concentrations of copper, the length of the lag phase tends to be related to the
time of α-tocopherol depletion from LDL (50). The role of γ-tocopherol is less
clear. Studies of in vitro incubation of LDL with α- or γ-tocopherol in plasma have
shown α-tocopherol to be more potent than γ-tocopherol in increasing the lag
phase of copper-mediated oxidation (51).

α-Tocopherol is generally considered to associate with LDL surface lipids
(52). The shorter lag phase in the formation of PCOOH in EPA/DHA-enriched
LDL could be related to the slightly decreased α-tocopherol content in EPA/DHA-
enriched LDL particles compared to linoleate and oleate-enriched LDL particles.
However, closer examination of the lag time for PCOOH formation and loss of
LDL vitamin E during copper-mediated oxidation revealed that, the maximal rate
of PCOOH formation began prior to the depletion of α-tocopherol from LDL.
Moreover, the time at which α-tocopherol was no longer detectable in oxidizing
LDL was positively correlated with the lag time for PCOOH formation in oxidizing
LDL in the oleate- and linoleate-enriched LDL but not in EPA/DHA-enriched
LDL. These data suggest that increasing the EPA and DHA content of LDL
phospholipids resulted in more rapid loss of LDL α-tocopherol. The lower rate of
PCOOH formation and lower maximal concentration of PCOOH in EPA/DHA-
enriched LDL could be explained by tocopherol mediated peroxidation (TMP)
where α-tocopherol is the primary lipid peroxidation chain carrying radical when
present in the LDL particle (53).
Although the exact mechanism for copper-mediated LDL oxidation remains controversial, LDL oxidation kinetic patterns have been found to differ when differing ratios of Cu\(^{2+}/LDL\) are present in the oxidation system. When the Cu\(^{2+}/LDL\) molar ratio is greater than 10-12, the formation of LDL oxidation products tends to follow the pattern, described by Esterbauer et al (44). This pattern is characterized by a lag phase during which LDL antioxidants are depleted, followed by a rapid propagation phase, a plateau at which time the rate of decomposition of hydroperoxides equals the rate of formation, and finally a rapid decomposition phase.

Another pattern of copper-mediated LDL oxidation occurs when the Cu\(^{2+}/LDL\) molar ratio is ≤ 2-3 (50,53). In this pattern of LDL oxidation there is no clear lag phase and LDL oxidation occurs in the presence of α-tocopherol. Additionally, under these latter conditions, oxidation is increased when the α-tocopherol content of LDL is increased through supplementation. This phenomenon has been well documented by Upston et al (53), and termed TMP. The Cu\(^{2+}/LDL\) ratio used in the present study was ~ 5 mol Cu\(^{2+}/mol\) LDL. During this study LDL phosphatidylcholine oxidation occurred in the presence of α-tocopherol with no apparent lag phase, while oxidation of LDL cholesteryl linoleate demonstrated a significant lag phase and propagation phase. Both kinetic patterns of lipid peroxidation can be explained using the TMP model.

Although the loss of n-6 PUFA was lower and the loss of n-3 PUFA was higher in LDL phospholipids after EPA/DHA supplementation than after oleate and
linoleate supplementation, the loss of total PUFA from LDL phospholipids after 6 h of oxidation did not differ among the 3 supplementation groups. Together with the PCOOH results, these data suggest that LDL surface lipids were initially oxidized more rapidly in EPA/DHA-enriched LDL than in oleate- and linoleate-enriched LDL, but the extent of oxidation, as indicated by the loss of total PUFA from LDL phospholipids after 6 h of oxidation, was not greater in EPA/DHA-enriched LDL than in oleate or linoleate-enriched LDL.

Monitoring the formation of CEOOH provides information regarding lipid peroxidation in the hydrophobic core of the LDL particle. In the present study we were able to quantify hydroperoxides formed from the following cholesteryl esters: CE18:1n-9, CE18:2n-6, CE20:4n-6, and CE22:6n-3. Even in LDL enriched with oleate or EPA/DHA, CE18:2O0H made up approximately 87% of the total CEOOH that could be quantified using the HPLC/chemiluminescence assay. Because the majority of CEOOH measured was made up of CE18:2O0H in all 3 supplement groups, differences among the groups were unchanged whether the lag time, rate, maximal concentration, or time to one half maximal concentration was based solely on CE18:2O0H or on the sum of all species of CEOOH quantified.

Almost all of the kinetic curves generated by monitoring CE18:2O0H formation demonstrated an obvious lag phase and a propagation phase, no matter which oil supplement had been consumed. Unlike the kinetic curves generated by monitoring PCOOH formation, the propagation phase for CEOOH formation did
not begin until the LDL particle had been depleted of \( \alpha \)-tocopherol in all 3 supplement groups.

Oleate-enriched LDL demonstrated a significantly longer lag phase in the formation of CE18:2OOH than linoleate- or EPA/DHA-enriched LDL, while the rate of CE18:2OOH formation was slower in oleate- than in linoleate-enriched LDL. In the LDL cholesteryl ester fraction the concentration of 18:1n-9 was 28% higher after oleate supplementation than linoleate and 24% higher than after EPA/DHA supplementation. After linoleate supplementation LDL cholesteryl ester content of 18:2n-6 was 19% higher than after oleate. Therefore a decrease in oxidative susceptibility of oleate-enriched LDL compared with linoleate-enriched LDL could be related to the increase in 18:1n-9 concentrations or the decrease in 18:2n-6 concentrations in the LDL core. Because CE18:1OOH could not be detected in all samples, comparisons of the parameters of oxidative susceptibility for CE18:1OOH formation could not be made among supplement groups. However, calculation of the time to one half maximal concentration allowed for comparison between CE18:1OOH and CE18:2OOH within the oleate supplementation group. In this case the oxidation of CE18:1n-9 appeared significantly slower than that of CE18:2n-6 within the oleate-supplementation group.

Unlike the findings for PCOOH formation, the lag phase in CE18:2OOH formation did not differ between EPA/DHA-enriched LDL and linoleate-enriched LDL. Moreover, the rate of CE18:2OOH formation, as well as the maximal
concentration of CE18:2OOH, was lower in EPA/DHA-enriched LDL than oleate- and linoleate-enriched LDL. Prior to oxidation, the CE18:2n-6 content of EPA/DHA-enriched LDL was 23% less than that of linoleate-enriched LDL. However, EPA/DHA-did not differ from oleate- enriched LDL in CE18:2n-6 content, making it difficult to attribute the decreased rate and maximal concentrations of CE18:2OOH in EPA/DHA-enriched LDL to decreased CE18:2 content. Within the EPA/DHA-supplementation group, CE22:6OOH reached one half maximal concentration significantly faster than CE18:2OOH, suggesting that CE22:6n-3 was being oxidized more rapidly than CE18:2n-6.

Yazu et al (7) demonstrated that the presence of methyl esters of EPA and linoleate in a 1:1 ratio in aqueous micelles reduced the oxidation of total substrate (EPA and linoleate) 5-fold compared with micelles of linoleate alone. The measurement of O2 uptake during the oxidation of aqueous micelles made of methyl linoleate and methyl EPA indicated that EPA reacted with twice as much O2 as linoleate. This would suggest that EPA formed mainly bicycloendoperoxides, containing 2 molecules of O2, while linoleate formed mainly hydroperoxides, containing 1 molecule of O2. The higher polarity of such an EPA-derived peroxyl radical would enhance its diffusion to the surface of the aqueous micelle. In fact, the same investigators conducted a subsequent study of the effect of 3 different antioxidants, with different site reactivities in aqueous micelles, on the oxidizability of methyl linoleate and methyl EPA. Their findings supported the idea that peroxyl radicals derived from EPA tended to localize at the surface of the micelle rather
than in the core (54). Together these studies indicated that in the presence of linoleate, the increased polarity of EPA-derived peroxyl radicals enhanced their diffusion to the micelle surface, increasing the termination reaction rate, decreasing the propagation rate, and ultimately decreasing the rate of total substrate oxidation in the EPA/linoleate micelles (7).

The LDL particle consists of an amphipathic surface monolayer and a hydrophobic core, suggesting it is more likely to behave similarly to the biphasic system of the aqueous micelle than to homogeneous solutions of fatty esters. Moreover, a number of fatty acids with 3 or more double bonds (including EPA, DHA, and AA) have been found to form bicyclic endoperoxyl radicals (43), which contain 2 molecules of O₂ and may be more polar than the peroxyl radicals formed from fatty acids with ≤ 2 double bonds. Therefore, in EPA/DHA-enriched LDL, the highly unsaturated n-3 fatty acids might also form more polar radicals, which would be more likely to localize at the surface of LDL, resulting in an increased rate of termination and a slower propagation rate. In this manner EPA/DHA-enrichment of LDL might explain the lower rate of CE18:2OOH formation in EPA/DHA-enriched LDL compared to linoleate-enriched LDL found in the present study.

Although the above hypothesis might explain the findings of decreased rate and maximal concentrations of PCOOH and CE18:2OOH during the oxidation of EPA/DHA-enriched LDL, several methodological issues must also be considered. EPA/DHA-enriched LDL contained greater concentrations of n-3 fatty acids, which
have 5 and 6 double bonds, respectively. Fatty acids with 3 or more double bonds are likely to form bicycloendoperoxides (43). Endoperoxides cannot be detected by the chemiluminescence technique used in this study (42). Thus, an unknown quantity of the hydroperoxides formed from the oxidation of EPA/DHA were not be measured by the HPLC/chemiluminescence assay. When PUFA are oxidized in vitro, as the number of double bonds in each fatty acid increases complex mixtures of hydroperoxides are produced. These hydroperoxides may follow a number of different decomposition pathways depending on the conditions of oxidation. Frankel (43) believes that comparing the relative rates of PUFA oxidation by monitoring hydroperoxide formation will result in lower yields for more highly unsaturated PUFA because they are decomposed more rapidly in the presence of metal ion-catalysts. More rapid decomposition of the hydroperoxides formed from n-3 PUFA during copper-mediated LDL oxidation might explain lower net rates of formation and lower maximum PCOOH and CE18:2OOH concentrations found in EPA/DHA-enriched LDL.

Although highly sensitive and specific compared to other assays purported to measure lipid hydroperoxides, the HPLC/chemiluminescence technique used in the present study had several limitations when used to measure the hydroperoxides formed from cholesterol esters of specific fatty acids. The HPLC separation, as modified for the present study, was not able to resolve all of the peaks representing CE20:4OOH, CE22:6OOH, and CE18:1OOH. Moreover, CE20:5OOH could not
be adequately resolved at all. Thus, the assay could not be considered quantitative for hydroperoxides of several of the cholesteryl ester species of interest.

Epidemiological research suggests that traditional Mediterranean diets which are, among other characteristics, rich in oleate are associated with a reduced risk of cardiovascular mortality (55,56). In the present study oleate-enrichment of LDL either decreased or had no adverse effect on all measures of oxidative susceptibility examined. The cardioprotective effects associated with diets rich in oleate might result from the increased resistance of oleate-enriched LDL to oxidation. However, such a mechanism depends on the assumption that resistance to copper-mediated oxidation ex vivo is retained in vivo, where the mechanism of LDL oxidation may differ.

A number of studies have shown the consumption of fish to be associated with decreased mortality from cardiovascular disease (57-59). This effect is thought to derive from the n-3 fatty acids, EPA and DHA, which are found in fatty fish. In the present study EPA/DHA-enrichment did not result in increased oxidative susceptibility of LDL, when compared to linoleate-enrichment, with the exception of a significantly shorter lag phase in the formation of PCOOPH. EPA/DHA supplementation also resulted in decreased rates of PCOOPH and CE18:2OOH formation and decreased maximal concentrations of PCOOPH and CE18:2OOH during copper-mediated oxidation compared with linoleate supplementation. While it is not clear from our findings that the susceptibility of EPA/DHA-enriched LDL to copper-mediated oxidation is actually decreased, it
appears that the oxidation of 18:2n-6 is diminished, at least in the LDL core. The mechanism for the observed cardioprotective effects of increased fish consumption is not yet clear. It is possible that the reduced 18:2n-6 oxidation in LDL could contribute to that cardioprotective effect, if LDL behave similarly in vivo. Based on the results of this study, the beneficial effects of increased fish consumption do not appear to be offset by an increase in the oxidative susceptibility of LDL.
References


CHAPTER 5

CONCLUSION

The purpose of the research described in the preceding manuscripts was to determine the effects of increased consumption of oleate, linoleate, and EPA/DHA by postmenopausal women on in vivo lipid peroxidation, as well as the susceptibility of LDL to ex vivo oxidation. Because no single assay provides an accurate index of overall lipid peroxidation or oxidative stress, this research also became an investigation of a number of techniques used to assess lipid peroxidation when the consumption of specific unsaturated fatty acids was increased.

For obvious reasons, the assessment of LDL oxidation in the arterial wall is virtually impossible to accomplish in humans. However, LDL can be isolated from human plasma and oxidized ex vivo to provide some insight into the relative oxidative susceptibility of LDL in individuals whose diets are rich in specific unsaturated fatty acids. Increased oleate intake resulted in oleate-enriched LDL that were more resistant to copper-mediated oxidation than linoleate-enriched LDL, and probably more resistant to oxidation than EPA/DHA-enriched LDL. Consumption of fish oil resulted in EPA/DHA-enriched LDL that appeared to oxidize earlier than oleate-and linoleate-enriched LDL, at least with respect to its phospholipids (surface lipids). However, there was no evidence that the extent of copper-mediated LDL oxidation over 6 hours, based on loss of total PUFA and
lipid hydroperoxide formation, was any greater in EPA/DHA-enriched LDL than oleate or linoleate-enriched LDL.

Studies of LDL oxidative susceptibility ex vivo are limited in the insight they can provide regarding actual in vivo lipid peroxidation when consumption of specific fatty acids is increased. Physiological responses in a living organism to changes in PUFA consumption may include alterations in cell membrane structure and function (Stillwell, Jenski, Crump, & Ehringer, 1997), as well as changes in a number of endogenous antioxidant defense systems (Venkatraman, Angkeow, Satsangi, & Fernandes, 1998). In contrast to the findings regarding LDL susceptibility to ex vivo oxidation, the most sensitive and specific assays of lipid peroxidation in vivo (F2-isoprostanes and MDA by GC/MS) suggested slightly decreased oxidative stress during EPA/DHA supplementation compared to oleate and linoleate supplementation. Thus, the potentially beneficial effects of diets rich in EPA and DHA in preventing or ameliorating chronic conditions such as cardiovascular disease may not be offset by an increased risk of lipid peroxidation in vivo.

The fact that two assays (TBA and MDA by GC/MS), which are supposed to measure the same index of oxidative stress (MDA), suggested opposite conclusions regarding the effect of EPA/DHA on in vivo lipid peroxidation emphasizes the importance of the measurement issues raised by this research. Many of the assays available for the measurement of lipid peroxidation lose their utility when specific PUFA concentrations in plasma or tissue vary due to changes
in dietary intake. Instead of measuring overall lipid peroxidation, different assays measure the oxidation or decomposition of specific PUFA. For example, assays of MDA tend to measure the decomposition of lipid peroxides with 3 or more double bonds, neglecting oleate and linoleate, while F₂-isoprostanes measure only a product of arachidonate oxidation. Presently, it is difficult to determine whether the results of such measurement techniques reflect actual differences in lipid peroxidation or merely differences in tissue and plasma PUFA concentrations.

It is unlikely that a single reliable indicator of total lipid peroxidation in vivo will ever be developed. Therefore different assessment strategies will be needed to better explore the effects of increased consumption of specific PUFA on oxidative stress. One strategy would be to assess a series of lipid oxidation products resulting from each of unsaturated fatty acid of interest. Assessing F₂-, F₃-, and F₄-isoprostanes arising from peroxidation of arachidonate, EPA, and DHA, respectively, or assessing exhaled hydrocarbon gases such as ethane and pentane arising from peroxidation of n-3 and n-6 PUFA, respectively, represent strategies of this nature. Additionally, using recently developed sensitive and specific GC/MS methods to determine concentrations of aldehydes arising from the oxidation of different fatty acids might provide insight into oxidative susceptibility as well as into atherogenic properties of specific aldehydic profiles.

The preceding study of LDL susceptibility to ex vivo oxidation was an attempt to examine effects of changing concentrations of specific fatty acids on the resistance of LDL to oxidative modification, which is likely to play a role in the
pathology of atherosclerosis. However, it is not clear whether oxidatively modified LDL that are enriched with linoleate or oleate are more or less likely to promote atherosclerosis than oxidatively modified LDL that are enriched with EPA/DHA. For example, specific decomposition products of lipid peroxidation, like MDA, have been implicated in the cytotoxic effects of LDL that may lead to necrosis and instability of atherosclerotic plaques (Diaz, Frei, Vita, & Keaney, 1997). The extent to which decomposition products resulting from the peroxidation of different PUFA affect the cytotoxic properties of LDL are not known. In other words, one strategy is to examine the susceptibility or resistance of different PUFA to oxidation in physiologically relevant systems, but another strategy is to determine whether oxidation of specific PUFA results in potentiated or attenuated effects with respect to atherogenesis.

The results of the preceding investigations suggested that highly unsaturated n-3 PUFA in EPA/DHA-enriched LDL might oxidize more rapidly ex vivo than PUFA with fewer double bonds. However, the increased oxidation of LDL n-3 fatty acids was accompanied by evidence of decreased oxidation of the n-6 PUFA, linoleate, the most abundant PUFA in LDL. The relevance of these findings to the pathology of atherosclerosis is unclear, but it is interesting to note that the predominant lipid oxidation product encountered in atherosclerotic lesions is hydroxy cholesteryl linoleate (Suarna, Dean, May, & Stocker, 1995). It is possible that decreased LDL cholesteryl linoleate oxidation could result in decreased atherosclerotic lesion formation. The results of our investigation into the effects of
increased unsaturated fatty acid consumption on in vivo oxidative stress indicated that lipid peroxidation in vivo was not increased even when plasma n-3 PUFA concentrations were more than tripled. Thus increased n-3 PUFA consumption may not result in increased lipid peroxidation in vivo, or in LDL particles that are more atherogenic.

The potential for dietary intake of specific fatty acids to affect the development and progression of atherosclerosis and other chronic diseases is substantial. The preceding studies represent an initial attempt to understand the effect of increasingly unsaturated fatty acids on systemic lipid peroxidation in postmenopausal women as well as the susceptibility of their LDL to oxidative modification. The results of the studies make it clear that any assessment of oxidative stress with respect to changes in dietary fat intake must take into consideration specific fatty acid concentrations in the tissue of interest, as well as the ultimate goal of the dietary intervention. In the case of the preceding studies, increasing LDL content of highly unsaturated n-3 fatty acids may have increased initial susceptibility of LDL lipids to ex vivo oxidation, but sensitive and specific measures failed to detect any evidence of increased lipid peroxidation in vivo. Future research depends on continued development of lipid peroxidation assays that are sensitive to changes in tissue concentrations of specific unsaturated fatty acids, as well as physiologically relevant assays of the pro- or anti-atherogenic properties of LDL and cell membranes enriched in those fatty acids.


APPENDICES
### Appendix 1

Effect of oil supplements on selected fatty acids in plasma (mmol/L). *

<table>
<thead>
<tr>
<th></th>
<th>Oleate</th>
<th>Linoleate</th>
<th>EPA/DHA</th>
</tr>
</thead>
<tbody>
<tr>
<td>16:0</td>
<td>3.19 ± 0.21a</td>
<td>2.79 ± 0.12b</td>
<td>2.67 ± 0.09b</td>
</tr>
<tr>
<td>18:0</td>
<td>0.86 ± 0.04</td>
<td>0.82 ± 0.03</td>
<td>0.81 ± 0.02</td>
</tr>
<tr>
<td>ΣSFA†</td>
<td>4.52 ± 0.26a</td>
<td>4.01 ± 0.17b</td>
<td>3.83 ± 0.12b</td>
</tr>
<tr>
<td>16:1n-7</td>
<td>0.34 ± 0.04a</td>
<td>0.24 ± 0.02b</td>
<td>0.26 ± 0.02b</td>
</tr>
<tr>
<td>18:1n-9</td>
<td>2.66 ± 0.20a</td>
<td>1.89 ± 0.06b</td>
<td>1.64 ± 0.06b</td>
</tr>
<tr>
<td>18:1n-7</td>
<td>0.24 ± 0.02a</td>
<td>0.20 ± 0.01b</td>
<td>0.18 ± 0.01b</td>
</tr>
<tr>
<td>ΣMUFA‡</td>
<td>3.64 ± 0.26a</td>
<td>2.70 ± 0.12b</td>
<td>2.43 ± 0.08b</td>
</tr>
<tr>
<td>18:2n-6</td>
<td>3.63 ± 0.15a</td>
<td>4.14 ± 0.20b</td>
<td>3.08 ± 0.12c</td>
</tr>
<tr>
<td>18:3n-3</td>
<td>0.10 ± 0.01</td>
<td>0.08 ± 0.01</td>
<td>0.10 ± 0.01</td>
</tr>
<tr>
<td>20:3n-6</td>
<td>0.22 ± 0.02a</td>
<td>0.20 ± 0.02a</td>
<td>0.10 ± 0.01b</td>
</tr>
<tr>
<td>20:4n-6</td>
<td>0.90 ± 0.07a</td>
<td>0.89 ± 0.04a</td>
<td>0.73 ± 0.03</td>
</tr>
<tr>
<td>20:5n-3</td>
<td>0.07 ± 0.02a</td>
<td>0.05 ± 0.02a</td>
<td>0.71 ± 0.13b</td>
</tr>
<tr>
<td>22:5n-3</td>
<td>0.06 ± 0.01a</td>
<td>0.05 ± 0.01b</td>
<td>0.12 ± 0.01c</td>
</tr>
<tr>
<td>22:6n-3</td>
<td>0.19 ± 0.02a</td>
<td>0.18 ± 0.02a</td>
<td>0.49 ± 0.03b</td>
</tr>
<tr>
<td>ΣPUFA§</td>
<td>5.60 ± 0.22</td>
<td>6.00 ± 0.23</td>
<td>5.67 ± 0.11</td>
</tr>
<tr>
<td>Σ n-6 PUFA</td>
<td>5.07 ± 0.19a</td>
<td>5.61 ± 0.22b</td>
<td>4.20 ± 0.13c</td>
</tr>
<tr>
<td>Σ n-3 PUFA</td>
<td>0.43 ± 0.03a</td>
<td>0.35 ± 0.02a</td>
<td>1.42 ± 0.06b</td>
</tr>
<tr>
<td>n-6/n-3 ratio</td>
<td>12.64 ± 0.96a</td>
<td>16.20 ± 1.29b</td>
<td>2.98 ± 0.18c</td>
</tr>
<tr>
<td>Peroxidation index'</td>
<td>9.46 ± 0.42a</td>
<td>9.69 ± 0.33a</td>
<td>12.36 ± 0.25b</td>
</tr>
</tbody>
</table>

*LSM ± SEM. Different letters represent significant differences among supplement groups (P < 0.05).
†Sum of the saturated fatty acids 13:0 + 14:0 + 15:0 + 16:0 + 18:0 + 19:0 + 20:0 + 21:0 + 22:0 + 23:0 + 24:0.
‡Sum of the monounsaturated fatty acids 16:1(n-7) + 18:1(n-9)t + 18:1(n-9)c + 18:1(n-7) + 20:1(n-9) + 24:1.
§Sum of the polyunsaturated fatty acids 18:2(n-6)t,t + 18:2(n-6)c,c + 18:3(n-3) + 18:4(n-3) + 20:2(n-6) + 20:3(n-6) + 20:3(n-3) + 20:4(n-6) + 20:5(n-3) + 22:5(n-3) + 22:6(n-3).
'Peroxidation index = (ΣPUFA with 2 double bonds x 1) + (ΣPUFA with 3 double bonds x 2) + (ΣPUFA with 4 double bonds x 3) + (ΣPUFA with 5 double bonds x 4) + (ΣPUFA with 6 double bonds x 5)
Appendix 2

Effect of oil supplements on measures of in vivo oxidative stress.*

<table>
<thead>
<tr>
<th></th>
<th>Oleate</th>
<th>Linoleate</th>
<th>EPA/DHA</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Plasma free F₂-isoprostanes</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(pmol/L)</td>
<td>193.2 ± 10.2ᵃ</td>
<td>181.9 ± 12.6ᵃ</td>
<td>168.7 ± 12.8ᵇ</td>
</tr>
<tr>
<td><strong>Plasma free F₂-isoprostanes</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(nmol/mol 20:4n-6)</td>
<td>221.3 ± 16.8ᵃᵇ</td>
<td>205.9 ± 17.2ᵃ</td>
<td>233.5 ± 18.3ᵃ</td>
</tr>
<tr>
<td><strong>Plasma malondialdehyde</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(nmol/L)</td>
<td>131.7 ± 6.7ᵃ</td>
<td>127.1 ± 5.1ᵃ</td>
<td>112.7 ± 6.2ᵇ</td>
</tr>
<tr>
<td><strong>Plasma TBARS†</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(µmol/L)</td>
<td>1.01 ± 0.05ᵃ</td>
<td>0.99 ± 0.04ᵃ</td>
<td>1.22 ± 0.07ᵇ</td>
</tr>
<tr>
<td><strong>Plasma α-tocopherol</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(µmol/L plasma)</td>
<td>32.5 ± 1.7ᵃ</td>
<td>31.1 ± 1.4ᵃᵇ</td>
<td>28.8 ± 1.4ᵇ</td>
</tr>
<tr>
<td><strong>Plasma α-tocopherol</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(mmol/mol plasma lipid)</td>
<td>5.08 ± 0.23</td>
<td>5.30 ± 0.38</td>
<td>4.98 ± 0.24</td>
</tr>
<tr>
<td><strong>Plasma γ-tocopherol</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(µmol/L plasma)</td>
<td>4.6 ± 0.3ᵃ</td>
<td>4.2 ± 0.4ᵃ</td>
<td>3.1 ± 0.5ᵇ</td>
</tr>
<tr>
<td><strong>Plasma γ-tocopherol</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(mmol/mol plasma lipid)</td>
<td>0.73 ± 0.06ᵃ</td>
<td>0.69 ± 0.07ᵃ</td>
<td>0.54 ± 0.09ᵇ</td>
</tr>
</tbody>
</table>

*LSM ± SEM. Different letters represent significant differences among supplement groups (P < 0.05).
†Plasma thiobarbituric acid reacting substances; lack of specificity of the assay precludes expression in nmol/L malondialdehyde.
## Appendix 3

Change in PUFA content (nmol/mg LDL protein) of LDL phospholipid (PL) and cholesterol ester (CE) fractions after 6 hours of oxidation.*

<table>
<thead>
<tr>
<th></th>
<th>Sunflower oil (oleate)</th>
<th>Safflower oil (linoleate)</th>
<th>Fish oil (EPA/DHA)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Prior to oxidation</td>
<td>Δ after 6 h oxidation</td>
<td>Prior to oxidation</td>
</tr>
<tr>
<td>PL18:1n-9</td>
<td>131 ± 5^a</td>
<td>14 ± 5</td>
<td>94 ± 6^b</td>
</tr>
<tr>
<td>PL18:2n-6</td>
<td>222 ± 19^a</td>
<td>-56 ± 8^1</td>
<td>239 ± 14^a</td>
</tr>
<tr>
<td>PL20:4n-6</td>
<td>96 ± 8^a</td>
<td>-55 ± 5</td>
<td>93 ± 9^a</td>
</tr>
<tr>
<td>PL20:5n-3</td>
<td>5 ± 1^a</td>
<td>-5 ± 1^1</td>
<td>4 ± 1^a</td>
</tr>
<tr>
<td>PL22:6n-3</td>
<td>26 ± 3^a</td>
<td>-18 ± 2^1</td>
<td>24 ± 2^a</td>
</tr>
<tr>
<td>PLΣPUFA</td>
<td>399 ± 19</td>
<td>-159 ± 16</td>
<td>407 ± 24</td>
</tr>
<tr>
<td>PLΣ n-6</td>
<td>354 ± 26^d</td>
<td>-127 ± 14^1</td>
<td>367 ± 21^a</td>
</tr>
<tr>
<td>PLΣ n-3</td>
<td>45 ± 4^a</td>
<td>-32 ± 3^1</td>
<td>39 ± 3^a</td>
</tr>
<tr>
<td>PLPI</td>
<td>774 ± 55^a</td>
<td>-392 ± 35^1</td>
<td>761 ± 50^a</td>
</tr>
</tbody>
</table>

|                | Prior to oxidation     | Δ after 6 h oxidation     | Prior to oxidation | Δ after 6 h oxidation | Prior to oxidation | Δ after 6 h oxidation |
| CE18:1n-9      | 393 ± 22^a             | -12 ± 13                  | 307 ± 11^b         | -17 ± 9               | 317 ± 15^b         | -16 ± 8               |
| CE18:2n-6      | 964 ± 61^a             | -452 ± 42^1,2             | 1147 ± 44^b        | -490 ± 54^1           | 932 ± 46^a         | -321 ± 37^2           |
| CE20:4n-6      | 142 ± 12               | -109 ± 9                  | 146 ± 16           | -101 ± 15             | 131 ± 9            | -83 ± 8               |
| CE20:5n-3      | 11 ± 1^a               | -10 ± 1^1                 | 9 ± 2^a            | -8 ± 2^1              | 133 ± 6^b          | -99 ± 6^2             |
| CE22:6n-3      | 10 ± 2^a               | -10 ± 2^1                 | 12 ± 2^a           | -8 ± 2^1              | 27 ± 3^b           | -22 ± 3^2             |
| CEΣPUFA        | 1170 ± 71^a            | -614 ± 47                 | 1346 ± 52^b        | -633 ± 62             | 1259 ± 53^ab       | -545 ± 45             |
| CEΣ n-6        | 1128 ± 67^a            | -579 ± 46^1               | 1308 ± 51^b        | -602 ± 62^1           | 1072 ± 50^a        | -407 ± 42^2           |
| CEΣ n-3        | 42 ± 6^d               | -35 ± 2^1                 | 39 ± 5^a           | -30 ± 6^1             | 187 ± 10^b         | -138 ± 10^2           |
| CEPI           | 1575 ± 99^a            | -940 ± 65                 | 1754 ± 81^a        | -925 ± 88             | 2074 ± 80^b        | -1119 ± 80            |

*Values represent least square mean (LSM) ± SEM. Different letters represent significant differences in fatty acid content prior to oxidation among oil supplement groups (p < 0.05). Different numbers represent significant differences in the change in fatty acid content after 6 h of oxidation among oil supplement groups (P < 0.05).

† Sum of the polyunsaturated fatty acids 18:2(n-6)t,t + 18:2(n-6)c,c + 18:3(n-3) + 18:4(n-3) + 20:2(n-6) + 20:3(n-6) + 20:3(n-3) + 20:4(n-6) + 20:5(n-3) + 22:5(n-3) + 22:6(n-3)

‡ Sum of the n-6 fatty acids

§ Sum of the n-3 fatty acids

* Peroxidation index = (ΣPUFA with 2 double bonds x 1) + (ΣPUFA with 3 double bonds x 2) + (ΣPUFA with 4 double bonds x 3) + (ΣPUFA with 5 double bonds x 4) + (ΣPUFA with 6 double bonds x 5)
Appendix 4

Change in PUFA content (mole %) of LDL phospholipid (PL) and cholesterol ester (CE) fractions after 6 hours of oxidation.*

<table>
<thead>
<tr>
<th></th>
<th>Sunflower oil (oleate)</th>
<th>Safflower oil (linoleate)</th>
<th>Fish oil (EPA/DHA)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Prior to oxidation</td>
<td>Δ after 6 h oxidation</td>
<td>Prior to oxidation</td>
</tr>
<tr>
<td>PL 18:1n-9</td>
<td>10.6 ± 0.5°</td>
<td>+2.5 ± 0.4</td>
<td>8.1 ± 0.7</td>
</tr>
<tr>
<td>PL 18:2n-6</td>
<td>17.3 ± 0.5°</td>
<td>-2.7 ± 0.4</td>
<td>19.9 ± 0.6</td>
</tr>
<tr>
<td>PL 20:4n-6</td>
<td>7.3 ± 0.4°</td>
<td>-4.0 ± 0.3</td>
<td>7.7 ± 0.5</td>
</tr>
<tr>
<td>PL 20:5n-3</td>
<td>0.4 ± 0.1°</td>
<td>-0.4 ± 0.1</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>PL 22:n-6</td>
<td>2.0 ± 0.1°</td>
<td>-1.4 ± 0.1</td>
<td>2.0 ± 0.2</td>
</tr>
<tr>
<td>PL ΣPUFA</td>
<td>31.2 ± 0.7°</td>
<td>-10.2 ± 0.8</td>
<td>33.8 ± 0.7</td>
</tr>
<tr>
<td>PL Σn-6</td>
<td>27.7 ± 0.6°</td>
<td>-7.8 ± 0.8</td>
<td>30.6 ± 0.6</td>
</tr>
<tr>
<td>PL Σn-3</td>
<td>3.5 ± 0.2°</td>
<td>-2.4 ± 0.2</td>
<td>3.2 ± 0.2</td>
</tr>
<tr>
<td>PL PI</td>
<td>60.3 ± 1.7°</td>
<td>-27.3 ± 1.9</td>
<td>63.0 ± 1.8</td>
</tr>
</tbody>
</table>

|                | Prior to oxidation     | Δ after 6 h oxidation     | Prior to oxidation | Δ after 6 h oxidation |
| CE 18:1n-9     | 18.9 ± 0.6°            | +7.2 ± 0.7                | 14.3 ± 0.8         | +5.4±0.8°              |
| CE 18:2n-6     | 46.1 ± 1.4°            | -12.2 ± 1.3               | 52.6 ± 1.1         | -11.0 ± 1.2            |
| CE 20:4n-6     | 6.8 ± 0.4              | -4.7 ± 0.3                | 6.6 ± 0.6          | -4.0 ± 0.6             |
| CE 20:5n-3     | 0.5 ± 0.1°             | -0.5 ± 0.1                | 0.4 ± 0.1°         | -0.4 ± 0.1°            |
| CE ΣPUFA      | 55.9 ± 1.4°            | -19.2 ± 1.3               | 61.6 ± 0.9         | -16.7 ± 1.4            |
| CE Σn-6       | 53.9 ± 1.3°            | -17.7 ± 1.4               | 59.9 ± 1.0         | -15.5 ± 1.5            |
| CE Σn-3       | 2.0 ± 0.2°             | -1.5 ± 0.2                | 1.7 ± 0.2°         | -1.2 ± 0.3°            |
| CE PI         | 75.1 ± 1.7°            | -33.4±1.6°                | 80.0 ± 1.8°        | -28.2 ± 2.5°            |

*Values represent least square mean (LSM) ± SEM. Different letters represent significant differences in fatty acid content prior to oxidation among oil supplement groups (p ≤ 0.05). Different numbers represent significant differences in the change in fatty acid content after 6 h of oxidation among oil supplement groups (P ≤ 0.05).

† Sum of the polyunsaturated fatty acids 18:2(n-6)t,t + 18:2(n-6)c,c + 18:3(n-3) + 18:4(n-3) + 20:2(n-6) + 20:3(n-6) + 20:3(n-3) + 20:4(n-6) + 20:5(n-3) + 22:5(n-3) + 22:6(n-3)

‡ Sum of the n-6 fatty acids

§ Sum of the n-3 fatty acids

Peroxidation index = (ΣPUFA with 2 double bonds x 1) + (ΣPUFA with 3 double bonds x 2) + (ΣPUFA with 4 double bonds x 3) + (ΣPUFA with 5 double bonds x 4) + (ΣPUFA with 6 double bonds x 5)
Appendix 5

LDL composition by major lipid class.*

<table>
<thead>
<tr>
<th>Lipid Class</th>
<th>Oleate</th>
<th>Linoleate</th>
<th>EPA/DHA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(μmol/mg LDL protein)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Triacylglycerols</td>
<td>0.49 ± 0.05&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.43 ± 0.03&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.34 ± 0.03&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Free Cholesterol</td>
<td>0.77 ± 0.03</td>
<td>0.79 ± 0.03</td>
<td>0.79 ± 0.02</td>
</tr>
<tr>
<td>Cholesteryl Esters</td>
<td>2.19 ± 0.06</td>
<td>2.26 ± 0.07</td>
<td>2.23 ± 0.05</td>
</tr>
<tr>
<td>Phospholipids</td>
<td>0.96 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.01 ± 0.02&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.95 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

*Values represent least square mean (LSM) ± SEM. Different letters represent significant differences among supplement groups (P < 0.05).
Appendix 6

Loss of vitamin E from LDL during oxidation.*

<table>
<thead>
<tr>
<th></th>
<th>Oleate</th>
<th>Linoleate</th>
<th>EPA/DHA</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \alpha )-tocopherol (nmol/mg LDL protein)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 min oxidation</td>
<td>12.20 ± 0.78(^a)</td>
<td>12.39 ± 0.79(^ab)</td>
<td>10.40 ± 0.65(^b)</td>
</tr>
<tr>
<td>10 min oxidation</td>
<td>7.92 ± 0.62(^a)</td>
<td>6.26 ± 0.99(^b)</td>
<td>2.70 ± 0.46(^c)</td>
</tr>
<tr>
<td>20 min oxidation</td>
<td>2.49 ± 0.50(^a)</td>
<td>1.23 ± 0.40(^b)</td>
<td>0.19 ± 0.11(^c)</td>
</tr>
<tr>
<td>Rate of loss (min(^{-1}))</td>
<td>0.50 ± 0.05(^a)</td>
<td>0.66 ± 0.05(^b)</td>
<td>0.74 ± 0.07(^b)</td>
</tr>
<tr>
<td>Time until depletion (min)</td>
<td>25.8 ± 1.6(^a)</td>
<td>19.5 ± 1.5(^b)</td>
<td>15.7 ± 1.2(^c)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Oleate</th>
<th>Linoleate</th>
<th>EPA/DHA</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \alpha )-tocopherol (mmol/mol LDL lipid)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 min oxidation</td>
<td>2.78 ± 0.18</td>
<td>2.80 ± 0.22</td>
<td>2.39 ± 0.14</td>
</tr>
<tr>
<td>10 min oxidation</td>
<td>1.81 ± 0.14(^a)</td>
<td>1.33 ± 0.14(^b)</td>
<td>0.63 ± 0.10(^c)</td>
</tr>
<tr>
<td>20 min oxidation</td>
<td>0.56 ± 0.12(^a)</td>
<td>0.29 ± 0.10(^b)</td>
<td>0.04 ± 0.01(^c)</td>
</tr>
<tr>
<td>Rate of loss (min(^{-1}))</td>
<td>0.11 ± 0.01(^a)</td>
<td>0.15 ± 0.01(^b)</td>
<td>0.17 ± 0.01(^b)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Oleate</th>
<th>Linoleate</th>
<th>EPA/DHA</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \gamma )-tocopherol (nmol/mg LDL protein)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 min oxidation</td>
<td>1.45 ± 0.15(^a)</td>
<td>1.38 ± 0.16(^a)</td>
<td>1.12 ± 0.16(^b)</td>
</tr>
<tr>
<td>10 min oxidation</td>
<td>1.39 ± 0.12(^a)</td>
<td>1.21 ± 0.21(^a)</td>
<td>0.74 ± 0.11(^b)</td>
</tr>
<tr>
<td>20 min oxidation</td>
<td>0.82 ± 0.11(^a)</td>
<td>0.52 ± 0.12(^b)</td>
<td>0.15 ± 0.05(^c)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Oleate</th>
<th>Linoleate</th>
<th>EPA/DHA</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \gamma )-tocopherol (mmol/mol LDL lipid)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 min oxidation</td>
<td>0.33 ± 0.03(^a)</td>
<td>0.31 ± 0.04(^a)</td>
<td>0.25 ± 0.03(^b)</td>
</tr>
<tr>
<td>10 min oxidation</td>
<td>0.31 ± 0.02(^a)</td>
<td>0.26 ± 0.05(^a)</td>
<td>0.17 ± 0.02(^b)</td>
</tr>
<tr>
<td>20 min oxidation</td>
<td>0.18 ± 0.02(^a)</td>
<td>0.12 ± 0.03(^b)</td>
<td>0.03 ± 0.01(^c)</td>
</tr>
</tbody>
</table>

*Values represent least square mean (LSM) ± SEM. Different letters represent significant differences among supplement groups (P ≤ 0.05).
Appendix 7

Susceptibility of LDL surface and core lipids to oxidation assessed by the formation of PCOOH and CE18:2OOH. *

<table>
<thead>
<tr>
<th></th>
<th>Oleate</th>
<th>Linoleate</th>
<th>EPA/DHA</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PCOOH</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lag time (min)</td>
<td>32.2 ± 4.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>22.8 ± 5.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.1 ± 1.7&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Maximal rate (nmol/mg LDL protein·min)</td>
<td>0.12 ± 0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.16 ± 0.01&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.12 ± 0.01&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Maximal concentration (nmol/mg LDL protein)</td>
<td>17.9 ± 1.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>19.0 ± 2.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>13.9 ± 1.9&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Time to half maximal concentration (min)</td>
<td>110 ± 10.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>82 ± 7.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>64 ± 7.0&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>CE18:2OOH</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lag time (min)</td>
<td>51.7 ± 4.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>38.3 ± 5.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>33.8 ± 3.8&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Maximal rate (nmol/mg LDL protein·min)</td>
<td>0.51 ± 0.04&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.67 ± 0.06&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.38 ± 0.05&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Maximal concentration (nmol/mg LDL protein)</td>
<td>66.8 ± 3.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>76.6 ± 4.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>51.4 ± 3.8&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Time to half maximal concentration (min)</td>
<td>124 ± 9.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>101 ± 6.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>113 ± 5.1&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

*Values represent least square mean (LSM) ± SEM. Different letters represent significant differences among supplement groups (P ≤ 0.05).
Appendix 8

Time to one half maximal concentration (min) for CEOOH of specific fatty acids within oleate, linoleate, and EPA/DHA supplement groups.*

<table>
<thead>
<tr>
<th></th>
<th>CE18:1OOH</th>
<th>CE18:2OOH</th>
<th>CE20:4OOH</th>
<th>CE22:6OOH</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Oleate</strong></td>
<td>171 ± 13.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>124 ± 9.9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>146 ± 10.7&lt;sup&gt;c&lt;/sup&gt;</td>
<td>NA&lt;sup&gt;†&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Linoleate</strong></td>
<td>137 ± 14.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>101 ± 6.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>122 ± 7.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>NA</td>
</tr>
<tr>
<td><strong>EPA/DHA</strong></td>
<td>181 ± 12.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>113 ± 5.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>141 ± 13.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>57 ± 8.7&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

*Values represent least square mean (LSM) ± SEM. Different letters represent significant differences among CEOOH species, within supplement groups (P < 0.05).

<sup>†</sup>No mean value for CE22:6OOH is given because CE22:6OOH was not reliably detected in oleate or linoleate supplement groups.
Appendix 9

Specific fatty acid concentrations in LDL phospholipids (nmol/mg LDL protein). N = 15 (40 observations)

<table>
<thead>
<tr>
<th></th>
<th>Prior to Oxidation</th>
<th>After 6 h Oxidation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>oleate</td>
<td>linoleate</td>
</tr>
<tr>
<td>18:1n-9</td>
<td><em>131 ± 5</em></td>
<td>94 ± 6</td>
</tr>
<tr>
<td>18:2n-6</td>
<td>222 ± 20</td>
<td>238 ± 14</td>
</tr>
<tr>
<td>20:4n-6</td>
<td>95.5 ± 7.6</td>
<td>93.4 ± 9.0</td>
</tr>
<tr>
<td>20:5n-3</td>
<td>5.4 ± 0.8</td>
<td>4.0 ± 0.8</td>
</tr>
<tr>
<td>22:6n-3</td>
<td>25.8 ± 2.7</td>
<td>24.2 ± 2.2</td>
</tr>
<tr>
<td>ΣSFA</td>
<td>685 ± 45</td>
<td>648 ± 30</td>
</tr>
<tr>
<td>ZMUFA</td>
<td>191 ± 10</td>
<td>143 ± 8</td>
</tr>
<tr>
<td>ΣPUFA</td>
<td>399 ± 29</td>
<td>407 ± 24</td>
</tr>
<tr>
<td>Σn-6 PUFA</td>
<td>354 ± 26</td>
<td>367 ± 21</td>
</tr>
<tr>
<td>Σn-3 PUFA</td>
<td>45 ± 4</td>
<td>39 ± 3</td>
</tr>
<tr>
<td>n6/n3</td>
<td>8.1 ± 0.5</td>
<td>9.5 ± 0.4</td>
</tr>
<tr>
<td>P1</td>
<td>774 ± 55</td>
<td>761 ± 50</td>
</tr>
<tr>
<td>UI</td>
<td>1364 ± 91</td>
<td>1310 ± 75</td>
</tr>
<tr>
<td>14:0</td>
<td>16.5 ± 1.7</td>
<td>17.1 ± 4.2</td>
</tr>
<tr>
<td>16:0</td>
<td>387 ± 24</td>
<td>361 ± 17</td>
</tr>
<tr>
<td>16:1n-7</td>
<td>5.7 ± 1.9</td>
<td>3.0 ± 0.9</td>
</tr>
<tr>
<td>16:2n-4</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>18:0</td>
<td>235 ± 16</td>
<td>228 ± 11</td>
</tr>
<tr>
<td>18:1n-9t</td>
<td>10.3 ± 1.3</td>
<td>8.0 ± 1.5</td>
</tr>
<tr>
<td>18:1n-7</td>
<td>10.0 ± 2.7</td>
<td>7.3 ± 2.4</td>
</tr>
<tr>
<td>16:4n-1</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>16:2n-6</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>20:0</td>
<td>7.8 ± 0.6</td>
<td>8.0 ± 0.9</td>
</tr>
<tr>
<td>18:3n-3</td>
<td>2.7 ± 0.8</td>
<td>2.4 ± 0.8</td>
</tr>
<tr>
<td>20:1n-9</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>20:2n-6</td>
<td>6.1 ± 0.9</td>
<td>5.8 ± 0.4</td>
</tr>
<tr>
<td>20:3n-6</td>
<td>32.9 ± 1.8</td>
<td>29.4 ± 1.7</td>
</tr>
<tr>
<td>22:0</td>
<td>15.5 ± 1.8</td>
<td>13.1 ± 1.2</td>
</tr>
<tr>
<td>22:1n-9</td>
<td>15.6 ± 3.8</td>
<td>13.7 ± 3.9</td>
</tr>
<tr>
<td>24:0</td>
<td>16.5 ± 2.0</td>
<td>15.5 ± 1.3</td>
</tr>
<tr>
<td>24:1</td>
<td>16.9 ± 2.1</td>
<td>15.1 ± 1.7</td>
</tr>
<tr>
<td>22:5n-3</td>
<td>11.4 ± 1.2</td>
<td>8.8 ± 1.3</td>
</tr>
<tr>
<td>ΣFA</td>
<td>1275 ± 80</td>
<td>1198 ± 55</td>
</tr>
</tbody>
</table>

* Values are LSM ± SEM. Different letters represent significant differences among values prior to oxidation (P ≤ 0.05). Different numbers represent significant differences among values after 6 h of oxidation (P ≤ 0.05).
† Not detected in the majority of samples.
## Appendix 10

Specific fatty acid concentrations in LDL cholesteryl esters (nmol/mg LDL protein).

N = 15 (41 observations)

<table>
<thead>
<tr>
<th>Prior to Oxidation</th>
<th>After 6 h Oxidation</th>
</tr>
</thead>
<tbody>
<tr>
<td>oleate</td>
<td>linoleate</td>
</tr>
<tr>
<td>18:1n-9</td>
<td><em>393 ± 21</em></td>
</tr>
<tr>
<td>18:2n-6</td>
<td>964 ± 61</td>
</tr>
<tr>
<td>20:4n-6</td>
<td>142 ± 12</td>
</tr>
<tr>
<td>20:5n-3</td>
<td>10.6 ± 1.2</td>
</tr>
<tr>
<td>22:6n-3</td>
<td>10.4 ± 2.3</td>
</tr>
<tr>
<td>ΔSFA</td>
<td>424 ± 36</td>
</tr>
<tr>
<td>ΔMUFA</td>
<td>498 ± 32</td>
</tr>
<tr>
<td>ΔPUFA</td>
<td>1170 ± 71</td>
</tr>
<tr>
<td>Δ-6 PUFA</td>
<td>1128 ± 67</td>
</tr>
<tr>
<td>Δn-3 PUFA</td>
<td>42.3 ± 6.1</td>
</tr>
<tr>
<td>n6/n3</td>
<td>28.6 ± 2.5</td>
</tr>
<tr>
<td>PL</td>
<td>1575 ± 99</td>
</tr>
<tr>
<td>UI</td>
<td>3243 ± 190</td>
</tr>
<tr>
<td>14:0</td>
<td>43.6 ± 5.2</td>
</tr>
<tr>
<td>16:0</td>
<td>254 ± 12</td>
</tr>
<tr>
<td>16:1n-7</td>
<td>74.1 ± 6.3</td>
</tr>
<tr>
<td>16:2n-4</td>
<td>ND</td>
</tr>
<tr>
<td>18:0</td>
<td>92 ± 21</td>
</tr>
<tr>
<td>18:1n-9t</td>
<td>8.8 ± 2.3</td>
</tr>
<tr>
<td>18:1n-7</td>
<td>7.2 ± 3.7</td>
</tr>
<tr>
<td>16:4n-1</td>
<td>ND</td>
</tr>
<tr>
<td>18:2n-6t</td>
<td>ND</td>
</tr>
<tr>
<td>20:0</td>
<td>13.9 ± 2.9</td>
</tr>
<tr>
<td>18:3n-3</td>
<td>16.7 ± 1.7</td>
</tr>
<tr>
<td>20:1n-9</td>
<td>10.8 ± 4.7</td>
</tr>
<tr>
<td>20:2n-6</td>
<td>ND</td>
</tr>
<tr>
<td>20:3n-6</td>
<td>16.2 ± 1.5</td>
</tr>
<tr>
<td>22:0</td>
<td>16.3 ± 5.6</td>
</tr>
<tr>
<td>22:1n-9</td>
<td>ND</td>
</tr>
<tr>
<td>24:0</td>
<td>4.6 ± 2.2</td>
</tr>
<tr>
<td>24:1</td>
<td>ND</td>
</tr>
<tr>
<td>22:5n-3</td>
<td>ND</td>
</tr>
<tr>
<td>ΣFA</td>
<td>2093 ± 123</td>
</tr>
</tbody>
</table>

* Values are LSM ± SEM. Different letters represent significant differences among values prior to oxidation (P ≤ 0.05). Different numbers represent significant differences among values after 6 h of oxidation (P ≤ 0.05).

† Not detected in the majority of samples.
Appendix 11

Specific fatty acid concentrations in plasma (mmol/L).*
N = 15 (43 observations)

<table>
<thead>
<tr>
<th></th>
<th>oleate</th>
<th>linoleate</th>
<th>EPA/DHA</th>
</tr>
</thead>
<tbody>
<tr>
<td>18:1n-9</td>
<td>2.66 ± 0.20&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.89 ± 0.09&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.64 ± 0.06&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>18:2n-6</td>
<td>3.63 ± 0.15&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.14 ± 0.20&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.08 ± 0.12&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>20:4n-6</td>
<td>0.90 ± 0.07&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.89 ± 0.04&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.73 ± 0.03&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>20:5n-3</td>
<td>0.07 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.05 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.71 ± 0.13&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>22:6n-3</td>
<td>0.19 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.18 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.49 ± 0.03&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>ΣSFA</td>
<td>4.52 ± 0.26&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.01 ± 0.17&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.83 ± 0.12&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>ΣMUFA</td>
<td>3.64 ± 0.26&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.70 ± 0.12&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.43 ± 0.08&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>ΣPUFA</td>
<td>5.60 ± 0.22</td>
<td>6.00 ± 0.23</td>
<td>5.67 ± 0.11</td>
</tr>
<tr>
<td>Σn-6 PUFA</td>
<td>5.07 ± 0.19&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.61 ± 0.22&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.20 ± 0.13&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Σn-3 PUFA</td>
<td>0.43 ± 0.03&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.35 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.42 ± 0.06&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>n6/n3</td>
<td>12.64 ± 0.96&lt;sup&gt;a&lt;/sup&gt;</td>
<td>16.20 ± 1.29&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.98 ± 0.18&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>PI</td>
<td>9.46 ± 0.42</td>
<td>9.69 ± 0.33</td>
<td>12.36 ± 0.25</td>
</tr>
<tr>
<td>UI</td>
<td>18.71 ± 0.86&lt;sup&gt;a&lt;/sup&gt;</td>
<td>18.39 ± 0.62&lt;sup&gt;a&lt;/sup&gt;</td>
<td>20.46 ± 0.34&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>14:0</td>
<td>0.19 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.15 ± 0.01&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.13 ± 0.01&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>16:0</td>
<td>3.19 ± 0.21&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.79 ± 0.12&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.67 ± 0.09&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>16:1n-7</td>
<td>0.34 ± 0.04&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.24 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.26 ± 0.02&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>16:2n-4</td>
<td>0.07 ± 0.03&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.03 ± 0.01&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.05 ± 0.02&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>18:0</td>
<td>0.86 ± 0.04</td>
<td>0.82 ± 0.03</td>
<td>0.81 ± 0.02</td>
</tr>
<tr>
<td>18:1n-9t</td>
<td>0.15 ± 0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.12 ± 0.01&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.09 ± 0.01&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>18:1n-7</td>
<td>0.24 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.20 ± 0.01&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.18 ± 0.01&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>16:4n-1</td>
<td>0.03 ± 0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.01 ± 0.01&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.01 ± 0.004&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>18:2n-6t</td>
<td>0.02 ± 0.01</td>
<td>0.03 ± 0.01</td>
<td>0.001 ± 0.001</td>
</tr>
<tr>
<td>20:0</td>
<td>0.07 ± 0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.07 ± 0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.04 ± 0.01&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>18:3n-3</td>
<td>0.10 ± 0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.08 ± 0.01&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.10 ± 0.01&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>20:1n-9</td>
<td>0.023 ± 0.006&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.009 ± 0.004&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.003 ± 0.002&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>20:2n-6</td>
<td>0.037 ± 0.004</td>
<td>0.037 ± 0.004</td>
<td>0.006 ± 0.003</td>
</tr>
<tr>
<td>20:3n-6</td>
<td>0.22 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.20 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.10 ± 0.01&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>22:0</td>
<td>0.06 ± 0.01&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.05 ± 0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.07 ± 0.01&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>22:1n-9</td>
<td>0.14 ± 0.01</td>
<td>0.13 ± 0.01</td>
<td>0.14 ± 0.02</td>
</tr>
<tr>
<td>24:0</td>
<td>0.06 ± 0.01</td>
<td>0.08 ± 0.02</td>
<td>0.06 ± 0.01</td>
</tr>
<tr>
<td>22:4n-6</td>
<td>0.26 ± 0.05</td>
<td>0.31 ± 0.06</td>
<td>0.28 ± 0.05</td>
</tr>
<tr>
<td>24:1</td>
<td>0.09 ± 0.01</td>
<td>0.11 ± 0.01</td>
<td>0.11 ± 0.01</td>
</tr>
<tr>
<td>22:5n-3</td>
<td>0.06 ± 0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.05 ± 0.01&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.12 ± 0.01&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

*Values are LSM ± SEM. Different letters represent significant differences among oil supplement groups (P ≤ 0.05).
Appendix 12

SAS PROGRAM FOR ANALYSIS OF CARRYOVER

libname whd 'c:sas\whd';

data cefapnm;
set whd.cefapnm;

if per=1 then co='0';
if per=2 and seq=1 then co='A';
if per=3 and seq=1 then co='B';
if per=2 and seq=2 then co='C';
if per=3 and seq=2 then co='A';
if per=2 and seq=3 then co='B';
if per=3 and seq=3 then co='C';
if per=2 and seq=4 then co='A';
if per=3 and seq=4 then co='C';
if per=2 and seq=5 then co='C';
if per=3 and seq=5 then co='B';
if per=2 and seq=6 then co='B';
if per=3 and seq=6 then co='A';
run;
/*title 'vivo file';
proc print;
run;*/

title 'Crossover analyses accounting for carryover effects';
title2 'model cnpid = seq id(seq) per trt co';

proc glm;
class seq id per trt co;
model cnpid = seq id(seq) per trt co /solution;
lsmeans trt co;

estimate 'trt A vs B' trt 1 -1 0;
estimate 'trt A vs C' trt 1 0 -1;
estimate 'trt B vs C' trt 0 1 -1;

estimate 'co A vs B' co 0 1 -1 0;
estimate 'co A vs C' co 0 1 0 -1;
estimate 'co B vs C' co 0 0 1 -1;
contrast 'per 2 vs 3' per 0 1 -1;
run;

/*title 'Crossover analyses with different carry-over effects within periods';
title2 'model cnpid = seq id(seq) group*seq per trt co per*co';

proc glm;
class seq id per trt co;
model cnpid = seq id(seq) per trt co per*co;
lsmmeans trt co;

estimate 'trt A vs B' trt 1 -1 0;
estimate 'trt A vs C' trt 1 0 -1;
estimate 'trt B vs C' trt 0 1 -1;
estimate 'co A vs B' co 0 1 -1 0;
estimate 'co A vs C' co 0 1 0 -1;
estimate 'co B vs C' co 0 0 1 -1;

*per 1 2 3 1 2 3;
co 0 A B C A B C;
estimate 'per 2 vs 3 for co=A' per 0 1 -1 per*co 0 0 0 -1 0 0;
estimate 'per 2 vs 3 for co=B' per 0 1 -1 per*co 0 0 1 0 0 -1 0;
estimate 'per 2 vs 3 for co=c' per 0 1 -1 per*co 0 0 0 1 0 0 -1;
run;

title 'Crossover analyses with different carry-over effects within periods';
title2 'model cnpid = id per trt co(per)';
proc glm;
class seq id per trt co;
model cnpid = id per trt co(per) /solution;
lsmmeans trt co(per);
estimate 'trt A vs B' trt 1 -1 0;
estimate 'trt A vs C' trt 1 0 -1;
estimate 'trt B vs C' trt 0 1 -1;
estimate 'co A vs B within per 2' co(per) 0 1 -1 0 0 0 0;
estimate 'co A vs C within per 2' co(per) 0 1 0 -1 0 0 0;
estimate 'co B vs C within per 2' co(per) 0 0 1 -1 0 0 0;
estimate 'co A vs B within per 3' co(per) 0 0 0 0 1 -1 0;
estimate 'co A vs C within per 3' co(per) 0 0 0 0 1 0 -1;
estimate 'co B vs C within per 3' co(per) 0 0 0 0 1 -1;
run;/*

title 'Crossover ANOVA ignoring carry over';
title2 'model cnpid = seq id(seq) per trt';
proc glm;
class seq id per trt;
model cnpid = seq id(seq) per trt;
lsmeans per trt;
estimate 'trt A vs B' trt 1 -1 0;
estimate 'trt A vs C' trt 1 0 -1;
estimate 'trt B vs C' trt 0 1 -1;

output out=whd.rescnpi predicted= precnpi
   residual= rescnpi;

proc print data=whd.rescnpi;
run;