EFFECTS OF FLAX AND HEMP SEED OILS ON ERYTHROCYTE CONCENTRATIONS OF EICOSAPENTAENOIC AND DOCOSAHEXAENOIC ACIDS IN VEGETARIANS

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DEDICATION

This thesis is dedicated to my loving wife Kirstin who gave me endless support and encouragement throughout this entire project and to our son Kai who inspired me to bring it to completion.

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ABSTRACT

Flax and hemp seed oils are rich sources of alpha-linolenic acid (ALA; 18:3n-3), which can be converted to eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3) in humans. We investigated the effects of these oils on erythrocyte concentrations of EPA, DHA, other fatty acids, and plasma lipids in healthy vegetarians. Twenty-two subjects consumed either one tablespoon of flax (7.1 g ALA) or hemp seed oil (2.2 g ALA) daily for twenty-eight days. Diet records were collected at baseline and during supplementation. Blood samples were collected at baseline and post-supplementation. No significant increases in plasma EPA, DHA or dihomo-gamma-linolenic acid (DGLA; 18:3n-6) were observed in either group. Significant increases in plasma high-density lipoprotein cholesterol (HDL) concentrations were observed in both groups. Flax and hemp seed oils were not effective at increasing erythrocyte EPA, DHA or DGLA concentrations but were effective at increasing HDL concentrations.

TABLE OF CONTENTS

Acknowledgements.....v

Abstract.....viii

List of Tables.....xiii

List of Figures.....xiv

List of Abbreviations and Symbols.....xv

Chapter 1: Introduction.....1

Chapter 2: Literature Review.....4

Essential Fatty Acids.....4

Essentiality.....4

Linoleic Acid.....5

Alpha-Linolenic Acid.....8

Essential Fatty Acid Metabolism.....8

Epidemiology and Essential Fatty Acids.....12

Eicosapentaenoic Acid and Docosahexaenoic Acid.....13

Eicosapentaenoic Acid.....13

Docosahexaenoic Acid.....14

Epidemiology and Eicosapentaenoic and Docosahexaenoic Acids.....15

Gamma-Linolenic Acid.....18

Food Sources of n-3 Fatty Acids.....18

Alpha-Linolenic Acid.....18

Eicosapentaenoic Acid and Docosahexaenoic Acid.....20

Polyunsaturated Fatty Acid Intakes of Vegans.....23
Ratios of Linoleic Acid to Alpha-Linolenic Acid.....23
Fatty Acid Status in Vegans.....24
Effects of ALA Supplementation on EPA and DHA Concentrations.....25
Conclusion.....29
Chapter 3: Subjects and Methods.....30
Study Population.....30
Study Design.....31
Anthropometric Measures and Lifestyle Assessment.....32
Dietary Intake.....33
Blood Samples.....35
Erythrocyte Fatty Acids.....36
Plasma Lipids.....36
Statistical Analysis.....37
Chapter 4: Results.....39
Subjects, Anthropometric Measures and Lifestyle Assessment.....39
Dietary Intake.....40
Eating Patterns.....41
Energy and Macronutrients.....41
Fatty Acids.....44
Micronutrients.....47
Erythrocyte Total Phospholipid Fatty Acids.....49

Multiple Regression Model.....51
Plasma Lipids.....53
Multiple Regression Model.....55
Chapter 5: Discussion.....56
Effects of Flax and Hemp Seed Oils On Erythrocyte EPA and DHA.....56
Effects of Hemp Seed Oil On Erythrocyte DGLA.....63
Effects of Flax and Hemp Seed Oils On Plasma Lipids.....65
Dietary Intake of Vegetarians.....68
Essential Fatty Acids.....68
Ratios of LA : ALA.....68
Eating Patterns.....69
Macronutrients.....70
Micronutrients.....70
Conclusion.....71
Appendix A: Vegetarian Diet Overview.....73
Vegetarian Diets Explained.....73
Health Aspects of Vegan Diets.....74
Nutritional Considerations For Vegans.....76
Vitamin B-12.....76
Vitamin D.....77
Iron.....77
Calcium.....78

Zinc.....79

Maternal and Infant Issues.....79

Appendix B: Instruments Developed & Used 81

Personal Information.....81

Diet History.....82

Medical History.....85

General Information.....89

Food Record Instructions.....90

24-Hour Food Record.....93

3-Day Food Record.....94

Oil Intake Record.....95

Tips for Oil Use.....96

Exit Interview Questionnaire.....97

References.....98

LIST OF TABLES

Table		Page
1.	Entrance Characteristics	40
2.	Energy, Macronutrient, and Fiber Intake	43
3.	Fatty Acid Intake and Ratios	46
4.	Micronutrient Intake	48
5.	Erythrocyte Fatty Acid Concentrations	50
6.	Plasma Lipid Concentrations	54

LIST OF FIGURES

Figure **Page**

1. Eicosanoid Structures.....7

2. Metabolism of Polyunsaturated Fatty Acids 10

LIST OF ABBREVIATIONS AND SYMBOLS

AA = arachidonic acid

ALA = alpha-linolenic acid

BMI = body mass index

DGLA = dihomo-gamma-linolenic acid

DHA = docosahexaenoic acid

EFA = essential fatty acid

EPA = eicosapentaenoic acid

GLA = gamma-linolenic acid

HDL = high-density lipoprotein

LA = linoleic acid

LCPUFA = long chain polyunsaturated fatty acid

LDL = low-density lipoprotein

LT = leukotriene

PG = prostaglandin

PUFA = polyunsaturated fatty acid

TX = thromboxane

α = alpha

β = beta

χ = gamma

Δ = delta

CHAPTER 1. INTRODUCTION

The interest in n-3 fatty acids began in the early 1980s when Holman , *et al.*, reported the first case of what they thought was alpha-linolenic acid (ALA; 18:3n-3) deficiency (1). It is now well known that ALA is an essential fatty acid (EFA) for normal growth and development and that n-3 fatty acids may play important roles in the prevention and treatment of several chronic diseases.

The importance of the n-3 fatty acids ALA, eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3) in health is unequivocal. ALA is an essential fatty acid, an energy source, a minor component of tissues (2), is needed for normal skin integrity (3-5) and is the precursor for endogenous production of EPA and DHA (5, 6). Deficiency of ALA results in decreased visual acuity, abnormal electroretinograms, scaly dermatitis and a variety of neurological abnormalities (1, 4, 5, 7).

EPA is a long chain polyunsaturated fatty acid (LCPUFA) that is a component of phospholipids in cell membranes throughout the body (2, 8). The main function of EPA is to serve as the direct precursor of eicosanoids, including series 3 prostaglandins (PGs) and thromboxanes (TXs), and series 5 leukotrienes (LTs). The eicosanoids produced from EPA are responsible for regulating a variety of critical physiological processes including platelet aggregation, blood pressure, bronchoconstriction, and bronchodilation (9).

DHA is a major structural component of cell membrane phospholipids throughout the body and is especially concentrated in the brain, retina, testes and sperm where it comprises up to 60% of total fatty acids (2, 7, 10). DHA is required for the function of

the normal visual cycle, is an important cell signaling molecule and has been shown to be directly involved in gene expression (2, 7, 10, 11).

Dietary supplementation with ALA has been shown to be beneficial for eicosanoid production and platelet aggregation (12, 13). While a number of large studies have found an association between a high intake of ALA and decreased risk for cardiovascular disease (14-17), other studies have failed to observe an association (18, 19).

In contrast, there is strong consensus in the literature to support an inverse relationship between dietary intake of EPA and DHA and the risk of cardiovascular disease (11, 20). Specifically, supplementation with EPA and DHA consistently increases the concentrations of these fatty acids in the blood, reduces plasma triacylglycerol concentrations, increases plasma high-density lipoprotein (HDL) concentrations, favorably affects eicosanoid production (anti-inflammatory and antithrombotic), and can prevent fatal arrhythmias (11, 20, 21). Additionally, these n-3 fatty acids have been shown to have beneficial effects in the prevention and management of autoimmune disorders, type 2 diabetes, Crohn disease, mild hypertension, and rheumatoid arthritis (11, 20).

Despite their importance in health and disease, intake of n-3 fatty acids is low in Western diets (11, 22). This is primarily due to low fish consumption and a food supply that is low in n-3 fatty acids and instead high in n-6 fatty acids (11).

Concentrated sources of ALA are limited to a small number of plant foods, such as flax, hemp, canola, and soybean oils, and walnuts. The most concentrated sources of

n-3 fatty acids are marine animals, such as fish and shellfish, which are extremely rich in EPA and DHA. However, fish is not included in a vegetarian diet (see **Appendix A**) and many omnivores avoid eating fish for various reasons. Thus, people who either eat vegetarian diets or eat little or no fish rely almost exclusively on endogenous production of both EPA and DHA from ALA.

However, biosynthesis of EPA and DHA from ALA is known to have several limitations, such as desaturase enzyme competition and tissue buffering (3, 23, 24). Nonetheless, consumers spend millions of dollars annually on a variety of ALA supplements, including flax and hemp seed oils in an effort to improve their n-3 status. In fact, The American Dietetic Association's position paper on vegetarian diets states that those who do not eat preformed sources of EPA or DHA require larger amounts of n-3 fatty acids, namely ALA (25). Although ALA supplementation has been shown to increase EPA concentrations readily, it has not proven to be a reliable means of increasing DHA concentrations (12, 13, 26-31). Therefore, it remains unknown what amount of ALA, what source of ALA, what duration of ALA supplementation, or what ratio of linoleic acid (LA; 18:2n-6) to ALA is necessary to produce an increase in DHA concentration.

The primary purpose of this research was to study the effects of ALA rich flax and hemp seed oils on erythrocyte concentrations of EPA and DHA in a group of healthy people consuming vegetarian diets. Additionally, we sought to determine the effects of these oils on other erythrocyte fatty acids and plasma lipids. Lastly, we intended to characterize current vegetarian eating patterns in a group of healthy subjects in Hawaii.

CHAPTER 2. LITERATURE REVIEW

The information presented below provides history and current information on essential fatty acid metabolism and intake; provides information on the tissue status of EPA and DHA observed in vegetarians; considers food sources of essential fatty acids, EPA, and DHA; and discusses attempts that have been made to increase EPA and DHA concentrations in both vegetarians and omnivores.

Essential fatty acids

Essentiality

George and Mildred Burr discovered in 1929 that fat is essential for normal growth in rats (32). After testing a variety of oils and purified fatty acids they found that linoleic acid (LA; 18:2n-6) restored the impaired growth and cured the scaly dermatitis observed in rats fed a fat-free diet (33). Burr and Burr advised using caution in applying these data to humans. However, through series of studies spanning decades, Hansen and other investigators subsequently confirmed the concept of LA being a dietary essential for humans (34-37).

Later, Holman, *et al.*, reported the first case of ALA deficiency (ALAD) in a six year old girl who was being maintained on a total parenteral nutrition (TPN) preparation nearly devoid of ALA (1). At that time, the findings of Holman and coworkers, and the concept of ALAD were disputed by other scientists (38). Nonetheless, Bjerve and colleagues proceeded to document several cases of ALAD in TPN patients (3-5, 39). Substantial evidence now exists to support ALA as a dietary essential fatty acid (7).

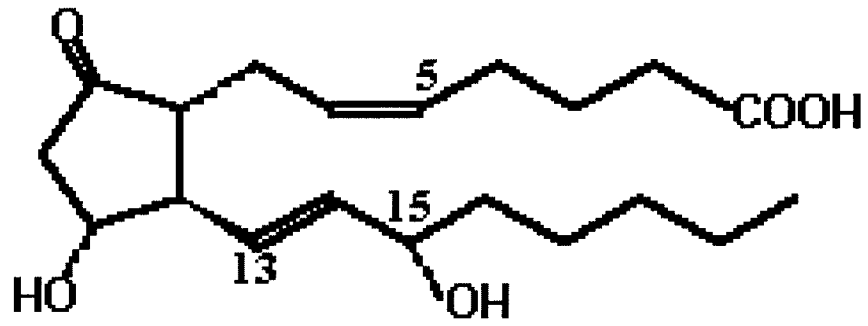
Linoleic acid

Linoleic acid is a component of cell membranes in tissues throughout the body (2) and its concentration in plasma lipids and adipose tissue is dependent upon dietary fatty acid composition (40, 41). Besides being necessary for normal growth and reproduction (42), linoleic acid serves as a precursor to important LCPUFAs, such as dihomo-gamma-linolenic acid (DGLA; 20:3n-6) and arachidonic acid (AA; 20:4n-6) (43). DGLA and AA then serve as direct precursors to eicosanoids, thus, making LA an indirect precursor.

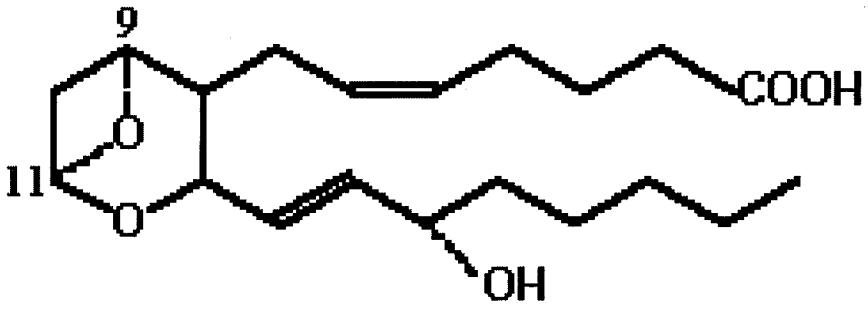
PGs, TXs, and LTs comprise the highly oxygenated family of lipids called eicosanoids, which are derived from DGLA, AA, and EPA, all of which are PUFAs containing twenty carbon atoms (**Figure 1**) (9). Eicosanoids are produced in virtually every cell in the body and exert their extremely powerful actions locally in contrast to traveling through the bloodstream like hormones. Eicosanoids are intimately involved in the inflammatory response and smooth muscle functions. As a result, they are responsible for regulating a variety of critical physiological processes including platelet aggregation, blood pressure, bronchoconstriction, and bronchodilation. Eicosanoids regulate their actions by having opposing effects from one another, which results in both activation and inhibition of the same processes. These actions are dependent upon the specific fatty acid from which the eicosanoids are derived. For example, TXs derived from AA promote platelet aggregation while those derived from EPA decrease aggregation.

An intake of LA acid below 0.1% of ingested kcals produces deficiency symptoms (37). For adults, it is estimated that intakes between 1-3% of kcals are required to avoid

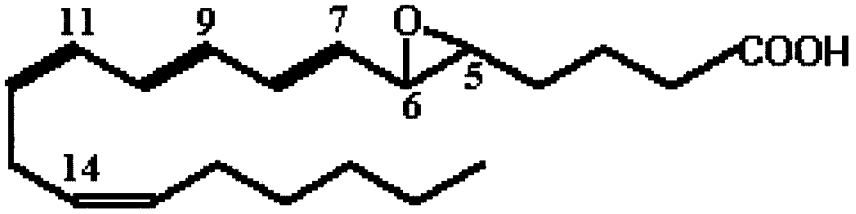
deficiency (7). According to Dietary Reference Intakes, 12-17 g/d of LA is considered adequate (44). While most vegetable foods and oils contain substantial amounts of LA, safflower, sunflower, and corn oils represent more concentrated sources of this EFA (6).



PGE_2



TXA_2



LTA_4

FIGURE 1. Eicosanoid Structures Produced from Arachidonic Acid. PGE_2, prostaglandin E_2; TXA_2, thromboxane A_2; LTA_4, leukotriene A_4.

Alpha-linolenic acid

ALA, an essential long chain polyunsaturated fatty acid, is located in various tissues but is not a major structural component (2). Although ALA is needed for normal skin integrity (3-5), its main role is to serve as a precursor to EPA and DHA, and thus to eicosanoids via EPA (5, 6). However, the efficiency of this conversion process is questionable (23, 24).

ALAD symptoms appear in humans when ALA comprises only 0.02-0.09% of caloric intake (1, 3-5, 39). Animal models of ALAD produce altered learning behaviors in rats as well as decreased visual acuity and abnormal electroretinograms in primates and rats (7). Symptoms of ALAD in humans include scaly dermatitis and a variety of neurological abnormalities (1, 4, 5). Minimum requirements for ALA, which are largely derived from studies that determined the amounts needed to reverse symptoms of ALAD, are estimated to be between 0.2-1.0% of kcal intake for adults (3-5) and between 0.54-1.2% for children (1, 39). Based on these requirements, it is generally recommended that ALA comprise 0.5-1.0% of kcal intake (6, 45). Additionally, Adequate Intake for ALA has been set at 1.1-1.6 g/d for adults (44).

Essential fatty acid metabolism

Human cells are known to convert dietary ALA to EPA and DHA (43, 46). In a series of subsequent reactions (**Figure 2**), dietary LA is converted to gamma-linolenic acid (GLA; 18:3n-6), DGLA, AA, and finally 22:5n-6. Hence, dietary ALA and LA give rise to two main families of fatty acids, namely the n-3 and n-6 families, respectively. It

is important to note that no interconversion takes place between these two families. These metabolic cascades occur through desaturation, chain elongation, and partial β-oxidation. The desaturation reactions are catalyzed by a series of enzymes named for the position of the fatty acid chain, counting from the carboxyl end, which is desaturated. For instance, Δ6 desaturase catalyzes ALA to 18:4n-3. Then an elongase converts 18:4n-3 to 20:4n-3, which is then converted by Δ5 desaturase to EPA. Although there is speculation that multiple chain length-specific desaturases exist, work in this area has been inconclusive (46). At this time, therefore, it is believed that the same enzymes that catalyze the conversion of ALA to EPA also catalyze the conversion of LA to AA. Hence, there is competition between the n-3 and n-6 families for the desaturase enzymes. For example, a high intake of LA is thought to impair the conversion of ALA to EPA through competition for Δ6 desaturase (22, 23, 42, 47-50). It was previously assumed that a Δ4 desaturase catalyzed the last step of the n-3 and n-6 pathways (51). This was a false assumption and the details of the final steps in the synthesis of DHA and 22:5n-6 are now more clearly understood (46, 51, 52). EPA undergoes elongation to 22:5n-3, which is elongated to 24:5n-3. Δ6 desaturase then converts 24:5n-3 to 24:6n-3 which undergoes partial β-oxidation to become DHA. Arachidonic acid is converted by the same enzymes to 22:5n-6 in an identical manner.

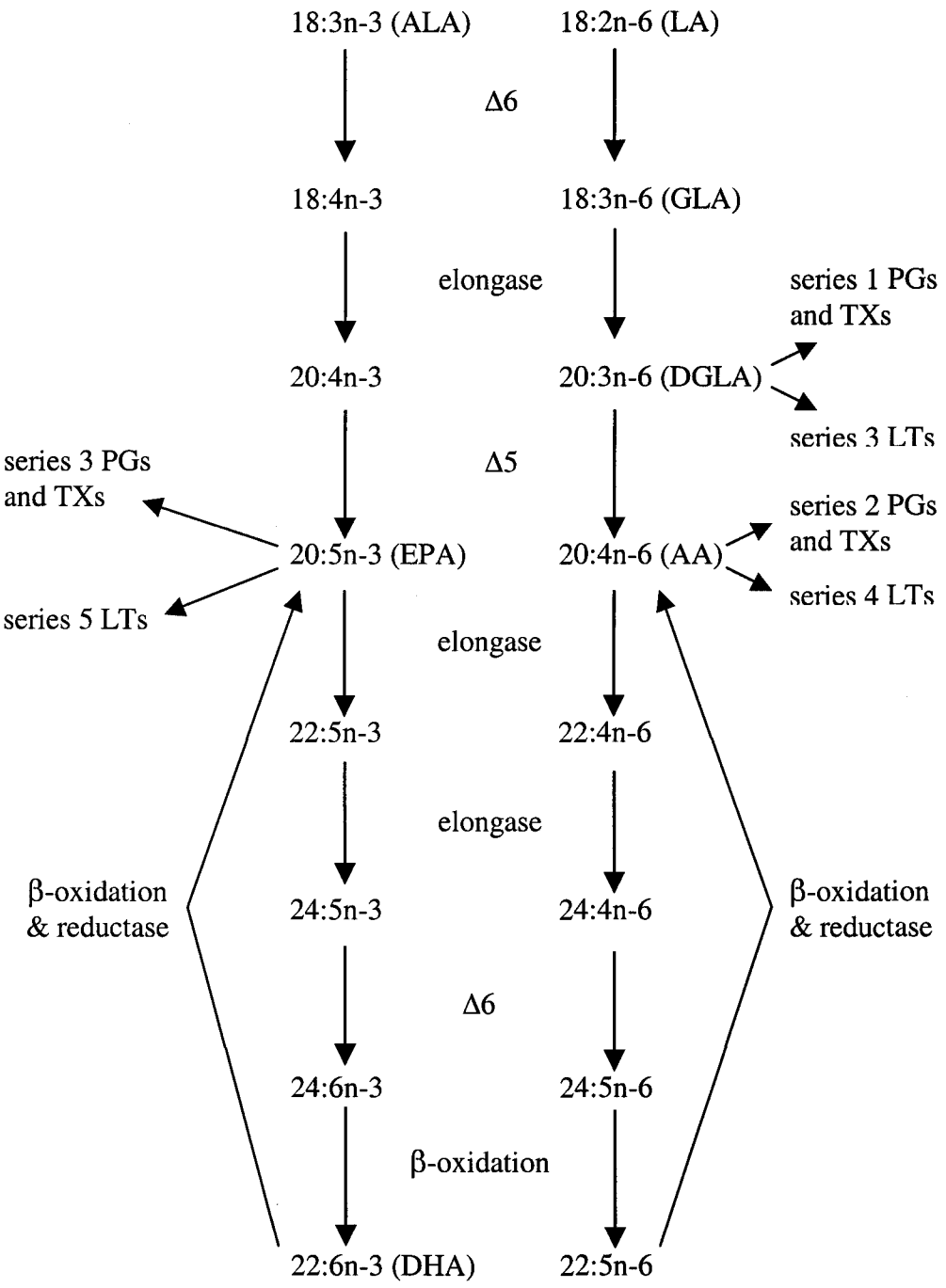


FIGURE 2. Metabolism of Polyunsaturated Fatty Acids and Retroconversion of DHA and 22:5n-6. ALA, alpha-linolenic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; LA, linoleic acid; GLA, gamma-linolenic acid; DGLA, dihomo-gamma-linolenic acid; AA, arachidonic acid; Δ6, Δ6-desaturase; Δ5, Δ5-desaturase; PG, prostaglandins; TX, thromboxanes; LT, leukotrienes.

The n-3 and n-6 pathways do not occur exclusively in one direction. It is now known that DHA can be converted back to EPA and likewise 22:5n-6 can be converted to AA by a process called retroconversion (**Figure 2**) (46, 53-57). It is estimated that about 1.4% of DHA is converted to EPA in humans without supplementation (54). In contrast, studies using an algae-derived form of DHA supplement have achieved retroconversion rates between 9.4-12% (56, 57). Despite this, the biological importance of retroconversion is not yet understood (54, 55). However, it has been suggested that because DHA can retroconvert to EPA, DHA may act as a storage pool for EPA and its eicosanoids, thus giving retroconversion a purpose (2).

Epidemiology and essential fatty acids

Linoleic acid. The relationship of LA to cardiovascular diseases remains unclear. Although plasma and adipose tissue LA has been inversely associated with the prevalence of coronary heart disease (CHD) in a number of populations (58), more recently, Kark, *et al.,* did not observe an association between adipose tissue LA and risk for acute myocardial infarction (59). Dietary LA has been reported to decrease both total and LDL cholesterol concentrations moderately (60, 61). Since a decrease in serum cholesterol is associated with decreased CHD risk, it is plausible that LA may decrease CHD risk (62, 63). Conversely, two large prospective, cohort studies found no significant relationship between LA intake and risk for CHD (15, 19). However, more recently a high intake of LA was associated with decreased risk of coronary artery disease (17). In addition, although there is a high intake of LA in Israel, Yam, *et al.,* report that this population has a high prevalence of cardiovascular disease and a high incidence of cancer compared to Western countries (64).

These paradoxical observations combined with animal and cell culture studies in which LA has been shown to enhance tumorigenesis have led to the question of whether or not high intakes of LA increase cancer risk (65). Although some data indicate that diets high in LA may slightly increase the risk of cancer, many researchers agree that there is not any substantial relationship between dietary LA and the risk of breast, colon, or prostate cancers (62, 65, 66). However, conclusions on this subject are difficult owing to a variety of methodological limitations and inconsistencies paired with the limitations of correlative studies (65, 67). Furthermore, Willett cautions that the high intake of LA in

Western society is a recent phenomenon and its relation to cancer should continue to be monitored (66).

Alpha-linolenic acid. A direct relationship between ALA and cardiovascular disease continues to be debated due to conflicting study results. Prospective cohort studies have observed an inverse relationship between dietary ALA intake and risk of CHD (15) and fatal ischemic heart disease (16). Conversely, two large prospective studies did not find an asociation between dietary ALA intake and risk of CHD (18, 19). In the Lyon Heart Study, de Lorgeril, *et al.*, reported a significant decrease in secondary cardiac events and death in subjects with a high intake of ALA compared to controls with a low intake (68). In a large cross-sectional study, dietary ALA was inversely associated with CHD (17). Dietary ALA has also been associated with a lower prevalence of carotid atherosclerosis (69). In addition, adipose tissue ALA has been inversely associated with nonfatal acute myocardial infarction (70). However, a high intake of ALA has been associated with increased risk for age-related macular degeneration (AMD), which is in contrast to the intake of EPA and DHA, which have been inversely associated for risk of AMD (71).

Eicosapentaenoic acid and docosahexaenoic acid

Eicosapentaenoic acid

EPA is a long chain polyunsaturated fatty acid that circulates in plasma and is a minor component of phospholipids in cell membranes throughout the body (2, 8). The major function of EPA is to serve as the direct precursor to series 3 PGs, TXs, and series

5 LTs (**Figure 2**) (6). Eicosanoids produced from EPA are known to decrease inflammation and platelet aggregation in contrast to series 2 PGs, TXs, and series 4 LTs (produced from arachidonic acid) that increase inflammation and platelet aggregation (6, 9, 13, 43, 72).

There is no official requirement established for the intake of EPA because it is synthesized from dietary ALA. However, Bjerve and coworkers estimated that 350-400 mg per day (or 0.4% of kcals) of EPA and DHA are necessary to maintain midnormal concentrations of these fatty acids in plasma and erythrocyte lipids of adults (5). EPA is synthesized by unicellular phytoplankton (73). Therefore, once EPA travels up the food chain it can become quite concentrated in some species of fish. Thus, seafood is one of the best dietary sources of EPA (74). As a result, a vegetarian diet is virtually devoid of preformed EPA.

Docosahexaenoic acid

In contrast to EPA, DHA is a major constituent of membrane phospholipids in cerebral gray matter, retinal photoreceptors, testes, and sperm (2, 7, 10). DHA occupies up to one third of the available positions on ethanolamine and serine phospholipids in cerebral gray matter (2, 10). DHA also occupies about 45-60% of the available positions of ethanolamine and serine phospholipids of rod outer segments of photoreceptor cells in the retina (7). In terms of biological function, DHA is thought to be involved in modulating the kinetics of carrier-mediated transport systems by altering membrane-bound enzyme activity and in modifying the properties of membrane receptors (10).

Therefore, it is suggested that DHA plays a vital role in signal transduction (2). In addition, DHA influences thermal adaptation of biological membranes (75) and is thought to inhibit platelet aggregation (76, 77). Although the details remain to be elucidated, it is now generally accepted that the major biological role of DHA is its function in the normal visual cycle (2, 7, 10). One of the proposed mechanisms for DHA function in the retina is the Molecular Spring Model (78). According to this model, DHA expands like a spring to accommodate the conformational change undergone by the transmembrane protein, rhodopsin, which allows a nerve impulse to be sent and vision to occur. Other researchers have proposed that DHA assists rhodopsin in its conformational change by destabilizing the lipid bilayer (79) or by altering the free energy of rhodopsin (80). Although many questions remain unanswered in DHA research, it is clearly a primary component of the central nervous system (CNS) and Salem, *et al.,* contend that preformed DHA may even be essential in the diet (81).

As with EPA, there are no official requirements for DHA because it can be synthesized endogenously from dietary ALA (**Figure 2**). However, estimations have also been made for adequate intakes of DHA to maintain stable tissue levels (5). DHA is also synthesized by phytoplankton making some marine animals an excellent source of this fatty acid. Like EPA, naturally occurring DHA is not part of most vegetarian diets.

Epidemiology and eicosapentaenoic and docosahexaenoic acids

Much of the interest in fatty acids relative to cardiovascular disease (CVD) was sparked by the classic work of Bang and Dyerberg (72, 82). In the late sixties they began

to study the diets and lipid profiles of Greenland Eskimos. Paradoxically, they found that Greenland Eskimos consuming a diet extremely high in animal fat (mostly fish) had a very low incidence of ischemic heart disease compared to Danish controls (82). The Eskimos also had lower serum triacylglycerols, lower total cholesterol, and increased HDL cholesterol concentrations than controls. Dyerberg and others later found that EPA does not promote platelet aggregation in vivo, which supported their hypothesis that EPA found in fish was most likely responsible for the cardioprotective effects they observed (72).

In the Multiple Risk Factor Intervention Trial, which studied a cohort of men with CHD compared to those without, it was found that men with CHD had significantly lower serum phospholipid DHA levels (83). The authors concluded that higher serum levels of DHA are associated with decreased risk of CHD. In a cross-sectional study, Bonaa, *et al.,* found that higher plasma phospholipid EPA levels from regular fish consumption were associated with lower triacylglycerols and increased HDL concentrations (84). Based on the weight of scientific evidence, the U.S. Food and Drug Administration recently authorized a qualified health claim for use on dietary supplements that suggests a beneficial relationship between the n-3 fatty acids EPA and DHA (but not ALA) and CHD. More recently, Leaf, *et al.,* have proposed a mechanism to explain how n-3 fatty acids prevent the fatal arrhythmias that cause sudden cardiac death (21).

Holman compared the serum phospholipid total n-3 fatty acid concentrations (includes EPA and DHA) of healthy adults from 7 different populations, spanning four continents, to those of healthy control subjects in Minnesota (22, 85). It was discovered

that all of the populations studied had higher serum concentrations of total n-3 fatty acids than the Minnesota controls, with the exception of Australian aborigines and Bulgarians that were lower. Nigerians and Northern Swedes had the highest n-3 concentrations, probably as a result of their high levels of fish consumption. Holman also compared the n-3 concentrations of Minnesota controls to groups of subjects having a variety of diagnosed neurological and immune diseases (e.g. multiple sclerosis, Crohn disease, and others). This comparison revealed that the n-3 concentrations of Minnesota controls were in the same lower half of the range as the groups of diseased subjects. Holman concluded that the Minnesota controls might have decreased disease resistance resulting from their lower n-3 status.

Decreased tissue concentrations of EPA and/or DHA have also been reported in other diseases. Soderberg, *et al.,* found that patients diagnosed with Alzheimer's disease had significantly lower brain DHA levels (measured by autopsy after death) compared to controls who died of natural causes not related to the nervous system and who had no clinical symptoms of dementia or other mental disorders (86). In addition, Hibbeln and Salem noted that decreased serum DHA concentrations are associated with increased depression (87). Furthermore, Stevens, *et al.,* found that young boys diagnosed with attention-deficit hyperactivity disorder (ADHD) had lower serum concentrations of both EPA and DHA compared to controls without ADHD (88).

Gamma-linolenic acid

GLA is an intermediate in the n-6 fatty acid pathway between LA and DGLA. Although food sources of GLA are limited, it is quite concentrated in hemp seed oil (1-4 g/100g GLA), evening primrose oil (EPO) (7-10 g/100g GLA), black currant oil (15-20 g/100g GLA), and borage oil (18-26 g/100g GLA) (89). In the body, GLA is rapidly converted to DGLA, which is slowly converted to AA, due to the limited activity of Δ5-desaturase, the enzyme needed for this reaction (**Figure 2**) (89, 90). A 15-hydroxyl derivative of DGLA is thought to competitively inhibit the production proinflammatory eicosanoids from AA (90). Thus, clinical studies have used rich sources of GLA, such as EPO and borage oil with success to manipulate eicosanoid production and improve inflammatory conditions in rheumatoid arthritis and atopic eczema (89-91). However, hemp seed oil as a source of GLA has not been studied in this manner.

Food sources of n-3 fatty acids

Alpha-linolenic acid

Since ALA is the only fatty acid vegans consume that allows them to synthesize EPA and DHA, it is prudent that a vegan diet contain rich sources of ALA (92). Unfortunately, concentrated sources of ALA are limited. Flax seed oil (linseed oil), flax seeds, hemp oil, walnuts, canola oil (low erucic acid rapeseed oil), and soybean oil represent the richest sources of commonly available ALA for both vegetarians and omnivores (74). To gain an idea of the range of ALA available from these sources, 1 tablespoon (Tbsp) of flax seed oil and 0.5 cup of tofu provide approximately 7.5 g and

0.4 g of ALA, respectively. To meet the upper end of the ALA requirement for a 2000 kcal diet (1% of kcals or 2.2g) estimated by Bjerve and coworkers (5), a person would have to eat about 1 teaspoon (tsp) of flax oil or 2.8 cups of tofu per day. It would take about 1.5 tsp of flax oil or 4.1 cups of tofu per day to meet the same requirement for a 3000 kcal diet.

Per capita availability of ALA from vegetable oil products is approximately 1.2 grams per day (g/d) in the United States, which has increased since 1970, mainly due to the increased use of canola and soybean oils (93). This estimate increases to approximately 1.7-2.2 g/d if consumption of dairy products, beans, broccoli, walnuts, and butterhead lettuce are included, all of which contain very small amounts of ALA. When fish and other foods are included in the calculation, the estimate rises to 2.8 g/d (94). These calculations of per capita availability of ALA appear to just meet the reported ALA requirement (2.2 g/d) needed to avoid symptoms of ALAD for a 2000 kcal diet. However, it is probably unwise to equate a lack of overt clinical symptoms with proper membrane function, eicosanoid metabolism, or perhaps prevention of certain diseases (22). Furthermore, the amount of ALA available per capita may not be adequate to meet the upper end of the ALA requirement for a 3000 kcal diet (1% of kcals or 3.3 g/d). In view of this potentially marginal ALA supply in the U.S., Holman recommends shifting the use of oils high in n-6 fatty acids to oils high in ALA such as flax, canola, and soybean oils (85). Moreover, Cunnane found that 50 g/d of ground flax seeds incorporated into bread was palatable and provided 12 g/d of ALA (31). Also, the

bioavailability of ALA from the bread, assessed by serum ALA concentrations, was similar to that of a flax seed oil supplement that provided an equal amount of ALA.

Eicosapentaenoic acid and docosahexaenoic acid

As EPA and DHA are not synthesized in higher plants, there are no naturally occurring preformed dietary sources of these fatty acids available to a vegan diet. However, there are a number of concentrated sources of them available to omnivores, and a smaller but potentially significant amount available to ovovegetarians. For omnivores, mackerel, bluefin tuna, and salmon are rich sources of EPA and DHA (74). As a result, fish is the main source of these fatty acids in the American diet (94). For instance, a 100 g serving of Atlantic mackerel contains approximately 900 mg of EPA and 1600 mg of DHA (74). Thus a 100 g serving of mackerel would far exceed the EPA and DHA needs of 350-400 mg/d estimated by Bjerve, *et al.,* (5). Range-fed chicken eggs are the main source of EPA and DHA for ovovegetarians providing approximately 20 mg and 110 mg per egg yolk, respectively (95). However, range-fed chicken eggs are not widely available in U.S. supermarkets. Instead, standard United States Department of Agriculture (USDA) eggs are commonly available which provide 0 mg of EPA and only 18.3 mg of DHA per yolk (95).

Perhaps inspired by research on the potential health benefits of n-3 fatty acids, successful attempts have been made to increase the concentration of EPA and DHA in beef (96, 97), poultry (98) and eggs (95, 99) by including varying levels of either flax meal or fish meal in the feed of animals. By feeding flax meal, which contains ALA,

animal tissues can convert ALA to EPA and DHA. Feeding fish meal, which already contains EPA and DHA, efficiently concentrates these fatty acids into the tissues of the livestock consuming them. For example, eggs from chickens fed flax meal, fish meal, or fish oil have much higher concentrations of EPA and DHA per yolk compared to USDA eggs (95, 99). In addition, concentrations of EPA but not DHA have been increased in the milk of range-fed cows consuming fresh grass compared to cows fed dried grass (100). Although this type of milk is not commonly available in U.S. supermarkets, it does represent a potential dietary source of preformed EPA for lactovegetarians. Any inferences with respect to increased concentrations of EPA and DHA in animal tissues must be drawn with caution. Highly unsaturated fatty acids, such as EPA and DHA are readily destroyed by heat, light, and oxygen. Thus, cooking may degrade much of the available n-3 fatty acids found in animal tissues. There are few data for the concentrations of EPA and DHA in animal products after they are cooked. However in one study, Sinclair and others found that beef grilled for 10-15 minutes lost approximately 20-30% of its original EPA and DHA content (96). Furthermore, increasing the tissue concentrations of these fatty acids in livestock may result in unfavorable taste properties. For example, meat from chickens fed fish meal and eggs from chickens fed fish oil have produced slightly abnormal flavors (98, 99). In contrast, in a study by Hulan, *et al.*, the taste panel did not describe the flavors as "fishy" or objectionable (98).

Attempts have been made to incorporate purified DHA into breads. Morita and Shirai found that adding microcapsulated DHA powder derived from fish oils into bread

ingredients produced high volume loaves with a good appearance and no fish odor (101). The DHA concentration also remained quite stable during baking, providing 25 mg per two slices of bread. In another study, Becker and Kyle measured the oxidative stability and organoleptic properties of bread standardized to contain 100 mg of either algae-derived or fish-derived DHA per slice (102). They determined that the bread made with algae-derived DHA had higher oxidative stability and less of an off-flavor than the bread made with fish oil-derived DHA over a 13 day period. Since algae-derived DHA is suitable to for a vegan diet, bread or other products fortified with this fatty acid may soon represent dietary sources of DHA for vegans.

Over the last half century most of our best sources of ALA have been replaced with oils rich in n-6 fatty acids or have been hydrogenated in order to improve shelf-life and retain flavor (22). In fact, the estimated U.S. intake of trans fatty acids (produced from hydrogenation) is 8 g/d (or 3% of total kcals) (103). This estimate is triple the amount of ALA intake recommended by Lasserre and coworkers (45). Dietary trans fatty acids have no known health benefits and may increase the risk of CHD (103). However, since the introduction of hydrogenation, effective alternate methods have been developed to maintain and deliver EFAs to consumers by preventing rancidity (42). In addition, much of the feed for livestock in the U.S. is corn based which is high in LA and low in ALA, producing meat low in n-3 fatty acids. However, as mentioned above, the levels of n-3 fatty acids can be increased in meat products by altering the diets of livestock (95-100). Given the importance of n-3 fatty acids in the CNS and our ability to enhance them

in foods, it seems contrary to continue destroying these important fatty acids in our food supply for the purposes of extending shelf-life or improving flavor (42).

Polyunsaturated fatty acid intake of vegans

Vegans choose to eat no naturally occurring, preformed EPA, DHA, or arachidonic acid whatsoever (104). Conversely, the mean LA intake of vegans has been observed to be two to five times higher than that of omnivore controls (27, 41, 50, 104-107). In one study (50), male vegans had a mean LA intake of 28.5 g/d compared to omnivores who ate 10.2 g/d. Studies measuring the ALA intake of vegans have found no significant differences from omnivore intake of ALA (27, 41, 105, 106). However, two studies did report a higher intake of ALA in vegans compared to omnivorous controls (50, 104). In one of these studies (50), the mean ALA intake of vegan males was 1.8 g/d and 1.0 g/d for omnivores. It is well known that high intakes of LA inhibit the conversion of ALA to its longer chain derivatives (42). With this in mind, it appears important to consider the ratio of LA to ALA in the diet.

Ratios of linoleic acid to α-linolenic acid

The issue of defining ratios of LA to ALA in the diet is complex . Several investigators agree that an optimal ratio should be determined (7, 13, 42) but few agree on similar numbers for this ratio. Lasserre and colleagues proposed that a ratio of 3:1 minimizes competition between LA and ALA to their respective metabolites (45). Yehuda and Carasso found that a ratio of 4:1 was optimal for enhancing learning

performance in rats. Conversely, Holman suggests that a ratio of 14:1 creates equal competition between the n-3 and n-6 families (22). However, in support of the work of Yehuda and Carasso (108), Holman argues that equal competition between the n-3 and n-6 fatty acid families might not be the only measure of optimal function, and that we should attempt to eat LA and ALA in ratios closer to 4:1 (22). The work of de Lorgeril and others strengthens Holman's argument (68). In their study, a ratio of 4:1 was associated with a greater than fifty percent decrease in cardiovascular mortality compared to a 20:1 ratio. With the exception of one study, which detected a ratio of 23:1 (105), a number of studies have found ratios between 5:1 and 10:1 in omnivorous diets (27, 41, 50, 104, 106, 109). In contrast, various findings indicate that the LA to ALA ratio of vegan diets ranges from 16:1 to 28:1 (27, 50, 104-106). Despite one study that observed a ratio of 5:1 (41), this range of LA to ALA ratios found in vegan diets far exceeds any recommendations that have been made. In a joint statement, the World Health Organization (WHO) and the Food and Agriculture Organization (FAO) recommend that this ratio should fall between 5:1 and 10:1 and that individuals with a ratio greater than 10:1 should increase their consumption of n-3 rich foods (61).

Fatty acid status in vegans

The fatty acid composition of various blood lipids, adipose tissue, and breast milk reflects the intake of dietary fatty acids (40, 41, 106, 110). Thus, as would be expected from their high intakes of LA and high ratios of LA to ALA, several studies have reported that vegans have much higher concentrations of LA in serum erythrocytes, platelets, free

fatty acids, phospholipids, cholesterol esters, adipose tissue, and breast milk as compared to omnivores (41, 49, 50, 106, 111). They also invariably have much lower concentrations of EPA and DHA compared to omnivores across all of these lipid fractions (41, 49, 50, 106, 111, 112). In contrast, levels of arachidonic acid in blood lipids vary considerably (41, 49, 50, 106, 111, 112). However, one consistent finding among these studies is that platelet levels of AA appear to be lower in vegans (41, 50, 111). The health consequences of these distinctly different fatty acid concentrations, especially that of DHA in adult vegans are unknown (41). However, as mentioned above, Holman found that the n-3 fatty acid status of a group of Minnesota control subjects was similar to the n-3 concentrations observed in a number of diseases (22, 85). Adding to this, the concentrations of serum phospholipid EPA and DHA of vegans reported by Agren, *et al.,* (41) are lower than the concentrations reported by Holman for the Minnesota control subjects. Furthermore, if it is assumed that the LA to ALA intake ratio between 4:1 and 10:1 recommended by WHO and FAO (61) is beneficial for health, vegans may need to alter the quality of their fat intake in order to decrease this ratio and improve their n-3 fatty acid status.

Effects of ALA supplementation on EPA and DHA concentrations

It has been demonstrated that supplementing EPA and DHA more efficiently incorporates these fatty acids into blood lipids compared to supplementing ALA (27, 28). However, some diets do not include preformed sources of EPA and DHA. Therefore, considering the importance of these two fatty acids, it is critical to question whether or

not dietary ALA reliably converts to EPA and DHA in humans. Several investigators have attempted to determine this by increasing ALA intake via supplements or dietary sources in both vegans and omnivores.

In a study of healthy adult omnivores it was found that flax seed oil capsules providing a mean of 5.9 g/d of ALA slightly increased platelet EPA concentrations but did not increase DHA concentrations (28). Similarly, Allman, *et al.,* found that healthy adult male omnivores fed nearly 3 Tbsp/d of flax seed oil doubled their platelet EPA concentrations in 23 days, while DHA concentrations were unchanged (13). Increases in serum phospholipid EPA, without increases in DHA, were also observed by Mantzioris and colleagues in subjects consuming 13.0-13.7 g/d of ALA from flax seed oil (30, 113). Such increases in EPA would be expected since the LA to ALA intake ratio in these studies was approximately 1:1. In one of these studies (113), phospholipid DHA actually decreased from an increase in dietary ALA. To explain this phenomenon, the authors suggested that the resultant increases in EPA might have displaced DHA from phospholipids. In another study, vegans were supplemented with flax seed oil providing 6.5 g/d of ALA, which produced a 300% increase in plasma phospholipid EPA after only two weeks (27). This would also be expected given that the flax seed supplement decreased the ratio of LA to ALA from 16:1 to 3:1. In contrast, no rise in DHA was detected in vegans or omnivores taking the flax seed oil. For comparison, omnivores in the study experienced large increases of both EPA and DHA in plasma phospholipids and platelets from a fish oil supplement (MaxEPA) containing these two fatty acids. The authors of this study concluded that humans may be slow to convert EPA to DHA and

that high levels of LA in adipose tissue of vegans may buffer the short term effect of lowering the ratio of LA to ALA. In another study, females taking flax oil supplements providing 12 g/d of ALA showed increases in plasma phospholipid EPA (31). Although no changes in plasma phospholipid were observed for DHA, there was an 80% increase in erythrocyte phospholipid DHA after four weeks of supplementation. Francois and colleagues also observed a significant increase in plasma EPA, without changes to DHA, after supplementing 10.7 g/d of ALA from flax oil for four weeks (29).

Chan and coworkers compared the conversion of ALA to EPA and DHA between a variety of vegetable oils in male omnivores (12). They discovered that a diet rich in canola oil and a diet rich in a mixture of sunflower, olive and flax oils produced double the amount of platelet and plasma phospholipid EPA compared to a diet high in soybean oil or a diet high in a mixture of sunflower and olive oils. In contrast to EPA, there were no increases in platelet or plasma DHA detected as a result of any of the four diets.

Kwon, *et al.,* found that a diet providing between 7.8-8.6 g/d of ALA from canola oil produced a significant increase in platelet EPA, without an increase in DHA (114). Similarly, subjects fed a diet rich in canola oil containing a mean of 8.5 g/d (2.8% of energy) of ALA for 18 days doubled platelet phospholipid EPA without changing DHA concentrations (115).

In healthy female subjects consuming 50 g/d of ground flax seeds (providing 12 g/d ALA) added to breakfast cereal, soup, juice, or yogurt there were increases of total n-3 fatty acids (EPA, DPA, and DHA combined) in plasma triacylglycerols, but not plasma phospholipids (31). In contrast, Chisholm and coworkers found that moderately

hyperlipidemic omnivore men consuming 78 g/d of walnuts, providing 5.9 g/d of ALA, had no increases of EPA or DHA in plasma triacylglycerols, cholesterol esters, or phospholipids (116). A lack in change of EPA and DHA status was also reported in another study of males consuming a diet rich in walnuts (117). Chisholm and coworkers concluded that in order to increase EPA concentrations from ALA sources, the ratio of n-6 to n-3 fatty acid intake must be low and ALA intake must be high (116). They further concluded that these are difficult criteria to meet by simply adding walnuts to the diet.

In a novel approach, Emken, *et al.,* determined by use of deuterium-labeled ALA that approximately only 15% of dietary ALA is converted to n-3 LCPUFAs when LA intake is held constant (23). They estimated that 15% of 2g of ALA would provide only 75-85% of the amount of n-3 LCPUFAs estimated by Bjerve to be required (350-400 mg/d). The percent ALA conversion ranged from 7.9-25.7%. This indicates that some subjects were only making about 50% of their n-3 fatty acid needs.

Taken together, the results of these studies suggest that providing rich sources of ALA can significantly increase EPA concentrations in serum lipids. However, increasing dietary ALA does not appear to increase DHA concentrations, and may actually lower them. If rising EPA concentrations resulting from increased dietary ALA displace phospholipid DHA, as suggested by Mantzioris and coworkers (113), it seems plausible that preformed sources of DHA may be necessary in the diet (81).

Conclusion

A vegetarian diet is thought to be adequate, especially if foods are chosen wisely and select fortified products are included. However, since a vegetarian diet excludes the richest sources of EPA and DHA, vegetarians rely mainly on endogenous production of these fatty acids from their essential precursor, ALA. Despite the low ALA requirements necessary for avoiding frank deficiency, there are few rich sources of ALA and the process of its conversion to EPA and DHA appears to be limited in humans. The high intake of LA observed in vegans creates a LA to ALA ratio that further impairs production of EPA and DHA. As a result, much lower blood lipid concentrations of EPA and DHA have been reported in vegans compared to omnivores. EPA and DHA are required for a host of normal physiological functions. In addition, lower levels of these fatty acids have been observed across a number of degenerative diseases whereas higher levels have been associated with decreased cardiovascular disease risk. Increasing ALA intake appears to predictably increase EPA but not DHA concentrations. In contrast, increasing DHA intake leads to an efficient rise of both EPA and DHA concentrations. Although it seems plausible that DHA may be a dietary essential, more research is needed to clarify this issue. Until DHA fortified products become available, it seems prudent that vegans and people who avoid or limit their fish consumption should meet recommendations for ALA intake, decrease their LA to ALA ratios, and omnivores should include sources of EPA and DHA in their diets.

CHAPTER 3. SUBJECTS AND METHODS

Study population

Healthy, non-smoking, male and female subjects between the ages of 21-80 y who had been following a vegetarian diet (a plant based diet excluding the consumption of red meat, poultry, pork, and fish but including small amounts of dairy and/or egg products) for at least six months prior to the study were recruited from the island of Oahu, Hawaii. To recruit volunteers, flyers were posted on several occasions at colleges, natural food stores, gyms, yoga studios, alternative healthcare practitioner offices, vegetarian restaurants, Seventh Day Adventist churches and Buddhist temples. Also, public announcements were made at Vegetarian Society of Hawaii meetings, cooking classes, hospitals, Seventh Day Adventist churches and health lectures. Advertisements for the study were placed in a variety of local publications, and were included in retail packages distributed by a local produce distribution company. Announcements were also made on three separate local radio stations.

Each potential subject was screened with diet and medical history questionnaires (see **Appendix B**). The diet history questionnaire was designed to identify if a person was truly adhering to a vegetarian diet and to identify if he/she had any dietary, supplement or drug patterns that could potentially interfere with regular fatty acid metabolism. Any subject with a history of regular consumption of red meat, poultry, pork, fish, walnuts, flax seeds, flax seed oil, hemp seed oil, or any oils known to be high in ALA, EPA, or DHA within four months of screening also were not eligible to participate. Any subjects with a history of regular use of nonsteroidal antiinflammatory,

antihypertensive, or lipid-lowering drugs, or other drugs known to affect lipid metabolism were not eligible for the study. The medical history questionnaire was designed to identify any health conditions that could potentially interfere with regular fatty acid metabolism. Exclusion criteria included pregnancy or irregular menstrual cycle (in women), a history of cardiovascular disease, cancer, diabetes, renal disease, hepatitis, obesity, or eating disorders. Twenty-four of the 56 subjects screened were enrolled. The study was approved by the Committee on Human Studies of the University of Hawaii at Manoa, and all subjects gave written informed consent.

Study design

In a parallel, double-blind design, two groups matched for age, sex, and type of vegetarian diet eaten were randomly allocated to add one tablespoon (14g) of either flax seed oil or hemp seed oil to their diet, with meals, daily for 28 consecutive days. The flax seed and hemp seed oils (oils and polyunsaturated fatty acid composition analysis were provided by Omega Nutrition, Bellingham, Washington) were unrefined, and cold pressed from seeds that were organically grown in accordance with the California Organic Foods Act of 1990 (California Food and Agriculture Code 46000). The flax seed oil contained approximately 15.9% LA (2.2 g/tablespoon) and 50.7% ALA (7.1 g/tablespoon) as percent of the total fatty acids. The hemp seed oil contained approximately 54.2% LA (7.6 g/tablespoon), 15.5% ALA (2.2 g/tablespoon), and 4.6% GLA (0.6 g/tablespoon) as percent of the total fatty acids.

Enough oil for the 28-day supplement period was provided to each subject in a pre-weighed, coded, black plastic bottle. Neither the subjects nor the study personnel were alerted as to the contents of the bottles at the time of their distribution, or during the supplement period. Because the oils were taken with food and not in capsules, it is possible that some subjects could determine which oil they were taking, due to preexisting familiarity with flax or hemp seed oils. However, great care was taken by the study personnel to maintain blinding throughout the study. In addition, all subjects completed an exit interview questionnaire, which assessed their ability to identify accurately which oil they were consuming during the study.

Subjects were instructed to keep the oils refrigerated during the supplement period to prevent development of rancidity. Compliance to oil intake was checked by having participants keep a log (see **Appendix B**) of their daily oil intake over the 28-day supplement period. This log was recorded separately from any other food records kept during the study. The bottles of oil were also weighed upon study completion, however, these data proved questionable and incomplete due to slight oil leakage and some subjects accidentally discarding their empty bottles prior to collection. In addition, subjects were called at least twice weekly to remind them to take their oil daily and to assess their tolerance for consuming the oil.

Anthropometric measures and lifestyle assessment

Heights and weights were measured without shoes once at baseline and weights were measured again at post-supplement. A single height measurement was taken with

each subject standing against a tape measure mounted vertically to a flat wall. The subjects stood with their heels, buttocks, and shoulders touching the wall with their faces pointed forward. A ninety-degree angle square was placed flat on the subjects' heads and matched to the tape measure on the wall to obtain a height measurement. Weight measurements were obtained by using an electronic scale (A&D model UC-300, load cell). Subjects were instructed to wear minimal clothing (socks, underwear, shorts, pants or skirt, shirt or dress, without any form of jacket, sweater or sweatshirt) at each weight measurement. Height and weight measurements were used to calculate body mass index (BMI). Any major changes in physical activity during the study period were recorded in an exit interview questionnaire after the final blood collection.

Dietary intake

Subjects filled out a total of three separate 3-day food records (see **Appendix B**), one at baseline, one during week two, and one during week four. Each 3-day record consisted of two weekdays and at least one weekend day. To encourage compliance, participants were allowed flexibility in choosing which three days of the week they recorded. Thus, the 3-day records do not necessarily consist of three consecutive days. The two separate 3-day records for weeks two and four were combined into a mean of six days for statistical analysis. Food records were analyzed for macronutrient, selected micronutrient, and essential fatty acid intake using the Food Processor software program (version 7.4, ESHA Research, Salem, Oregon).

Foods not listed in the Food Processor database, such as restaurant food, personal recipes, and packaged foods, were entered manually. Recipes were obtained from restaurants and participants and could then be added to the Food Processor database. For packaged foods, nutrition facts labels served as the primary source of data. Since quantities of LA and ALA are not required on nutrition facts labels, values for these two fatty acids were estimated by the following procedure. The primary source of total fat was identified from the ingredients statement on the packaging. Percentages of LA and ALA of selected foods, published by Hepburn and others (74), were used to calculate quantities of these two fatty acids, which were then entered into the Food Processor database.

Subjects were instructed to maintain their normal dietary habits throughout the study period and were instructed to avoid consuming flax or hemp products exclusive of their normal dietary patterns and of the oils being supplied during the supplement period. They were instructed to record all food and beverages consumed in common household measures. Supplement use also was recorded on the 3-day food records. Each subject was given a complementary set of household measuring cups and spoons in order to help them estimate their dietary intake. The subjects were instructed to avoid measuring out the food they ate but instead to estimate their intake in terms of household measures, recognizing that measuring food is often impractical for free-living subjects.

All subjects attended an instruction meeting prior to completing the baseline food record. At the meeting, they were taught by the principal investigator how to estimate their dietary intake and fill out their food records correctly. In addition, several stations

were set up with different quantities of common vegetarian food displayed. Subjects spent time at each station attempting to estimate the correct quantity of food or beverage displayed. The purpose of this exercise was to familiarize the subjects with estimating amounts of food as they see them in practical situations.

On all three separate occasions, the subjects were reminded, at least one day prior by telephone, as to which dates they needed to fill out their food records. Furthermore, as an optional resource for participants, a web site (see **Appendix B**) was constructed on the Internet that contained all the necessary information for filling out food record forms, scheduling, directions to the blood collection site, contact numbers, and the ability to print extra copies of food record forms.

Blood samples

Approximately 12-17 ml of venous blood was collected by a trained phlebotomist from the antecubital area of each subject into one 8.5 ml SST® vacuum tube and one 8.5 ml EDTA vacuum tube on two separate occasions, first at day 0 (baseline) and second at day 29 (post-supplement). The samples were drawn with 21-gauge needles while subjects sat upright in a chair. Subjects were instructed to fast 12 hours prior to blood collection. Upon arrival to the blood draw site, on both occasions, each participant verbally confirmed they had fasted the recommended number of hours prior to collection. In addition, each participant recorded on the written exit interview questionnaire that they had fasted the recommended amount of time for both blood collections.

The SST® tubes were immediately centrifuged and taken to Clinical Laboratories of Hawaii that same day for plasma lipid analysis. The EDTA tubes were inverted several times and immediately placed on wet ice in a dark container to avoid light and temperature damage. They were then taken within two hours to the Cancer Research Center of Hawaii laboratory where they were immediately separated into erythrocytes and plasma in preparation for later fatty acid analysis. To achieve this separation, the EDTA tubes were first centrifuged, the plasma (approximately 5 ml) was drawn off, placed in cryovials, and frozen at -70° C. The remaining erythrocytes (approximately 3.5 ml) were washed in an equal amount of saline twice, placed into cryovials, and frozen at -70° C.

Erythrocyte fatty acids

Erythrocyte fatty acid analysis took place approximately three months later at the Hormel Institute of the University of Minnesota after being shipped overnight from the Cancer Research Center of Hawaii packed on dry ice. At the Hormel Institute, the samples were stored at -70° C for approximately one month, at which point they were analyzed for erythrocyte total phospholipid fatty acid content by the methods described by Holman and colleagues (118).

Plasma lipids

Plasma lipid samples were analyzed at Clinical Laboratories of Hawaii on the same day they were drawn on both occasions. Plasma total cholesterol and triacylglycerols were determined enzymatically on a Roche/Hitachi analyzer. Total

cholesterol was determined using the Trinder-modified Boehringer Mannheim high performance method (119). Triacylglycerols were determined by the methods of Wahlfeld and Bergmeyer (120). Direct measurement of HDL cholesterol was determined by the methods of Sugiuchi (121). LDL cholesterol was calculated by using the Friedwald equation (122).

Statistical analysis

Statistical analysis of the data was done using the SAS software program (version 7, SAS Institute, Cary, North Carolina). Multivariate analysis of variance (MANOVA) was used to test the compatibility of the two groups based on LA and ALA intake, and erythrocyte EPA and DHA concentrations at baseline. Chi-square was used to determine any differences in sex between the two groups at baseline. Repeated measures t-tests were used to identify within group differences in erythrocyte fatty acids, plasma lipids, dietary factors, body weight, and BMI from baseline to post-supplement. Two-sample t-tests were used to identify differences in erythrocyte fatty acids, plasma lipids, body weight, and BMI between groups at baseline and post-supplementation, and also for dietary factors at baseline and during the supplement period. The Bonferroni correction was made to guard against type I error associated with doing multiple tests (123). In this study, four t-test comparisons were made (within group comparisons for both groups = 2, between group comparisons at two time points = 2), so the standard α value of 0.05 was divided by four to arrive at the adjusted α value of 0.0125. For all t-tests, any P value of < 0.0125 was considered significant. However, it is important to note that under the

Bonferroni correction, changes can only be considered significant at the α value of 0.05, not the adjusted α value 0.0125, and not at any P value calculated to be less than 0.0125. In keeping with this concept, although the specific P values for t-tests are reported throughout this paper (e.g. P = 0.008), they must be considered significant only at P = 0.05. Multiple regression as analysis of covariance (ANCOVA) was used to determine the variables responsible for predicting the variability in post-supplement erythrocyte EPA, DHA, DGLA, arachidonic acid and the plasma lipids. P values of < 0.05 were considered significant for these tests.

CHAPTER 4. RESULTS

Subjects, anthropometric measures and lifestyle assessment

Twenty-two of the 24 subjects enrolled completed the study. One subject withdrew because of scheduling difficulties and one withdrew due to gastrointestinal symptoms. The entrance characteristics, body weight, and BMI for the 22 subjects who completed the study are reported in **Table 1**.

No significant differences in age, sex, body weight, BMI, or number of years eating a vegetarian diet were found between the groups at baseline or post-supplement. No significant changes in body weight or BMI were observed from baseline to post-supplement within either group. The mean age in years (yr) ± SD was 43.8 ± 17.6 in the flax group and 42.0 ± 12.5 in the hemp group. The mean number of years eating a vegetarian diet ± SD was 6.2 ± 3.9 (range: 2-15 yr) in the flax group and 4.1 ± 5.0 (range: 0.5-15 yr) in the hemp group. The flax group consisted of 5 vegans, 4 lacto-vegetarians, and 1 lacto-ovo-vegetarian. The hemp group consisted of 4 vegans, 2 lacto-vegetarians, and 6 lacto-ovo-vegetarians. The reasons cited by the subjects for following a vegetarian diet included health, well being, animal rights, ethics, environmental, economical, spiritual, and religious considerations (see **Appendix B** for instrument used to identify these reasons).

TABLE 1.
Comparison of entrance characteristics, body weight, and BMI of subjects in both groups at baseline and post-supplement*, **

	Flax oil (n = 10) (M = 5, F = 5)		Hemp oil (n = 12) (M = 5, F = 7)	
	Baseline	**Post-supplement**	**Baseline**	**Post-supplement**
Body weight (kg)	64.9 ± 12.8	64.5 ± 13.0	65.8 ± 13.4	65.7 ± 14.0
BMI (kg/m^2)	21.7 ± 3.8	21.6 ± 3.9	22.7 ± 3.1	22.6 ± 3.3

* Mean ± SD.
** No significant (NS) differences were found within either group or between groups at any point.

Seven (32%) of the 22 subjects who completed the study reported on the exit interview questionnaire that they knew which oil they were taking during the study. Five (23%) of those seven were able to correctly identify which oil they were actually taking.

One subject reported experiencing gas and one reported experiencing diarrhea as possible side effects of taking the flax oil. Four subjects reported experiencing either upset stomach, mild nausea, acne, or bloating, respectively, as possible side effects of taking the hemp oil.

Dietary intake

On the exit interview questionnaire 18 (82%) of the 22 participants that completed the study reported that they thought the food records they kept accurately reflected their usual dietary intake. Travel, increased intake, major change in dietary pattern, and lower intake on recording days during the study were the general reasons why the other 4 (18%) participants thought the food records did not represent their usual dietary intake.

Eating patterns

The plant-based diets of the subjects were largely comprised of a combination of whole grains and whole grain products, fresh fruits and vegetables, legumes, fresh herbs, nuts and seeds. Their diets were devoid of any red meat, poultry, pork or fish but did include, in only a few subjects, small amounts of dairy products and eggs. Their diets also included a wide variety of processed foods that are not always included in a traditional vegetarian diet. Some of these foods included the following: canned or jarred sauces and soups, desserts (cookies, ice cream), beverages (tea, fruit juice, diet and regular soda, sports drinks, beer, wine), condiments (catsup, mayonnaise, mustard), salad dressings, boxed pilaf mixes, frozen vegetables, snack foods (tortilla and potato chips, pretzels, crackers, energy bars), ready-to-eat cereals, breads (bagels, muffins), margarine, vegetable oils, dried herbs and seasonings, sweetener (sugar, maple syrup, brown rice syrup), and candy. These diets also contained an assortment of meat analogs, cow milk and cheese substitutes including tofu, texturized vegetable protein, vegetable burgers, analog sausage patties, analog ground beef, analog sliced meat, analog hot dogs, soy milk, rice milk, and mock cheese slices, all of which are primarily derived from soy or wheat proteins and combined with other ingredients. In addition, these diets included food from traditional restaurants and fast food chains.

Energy and macronutrients

Changes in energy, macronutrient, and fiber intake from baseline to during the supplement period are reported in **Table 2**. The average intake of flax oil contributed

approximately 126 kcal/d and the hemp oil provided approximately 125 kcal/d. There were no significant differences in energy, macronutrient, or fiber intake detected between the groups at baseline, during the supplement period or within either group. The hemp group experienced a significant decrease ($P = 0.008$) from baseline in the percentage of energy from carbohydrates from 68.9 to 60.7%. There were no other significant differences in the percentage of energy from macronutrients detected between the groups at either time point or within either group.

TABLE 2.
Comparison of energy, macronutrient, and fiber intake of subjects in both groups at baseline and during the supplement period [*], [**], [***]

	Flax oil (n = 10)		Hemp oil (n = 12)		DRIs	
	Baseline	**During**	**Baseline**	**During**	**Male**	**Female**
Energy (kcal)	2162.4 ± 556.9	2299.9 ± 456.5	1994.7 ± 773.4	1923.4 ± 652.1	2,566	2,202
Protein (g/d)	68.3 ± 21.0	67.0 ± 11.0	61.0 ± 26.2	62.2 ± 24.6	56	46
Protein (% energy)	12.7 ± 1.9	13.0 ± 3.1	12.4 ± 2.1	13.0 ± 2.9	10-35	10-35
CHO (g/d)	352.5 ± 73.9	352.8 ± 59.6	336.2 ± 120.4	292.9 ± 109.5	130	130
CHO (% energy)	66.6 ± 12.4	62.2 ± 8.8	68.9 ± 9.3	60.7 ± 9.2[a]	45-65	45-65
Fat (g/d)	57.2 ± 35.7	70.9 ± 31.8	52.1 ± 30.9	59.9 ± 23.27	N/A	N/A
Fat (% energy)	22.4 ± 10.5	26.9 ± 7.9	22.2 ± 8.2	26.7 ± 7.0	20-35	20-35
Fiber (g/d)	44.4 ± 15.6	42.1 ± 11.1	40.0 ± 15.5	33.3 ± 9.9	25	38

[*] Mean ± SD. CHO, carbohydrate.
[a] Significantly different from baseline (repeated measures t-test):, [a] P = 0.008.
[**] No significant (NS) differences were found between the groups at either baseline or during the supplement period (two-sample t-test).
[***] DRIs, Dietary Reference Intakes (44). Energy provided as Estimated Energy Requirements (EER) for low active, 65-inch height, 68.0 kg weight, 24.99 BMI men and women 30 years of age. Total protein and carbohydrate are provided as Recommended Dietary Allowance (RDA) for males and females aged 31-50 y. Protein, carbohydrate and fat as percent of energy are provided as Acceptable Macronutrient Distribution Ranges (AMDRs) for adults. Fiber is provided as Adequate Intakes (AIs) for males and females aged 31-50 y. N/A = not available as neither AI or RDA have been set for total fat.

Fatty acids

The changes in fatty acid intake of the groups from baseline to during the supplement period are reported in **Table 3**. There were no significant differences in LA and ALA intake between the groups at baseline (by MANOVA). The flax and hemp oil supplements provided an additional 2.2 g/d LA and 7.1 g/d ALA, and 7.6 g/d LA and 2.2 g/d ALA, respectively. Significant increases in LA ($P = 0.004$), n-6 ($P = 0.002$) and PUFA ($P = 0.001$) were observed in the hemp group. The intake of ALA increased significantly ($P < 0.0001$ for both) from 2.1 to 8.8 g/d in the flax group and 1.6 to 3.9 g/d in the hemp group. There was also a significant increase ($P < 0.0001$ for both) in the n-3 intake of both groups.

The increased intake of ALA in the groups resulted in a significant decrease in the ratio of LA:ALA from 8.6 to 2.2 in the flax group ($P = 0.001$) and 9.4 to 5.6 in the hemp group ($P = 0.0003$). Likewise, the ratio of total n-6:n-3 intake decreased significantly in the flax group ($P = 0.001$) and the hemp group ($P = 0.0004$).

Significant differences in GLA, ALA, and total n-3 intake (all $P < 0.0001$) were found between the groups during the supplement period. There were also significant differences in the intake ratios of n-6:n-3 and LA:ALA (both $P < 0.0001$) calculated between the groups during the supplement period.

The mean intakes of EPA, DHA, and AA were all zero for both groups at baseline and during the supplement period (these only occur in animal foods). In addition, GLA, which is found only in a limited number of foods and supplements (e.g. hemp oil, borage oil, black currant oil, evening primrose oil), was absent from the diet of both groups at

baseline and during the supplement period. However, the mean intake of GLA (via the hemp seed oil) in the hemp group was approximately 0.64 g/d during the supplement period

TABLE 3.
Comparison of dietary fatty acid intake and ratios of subjects in both groups at baseline and during the supplement period[*, **]

	Flax oil (n = 10)		Hemp oil (n = 12)		DRIs	
	Baseline	**During**	**Baseline**	**During**	**Male**	**Female**
SFA (g/d)	11.0 ± 8.2	11.6 ± 6.4	9.8 ± 5.7	10.1 ± 6.5	N/A	N/A
MUFA (g/d)	18.1 ± 13.4	22.9 ± 14.9	17.0 ± 10.7	15.4 ± 6.8	N/A	N/A
PUFA (g/d)	19.1 ± 12.6	27.8 ± 9.2	15.3 ± 10.2	25.9 ± 6.7[a]	N/A	N/A
n-6 (g/d)	17.0 ± 11.5	19.1 ± 9.1	13.7 ± 8.9	22.1 ± 5.9[b]	14.3-28.5	12.2-24.5
n-3 (g/d)	2.1 ± 1.3	8.8 ± 0.9[c, l]	1.6 ± 1.4	3.9 ± 1.1[c]	1.7-3.7	1.5-2.9
n-6 : n-3	8.6 ± 3.9	2.2 ± 1.1[d, l]	9.4 ± 2.2	5.8 ± 1.3[e]	N/A	N/A
LA (g/d)	17.0 ± 11.5	19.1 ± 9.1	13.7 ± 8.9	21.4 ± 5.9[f]	17	12
ALA (g/d)	2.1 ± 1.3	8.8 ± 0.9[g, l]	1.6 ± 1.4	3.9 ± 1.1[g]	1.6	1.1
LA : ALA	8.6 ± 3.9	2.2 ± 1.1[h, l]	9.4 ± 2.2	5.6 ± 1.3[i]	N/A	N/A

[*] Mean ± SD. SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; LA, linolenic acid; ALA, alpha-linolenic acid.

[a-k] Significantly different from baseline (repeated measures t-test): [a] $P = 0.001$, [b] $P = 0.002$, [c] $P < 0.0001$, [d] $P = 0.001$, [e] $P = 0.0004$, [f] $P = 0.004$, [g] $P < 0.0001$, [h] $P = 0.001$, [i] $P = 0.0003$.

[l] Significantly different from corresponding value in hemp oil group (two-sample t-test), $P < 0.0001$.

[**] DRIs, Dietary Reference Intakes (44). N/A = not available as neither AI nor RDA have been set for SFA, MUFA, PUFA, n-6 : n-3, and LA : ALA. n-6 and n-3 are provided as Acceptable Macronutrient Distribution Ranges (AMDRs) based on Estimated Energy Requirements (EER) for low active, 65-inch height, 68.0 kg weight, 24.99 BMI men and women 30 years of age. LA and ALA are provided as AI.

Micronutrients

Values for selected micronutrient intake (based on relationship to vegetarian diets and/or fatty acid metabolism) are shown in **Table 4**. No significant differences in micronutrient intake were found between the groups at either baseline or during the supplement period. In addition, micronutrient intake did not change significantly in either group from baseline to during the supplement period.

TABLE 4.
Comparison of selected micronutrient intake of subjects in both groups at baseline and during the supplement period [*], [**], [***]

	Flax oil (n = 10)		Hemp oil (n = 12)		DRIs	
	Baseline	**During**	**Baseline**	**During**	**Male**	**Female**
Vitamin B12 (μg/d)	12.2 ± 26.2	11.4 ± 20.1	293.8 ± 643.9	11.8 ± 21.1	2.4	2.4
Vitamin E (mg/d)[1]	68.0 ± 178.0	62.3 ± 162.9	124.1 ± 260.2	66.1 ± 138.6	15	15
Calcium (mg/d)	891.1 ± 554.9	841.7 ± 363.8	1007.5 ± 630.5	769.1 ± 360.6	1000	1000
Iron (mg/d)	23.1 ± 11.8	21.5 ± 5.5	24.9 ± 20.1	19.8 ± 8.4	8	18
Sodium (mg/d)	3234.1 ± 1638.4	3058.8 ± 1219.7	3219.9 ± 1663.6	3282.9 ± 1730.8	N/A	N/A
Zinc (mg/d)	10.1 ± 8.5	11.3 ± 9.0	10.3 ± 4.5	8.4 ± 3.9	11	8

[*] Mean ± SD.
[**] No significant (NS) differences were found within either group or between groups for these micronutrients at any point.
[1] As alpha-tocopherol equivalents.
[***] DRIs, Dietary Reference Intakes (124-127). Vitamins B12 and E, iron, zinc are provided as RDAs. Calcium is provided as an AI. N/A = not available

Erythrocyte total phospholipid fatty acids

Erythrocyte total phospholipid fatty acid concentrations at baseline and at post-supplement are shown in **Table 5**. At baseline, there were no significant differences in EPA or DHA combined between the groups (by MANOVA). In addition, no significant differences in any individual erythrocyte fatty acids existed between the groups at baseline (by two-sample t-test).

EPA and DHA concentrations did not increase significantly in either group. There were no significant changes observed in LA, GLA, ALA, DGLA, or AA in either group. Total n-3 fatty acids increased significantly ($P = 0.009$) in the flax group. The resulting n-6 : n-3 ratio did not change significantly in either group. A significant difference in ALA ($P = 0.001$) was found between the groups at post-supplement.

TABLE 5.

Comparison of the erythrocyte total phospholipid fatty acid concentrations (expressed as percentage of the total fatty acids) in both groups at baseline and post-supplement*

	Flax oil (n = 10)		Hemp oil (n = 12)	
	Baseline	**Post-supplement**	**Baseline**	**Post-supplement**
16:0	31.3 ± 4.3	30.2 ± 3.8	30.7 ± 3.1	30.7 ± 2.5
18:0	12.8 ± 5.2	14.5 ± 3.1	13.8 ± 3.5	13.3 ± 1.9
18:1n-9	14.9 ± 1.4	14.4 ± 1.2	15.6 ± 2.2	14.4 ± 1.3
18:2n-6 (LA)	12.6 ± 1.7	12.2 ± 1.3	11.6 ± 1.0	11.6 ± 0.9
18:3n-6 (GLA)	0.2 ± 0.2	0.2 ± 0.3	0.2 ± 0.3	0.1 ± 0.1
18:3n-3 (ALA)	0.3 ± 0.1	0.4 ± 0.2[1]	0.2 ± 0.1	0.2 ± 0.1
20:3n-6 (DGLA)	1.7 ± 0.5	1.6 ± 0.4	1.4 ± 0.4	1.3 ± 0.5
20:4n-6 (AA)	12.1 ± 1.5	12.1 ± 1.3	11.6 ± 2.0	12.6 ± 1.7
20:5n-3 (EPA)	0.2 ± 0.1	0.4 ± 0.2	0.3 ± 0.2	0.3 ± 0.1
22:4n-6	3.1 ± 0.4	2.9 ± 0.6	3.2 ± 0.8	3.5 ± 0.6
22:5n-6	0.3 ± 0.2	0.3 ± 0.2	0.3 ± 0.2	0.3 ± 0.2
22:5n-3	2.5 ± 0.5	2.8 ± 0.7	2.8 ± 0.4	3.0 ± 0.7
22:6n-3 (DHA)	1.5 ± 0.7	1.6 ± 0.6	1.6 ± 0.6	1.7 ± 0.5
Total n-3	4.5 ± 0.7	5.2 ± 1.1[a]	4.8 ± 0.7	5.3 ± 0.9
Total n-6	30.3 ± 2.6	29.6 ± 2.3	28.5 ± 3.5	30.1 ± 2.5
n-6 : n-3	7.0 ± 1.3	6.0 ± 1.4	6.0 ± 1.1	5.9 ± 1.2

* Mean ± SD. LA, linoleic acid; GLA, gamma-linolenic acid; ALA, alpha-linolenic acid; DGLA, dihomo-gamma-linolenic acid; AA, arachidonic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid.

[a] Significantly different from baseline (repeated measures t-test): [a] P = 0.009.

[1] Significantly different from corresponding value in the hemp group (two-sample t-test): [1] P = 0.001

Multiple regression model

The variability in post-supplement erythrocyte EPA concentration was not significantly (P = 0.055) predicted by baseline erythrocyte EPA, LA and ALA intake during the supplement period, or the group.

The variability in post-supplement erythrocyte DHA concentration was significantly predicted by using baseline erythrocyte DHA concentration, LA and ALA intake during the supplement period, and the group (P = 0.002). The baseline erythrocyte DHA concentration was found to be the only statistically significant predictor of post-supplement DHA concentration (P = 0.0001).

Similarly, the variability in post-supplement erythrocyte total n-3 fatty acids was found to be significantly predicted only by baseline erythrocyte total n-3 fatty acid concentration (P = 0.002). ALA intake during the supplement period and the group had no statistically significant effects on post-supplement total n-3 concentrations.

The variability in post-supplement erythrocyte DGLA was not found to be predicted by using baseline erythrocyte DGLA, LA intake during the supplement period, or the group (P = 0.056).

However, the variability in post-supplement erythrocyte AA was predicted by using baseline erythrocyte AA, and LA intake during the supplement period (P = 0.027). Of these predictors, only baseline AA concentration contributed significantly to the prediction of post-supplement AA concentration (P = 0.009).

In summary, the statistical findings from multiple regression seem to be consistent in indicating that flax and hemp seed oils make no difference on the post-supplement erythrocyte concentrations of EPA, DHA, total n-3, DGLA, or AA.

Plasma lipids

Plasma lipid concentrations for both groups at baseline and post-supplement are shown in **Table 6**. There were no significant differences found in any plasma lipids or the ratios derived from these concentrations between the groups at baseline or post-supplement. The mean total cholesterol was 150.3 mg/dL in the flax group and 165.2 mg/dL in the hemp group at baseline. Total cholesterol and LDL remained unchanged in both groups from baseline to post-supplement. However, there were significant increases in HDL from 50.1 to 55.4 mg/dL in the flax group ($P = 0.004$) and 54.0 to 58.9 mg/dL in the hemp group ($P = 0.0004$). There was also a significant decrease in the ratio of total cholesterol : HDL in the hemp group only ($P = 0.006$).

TABLE 6.
Comparison of plasma lipids in both groups at baseline and post-supplement [*, **]

	Flax oil (n = 10)		Hemp oil (n = 12)	
	Baseline	**Post-supplement**	**Baseline**	**Post-supplement**
Total cholesterol (mg/dL)	150.3 ± 31.1	151.4 ± 31.1	165.2 ± 42.7	164.5 ± 32.6
LDL (mg/dL)	77.2 ± 19.9	79.6 ± 25.8	87.3 ± 32.7	85.8 ± 26.5
HDL (mg/dL)	50.1 ± 6.3	55.4 ± 6.6[a]	54.0 ± 20.0	58.9 ± 20.8[b]
Triacylglycerols (mg/dL)	114.2 ± 113.0	82.3 ± 36.0	119.3 ± 81.2	99.3 ± 64.9
Total cholesterol : HDL	3.1 ± 1.0	2.8 ± 0.6	3.3 ± 1.0	3.0 ± 0.9[c]
LDL : HDL	1.6 ± 0.5	1.5 ± 0.5	1.7 ± 0.7	1.6 ± 0.7

[*] Mean ± SD.
[a-c] Significantly different from baseline (repeated measures t-test): [a] P = 0.004, [b] P = 0.0004, [c] P = 0.006.
[**] NS differences were found between groups at baseline or post-supplement (two-sample t-test).

Multiple regression model

Through multiple regression it was found that the variability in post-supplement concentrations of total cholesterol, LDL, HDL, and the ratios of LDL : HDL and total cholesterol : HDL were significantly predicted only by their respective baseline concentrations, SFA and *trans* fatty acid intake during the treatment period, and the group (all $P < 0.0001$). Similarly, the variability in post-supplement triacylglycerol concentration was predicted only by baseline triacylglycerol concentration ($P = 0.0009$).

In summary, the statistical findings from multiple regression seem to be consistent in indicating that flax and hemp seed oils make no difference on the post-supplement plasma lipid concentrations of total cholesterol, LDL, HDL, triacylglycerol, or the ratios of LDL : HDL and total cholesterol : HDL.

CHAPTER 5. DISCUSSION

The primary goal of this study was to determine the effects of supplemental ALA from flax and hemp seed oils on erythrocyte EPA and DHA concentrations in a group of vegetarians. Additional goals were to determine the effects of these oils on plasma lipid concentrations, to determine the effects of GLA from hemp seed oil on erythrocyte DGLA and to characterize current vegetarian eating patterns in a Hawaii.

Effects of flax and hemp seed oils on erythrocyte EPA and DHA

The addition of flax and hemp seed oils containing 7.1 g/d and 2.2 g/d of ALA, respectively to the diets of vegetarians for four weeks did not significantly increase the erythrocyte concentrations of EPA or DHA. The results of the present study concur with the work of others that failed to observe increases in plasma and platelet DHA in vegetarians (26, 27) and in omnivores (12, 13, 28-31) as a result of supplementing the diet with ALA from flax oil. However, these studies did report significant increases in plasma and/or platelet EPA, which is in contrast with our findings. Furthermore, in a study by Sanders and Younger, the addition of 6.5g/d of ALA from flax oil to the diets of vegans led to a 3-fold increase in plasma phospholipid EPA after only two weeks (27). Li, *et al.*, found an intake of 15.4 g/d of ALA from flax oil and a flax oil-based margarine resulted in a 2.5-fold increase in platelet phospholipid EPA and a greater than 4-fold increase in plasma phospholipid EPA after four weeks in healthy vegetarian men (26).

The concentration of erythrocyte EPA nearly doubled in healthy adult omnivore women after two weeks of supplementation with 10.7 g/d of ALA from flax oil (29). In a

study of healthy adult omnivores, Freese, *et al.,* (28) found that flax oil capsules providing a mean of 5.9 g/d of ALA led to a 17% increase in platelet EPA concentrations after twelve weeks. Allman, *et al.,* (13) observed a more than 2-fold increase in platelet EPA concentration after adding approximately 22 g/d of ALA from flax oil to the diet of healthy omnivores for 23 days. Mantzioris, *et al.,* (30) also reported a more than 2-fold increase in plasma phospholipid EPA after the addition of 13.0-13.7 g/d of ALA from flax oil and a flax oil spread to the diet of healthy male omnivores for four weeks. After providing approximately 13.4 g/d ALA for 18 days from a flax-based oil mixture, Chan, *et al.,* (12) observed a significant increase in plasma and platelet phospholipid EPA concentrations in healthy omnivores. In another study, Cunnane, *et al.,* (31) supplemented the diet of healthy females with 12 g/d ALA via flax oil capsules and found significant increases in triacylglycerol EPA concentrations after four weeks. In contrast, supplements of algae-derived DHA typically lead to predictably large increases in both DHA and EPA concentrations in both vegetarians and omnivores (56, 57). The lack in rise of EPA and DHA concentration after ALA supplementation in the present study is striking, especially since it was carried out in conditions designed to increase synthesis of long chain n-3 PUFAs (i.e. high intake of ALA, low ratio of LA : ALA).

There are several possible reasons why EPA and DHA levels did not increase in this study. One possible reason is that human biosynthesis of EPA and DHA from ALA is known to be limited in healthy adults (3, 23, 24). Emken and others estimated by use of deuterium-labeled ALA that an average of only 15% of dietary ALA is converted to long chain n-3 PUFAs when LA intake is held constant (23). Based on their findings, it

would not have been possible to see a significant increase in EPA or DHA in our study. More recently, Pawlosky, *et al.,* quantified the biosynthesis of DHA from plasma ALA using isotope tracer methodology (24). They determined that only about 0.2% of plasma ALA was available for synthesis of EPA, and that about 63% of the plasma EPA was available for production of docosapentaenoic acid (DPA; 22:5n-3), an intermediate in the production of DHA, and that only 37% of DPA was accessible for the production of DHA. In our study, baseline concentrations of erythrocyte ALA were 0.3% and 0.2% for the flax and hemp groups, respectively. Based on the work of Pawlosky, *et al.*, it appears evident that a significant increase in DHA was indeed possible but did not occur.

A second likely reason for a lack of increase in EPA or DHA is that most ALA may have been oxidized instead of being converted to its longer chain metabolites. This effect has been observed in both animals (128) and humans (24, 29, 129) and Sinclair, *et al.,* propose that β-oxidation is the major metabolic route of ALA (130). To avoid β-oxidation of ALA, Allman, *et al.,* (13) advised their subjects to take the flax oil with meals and still did not observe an increase in DHA concentrations. Although we advised our subjects to take their oil with food we were not able to measure or enforce compliance with this guideline. Our results combined with the work of others suggest that ALA may be preferentially oxidized rather than converted to EPA or DHA.

A third possible reason for a lack of increase in EPA or DHA is enzyme competition and inhibition. The Δ6-desaturase enzyme is required for two separate steps in the bioconversion of ALA to DHA (**Figure 2**). The first step requiring this enzyme is conversion of ALA to 18:4n-3, which is known to be the rate-limiting step in the

synthesis of EPA from ALA (22). The second step requiring Δ6-desaturase is conversion of 24:5n-3 to 24:6n-3, which is thought to be the rate-limiting step in the synthesis of DHA from EPA (131). Δ6-desaturase is also responsible for catalyzing the corresponding reactions in the n-6 pathway. This creates competition for this enzyme and a high intake of LA is known to inhibit the conversion of ALA to EPA (22, 23, 42, 47-50). During the supplementation period, the mean LA intake of subjects increased. Although dietary LA : ALA decreased, competition for Δ6-desaturase may explain why we did not observe an increase in EPA. In addition, a high intake of ALA has been shown to inhibit the second Δ6-desaturase reaction in humans (29) and rats (51). The intake of ALA in our subjects was high during supplementation so this may partially explain why we did not detect an increase in DHA.

A fourth possible reason for not observing an increase in EPA and DHA is that EPA may have been used to produce its respective eicosanoids (43). This would mean that even if EPA concentration increased significantly at some point between the baseline and post-supplement, the additional EPA was used for eicosanoid production, resulting in no significant change between the two time points we measured. Although this explanation is plausible, since we did not analyze blood samples at any point in-between baseline and the end of the study, we have no way of knowing for sure if this would help explain our results.

A fifth possible reason for not observing an increase in EPA and DHA is that other tissues may buffer changes in DHA concentrations. Sanders and Younger suggested that the high levels of LA found in adipose tissue of vegetarians may act as a

buffer system, which prevents any rise in DHA over a short duration (27). Pawlosky, *et al.,* also postulated from their results that liver or adipose tissues might act as reservoirs that contribute to the maintenance of plasma DHA concentrations (24). Twelve weeks is the longest duration study that reported increases in EPA concentrations without a rise in DHA (28). If the buffering theory is correct, it may take longer than twelve weeks to see significant increases in DHA concentrations as a result of ALA supplementation. Hence, an increase in DHA as a result of four-week trial would not be expected.

Another possibility for not seeing a significant rise in EPA and DHA in this study is the small sample size. Our small sample necessitated a larger effect size in order to detect significant differences. However, Sanders and Younger reported a significant increase in EPA in a sample of only four vegans, so sample size does not fully explain our results (27).

Additionally, it is possible that type II error was the cause for not detecting a significant increase in EPA in the flax group. The concentration of EPA doubled in the flax group from baseline to post-supplement, which was significant at $P = 0.021$ prior to applying the Bonferroni correction. However, we used an adjusted P value of 0.0125 under the Bonferroni method, which rendered the increase in EPA non-significant at $P = 0.05$. Although the Bonferroni correction is frequently used in biomedical research to control for type I error, its use is not universally accepted (132). The Bonferroni correction, by its very nature of reducing type I error, introduces the possibility of too much type II error. Under this argument, it is possible that there was indeed a truly significant increase in EPA in the flax group in our study despite the fact we are reporting

it as non-significant. This line of thought is further strengthened by the fact that we did detect an increase in erythrocyte total n-3 in the flax group, which suggests a trend toward an increased EPA.

From multiple regression analysis, it appears that the lack in rise of DHA concentration was largely predicted by baseline DHA concentrations. In other words, baseline DHA concentrations of the subjects in this study were likely to remain unchanged. This may be explained by individual variation of the subjects, the quantity of ALA supplemented, or the short duration of the study. However, the baseline concentrations of DHA in our study were similar to those previously observed in vegans (49), which tend to be much lower than concentrations observed in omnivores (29, 41, 133). This indicates that our subjects may have indeed been susceptible to increases in DHA.

Numerous additional hypotheses have been proposed to explain the inefficiency of the conversion of ALA to EPA and DHA. Some evidence suggests that trans fatty acids (TFAs) may impair the conversion of ALA to LCPUFA (134). We estimated the intake of TFAs to be 0.44 g/d and 0.87 g/d in the flax and hemp groups, respectively during the supplement phase of our study, which is considered quite low compared to the estimated average intake of 5.3 g/d in the United States (135). Although the TFA intake of our subjects is probably underestimated due to nutrient database limitations, we do not suspect that TFA had much influence on our results. Vitamin B-6 deficiency has also been suspected of impairing the conversion of ALA to EPA and DHA (136). This does not provide much explanation for our results since the mean intake of vitamin B-6 was

5.4 mg/d and 9.2 mg/d in the flax and hemp groups, respectively, both of which are greater than the Recommended Dietary Allowance of 1.3 mg/d for men and women aged 31-50 y (125).

More reasons have been proposed that may help explain the inefficient conversion of ALA to its longer chain metabolites. Some recent evidence suggests that young women may convert ALA to EPA and DHA more efficiently than young men (137, 138). In addition, impaired bioconversion of ALA to DHA has been suspected in elderly populations (139). However, the mean age in our study was neither young nor elderly so this offers little explanation for our results. After reporting an inverse correlation between ALA intake and plasma phospholipid and platelet phospholipid DHA concentrations, Mantzioris, *et al.,* (113) concluded that the subsequent rise in EPA concentration from ALA supplementation might displace DHA from phospholipids. Simply stated, as EPA concentration increases DHA concentration decreases. This does not explain our results since we did not detect an increase in EPA or decrease in DHA. Infante and Huszagh theorized that deficiency of mitochondrial carnitine, which acts as a coenzyme in the synthesis of DHA, might play a role in impairing ALA conversion (140). However, since we did not measure plasma carnitine concentrations, we have no way of knowing how this may or may not have contributed to our results.

For hemp seed oil in particular, no studies have been published to date on its potential effects on erythrocyte fatty acid concentrations, making the present study unique in that respect. Due to the paucity of hemp research combined with the fact that hemp oil is a good source of ALA, the explanations provided above for a lack in rise of EPA or

DHA from flax oil should also be considered as explanations for hemp oil results. Despite the high percentage of ALA in hemp seed oil, it also contains a high percentage of LA, so in theory a large increase in EPA and DHA would not be expected. However, supplementation with hemp seed oil significantly increased the ALA intake and decreased the LA : ALA ratio in our study, so it seemed plausible to effect a significant increase in EPA or DHA. In addition, Brouwer, *et al.,* (141) hypothesized that, since GLA is thought to activate Δ6-desaturase, perhaps increasing ALA intake along with GLA may improve the production of EPA and DHA. They, too, failed to observe increases in EPA and DHA concentrations after increasing ALA and GLA intake for four weeks. Hence, our results concur with theirs.

Taken together, the many reasons summarized here may have contributed in part to the lack in rise in erythrocyte EPA and DHA concentrations observed in this study. These explanations highlight the many challenges in the biosynthesis of EPA and DHA from ALA and make it abundantly clear that several details about this pathway yet remain to be elucidated.

Effects of hemp seed oil on erythrocyte DGLA

The addition of hemp seed oil, which provided approximately 0.64 g/d of GLA for four weeks to the diets of vegetarians, did not significantly increase the erythrocyte concentrations of DGLA. Again, due to the novelty of this study using hemp seed oil, no other data exist for direct comparison relative to its potential effects on DGLA.

Consequently, we must rely on research published on the effects of supplementing GLA from other sources for comparison.

The results of the present study are in contrast with the work of others as supplementation with GLA from evening primrose oil (EPO) or borage oil typically results in significant increases in DGLA concentrations (89, 142-145). In fact, a dose of GLA from EPO as low as 0.18 g/d resulted in an increase of DGLA in hemodialysis patients (145). Therefore, it is somewhat surprising that we did not observe an increase in DGLA from hemp oil in this study. The failure to see a rise in DGLA in this study may be due to the small sample size, as previously discussed. Conversely, there may be other explanations for our results.

One possible reason is that the healthy subjects in our study were not susceptible to increases in DGLA. Two of the above studies (143, 145) that showed increases in DGLA were carried out in subjects with inflammatory disorders, which are known to be influenced by dietary fatty acids (89). Interaction with medication may somewhat explain why other studies have observed increases in DGLA. Belch and Hill (90) noted that patients involved in inflammatory trials are often simultaneously taking medications to control their inflammatory conditions. Such medications are known to alter eicosanoid and fatty acid metabolism, thus making direct comparison to the present study difficult, as our subjects did not report taking such medications. However, this does not fully explain our contrasting results since Horrobin, *et al.,* (142) did observe an increase in DGLA in healthy subjects. Our short study duration does not provide much explanation for our contrasting findings since increases in DGLA from evening primrose oil have been

achieved after only ten days of supplementation (142). Alternatively, Fan and Chapkin have hypothesized that the stereochemistry of GLA to its respective triacylglycerol may be important in determining its bioavailability, not just the absolute quantity of GLA (89). If their hypothesis is correct, it is possible that the GLA in hemp seed oil is not as bioavailable as it is from other sources such as borage or evening primrose oils. Collectively, these explanations may help to clarify why no increase in DGLA concentrations resulted from hemp oil supplementation in the present study. More research is needed to characterize the effects of hemp seed oil on fatty acid status.

Effects of flax and hemp seed oils on plasma lipids

The addition of flax and hemp seed oils to the diet of vegetarians resulted in significant increases in HDL concentrations but did not significantly alter the concentrations of any other plasma lipids or their resulting ratios. These results are in contrast with flax oil studies in vegetarians (26) and omnivores (28, 30, 146) that did not show any changes in HDL but in agreement with their findings of other plasma lipids. Our results are in agreement with several other studies that have reported low total cholesterol (49, 50, 104, 109, 111, 147-151), LDL cholesterol (50, 104, 111, 147-149) and triacylglycerol concentrations (49, 147, 148, 150) in vegetarians.

Through multiple regression analysis, it appears evident that baseline HDL concentrations predicted post-supplement HDL concentrations. However, since the mean HDL concentrations in both groups at baseline were not considered either low (<40 mg/dL) or high (≥60 mg/dL) based upon current guidelines put forth in the Third Report

of the National Cholesterol Education Program's (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (152), it seems plausible that they may have been more susceptible to the increases in HDL we observed. Despite this, additional explanations may provide insight to our findings. It is possible that HDL concentrations may have increased due to increases in exercise by some participants, as regular physical activity is known to increase HDL (152). Although we did not measure physical activity in this study, on the exit interview questionnaire 14% of the participants reported major increases in their level of exercise during the study period while the remaining 86% reported no changes in their level of activity.

A number of explanations may help explain why changes in other plasma lipids did not occur in this study. Both groups had desirable total cholesterol (<200 mg/dL), optimal LDL cholesterol (<100 mg/dL) and normal triacylglycerol (<150 mg/dL) concentrations at baseline, based on current guidelines (152). Thus, significant decreases in plasma lipids in these normolipidemic subjects would not be expected (30, 146). In addition, SFA intake, which is known to alter plasma cholesterol, was low in both groups (less than 5% of energy), and remained unchanged. Lastly, reduction in body weight, which is also known to reduce cholesterol levels (152), did not occur in either group.

In contrast, Cunnane, *et al.,* (31) observed significant reductions in total and LDL cholesterol concentrations in healthy subjects after four weeks of consumption of 50 g of flax seed meal, which provided approximately 12 g/d ALA. They hypothesized that these reductions were due to components in flax other than ALA. Additional studies conducted with partially defatted flax (very low in ALA content) have since supported their

hypothesis (153, 154). Our lack in change of cholesterol concentrations after ALA supplementation appears to support their findings. This is quite interesting since conflicting reports on the relationship of ALA to coronary artery disease have recently been published (17, 18). It seems that these large studies with conflicting results may benefit from qualifying the sources of ALA instead of focusing on the absolute quantity eaten by the participants.

For hemp oil in particular, as previously mentioned, we must rely on studies of GLA for comparison. EPO supplementation has resulted in an increased HDL concentration in rabbits (155). However, the effects of GLA supplementation on plasma lipids in humans are equivocal. Viikari and Lehtonen observed no changes in total or HDL cholesterol concentrations or triacylglycerol concentrations in hyperlipidemic subjects as a result of EPO supplementation (156). Chaintreuil, *et al.,* detected decreases in both total cholesterol and triacylglycerol concentrations in insulin-dependent diabetic patients from a GLA supplement of 2 g/d for six weeks (157). In contrast with our results, Ishikawa, *et al.,* (158) observed a significant decrease in low-density lipoprotein (LDL) cholesterol in hyperlipidemic subjects after eight weeks of an EPO supplement. In agreement with our results, Guivernau, *et al.,* (159) found an increase in HDL cholesterol in hyperlipidemic subjects with a GLA dose of 3g/d. Based on these observations, it is possible that HDL concentrations increased in the hemp group as a result of GLA intake.

Dietary intake of vegetarians

Essential fatty acids

In this study, supplementation with flax and hemp seed oils led to increases in the intake of ALA and n-3 fatty acids in both groups and increases of PUFA, n-6, and LA in the hemp group only. The estimated intake of LA in this study was similar to one other vegetarian study (41) but lower than that reported in other vegetarian studies (26, 27, 50, 104, 107). The intake of ALA in this study was similar to what has been reported in other vegetarian studies (26, 27, 41, 50, 104). In addition, the vegetarian diets characterized in this study appear to be adequate sources of both LA and ALA according to Dietary Reference Intakes (DRIs) values for these nutrients. Our results are limited by the fact that essential fatty acid data for processed foods are lacking in most nutrient databases. However, we did take precautions to reduce this limitation, which is shared by other researchers intending to quantify intake of fatty acids.

Ratios of LA : ALA

In this study, supplementation with flax and hemp seed oils led to decreases in the ratios of n-6 : n-3 and LA : ALA in both groups. Our estimates of the ratio of LA : ALA in this study are similar to a range of 5-8.5 reported in two vegetarian studies (26, 41) but much lower than a range of 16-28 reported in several other vegetarian studies (27, 50, 104-106). Lower ratios of LA : ALA would be expected in our study since LA intakes were lower while ALA intakes were similar to the above studies. The ratios of LA : ALA in both groups at baseline were within the recommended range of 5-10 set forth by the

WHO and FAO (61) but lower after supplementation in the flax group. This indicates that adequate ratios of LA : ALA can be achieved by vegetarian diets and can further be reduced via supplementation with both flax and hemp seed oils. Although most researchers agree that an ideal ratio for LA : ALA exists, it seems clear that few results support an agreeable number for that ratio and more work must done to elucidate it. Furthermore, it seems likely that more than one ideal ratio may exist, depending on the desired outcome.

Eating patterns

The vegetarian diets of the participants in this study contained a varied combination of whole foods, processed foods and restaurant-prepared foods. The level of detail we have provided here to describe the foods eaten by our participants is not common among other vegetarian studies. In contrast, vegetarian diets are more commonly described by foods that are excluded or only by the whole food groups that comprise them (26, 27, 41, 104, 160-163). The former description (excluded foods) tells very little about what actually comprises the diet and the latter (food groups) could lead to the conclusion that vegetarians do not eat any processed, packaged or refined foods whatsoever. Although this may be true in some vegetarian populations, our observations indicate the possibility that the authors failed to provide enough detail about the vegetarian diets recorded in their publications. This practice should be discouraged in order to avoid creating false assumptions and misunderstandings of vegetarian diets.

Furthermore, describing vegetarian diets with more specificity may provide additional insight when interpreting findings in other biological tests influenced by diet.

Macronutrients

Although the hemp group experienced a significant decrease in carbohydrates as a percentage of energy, supplementation with flax and hemp seed oils did not appreciably alter other macronutrient intake in this study. The macronutrient intake of the participants in this study is similar to intake reported in several other vegetarian studies (26, 50, 109, 147, 148, 160, 163, 164). Interestingly, although a variety of eating patters occur under the broad label of vegetarian, in terms of macronutrient intake they appear to be quite homogeneous. These results also indicate that a vegetarian diet can provide an adequate macronutrient intake based on current recommendations (44).

Micronutrients

Supplementation with flax and hemp seed oils did not significantly change the intake of any of the micronutrients reported in this study. The estimated intake of vitamins B12 and E were higher in our study compared to other studies in vegetarians (107, 147, 148, 160, 163). This is probably a result of supplementation by our participants as well as the fact that many prepackaged vegetarian foods are now fortified with these nutrients. The calcium intake of our participants was similar to previous observations in vegetarian studies (26, 147, 160, 163) but higher than others (107, 148). The higher values we observed are again most likely a result of supplementation and

additionally because most of the cow milk substitutes consumed by our participants were calcium fortified. The iron intake of our participants was also similar to what has been reported in other vegetarian studies (148, 160) but higher than that reported in others (26, 107, 147, 163). The higher intake of iron we observed was again most likely due to supplementation. We reported higher sodium intakes than other vegetarian studies (26, 148, 163). Our observations could be explained by the processed and restaurant food consumed by our subjects. The zinc intake of our subjects was similar to what has been observed in other vegetarian studies (26, 107, 147, 148, 160, 163). Collectively, these results seem to indicate that micronutrient intake varies more widely than does macronutrient intake as a result of differing vegetarian eating patterns. This emphasizes the importance of providing more detail when describing vegetarian diets in research. These results also indicate that a vegetarian diet can provide an adequate intake of these micronutrients based on current recommendations (124-127).

Conclusion

This study has additional limitations beyond those already cited. Food records were utilized to estimate dietary intake, which are known to have several limitations such as under or overestimation of intake, reflection of atypical intake, and reflecting only current intake (165). However, we did provide detailed instruction and adequate demonstration to the subjects in order to reduce these limitations. In addition, errors in food composition databases may have contributed to some of the variation we observed in

dietary intake. Lastly, the study population was extremely varied in terms of age, gender and ethnicity, which further complicates the interpretation of our results.

Regardless of these limitations, the results of this study indicate that 4 weeks of supplementation with flax and hemp seed oils does not appear to be an effective means of increasing erythrocyte EPA or DHA concentrations in healthy vegetarians. Additionally, hemp oil does not seem to influence erythrocyte DGLA concentrations. The results also suggest that flax and hemp oils have similar effects on plasma lipids and may help increase HDL concentrations. These results also imply that a modern, U.S. vegetarian diet can contain a wide variety of foods and as a result can supply adequate quantities of macronutrients, fatty acids and select micronutrients. While this research has resulted in a number of interesting findings, it also brings up several questions that provide opportunity for further investigation. First, why is the bioconversion of ALA to EPA and DHA so inefficient? Although several hypotheses exist to explain this inefficiency, the details of what influences this pathway are still largely unknown. Second, what are the health implications of relying on dietary ALA as the only source of n-3 fatty acids? Third, should vegetarians be advised include sources of preformed DHA in their diets? Lastly, do flax and hemp seed oils really increase HDL concentrations, and if so, what component or components of these oils are responsible for the mechanism? The answers to these questions will add to our knowledge of fatty acid metabolism and increase our understanding of the implications of lipid intake on human health and the prevention and treatment of disease.

APPENDIX A: VEGETARIAN DIET OVERVIEW

Vegetarians choose to eat minimal amounts of animal products. EPA and DHA, are absent from plant foods, and thus, are largely absent from a vegetarian diet. Because of this, vegetarians represent a unique population in which to study the metabolism and status of essential fatty acids and their longer chain polyunsaturated derivatives, which play important physiological roles. The information presented below defines vegetarian diets and provides perspective on using vegetarians as a study population.

Vegetarian diets explained

People may eat a vegetarian diet for the following reasons: religious, ethical, environmental, health, or economic (163). For example, the Seventh Day Adventist church strongly recommends following a lactoovovegetarian diet (166). Some people adhere to a vegetarian eating pattern because they believe that raising animals for food is unethical. Certainly other motives exist to explain why persons may follow a vegetarian diet.

The phrase, "vegetarian diet," is general and deserves explanation. The vegetarian diet can be subdivided into two main categories, lactoovovegetarian and vegan. The lactoovovegetarian diet can be further subdivided into lactovegetarian and ovovegetarian. Lactoovovegetarian diets exclude all animal products except dairy foods and eggs. Lactovegetarian diets exclude all animal products except dairy foods. Ovovegetarian diets exclude all animal products except eggs. Hence, any prefix to the word vegetarian

denotes the only animal product(s) eaten in that diet. Vegan diets exclude the consumption of all animal products. Although vegans are often referred to in older literature as "pure," "strict," or "extreme" vegetarians (105, 161), the use of these terms is no longer widely used.

A vegan diet has been found to consist of whole grains, legumes, nuts, seeds, fruits, vegetables, and can include variety of sea vegetables, herbs and spices (27, 107, 164). Due to this array of potential foods, a large degree of variation can occur even within a vegan diet (92). This variation becomes more evident when considering the differences in food availability and cultural practices among countries. In addition, some vegans can have other coexisting personal, religious, or economic restrictions to their eating patterns which may alter nutrient intake (162).

Health aspects of vegan diets

The health benefits of vegetarian diets are well documented (92, 162). However, these benefits should not be assumed to apply to vegans because most vegetarian studies are conducted using lactoovovegetarians, or lactovegetarians as subjects. In support of this concept, Dwyer found that risk reduction for cardiovascular disease was more dependent upon the type of vegetarian eating pattern rather than simply a general protective effect of vegetarianism (162). Despite this, several studies (40, 41, 49, 50, 104, 105, 107, 111, 112, 149-151, 163, 164, 167-172) have been conducted with vegan subjects and a number of positive attributes are associated with a vegan diet (162, 173).

Early studies revealed that a vegan diet provides generous quantities of fruits and vegetables and thus can be higher in vitamin C, iron, beta-carotene, and fiber compared to an omnivorous diet (167-169). Vegans were found to have low intakes of total fat, saturated fat, and had lower serum cholesterol concentrations than omnivorous controls (105, 168, 173). It was then concluded that a vegan diet along with a vitamin B-12 supplement is nutritionally adequate for adults, and that the health of vegans does not differ much from that of healthy omnivores (112, 150, 173).

Subsequent studies confirmed that vegans tend to have higher intakes of carbohydrates, vitamin C, folic acid, potassium, magnesium, and fiber than omnivores (40, 50, 104, 107, 163, 164, 170, 171). A vegan diet is very low in saturated fatty acids and has been observed to contain less alcoholic beverages compared to omnivorous diets (40, 41, 50, 104, 107, 164, 170). Vegans have also displayed lower BMIs than omnivores (40, 41, 163). Furthermore, several reports have shown that vegans have serum total and LDL cholesterol concentrations much lower than that of omnivores (40, 49, 50, 104, 111, 149-151, 164, 172).

Increased serum cholesterol concentrations are associated with increased risk for CHD and lowering serum cholesterol may decrease CHD risk (63). Since vegans tend to have very low cholesterol concentrations (148), it is logical to conclude that they may have an overall decreased risk of CHD. However, direct evidence is both lacking and contradictory. One British study found that lifelong vegans had a 57% lower incidence of CHD than omnivore controls (149). Other research suggests that due to their low cholesterol concentrations and lower percent body fat, vegans may be less likely to

develop ischemic heart disease (49, 172). In contrast, Phillips, *et al.,* found that a group of vegan women had the highest CHD risk compared to both omnivores and Seventh Day Adventist vegetarians in their study (166). Although other vegetarian subgroups have lower morbidity and mortality from coronary artery disease (162, 174), at this time there is a lack of substantial evidence to conclude that vegans are at lower risk for CHD.

Nutritional considerations for vegans

Following a vegan diet can be healthful (107, 151) but should not be undertaken without paying special attention to certain nutrients and food components. The major nutritional concerns, relative to risk of deficiency, of a vegan diet include the following: vitamin B-12, vitamin D, iron, calcium, zinc, and possibly ALA (92, 162). With careful choice of foods and use of some fortified products, sufficient quantities of these food components can be obtained from a vegan diet throughout the life cycle, even during pregnancy and lactation (92, 175-177).

Vitamin B-12

Since plant foods are not a dependable source of vitamin B-12 and because vegans tend to have lower serum levels of this nutrient compared to omnivores (178, 179), it is recommended that vegans supplement this vitamin (92). However, cow milk alternatives such as soy or rice milk, ready-to-eat breakfast cereals, and some other foods are now fortified with B-12. Yet, elderly people regardless of their eating style may have low serum B-12 concentrations and frequently exhibit impaired absorption of this vitamin

(180). This can be complicated by the fact that serum B-12 levels tend to be low in longtime vegans (178, 179). As a result of these issues, it is recommended that all vegans, vegetarians and people over age 50 use a vitamin B-12 supplement or fortified foods in their diets (25). Since clinical symptoms of vitamin B-12 deficiency can take several years to appear, some vegans, especially elderly or longtime vegans should consider using a B-12 supplement.

Vitamin D

Very few adequate food sources of vitamin D exist aside from fortified products. Fortunately for vegans, vitamin D is produced in the skin upon exposure to sunlight, and most of the vitamin D humans need can be produced in this manner (181). However, aging, use of sunscreen, dark skin pigmentation and poor location relative to the angle of the sun can diminish skin production of vitamin D (181). As a result, vegans living in areas of low sun exposure should eat more vitamin D fortified foods or take a supplement to compensate for their lack of endogenous production (25). Alternatively, most soy and rice milks are fortified with vitamin D.

Iron

Iron intake of vegans is often higher than that of omnivores (107, 171, 178). However, a vegan diet provides only nonheme iron. Absorption of nonheme iron is less efficient than heme iron, which may explain why some vegans have lower iron stores compared to omnivores (178). Yet, the outcome of lower iron storage in vegans is

equivocal because the incidence of iron-deficiency anemia in vegetarians is not any higher than that of omnivores (107, 162, 182). In addition, vegans appear to have a high intake of vitamin C (163), which is known to aid in the absorption of nonheme iron (182). If vegans choose their foods carefully and include some fortified foods in their diet, iron recommendations can be met (25).

Calcium

Vegan intake of calcium is consistently lower than that of omnivores (92, 107, 162, 163). Vegan diets also tend to be low in protein (107, 163), which has been shown to reduce intestinal calcium absorption over a short duration (183). Although the long term implications of this are unknown, epidemiological studies indicate that a low protein diet, and the resultant reduction in calcium absorption, may increase the rate of bone loss (183). In contrast, diets moderate in protein (1.0 – 1.5 g protein/kg) are associated with normal calcium metabolism (183). Based on this information, it seems that vegans may benefit from eating a diet more moderate in protein. In addition, the American Dietetic Association urges vegans to meet the AI for calcium set forth by the Institute of Medicine (IOM), which is 1000 mg/d for males and females aged 31-50 y (25, 124). To meet these recommendations, vegans should eat rich plant sources of calcium and include fortified products such as soymilk and calcium-precipitated tofu in their diets.

Zinc

Zinc intake of vegans has been found to be lower (163, 184) but also higher (107) than that of omnivores. However, some studies (107, 163) have found the zinc intake of vegans to be similar to the Recommended Dietary Allowances (RDAs) set forth by the IOM, which is 11 mg/d for males and 8 mg/d for males aged 31-50 y (127). Perhaps more importantly, zinc intakes below the Recommended Dietary Allowances (RDAs) have been reported in both vegans and omnivores. One study found that indeed lactoovovegetarians appear to be at increased risk for zinc deficiency compared to omnivores, but the severity of this risk is presently unclear (185). Regardless, a vegan diet rich in whole grains and legumes can provide adequate amounts of zinc (185). Although, due to the high fiber and phytate content of these foods, the bioavailability of zinc is most likely compromised in vegans (92, 186). Until this issue is resolved, vegans are encouraged to meet the RDAs for zinc (92).

Maternal and infant issues

The largest percentage of growth in the human brain begins during the last trimester of gestation and continues approximately up to 18 months after birth (7). During the last trimester, concentrations of DHA in the cerebrum and cerebellum increase three to five fold (2, 10). Similar increases are also typical during the first 12 weeks after birth. These facts highlight the importance of n-3 fatty acids in the perinatal period.

With this in mind, breast milk of vegan mothers has been reported to contain very little DHA compared to breast milk of omnivores (106). As a result, breast fed infants of

vegan mothers have low erythrocyte membrane concentrations of DHA (106, 176). A growing body of work shows that both preterm (187, 188) and term (189-191) infants exclusively fed non-DHA containing formula have lower erythrocyte DHA concentrations and exhibit decreased visual acuity in the first two months of life compared to infants fed breast milk from omnivore mothers or a formula fortified with DHA. Although, the long-term effects of these observations in humans are unknown, both rats and primates depleted of DHA produce abnormal electroretinograms and also display decreased visual acuity (2, 10). Conversely, some studies in term infants have found no differences in visual acuity between breast-fed or formula fed infants (192, 193).

Infant formulas sold in the U.S. are not required to be fortified with DHA on the premise that ALA, which is added to formulas, can be converted to DHA (194). However, since DHA is found in human milk and is an important structural component of brain and retinal tissue, it has recently been added to some infant formulas in the U.S. (195). Due to the importance of DHA, vegans are recommended to include a variety of sources of ALA or use a suitable vegetarian DHA supplement (algae-derived) while pregnant or lactating (25).

APPENDIX B: INSTRUMENTS DEVELOPED & USED

Personal information

Name (last, first):_____

Code #_____

Home Phone #:_____ Date:_____
Work Phone #:_____ Age:____
Fax #:_____ Gender: M F
E-mail Address:_____
Home Address:_____
_____

Which of the above is the best way to contact you? Circle the best two.

What is the best time of day / night to reach you?_____

Which time(s) and day(s) of the week would you prefer to meet for instruction and blood drawing?_____
_____
_____

Do you have a working refrigerator where you live? Yes No

Are you planning to travel during the months of June or July of 1999 Yes No
If yes, when will you be gone?_____

Diet history

Name (last, first)_____

Code #_____

Date_____

1. Do you follow a vegan diet? Yes No If yes, for how long?_____

2. Which type(s) of animal products, if any, do you include in your diet?
   _____
   _____
   _____

3. Do you currently use flax oil in your diet? Yes No
   If yes, how much and how often?_____
   _____
   _____

4. Do you currently use flax seeds in your diet? Yes No
   If yes, how much, how often, and it what form?_____
   _____
   _____

5. Do currently use any other Yes No Not Sure
   food products that have flax in them?
   If yes, what kind, how much and how often?_____
   _____
   _____
   _____

6. Do you currently take flax oil capsules or supplements? Yes No
   If yes, how much and how often?_____
   _____

7. Do you currently use hemp seed oil in your diet? Yes No
   If yes, how much and how often?_____
   _____
   _____

8. Do you currently use any hemp-based food products? Yes No Not Sure
   If yes, what kind, how much and how often?_____

_____

_____

9. Do you currently take hemp oil capsules or supplements? Yes No
   If yes, how much and how often?_____
   _____

10. Do you currently use canola oil in your diet? Yes No
    If yes, how much and how often?_____
    _____
    _____

11. Do you currently use soybean oil in your diet? Yes No
    If yes, how much and how often?_____
    _____
    _____

12. Do you currently use perilla oil in your diet? Yes No Not Sure
    If yes, how much and how often?_____
    _____

13. Do you currently use walnut oil in your diet? Yes No
    If yes, how much and how often?_____
    _____
    _____

14. Do you eat walnuts on a regular basis? Yes No
    If yes, how much and how often?_____
    _____

15. Do you currently use mustard oil in your diet? Yes No
    If yes, how much and how often?_____
    _____

16. Do you currently take docosahexaenoic acid (DHA)capsules or supplements? Yes No Not Sure
    If yes, how much and how often?_____
    _____
    _____

17. Do you currently use any kind of essential fatty acid oil or supplement? Yes No Not Sure
    If yes, what kind, how much and how often?_____
    _____

18. Do you eat any products that contain olestra or other types of synthetic fat replacements? Yes No Not Sure
If yes, what products, how much and how often?_____

19. Do you drink alcoholic beverages? Yes No
If yes, what type(s), how much, and how often?_____

20. Do you currently take any vitamin or mineral supplements? Yes No
If yes, what type(s), how much and how often?_____

21. Do you currently take any herbal supplements? Yes No
If yes, what type(s), how much and how often?_____

22. Are you currently following any type of weight loss diet? Yes No
If yes, please explain._____

23. Are you currently following any type of diet in order to lower your levels of cholesterol or triglycerides? Yes No
If yes, please explain._____

24. Are you currently following any type of special diet at all? Yes No
If yes, please explain._____

Medical history

Name (last, first)________________________________

- -

Code #_______________

Date_______________ Date of Birth__________________________

Name of emergency contact person____

Relationship_____________ Home Phone____________ Work Phone____________

Hospital Preference___________________________________ Phone____________

Doctor Preference________________________________ Office Phone____________

MEDICAL HISTORY

Have you ever been told by a doctor that you have, or had, any of the following conditions?
Please check all that apply and give the age at which each condition was first diagnosed.

Allergies	_____Yes, at age_____years old	_____No
Angina (coronary insufficiency)	_____Yes, at age_____years old	_____No
Asthma	_____Yes, at age_____years old	_____No
Cancer	_____Yes, at age_____years old	_____No
Cardiovascular disease	_____Yes, at age_____years old	_____No
Chronic fatigue	_____Yes, at age_____years old	_____No
Diabetes	_____Yes, at age_____years old	_____No
Eating Disorder	_____Yes, at age_____years old	_____No
Gallstones	_____Yes, at age_____years old	_____No
Heart attack (myocardial infarction, etc.)	_____Yes, at age_____years old	_____No
High blood cholesterol, hyperlipidemia	_____Yes, at age_____years old	_____No
High blood pressure (hypertension)	_____Yes, at age_____years old	_____No
Inflammatory disease (arthritis, etc.)	_____Yes, at age_____years old	_____No
Intestinal disorder	_____Yes, at age_____years old	_____No
Kidney problems	_____Yes, at age_____years old	_____No
Liver disease	_____Yes, at age_____years old	_____No
Metabolic disorder	_____Yes, at age_____years old	_____No
Obesity	_____Yes, at age_____years old	_____No
Skin disorder	_____Yes, at age_____years old	_____No
Stroke	_____Yes, at age_____years old	_____No

Upper GI complaints (nausea, vomiting) _____Yes, at age_____years old _____No

CURRENT THERAPY

Are you currently receiving long-term medical treatment (e.g. insulin injections, dialysis, intravenous therapy, etc.) for any illness or condition?

____Yes ____No

If yes, please give the details:

Illness or condition Treatment

_____

_____

_____

MEDICATIONS CURRENTLY TAKEN

Prescription medications:

_____

_____

_____

_____

Over-the-counter medications (aspirin, anti-inflammatories, antacids, stomach acid supressors):

_____

_____

_____

PAST TREATMENTS

In the past year, have you seen a physician or

_____Yes _____No

other health practitioner for any medical condition?

Diagnosis_____

Therapy_____

Have you ***ever*** had any surgeries/operations?____Yes ____No If yes, please list them.

_____ Year_____ or age_____

_____ Year_____ or age_____

_____ Year_____ or age_____

WEIGHT HISTORY

How much do you weigh at this time? _____ lbs

Since adopting a vegan diet, has your weight ____Stayed the same _____Increased _____Decreased

	lbs	Kg
By how much?:_____	5 or less	2.2 or less
_____	10-20	4.5 - 9.0
_____	21-30	10 - 13.5
_____	31-40	13.6 - 18.1
_____	41-50	18.2 - 22.7
_____	51-60	22.8 - 27.3
_____	61-75	27.4 - 34.1
_____	76-99	34.2 - 45.5
_____	over 100	>45.6

Is maintaining your weight a constant struggle? _____Yes _____No

If yes, is the struggle to keep your weight _____Down _____Up

How tall are you? _____Feet _____Inches or _____Centimeters

GASTROINTESTINAL HEALTH

FREQUENCY OF BOWEL MOVEMENTS:

_____More than 5 movements per day_____4-5 movements per day_____2-3 movements per day

_____1 movement per day _____1 movement every 2 days _____1 movement every 3-4 days

_____1 movement every 5 days or longer

CONSISTENCY OF STOOLS

_____Consistently loose – bordering on diarrhea

_____Consistently soft, but formed

_____Normal – firm, formed

_____Consistently hard, sometimes painful

_____Generally float in toilet water

_____Generally sink in toilet water

Symptoms of intestinal Dysfunction	Less than 1 time per year	3 to 5 times per year	More than 5 times per year
_____Blood in stool	_____	_____	_____
_____Excessive mucus in stool	_____	_____	_____
_____Excessive gas, bloating	_____	_____	_____
_____Cramps	_____	_____	_____
_____Diarrhea	_____	_____	_____

LIFESTYLE

Do you smoke cigarettes? _____Yes _____No
Do you exercise regularly? _____Yes _____No
If yes, what kind(s) and how often?_____

_____

_____

SKIN CONDITION

Is your skin dry – do you have flaking of the skin on your scalp, eyebrows, arms, legs, hands; cracking of skin on fingers? _____Yes _____No
Is your hair dry or brittle? _____Yes _____No
Do your fingernails split easily? _____Yes _____No

FEMALES ONLY

Are you pregnant?
Do you plan to become pregnant in the next three months? _____Yes _____No
Are you lactating and/or breast feeding currently? _____Yes _____No
Do you now experience monthly periods? _____Yes _____No
Do you consider your menstrual cycle to be regular? _____Yes _____No
Are you currently taking any birth control pills? _____Yes _____No
Are you currently going through menopause? _____Yes _____No
Are you post-menopausal? _____Yes _____No
If yes, how long have you been post-menopausal?_____

General information

- Aloha, and thank you for participating in the Vegetarian, Flax, and Hemp Seed Oils Study!
- Please take time after the first Instruction Meeting to read all forms provided to you in your folder, especially the instructions for filling out your food records.
- Please verify that you have all the proper forms in your folder.

Items That Should Be In This Folder

* Food Record Forms (5 total)
* Food Record Instructions
* Flax / Hemp Oil Intake Form
* Map / Directions to clinic
* Participant Scheduling Form
* Flax / Hemp oil use ideas
* Envelopes / stamps for mailing back food records

Your Main Responsibilities

- Attending this Instruction Meeting.
- Keeping accurate food records.
- Making it to the two blood tests as scheduled.

Contact & Information Numbers/Addresses

For general information, updates, help filling out your food record forms, map/directions to the blood drawing clinic, and scheduling information try looking at the website listed below. This website was created exclusively for you! If you can't find the information you need on the website, feel free to call Jeff during the hours listed.

Website Address: www2.hawaii.edu/~jefferyj/

Jeff Johnson (Principal Investigator)732-6515 (between 9am-10pm)

Dr. Dian Dooley (thesis committee chairperson) 956-7021

Dr. Alan Titchenal (thesis committee member) 956-7411

Food record instructions

- Record the exact amount of **all foods** you eat and **all beverages** you drink, **except water**. It's best to record what you eat at the time you eat it, or as close to that time as possible. This will help you avoid having to recall from memory the foods you ate earlier in the day and will also help us gain more accurate information.
- Please be as **honest** as possible. If you eat something that you don't normally eat, record it anyway, we need this information from you to be as accurate as possible.
- Please record all foods and beverages in **standard household measures**, such as cups, teaspoons, and tablespoons. If you feel comfortable recording the weight of the food, you may use this method also. Use either fluid ounces or cups for beverages.
- Please record how the food was prepared. For example, was it raw, baked, boiled, fried, steamed, microwaved, or stir-fried? If oil was used in cooking, please indicate which type, and how much.
- **Grains/Flours:** Record in level cup portions, after cooking. Specify what type of grain was used. For pastas, specify the shape, and whether or not it was made from enriched flour.
  - Dry/cold cereals: record level cup portions and specify the brand name.
  - Breads: record the number of slices and specify which types of grains are in the bread (rye, whole wheat, etc.). Specify whether or not the bread was eaten toasted. If you can, please specify whether or not oil is used to make the bread, and if so, which type.
  - Flours: record the type of flour and the amount in level tablespoons or cups.
- **Vegetables:** List the name and how prepared.
- Cooked vegetables: record the number and size of pieces, such as two carrot sticks, 4" long, or record by cup portions.
- Frozen vegetables: record by name and amount in cups.
- Canned vegetables: record by name and amount in cups, specify if the juice was consumed.
- **Fruits:**
  - Whole raw fruits: record the type of fruit, the size (small, medium, large), and the number eaten.
  - Frozen fruits: record by name and amount of cups.
  - Fruit juices: record in fluid ounces or cups. Specify the type of juice. Specify if it was sweetened, unsweetened, canned, frozen, fortified, or freshly juiced.
  - Canned fruit: record by name and amount of cups. Specify whether it was canned in light or heavy syrup.
- **Meat Alternates/Soy products:**
  - Tofu: record tofu in level cups, tablespoons, or ounces. Specify how the tofu was prepared. Specify if tofu is silken, soft, firm, or extra firm. Specify which type of precipitate is used to solidify the tofu (e.g. calcium sulfate, magnesium chloride), or the brand name.

    - Burger patties/slices: record the name brand, how many slices, and how it was prepared.
    - Tempeh/Seitan: record the number of ounces eaten, name brand, and how prepared.
    - Tofu wieners: record the name brand and the number of wieners eaten.
- **Milks** (soy, rice, almond, oat, etc.): record in cups or fluid ounces. Specify the type of milk, the flavor, and the brand name. Specify if the milk is or isn't fortified. For example, 1 cup Westsoy Plus soymilk, vanilla.
    - Soy yogurts/cheeses: record the amount eaten and the name brand.
- **Beans/Peas:** record the kind of bean/pea eaten, and record the number of cups after cooking. If canned beans are used, specify if the juice was used.
- **Nuts/Seeds(and their butters):** record the type of nut/seed and the amount in teaspoons, tablespoons, or cups.
- **Fats/Oils:** record in level teaspoons or tablespoons. Record the type of fat/oil that was used. Specify how it was used, in cooking, raw, as a marinade, etc. **Remember that you SHOULD NOT record any flax/hemp oil consumption on your food record forms, those amounts should be recorded on the *Oil Intake Form*.**
- **Beverages:** record the amount in cups or fluid ounces and specify the type of beverage consumed.
- **Snack Foods:** record the number of pieces of chips or crackers you eat, specify the type of snack and the name brand. Example, Bearito's Corn Chips, 15 pieces. For popcorn, record in level cups the amount of popped corn.
- **Desserts:** record the brand name and the amount eaten in cups. For example, frozen Rice Dream cocoa marble fudge, 1 cup.
- **Condiments/Sauces/Herbs:** for all syrups, sauces, gravies, toppings, jams, jellies, soy sauces, ketchup, mustard, and herbs, specify by teaspoon, tablespoon, or cup. For nutritional yeast, record the amount of teaspoons, tablespoons, or cups used, and please specify the brand if you know it.
- **Specialty Items:** for many special foods usually found at natural food stores, please do your best to estimate the amounts for each food in the product, record the brand name, and if possible save the label and turn it in with your food record forms.
- **Combination Foods:** for soups, stews, casseroles, pizzas, any mixed foods, list the amount of each ingredient separately. For homemade recipes, list the amount of each ingredient separately, and if possible, attach the recipe to your food record form. A photocopy of the recipe from a cookbook will work fine also.
- **Take-out/Restaurant Food:** please record the name of the restaurant, the name of the menu item(s), and how much was eaten. If you feel comfortable estimating the amount of the ingredients, please do so.
- **Supplements:** please record the number of all supplements that you take on the days you record your food intake. This includes all vitamins, minerals, herbs, powders, or other pills that you might take. Please include the name brand and if possible, save the label to turn it in with your food record forms.

Common Abbreviations:

cup = c ounce = oz
teaspoon = tsp pound = lb
tablespoon = Tbsp grams = g

Common Equivalents:

3 tsp = 1 Tbsp 4c = 1quart
16 Tbsp = 1 c 8 oz = 1 c
2 c = 1 pint 28.35 g = 1 oz

24-Hour food record

Name_____ Date_____

Meal/Snack Time	Food/Beverage Consumed	Amount Consumed	How The Food Was Prepared

3-Day food record

Name_____ Date_____

Meal/Snack Time	Food/Beverage Consumed	Amount Consumed	How The Food Was Prepared

Oil intake record

Name_____

Please record all of the flax/hemp oil you consume each day, beginning on_____.
Please follow the examples given on the first two lines of the table below.
This table is to be used **ONLY** for recording your intake of the oil provided to you by this study. **DO NOT** record your normal oil consumption on this form. That will be recorded on your 3-day and 24-hour food record forms.

Date	Amount Eaten/Used	How It Was Used
5-5-99	1.5 tablespoons	In a fruit smoothie
5-6-99	1 tablespoon	As a dressing on a salad

Tips for oil use

Remember that these oils contain high quality fatty acids that are easily damaged by heat, light, and oxygen. Keep your bottles tightly capped and refrigerated in between uses. Also, These oils are **not to be used for cooking**. You must use these oils raw or on only slightly warmed foods. Try some of the ideas listed below.

- Add some of the oil to a fruit smoothie.
- Add some of the oil to a soymilk shake.
- Mix the oil with some balsamic vinegar and herbs to make a tasty vinaigrette.
- Use the oil in place of other oils you normally use to make cold, deli-style salads with pasta, rice or beans.
- Drizzle over rice or baked potatoes (let them cool down before adding the oil).
- Drizzle over bread or toast, pancakes, or waffles (as long as they're not too hot).
- Mix oil into oatmeal or other cereals (after they've cooled a bit).
- Toss with a batch of lightly steamed veggies (after they've cooled a bit).
- Grind peppercorns over some of the oil and dip your favorite bread into the mixture.
- Mix the oil with a glass of juice.
- Use the oil in place of olive oil when making hummus or pesto.

Of course, many possibilities exist for taking the oils, feel free to experiment and make up your own recipes as you go along. Just remember to keep the above heat guidelines in mind when experimenting. If you're not feeling creative, it's also OK to just gulp it down by itself. I will try to post more uses for the oils on the web site as the study progresses.

Exit interview questionnaire

Name_____ Date_____

Did you know what type of oil you were given during the study? Yes No
If yes, how did you know?_____

Circle the name of the oil you think you were taking during the study. Flax Hemp

Did you forget to take the oil on any days during the study? Yes No
If yes, how many days out of the 28 did you miss?_____
If yes, did you make up for those days by taking more the next days? Yes No

Did you experience any side effects of using the hemp/flax oil? Yes No
If yes, please explain_____
_____

Did you take any supplements containing DHA oil during the study? Yes No
If yes, how much and how often?_____

Do you think the food records you kept during the study accurately reflect your usual dietary intake? Yes No
If no, please explain_____
_____
_____

Did you think it was difficult to record your food intake? Yes No
If yes, what did you find difficult about it?_____
_____

Did your exercise pattern change drastically during the study? Yes No
If yes, please explain how_____
_____

Did you fast for the required number of hours prior to donating blood? Yes No
If no, how many hours did you fast prior to donating blood the 1st time____ 2nd____

With which ethnic group do you most closely identify?_____

What is/are your reasons for following a vegetarian diet?_____
_____

Did you use the website during the study? Yes No

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