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University of Tennessee, Knoxville

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To the Graduate Council:

I am submitting herewith a thesis written by Laura Louise Jones entitled "Human Equivalent Dose Modeling for Omega-3 Fatty Acid Supplementation in C57BL/6J Mice." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Nutrition.

Jay Whelan, Major Professor

We have read this thesis and recommend its acceptance:

Gary E. Truett, Jung Han Kim

Accepted for the Council:
Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

To the Graduate Council:

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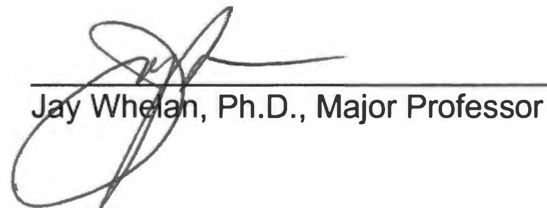

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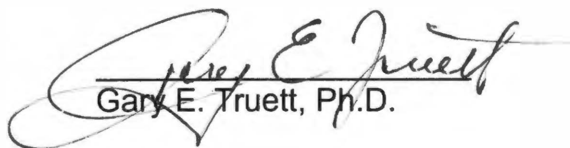

Gary E. Truett, Ph.D.

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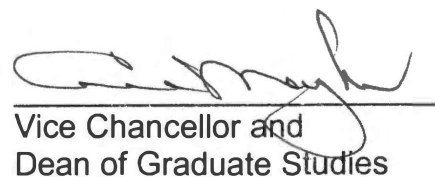

Vice Chancellor and
Dean of Graduate Studies

Thesis
2005
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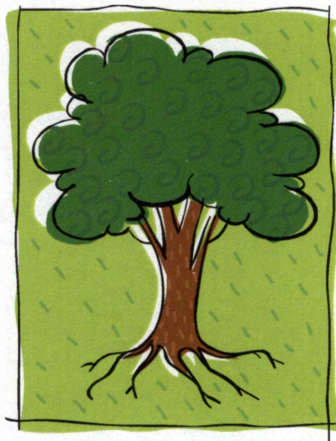
HUMAN EQUIVALENT DOSE MODELING FOR OMEGA-3 FATTY ACID SUPPLEMENTATION IN C57BL/6J MICE

A Thesis
Presented for the
Master of Science Degree
The University of Tennessee, Knoxville

Laura Louise Jones
December 2005

DEDICATION

I would like to dedicate this thesis to my husband, ***Anthony Kenneth Jones***.
Yesterday, today, and tomorrow: I choose you.



"Blessed is the man who trusts in the Lord, whose hope and confidence- the Lord is. He will be like a tree that is planted by the water, which stretches out its roots by the stream. It does not fear when heat comes, its leaves are always green. And it has no worries in a year of drought and never fails to bear fruit."

Jeremiah 17: 7-8

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Thank you **Lord**!

ABSTRACT

The rodent model is often used to study the impact of dietary n-3 fatty acids on a variety of biological endpoints, and the results of these studies have been used to explain anticipated effects of n-3 fatty acid intake in humans. However, supplemental levels of n-3 fatty acids that are commonly used in rodent studies do not represent reasonable human intake, by comparison. Currently there is no standard method for the addition of n-3 fatty acids to rodent diets. We tested a mathematical model for dosing supplemental levels of α-linolenic acid (ALA) and eicosapentaenoic acid (EPA) to rodent diets on the basis of energy%. C57Bl/6J mice were fed a background diet that modeled typical Western intake in both macronutrient and fatty acid composition. Three levels of ALA and EPA (0.3, 0.8, and 1.4 energy%) were supplemented to either a normal-ALA control diet (0.6 energy% ALA) or a high-ALA control diet (1.2 energy% ALA). Plasma and erythrocyte phospholipid fatty acid changes were determined and compared to archival human n-3 fatty acid supplementation studies reporting the same tissue endpoints. In mice, supplemental EPA had a greater effect than supplemental ALA on both plasma and erythrocyte phospholipid EPA. Docosahexaenoic acid (DHA) levels in mice were only minimally changed by either ALA or EPA supplementation. Use of the high- ALA control diet resulted in attenuated phospholipid fatty acid changes in both tissues compared to the normal-ALA control diet for both supplemented fatty acids. At each supplemented dose of ALA or EPA, changes in murine plasma or erythrocyte

phospholipid EPA exceeded changes observed in the same human tissues by 2-4 fold when compared to equivalent human supplemental doses in energy%. Tissue changes observed using the high-ALA control diet better modeled the results observed in humans at the same supplemental energy% for both ALA and EPA in plasma and erythrocyte phospholipids. This is the first study to use pharmacodynamic modeling to compare the effect of supplemental n-3 doses on mouse and human endpoints. The addition of n-3 fatty acids to rodent diets on the basis of energy% represents a reasonable improvement to current dosing strategies. This data is useful both as a guideline for n-3 fatty acid dosing in rodent studies and as a reference point for future calculated refinements in dosing.

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NOMENCLATURE

Abbreviations

AA	Arachidonic acid (20:4 n-6)
AI	Adequate intake
ALA	Alpha-linolenic acid (18:3 n-3)
CVD	Cardiovascular disease
COX	Cyclooxygenase enzyme
EPA	Eicosapentaenoic acid (20:5 n-3)
DGLA	Dihomogammalinolenic acid (20:3 n-6)
DHA	Docosahexaenoic acid (22:6 n-3)
DPA	Docosapentaenoic acid (22:5 n-3)
DRI	Dietary Reference Intakes
GLA	Gamma-linolenic acid (18:3 n-6)
HED	Human equivalent dose
LA	Linoleic acid (18:2 n-6)
LCn3	Long chain n-3 fatty acids
LOX	Lipoxygenase enzyme
n-3	Omega-3
PL	Phospholipid
PRP	Platelet rich plasma
TG	Triglyceride
TXB_2	Thromboxane B_2
RBC	Erythrocytes, red blood cells
sn	Stereochemical numbering/ nucleophilic substitution

1 REVIEW OF THE LITERATURE

1.1 Introduction

The generation of information regarding omega-3 (n-3) fatty acids has been prolific over the past thirty years subsequent to Dyerberg and Bang's discovery in 1971 attributing heart disease incidence reduction in Greenland Eskimos to their consumption of polyunsaturated fatty acids, particularly n-3 fatty acids (1-3). Data obtained from both animal studies and human clinical trials have been used to examine the mechanisms by which n-3 fatty acids convey health benefits and to make judgments about optimal dietary intake levels for general health and disease prevention. Although much knowledge has been gained, there are still several questions that have not been fully addressed. The following project grew out of a need for empirical data to address three primary questions:

1. What is the difference in biological impact between terrestrial n-3 fatty acid, α-linolenic acid (18:3 n-3; ALA), and long- chain n-3 fatty acids (LCn3), eicosapentaenoic acid (20:5 n-3, EPA) and docosahexaenoic acid (22:6 n-3, DHA)?
2. What is the impact of terrestrial or LCn3 in mice when given at levels that are comparable to human supplemental intake?
3. What methodological improvements can be made in the dosing of n-3 fatty acids in rodent studies that allows for a more direct comparison of mouse and human data and a standardization of dosing across different research studies using animals?

1.2 Overview of Omega-3 Fatty Acids

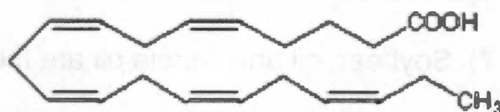
Description

N-3 fatty acids are polyunsaturated hydrocarbon chains in which the first double bond occurs at the third carbon from the methyl end of the molecule (4). The three predominant dietary n-3 fatty acids are: ALA, EPA, and DHA (**Figure 1-1**). The unique structure of n-3 fatty acids predicts, in part, their biological fate. Methylene-interrupted double bonds present within n-3 fatty acids make them a vulnerable target for hydrogen abstraction by reactive oxygen species due to both weakened carbon-hydrogen bonding at the methylene groups and to structural potential for radical stabilization via resonance (4).

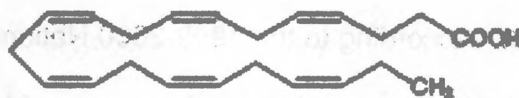
Dietary sources

ALA is an n-3 fatty acid of plant origin that is consumed primarily in the form of vegetable oils such as canola oil (9.3% ALA by weight) and soybean oil (6.8 % ALA by weight) (5). Other significant sources of ALA include walnuts, flaxseeds, flaxseed oil, soybeans, soybean oil, and wheat germ (**Table 1-1**). EPA and DHA are often called the "marine" n-3s because they are present in the highest concentration in fatty fish such as mackerel, herring, salmon, tuna, trout, and halibut (6). There are also a variety of oil capsule supplements on the market that contain varying concentrations of EPA and DHA (and in some cases, other polyunsaturated fatty acids as well).

18:3 n-3, α-Linolenic acid



20:5 n-3, Eicosapentaenoic acid



22:6 n-3, Docosahexaenoic acid

Figure 1-1: Structure and nomenclature of the three predominant n-3 fatty acids found in the Western diet.

Table 1-1

Alpha-linolenic acid content of selected food sources (5).

Food Source	ALA content (wt%)
Canola oil	9.3
Flax seeds	18.1
Flax seed oil	58
Soybeans	1.3
Soybean oil	6.8
Walnuts (English)	9.1
Wheat Germ	0.72

Western consumption

Kris-Etherton, et al. (6) report that the average daily intake of all n-3 fatty acids in the United States is ~1.6 grams (or 0.7 energy % based on a 2000 calorie diet). The average U.S. consumption of ALA is 1.1-1.4 grams per day (0.5-0.6 energy %) (6, 7). Soybean oil and canola oil are the most prevalent ALA sources in the U.S. diet (6). The remaining portion of average daily n-3 intake comes from roughly 75 mg EPA and 125 mg DHA for a total of ~200 mg LCn3 (0.09 energy %) (6-8). According to the 1999-2000 National Health and Nutrition Examination Survey data, the most frequently consumed fatty fish in the United States were tuna and salmon (9, 10).

Recommendations for consumption

At present, there is insufficient data necessary to establish a Recommended Dietary Allowance for ALA, EPA and DHA; however, several dietary recommendations have been made by a variety of national and international health organizations (**Table 1-2**). Recommendations are generally subdivided by the following population groups: healthy adults, pregnant mothers, and coronary heart disease patients. In September of 2004, the United States Food and Drug Administration (FDA) approved a petition requesting a qualified health claim for foods containing EPA and DHA for the prevention of coronary artery disease (11).

TABLE 1-2[1]

Selected national and international omega-3 fatty acid recommendations.

AGENCY/GROUP	COUNTRY	POPULATION GROUP	RECOMMENDATION
American Heart Association (12)	USA	Adults	Two servings fatty fish/ week Include oils rich in ALA
		CHD patients	~1 g EPA + DHA/ day
		Patients with ↑ TG	2-4 gms EPA + DHA/ day
Workshop on the Essentiality of and Intakes from Omega-6 and Omega-3 Fatty Acids (13)	International	Adults	ALA: 2.22 g/day EPA to be at least: 0.22 g/day DHA to be at least: 0.22 g/day
		Pregnancy/ Lactation	DHA to be at least: 0.3 g/day
International Society for the Study of Fatty Acids and Lipids (14)	International	Adults	ALA: 0.7 energy% (1.56 g/day; 2000 kcal) EPA + DHA: at least 0.5 g/ day
World Health Organization (15)	International	Adults	All ω-3 fatty acids: 1-2 energy% (2.2- 4.4 g/day; 2000 kcal)

TABLE 1-2[1]

Continued

AGENCY/GROUP	COUNTRY	POPULATION GROUP	RECOMMENDATION
National Heart Foundation of Australia (16)	Australia & New Zealand	Healthy adults and CHD patients	ALA: 2 g/day At least 2 fatty fish meals/week
Nordic Council of Ministers (17)	Denmark, Finland, Norway, & Sweden	Persons over 3 years of age	All ω-3 fatty acids: At least 0.5% (1.1 g/day; 2000 kcal)
British Nutrition Foundation (18)	United Kingdom	Adults	ALA: At least 0.2 energy% (0.44 g/day; 2000 kcal) EPA + DHA: 0.5 energy% (1.1 g/day; 2000 kcal)
Health Council of the Netherlands (19)	The Netherlands	Birth to 5 months	ALA: 0.08 g/ kg/day DHA: 0.02 g/ kg/ day
		Adults	ALA: 1.0 energy% (2.2 g/day; 2000 kcal) EPA + DHA: 0.2 g/ day
		Pregnancy & Lactation	ALA: 1.0 energy% EPA + DHA: 0.2 g/day

[1] Adapted from Source: (20).

It reads,

> *"Supportive but not conclusive research shows that consumption of EPA and DHA omega-3 fatty acids may reduce the risk of coronary heart disease. One serving of [Name of the food] provides [ ] gram of EPA and DHA omega-3 fatty acids. [See nutrition information for total fat, saturated fat, and cholesterol content.]"*

The use of this health claim will help to encourage public consumption of LCn3 and increase the likelihood of reaching intake recommendations.

Digestion, absorption, and transport

N-3 fatty acids are consumed in the form of triglycerides and phospholipids present in food. Phospholipids contain 85-90% fatty acids and triglyceride content is ~95% fatty acid (21). As unsaturated fatty acids, ALA, EPA and DHA are primarily found in the sn-2 position of both triglycerides and phospholipids (22). N-3 fatty acids follow the same course of digestion and absorption as other lipid components. The mechanical churning of the stomach (coupled with the action of gastric lipase) is the first step in lipid digestion (22). Some short and medium chain fatty acids are directly absorbed through the stomach wall. Longer fatty acids (all omega-3s), however, are primarily broken down in the small intestine. In the duodenum, bile salts from the liver, as well as physical hydrophobic interactions (23), help to emulsify fat droplets into manageable subsections, creating an increase in surface area for enzymatic action. Pancreatic lipase (activated by co-lipase) hydrolyzes fatty acids in the sn-1 and sn-3 positions on the glycerol backbone of food derived triglycerides

(24), leaving two non-esterified fatty acids and 2-monoacylglycerol.

Phospholipase A_2, also originating in the pancreas, cleaves fatty acids present in the sn-2 position of phospholipids, leaving a free fatty acid and a lysophospholipid (22) (**Figure 1-2**). The free fatty acids and partially digested acylglycerols and phosphoglycerols are transported through the hydrophilic layer that coats the intestinal lumen by micelles. Micelles are small groupings of phospholipids, free fatty acids, cholesterol, mono and diacylglycerols, and bile salts that act as amphipathic carriers of lipid soluble molecules to the enterocyte membrane surface.

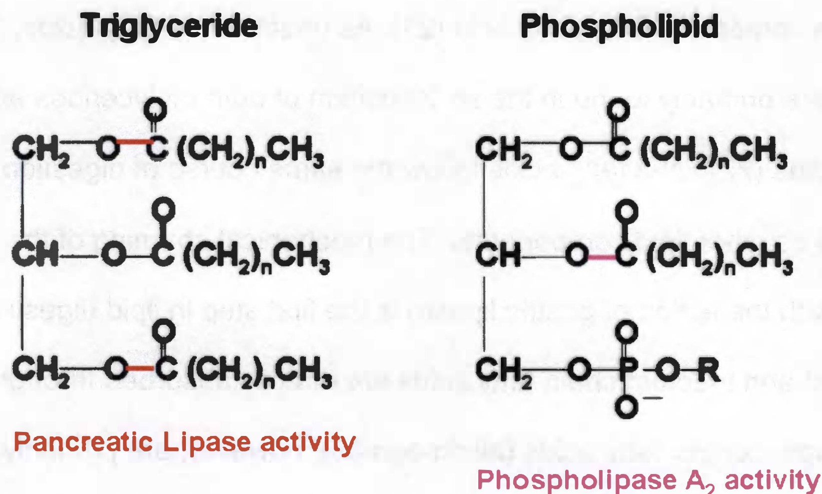


Figure 1-2: Targeted bonds of pancreatic lipase on dietary triglyceride and phospholipase A_2 on dietary phospholipids.

Particles of lipid digestion were at one time thought to be absorbed solely via passive diffusion, however, it is now believed that fat binding protein carriers help to mediate the transfer (25, 26). Within the enterocyte, fatty acids, monoacylglycerols and phosphoglycerols are reassembled into triglycerides and phospholipids and combined with apoprotein to be secreted into the lymphatic system as chylomicrons and eventually enter circulation through the superior vena cava. Chylomicrons are distributed in the circulation to adipose tissue for storage and other peripheral tissues for energy (22). Lipoprotein lipase attached to the endothelial wall of tissue capillaries cleaves the fatty acids in the sn-1 and sn-3 positions of triglycerides, in a similar fashion to pancreatic lipase. Monoacylglycerol lipase within the plasma membrane finishes the hydrolysis of 2-monoacylglycerol (27) and the fatty acids are carried across the membrane via a fatty-acid binding protein that co-transports with sodium ions (22). Free glycerol and chylomicron remnants are circulated back to the liver and absorbed. The metabolic fate of absorbed fatty acids depends on the tissue, the fatty acid type, and the energy state of the individual.

Endogenous long chain omega-3 formation

In addition to dietary intake, a minor amount of long chain n -3 fatty acids are synthesized de novo from dietary ALA (**Figure 1-3**). Mammals lack the ability to desaturate fatty acyl chains beyond the $\Delta 9$ carbon, making consumption

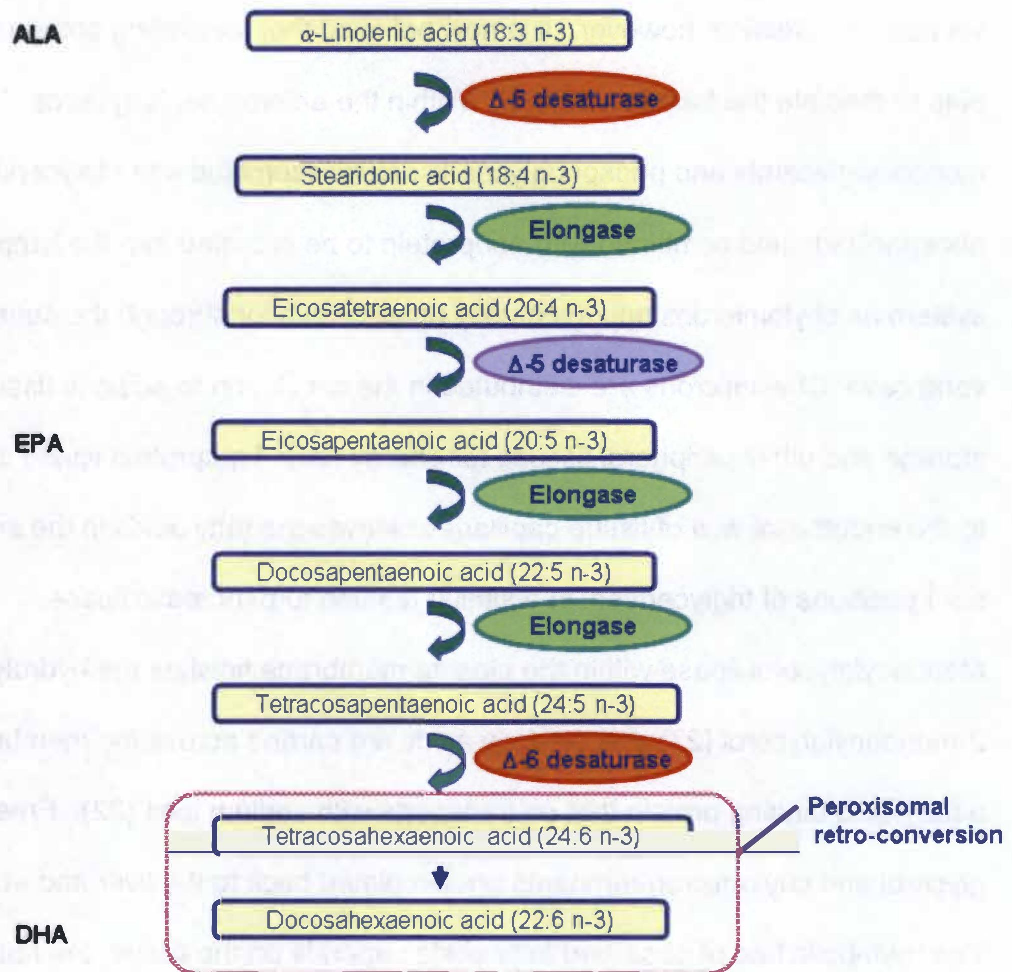


Figure 1-3: Enzymatic conversion of ALA to LCn3 metabolites. Conversion occurs on the cytosolic interface of the endoplasmic reticulum with the final two steps involving peroxisomal retroconversion (28-31).

of the parent compound ALA essential (32). Conversion of ALA to long chain metabolites takes place using enzymes found in the endoplasmic reticulum membrane (29-31) (examined as microsomes), with the final steps of DHA synthesis using peroxisomes (28). The majority of conversion takes place in the liver, however, the machinery for ALA desaturation has been found in a variety of tissues such as brain, skeletal muscle, lung, and heart (33, 34). **Table 1-3** compares several studies where ALA conversion rates were estimated using oral doses of radio-labeled ALA. Most studies report less than 5% conversion from ALA to EPA, and overall there appears to be very little conversion to DHA. This is supported by a study reporting an inverse relationship between the amount of dietary ALA consumed and DHA in several blood fractions (35). It was suggested that ALA is displacing DHA from membrane PLs, which would result in decreased absolute DHA content in the membrane. The highest conversion rates were seen in a study by Burdge et al. (36), where the rate of ALA to EPA conversion in young women was 21% and the conversion to DHA was 9%. Separating conversion rates by gender has led to the hypothesis that women may have a greater potential for conversion based on childbearing capacity and prenatal needs for DHA (36, 37).

Several factors influence the propensity for ALA conversion to long chain metabolites, including:

- The amount of ALA at a given time that is removed from conversion by β-oxidation (38).

TABLE 1-3

Comparison of estimated metabolic conversion rates of dietary α-linolenic acid to long chain metabolites.

Author/ Source	Subjects	Background Diet	Labeled fatty acid	Percent Conversion to EPA	Percent Conversion to DHA
Emken (39), 1994	Young adult males	LA- 15 g/d	Deuterated LA + ALA	8.0 ± 1.5	4.0 ± 1.6
		LA- 30 g/d		3.4 ± 0.5	3.6 ± 2.0
Hoffman (40), 2001	Healthy males	Low fat Low omega-3	C^{13} ALA		5.0
Pawlosky (38), 2001	Healthy adults	Beef based	Pentadeuterated ALA	0.2 ± .02	0.047
Pawlosky (41), 2003	Healthy adults	Self selected	Pentadeuterated ALA	0.2 ± 0.01	0.057
		Fish based		0.10 ± 0.02	0.018
		Beef based		0.19 ± 0.01	0.075

TABLE 1-3

Continued

Author/ Source	Subjects	Background Diet	Labeled fatty acid	Percent Conversion to EPA	Percent Conversion to DHA
Burdge (42), 2002	Young men	Habitual diet	C^{13} ALA	7.9	0
Burdge (36), 2002	Young women	Habitual diet	C^{13} ALA	21	9
Burdge (43), 2003	Older men	Control	C^{13} ALA	1.96[1]	0.03[1]
		ALA- 10g/d		2.6[1]	0.05[1]
		EPA + DHA- 1.5 g/d		4.0[1]	0.07[1]

[1] Based on cumulative amounts of EPA or DHA as a percent of total labeled ALA + EPA + DPA + DHA.

- The rate and sufficiency of desaturase enzymes (Ex: Δ-5 desaturase down regulation in retinitis pigmentosa (40)).
- The amount of linoleic acid (LA; 18:2n-6) in the diet (39, 44). LA competes with ALA for the rate limiting Δ-6 desaturase enzyme. Delta-6 desaturase is 19 times more selective for ALA than for LA (45), however, the ratio of LA content to ALA content in the typical Western diet is 10:1, thus presenting competitive inhibition of ALA conversion.
- The absolute amount of ALA, EPA, and DHA in the diet (46-48).

One limitation of current conversion studies is their reliance on plasma data. It is arguable that in tissues where DHA is particularly important (i.e. brain and retina), conversion rates may be greater to compensate for insufficient dietary DHA, and that this is not reflected in the conversion estimations from plasma. However, overall, the current estimates of ALA conversion suggest that dietary ALA intake may not be a sufficient substitute for preformed LCn3 (39), particularly if the benefits associated with LCn3 intake are desired. Furthermore, increasing ALA consumption from food sources in an attempt to maximize ALA conversion may yield a level of total fat intake that exceeds current dietary recommendations (49).

Critical location and timing of accumulation

The chief residence of n-3 fatty acids in biological organisms is the sn-2 position (50, 51) of phospholipids that compose cellular and organelle membranes. Polyunsaturated fatty acids are usually found within phospholipids on the cytosolic half of the membrane bilayer (52). ALA, DHA, and EPA can all be found in membranes, however, ALA is almost negligible in membrane lipids (Table 1 in (53) & (54)) due to its propensity to be oxidized or used for other metabolic purposes including its use a precursor to the other n-3 acids as described above. The most prominent membrane n-3 is DHA followed by DPA and EPA (Table 1 in (53)). The two tissues that accumulate the highest concentration of n-3 fatty acids in the body are retinal photoreceptor rod outer segments (53) and neural synaptosomes (49). DHA makes up 50% of the fatty acid content of rod outer segment membrane in the retina and can occupy both the sn-1 and sn-2 positions of the these phospholipids (55), allowing for maximal DHA concentration. Most of the DHA found in brain and retina accumulates in the last trimester of pregnancy and the early part of life after birth (50, 56-58). Prenatal accumulation of LCn3 is complex and involves several possible contributing sources. Maternal blood supply to the fetus (via the placenta) is the main supply of all fatty acids to the growing infant and lipid transfer is a reflection of maternal diet as well as maternal lipid stores (50, 59). Placental specificity for DHA binding has been reported (60), highlighting the priority that is placed on DHA acquisition for the fetus. Fetal conversion of ALA to LCn3 offers another possible route for accumulation. Conversion has been reported in fetal brain (56,

61) and liver (51). Delta 5 and Δ6 desaturase enzymes have also been found in human placenta (33). Lastly, it has been shown in rats that amniotic fluid swallowed in utero may also provide ALA to the fetus as a LCn3 precursor (51, 62).

In the initial period after birth, the infant is dependent on both body stores and external feeding for n-3 fatty acids. Breast milk is rich in DHA and AA (63). Synthetic formulas, however, were devoid of either fatty acid until 2002, when commercial formula companies began adding DHA and AA to their formula in an attempt to better mimic breast milk (64). Infants receiving formula without DHA are dependent on conversion from ALA in formula or adipose stores to obtain necessary DHA. Both term and preterm infants can convert ALA to DHA (65), however, this source of LCn3 is limited. Rat pup body stores of DHA have been shown to be a substantial source of DHA in rat brain (66), even when ALA is provided in the diet, suggesting that there is a preference for preformed DHA. This is a particularly important discovery for the treatment of preterm infants (66), who may miss the adipose accumulation associated with the later stages of gestation (67, 68) resulting in a reduced reserve of DHA from adipose.

Brain and retina are known to protectively retain DHA content (50, 69), and both are resistant to dietary n-3 deprivation (53, 70). Other membranes that are less dependent on stable n-3 fatty acid content such as liver, erythrocyte, and plasma membranes, readily undergo fatty acid turnover that is a directly influenced by changes in dietary intake.

Functional use of omega-3 fatty acids in the body

Function derived from structure

EPA in plasma membranes is mainly used as an eicosanoid precursor (see below), while DHA in membranes has a very specific structural function. The six double bonds of DHA impart flexibility (71, 72), disorder (73), and free volume (74) to regions of high DHA concentration. Steric hindrances and neighboring molecules exert external forces on the acyl chains (55) of phospholipid molecules, influencing the thermodynamic favorability of different structural conformations within the membrane. Molecular simulations show that DHA is able to make rapid conformational changes (72), and "interconversion between torsional states" (73, 75), and this is how it is able to confer acyl chain flexibility and fluidity to membranes. The space and flexibility provided by DHA content impacts the activation of certain membrane embedded receptors and enzymes, particularly those that undergo conformational changes upon activation requiring space for expansion.

In the rod outer segments of the retina, high DHA concentration facilitates the activation of rhodopsin and subsequent G protein-coupled receptor signaling that impacts the visual response (76). Anderson, et al. (77) have shown that the retina conserves DHA because of its importance in rhodopsin activation. In the event of n-3 deficiency, 22:5 n-6 accumulates in the retina in place of DHA (76, 78). However, even though 22:5 n-6 is almost structurally identical to DHA in that it lacks only 1 double bond, DHA replacement negatively

impacts electroretinogram measurements, implying that DHA provides a specific structural advantage for rhodopsin activation and vision (78).

The structural influence of membrane DHA on G protein-coupled receptors may also effect the neurotransmission of dopamine, serotonin, norepinephrine, acetylcholine, and GABA; all of which use G protein coupled-receptors (53). This is plausible based on the high concentration of DHA in synaptosmal membranes and the repeated observation that DHA concentration may affect psychological outcomes. It has also been shown that neurotransmitter reuptake may be increased by degree of membrane unsaturation (79) through enhancement of pinocytosis.

Ion channels and transport proteins are also affected by n-3 fatty acid content in the membrane. DHA has been shown to influence potassium channels (80), Na^+ K^+-ATPase activity (81, 82), and EPA influences Na^+ and Ca^{++} channels in cardiac myocytes (83, 84).

Some of the other structural characteristics that are influenced by the DHA content in the membrane include:

- Lipid rafts- Lack of affinity between DHA rich regions and cholesterol cause cholesterol rich/ DHA poor domains to form laterally within the membrane (85). Formation of lipid domains compartmentalize portions of the membrane for specific functions (32). Raft formation may also influence membrane embedded protein localization (73).
- DHA influences the speed of phospholipid flip-flops across the hydrophobic region within the plasma membrane(86).

- DHA may also intercept oxidative damage that would otherwise affect receptors and other membrane proteins (53).

Function derived from biological products

Polyunsaturated fatty acids (ex: DGLA (dihomogammalinolenic acid), AA, EPA, and DHA) are precursors to a diverse host of biological products including: prostaglandins, leukotrienes(87), thromboxanes, lipoxins(87, 88), hepoxilins, hydroxy and hydroperoxy compounds (89); and the more recently discovered: isoprostanes (90, 91), neuroprotectins (92), and resolvins (93). AA and EPA are the two main eicosanoid precursors which compete for sn-2 position placement in membrane phospholipids and subsequent cleavage for ultimate eicosanoid production. Phospholipid concentration of AA and EPA is a direct reflection of dietary intake (89). AA and EPA are substrates to several enzyme systems that produce eicosanoid products, such as: cyclooxygenases (COX), lipoxygenases (LOX), and epoxygenases (32, 94). Eicosanoids act as local hormones (23), having both autocrine and paracrine activities; they travel only short distances to their site of action (32, 95). This is, in part, due to their rapid inactivation and metabolism (96, 97). AA is a precursor to the series-2 prostaglandins (ex: PGE_2, PGI_2, TXA_2) and the series-4 leukotrienes (ex: LTA_4, LTB_4, etc..). EPA is a precursor to the series-3 prostaglandins (ex: PGE_3, TXA_3) and the series-5 leukotrienes (ex: LTA_5, LTB_5). In general, products derived from EPA are less potent and exhibit a less inflammatory effect than products derived from AA (97). For example, platelets produce the AA derived product

thromboxane A_2, which acts as a vasoconstrictor and stimulates platelet aggregation (98). If EPA is enriched in membrane phospholipids rather than AA, EPA will be released and used to produce thromboxane A_3, which is neither a strong vasoconstrictor or aggregator (**Figure 1-4**) (98). Enrichment of membranes with EPA as a competitor to AA has been suggested for the amelioration of several pathological conditions where AA derived products elicit undesirable effects, such as: rheumatoid arthritis, asthma, cancer, thrombosis, and other conditions involving n-6 eicosanoid stimulated inflammation.

In addition to the competitive effect between EPA and AA for enzymes involved in eicosanoid production, new research indicates that EPA and DHA are precursors to potent derivatives of their own, namely, neuroprotectins (92) and resolvins (93). Resolvins (short for "resolution phase interaction products") are produced from both EPA and DHA and have been shown to have anti-inflammatory properties through their involvement with polymorphonuclear leukocytes (93). Neuroprotectins are derived from DHA and are believed to protect brain cells from "oxidative stress-induced apoptosis" (92).

Function derived from genetic influence

In addition to their structural and eicosanoid functions, n-3 fatty acids have been shown to have substantial influence on gene expression (99). Omega-3 fatty acids act as ligands to several nuclear transcription factors involved in metabolism, such as: peroxisome proliferator activated receptors (PPAR-α, -β, and -γ), liver X receptors (LXR-α and LXR-β), and the retinoic X receptor (RXR)

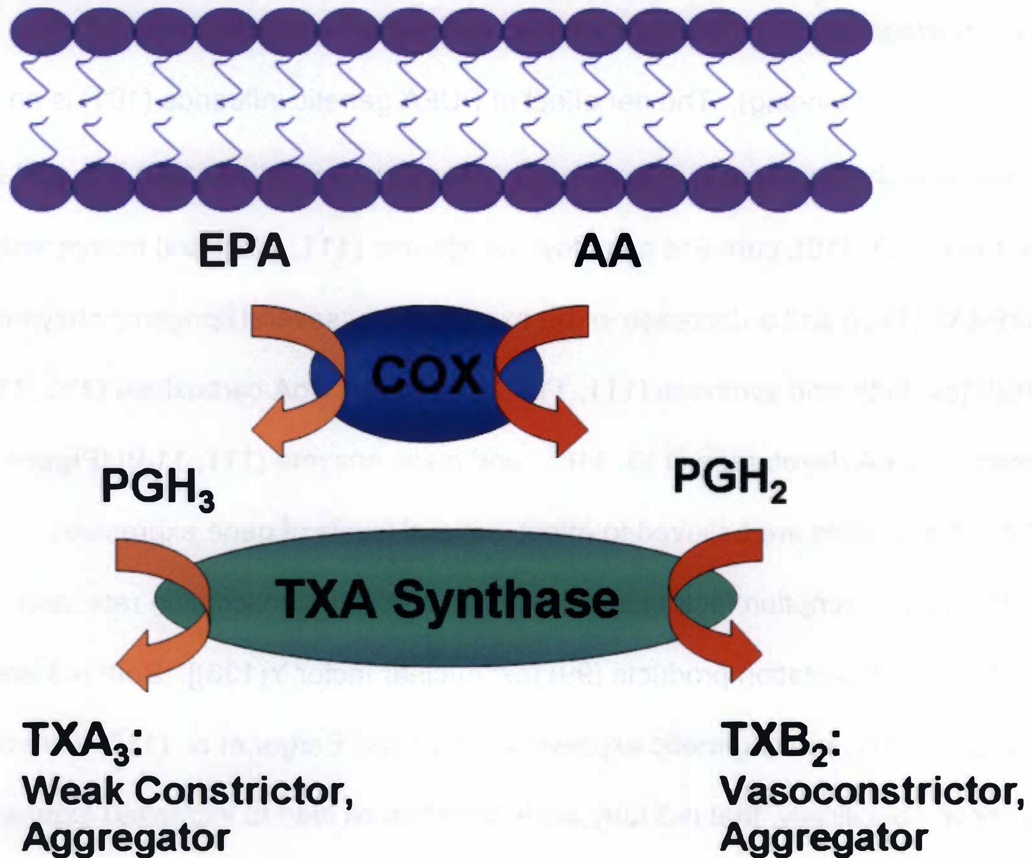


Figure 1-4: Eicosanoid derivatives of EPA or AA in stimulated platelets; Series-2 vs. Series-3 thromboxanes (98, 100).

(101-103). N-3 fatty acids have also been shown to influence (101) other transcription factors, namely, sterol regulatory binding proteins (1a, 1c, & 2) (104, 105), nuclear factor Y (106), and hepatic nuclear factor-4 (107) (though not through ligand binding). The net effect of PUFA genetic influence (101) is an increase in the expression of enzymes involved in β-oxidation (108) [ex: acyl CoA oxidase (109, 110), carnitine palmitoyl transferase (111, 112), and microsomal CYP4A2 (110)] and a decrease in the expression of several lipogenic enzymes (108) [ex: fatty acid synthase (111, 113-115), acetyl- CoA carboxlase (110, 114), stearoyl –CoA desaturase (113, 116), and malic enzyme (111, 113)] (**Figure 1-5**). Fatty acids are believed to effect several levels of gene expression including: transcription factor binding, mRNA stability, transcription rate, and alteration of translation products (99) [ex: nuclear factor Y(106)]. Both n-3 and n-6 fatty acids impact genetic expression; however, Berger et al. (117) were able to show, specifically, that n-3 fatty acids from fish oil lead to increased expression of genes favoring oxidation and decreased expression of genes favoring lipogenesis based on microarray measurement. Gene expression does not appear to be limited to LCn3. In rat brain, ALA, EPA, and DHA were shown to have similar influence on affected genes (118). In addition to the fatty acid effect on gene expression, eicosanoid products of PUFA are also a potential source of influence (119, 120). Mechanisms of n-3 fatty acid effect on gene expression are still being discovered and new findings will help to clarify what health benefits attributed to n-3 fatty acids are due to their genetic influence.

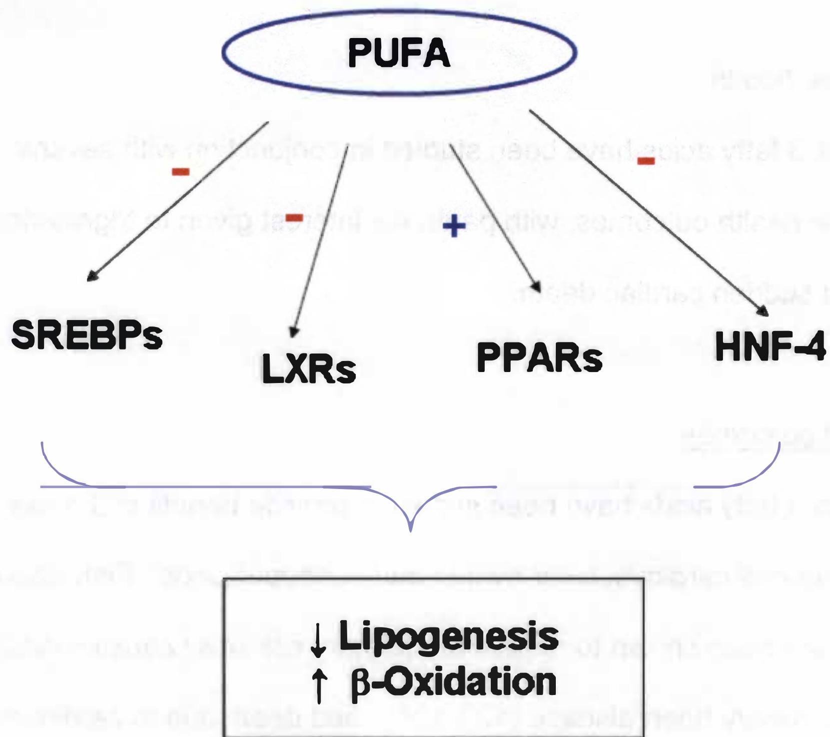


Figure 1-5: Net effect of polyunsaturated fatty acid influence on several nuclear transcription factors involved in lipid metabolism (101, 103, 121).

Benefits of dietary omega-3 fatty acids in humans

Cardiovascular health

Omega-3 fatty acids have been studied in conjunction with several cardiovascular health outcomes, with particular interest given to triglyceride reduction and sudden cardiac death.

General CVD outcomes

Omega-3 fatty acids have been shown to provide benefit in the risk reduction of several cardiovascular events and subpopulations. Fish intake and LCn3 intake has been shown to reduce the primary risk of all cause mortality (122, 123), coronary heart disease (123-125), and death due to cardiovascular disease (124, 126). Fish intake and LCn3 supplementation has been shown to provide secondary risk prevention of all cause mortality (127), death due to coronary heart disease (127, 128), non- fatal cardiovascular events (128, 129), and blood pressure reduction (129, 130). Dietary and supplemental ALA intake has also been implicated in the primary risk reduction of non-fatal myocardial infarction (131, 132), prevalence of coronary heart disease (133, 134), all cause mortality (134), and death from cardiovascular disease (131, 134). Secondary prevention from ALA intake and supplementation provided risk reduction for coronary deaths (135, 136), non-fatal myocardial infarction (128, 135, 136), and all cause mortality (136). In general, however, CVD benefit from ALA consumption is less definitive than LCn3 consumption (137). Along with

dietary and supplemental intake of n-3 fatty acids, the concentration of n-3 fatty acids in serum cholesterol esters and plasma phospholipids has been shown to provide risk reduction for fatal CHD (138, 139) and all cause mortality(139).

Not all studies, however, proved beneficial. Several studies reported no CHD reduction from ALA intake (140, 141) or LCn3 intake (142-144) or benefit in risk of restenosis from LCn3 (145), with one study even showing an increased risk in all cause mortality in those patients encouraged to increase fish consumption (146). Kris-Etherton,et al. attribute some of the inconsistency of outcomes across these studies to differences in the way cardiac events were defined, differences in the populations studied, differences in the measurement of fish intake, or simply variability in study design (147).

Stroke

The effect of omega-3 fatty acids on risk of stroke remains unclear. The etiology of this connection is also unclear, though the anti-thrombotic potential of LCn3 (via a shift in eicosanoid production) is likely a contributing factor. Several studies report a decreased risk based on fish intake or supplemental EPA + DHA (123, 148, 149), with one study showing an impact divide in gender with no effect in men, but a reduction in stroke risk in women who ate fish at least once per week (142). ALA intake has also been shown to decrease stroke risk (132, 135, 140). On the contrary, many studies have also reported either no benefit from fish intake (143, 150) or a trend toward an increased risk of stroke based on fish

intake (150) or supplementation with EPA + DHA (151) or EPA + gamma-linolenic acid (18:3 n-6, GLA) (129).

Sudden cardiac death and arrhythmias

The most consistent and sound correlation between the intake of n-3 fatty acids and cardiovascular health benefit is their effect on sudden cardiac death and cardiac arrhythmias. The precise mechanism by which n-3 fatty acids exert their effect on arrhythmias is not completely understood, however, the current body of evidence centers around two main hypotheses. The first explanation is related to the ability of polyunsaturated fatty acids to modify the electrophysiology of heart muscle cells. N-3 fatty acids increase the threshold for heart muscle action potential and subsequent contraction through their effect on myocyte ion channels (both Na^+ and Ca^{2+}), and this may in part explain their ability to provide stability and anti-arrhythmic benefit to the heart (83, 84). Another possible mechanism is specifically the dietary modification of myocyte phospholipid fatty acid composition by n-3 fatty acids which replaces AA in the myocyte membrane and shifts the potential away from the production of series-2 eicosanoids to series-3 eicosanoids (152). This has been shown to reduce arrhythmia and sudden cardiac death through the reduction of series-2 eicosanoid thromboxane A_2 (153). The majority of both observational studies and intervention studies found a decrease in sudden death with LCn3 intake from diet (122, 125, 154, 155) or supplementation (128, 151). Several studies also showed that ALA consumption is related to a reduced risk of sudden cardiac death (128, 132, 135,

136). One study reported a relative increase in sudden death based on advice to consume fish or fish oil capsules (156), however, this is an exception.

Blood triglyceride reduction

EPA and DHA (157) are consistently shown to reduce circulating triglyceride levels by 10-33% (158). Several mechanisms are believed to be involved in this reduction, including: a decrease in hepatic triglyceride synthesis via reduction of activity in enzymes involved in fatty acid and triglyceride synthesis; decreased secretion of triglyceride from the liver as VLDL; and an increase in peripheral lipoprotein lipase which results in an increase in triglyceride clearance from the blood (159-161). Fasting plasma triglyceride levels greatly effect the post-prandial rise in triglyceride concentration (160) and potential decrease with dietary n-3 fatty acid consumption, with higher basal triglyceride levels resulting in a greater potential reduction (158). Though triglyceride level itself has not emerged as a consistent independent risk factor for CVD, it has been suggested that the interdependent nature of triglyceride, LDL, and HDL as lipid carriers may mask the independent role of triglyceride levels (160). Increased dense LDL levels (162) and reduced HDL levels (163), both risk factors for CVD, are partially dependent on triglyceride levels and may actually prove a concurrent or secondary event to elevated triglyceride levels.

Cardiovascular summary

Though results and significance vary, it is generally accepted from many large studies that n-3 fatty acid intake (particularly LCn3) offers a beneficial effect through the reduction of specific cardiovascular health outcomes such as CVD mortality, sudden death (cardiac arrest), and myocardial infarction (137). It is also noteworthy that although the results of studies using LCn3 or ALA are varied and do not lend easily to clear cut recommendations, the potential benefits of increased ALA and LCn3 intake outweigh the slight, if any, potentially negative outcomes (137).

Cancer

At the present time, evidence supporting a beneficial role for n-3 fatty acids in the prevention and treatment of a variety of cancers is much stronger in animal models and cell culture studies than in human epidemiological studies (95). Benefit of n-3 fatty acids has been reported with human breast (164), colorectal (165-167), pancreatic (168, 169), lung (169-171), prostate (172-174), and skin cancer (175, 176), however, the conclusions have not always been consistent and the overall effect of n-3 fatty acids and cancer in humans remains uncertain. Several plausible mechanisms for potential benefit have emerged from cell and animal studies, with inhibition of AA derived eicosanoids representing the most promising explanation of benefit (177, 178). AA derived PGE_2 production has been implicated in several key pathways that promote proliferation of cancer cells, growth of a capillary support system (angiogenesis),

and metastasis of cancer cells to other tissues (179, 180). Other potential mechanisms include: inhibition of tumor proliferation through the agonism of PPARγ, reduction of NFκB activation and TNFα expression, reduction in *ras* oncogene induction, COX-2 supression, reduction in cytochrome p450 aromatase activity resulting in reduced estrogen production, and upregulation of antioxidant enzyme systems contributing to the amelioration of reactive oxygen species as potential pro- tumorigenic mutagens (178, 181). One of the potential reasons for contradictory findings between animals and humans may be that the doses given in animal studies looking at the association between n-3 fatty acids and cancer exaggerate the likely effects in humans (178). This problem emphasizes the motivation of the current study to determine "human equivalent" n-3 fatty acid doses for animals that can offer better predictive value to anticipated effects in human trials.

EPA intake has also been implicated as a treatment option for cancer related cachexia (182). Though initial findings looked as though EPA may improve appetite, weight status, and possibly even survival (183, 184); larger comparative trials have not shown any significant improvements (185-187) with administration of EPA.

Inflammation

Several disease states that have an underlying inflammatory component may benefit from the potential anti-inflammatory action of n-3 fatty acids. These include: cardiovascular disease (188), rheumatoid arthritis (189), air-way

inflammation (asthma), systemic lupus erythematosis, and inflammatory bowel disease (190). Eicosanoids derived from AA act as stimulants of inflammation and systemic immune response through cyclooxygenase and lipoxygenase products, particularly, PGE_2 and LTB_4 (191). EPA competes as a substrate for COX and LOX and the resultant 3-series prostaglandins and 5-series leukotrienes are less inflammatory than the AA derived products. In addition to modifying eicosanoid production, fish oil supplementation has been shown to suppress the expression of IL-1β (192-195), TNF -α (194, 195), and IL-6 (194), with ALA supplementation also decreasing IL-1β (195), TNF-α (195), and IL-6 expression (196). Habitual intake of EPA + DHA were also shown to be inversely associated with the expression of plasma TNF receptors (8). Though the ability of n-3 fatty acids to attenuate the inflammatory and immune response is desirable in these disease states, it should also be noted that a reduction in immune response elicited by n-3 intake may be an unwanted outcome in those individuals fighting an infectious disease (197), and particularly those who are already immunocompromised (ex: the elderly).

Diabetes

N-3 fatty acids do not appear to offer significant beneficial effects for the treatment of diabetes mellitus. A recent report investigating the effect of n-3 fatty acids on several cardiovascular disease risk factors concluded that n-3 fatty acids had no significant effect on hemoglobin A_1C levels, and had no consistent effect on fasting blood sugar or fasting insulin levels (158). While impaired

glycemic control has been reported in some studies with high supplemental n-3 doses (198, 199), n-3 fatty acids are generally found to have no significant effects on glycemic control (200-206), including an18 study meta-analysis which found no significant effects of n-3 fatty acid supplementation on fasting glucose or hemoglobin A_1C levels in Type 2 diabetics (207).

Psychological disorders

The high levels of DHA found in brain tissue coupled with the discovery that n-3 fatty acid status in various tissues (mainly RBC PL) is decreased in schizophrenia (208), adult (209) & childhood (210) attention-deficit/hyperactivity disorder, and depression (211, 212), has stimulated research looking at possible connections between n-3 fatty acid intake and several psychological disorders. Supplementation has been found to improve objective measures of unipolar depressive disorder (213), dark adaptation in dyslexia (214), and has been reported to lengthen the remission of symptoms in patients with bi-polar depression (215). In general, the majority of studies in schizophrenics have also shown a beneficial effect from EPA supplementation (216) when compared to placebo controls (217-219) with few exceptions (220). N-3 fatty acids have also been suggested for attention-deficit / hyperactivity disorder and other childhood developmental disorders, though two recent placebo-controlled trials supplementing DHA did not report an improvement in ADHD symptoms (221, 222), with another study reporting no improvement in physical aggression in children who were supplemented with EPA + DHA (223). An interesting

hypothesis put forth by Hibbeln et al., suggests that mortality from coronary heart disease and depression increases across several countries as n-3 fatty acid intake declines (224, 225). This further potentiates the role of n-3 fatty acids in psychological phenomena and this area of research calls for large scale definitive trials for conclusive answers.

Pre and postnatal development

Increased understanding of the important role that n-3 fatty acids (particularly DHA) play in brain and retina development has stimulated many studies examining the effect of n-3 supplementation during different stages of development, for example, the impact of maternal diet on prenatal development and the mode of feeding (breast milk, standard formula, or formula with added long chain PUFA) on postnatal development in term and pre-term infants. These studies were recently reviewed by an expert committee workshop of the Child Health Foundation (226) producing recommendations for maternal and infant n-3 intake based on available evidence of potential benefits. Thus far, it is generally considered unnecessary for pregnant women to supplement n-3 fatty acids (226), though it is encouraged that they include DHA rich foods in their diet. Attention to n-3 fatty acid status may prove particularly beneficial in specific subgroups of pregnant women, such as vegetarian women and women who are pregnant subsequent to a first birth (based on the finding that DHA status is best in the first child and maternal stores of DHA decline in subsequent pregnancies (59)). After birth, it is highly encouraged that infants are breast fed (226). It is also

encouraged that infants who are not breast fed receive formula supplemented with AA and DHA (226, 227), with higher concentrations of AA and DHA for preterm infants (226), based on a greater potential for benefit of supplementation in preterm infants (53, 64, 228, 229).

Safety of fish consumption

Parallel to increasing public and scientific interest in n-3 fatty acid benefits, regulatory agencies such as the Environmental Protection Agency and the FDA have expressed concern touting the link between fish consumption and methylmercury exposure. As a by-product of industrial emissions and volcanoes, mercury finds it way into water bodies through environmental precipitation (230). Predatory fish become the main repository of mercury accumulation through successive concentration at each level of the food chain (231). Nervous system tissue is the most vulnerable to mercury exposure, particularly fetal neural tissue (232). Research findings have been contradictory as to the impact of mercury from fish consumption on possible heart related benefits. Guallar et al. (233), found an association between mercury level in toenail clippings and risk of myocardial infarction incidence in men, while Yoshizawa et al. (234), did not find an association between mercury levels and incidence of coronary heart disease. Current advisories suggest that women who are planning a pregnancy or are currently pregnant or lactating and young children should avoid four types of fish with the highest mercury content: tilefish, swordfish, king mackerel, and shark (235), while other demographic groups

should balance fish intake between those with high and low mercury content (**Table 1-4**). It is likely that potential harm from mercury exposure based on current fish consumption patterns is outweighed by the potential benefits, particularly for those who are at risk of coronary heart disease (9, 230, 231). An alternative source of n-3 fatty acids is refined fish oil supplements, which have very low mercury content (231). More research is needed to fully understand the potential risk of mercury exposure and possible counterproductive impact on cardiovascular benefits provided by fish consumption.

1.3 Animal model research and omega-3 fatty acid supplementation

Animal models in research

According to the National Research Council (236), an animal model is "a living organism in which normative biology or behavior can be studied, or in which a spontaneous or induced pathological process can be investigated, and in which the phenomenon in one or more respects resembles the same phenomenon in humans or other species of animal (as modified in (237))." Several animal models are currently used in biological research, including: mice, rats, hamsters, rabbits, guinea pigs, monkeys, dogs, and swine. Animal models are not intended to perfectly mimic human function (238-240), but to provide a

Table 1-4

FDA/ EPA recommendations for fish intake[1]

Fish to avoid	**Fish to consume in limited quantities** (up to 6 ounces/wk.)	**Fish to consume in limited quantities** (up to 12 ounces/wk.)
Shark	Canned Albacore "White" Tuna	Canned Light Tuna
Swordfish		Sea Bass
King Mackerel		Gulf Coast Oysters
Tilefish (a.k.a. Golden bass, Golden snapper)		Marlin
		Halibut
		Pike
		Walleye
		White Croaker
		Largemouth Bass
		Mahi Mahi
		Blue Mussel
		Cod
		Eastern Oyster
		Channel Catfish (wild)
		Great Lakes Salmon
		Gulf Coast Blue Crab
		Lake Whitefish
		Pollack

Fish lowest in methyl mercury: Catfish (farmed), King Crab, Flounder (summer), Scallops, Trout (farmed), Salmon (wild Pacific), Shrimp, Tilapia, Sardines

[1] Recommendations are for women who are/ may become pregnant, are nursing, and small children.

"surrogate" (241, 242) that is often better controlled and better able to answer narrow research questions than could be determined from highly variable human activity (243). Specimens from inbred animal strains (ex: mice) used in research are almost genetically identical (244), thus eliminating much of the inherent genetic variability found in human studies performed on mixed populations. The choice of a particular animal model depends on the research question that is being investigated and how closely a species matches humans in the mechanism of inquiry (243). Selection of the appropriate animal model is also based on criteria such as: availability of a particular model to many investigators, handling ability of a particle animal, multiparous reproduction, adequate yield of specimens necessary for assessment (245), and precedence of research using a particular model in the field of study (246). In addition, proliferation of genomic research in recent years has increased the selection of models based on known genetic homology between humans and animals (243).

Advantages of the murine model

There are several advantages to using the mouse model in nutrition research. Mice are generally inexpensive to purchase and feed (though cost depends on genetic strain and diet selection). Small size allows for caging of multiple animals in groups and requires less space than other models (247). Mice reproduce quickly (247) and are multiparous (246), which has contributed to the formation of several inbred strains for purchase, which are nearly 100% homozygous (246). The wide use of rodents over the last century has produced

a plethora of publication precedence, making the mouse model more trusted than newer, less documented models. In recent years, both the mouse (248) and human genome (249) have been categorized, revealing a relative 70-90% genetic homology between mice and humans (250). This has made mice a particularly useful model for studying human biological processes that have been conserved between the two species (244, 251). Specifically, mice are useful for n-3 fatty acid research because the same fatty acids are essential in mice and humans. Mice also metabolize fats through the same pathway and house the same enzymatic machinery for polyunsaturated fat processing as humans (66).

Disadvantages of the murine model

Few disadvantages exist in the use of the mouse for nutrition research. In reference to their use in lipid metabolism, it has been reported that $\Delta 5$ and $\Delta 6$ desaturase activity in rodents is greater than the rate of human desaturases (252, 253). This detail is sometimes cited in an attempt to discredit the use of the mouse model as a surrogate for human fatty acid metabolism. However, expectation and quantification of this rate difference should not hinder reasonable extrapolation to humans from murine data. It has also been shown that DHA levels in several human tissues are reduced compared to rodents (254), further suggesting a possible difference in conversion capacity. Another possible disadvantage is that mice are not considered a high fidelity model for certain human diseases such as the development of atherosclerosis. This is due to the difference in predominant cholesterol carrying lipoproteins between

rodents and humans (255). This is also based on the resistance of rodents to dietary fat induced atherosclerosis (255).

Design improvements for murine model research with omega-3 fatty acids

Choice of control/background diet

We have observed that one of the significant methodological problems with rodent studies supplementing n-3 fatty acids is the use of a control (background) diet that is not representative of typical human consumption patterns. When supplemented groups are compared to control diets that are devoid of long chain polyunsaturated fatty acids EPA, DHA, and AA, the results will likely demonstrate exaggerated increases of n-3 fatty acids in a variety of tissues due to the lack of metabolic constraints enforced by the presence of other LCn3 and AA in the control diet. We believe the solution to this problem (as detailed below and in Chapters 2 and 3) involves the calculated addition of all polyunsaturated fatty acids in a manner that is representative of their relative proportion in the typical (Western) human diet. It has been suggested that, "...if the (experimental) design inadequately represents the 'normal' life conditions of the target species, inaccurate conclusions may be drawn, regardless of the value of the model itself" (240). When applied to nutrition studies, this statement highlights the need for the diet design of the model species (in this case, mice) to model the diet of the target species (humans) in order for accurate conclusions to be drawn from the analysis.

Relevant dosing

A recent survey of general studies using supplemental n-3 fatty acids in rodents shows that current supplemental doses are not representative of reasonable expected human consumption (**Table 1-5**). Standardization of supplemental n-3 fatty acid doses in mice that represent a “human equivalent dose” is necessary if animal studies are to have true predictive value for humans. The precedent for change, in part, comes from observations in toxicological studies utilizing, “physiologically based pharmacokinetic” (PBPK) modeling (256) (also called “biologically based dose-response (BBDR) (257, 258)). Physiologically based modeling is meant to reduce extrapolation of dosages between two species to a mathematical model (257) that is based on the metabolism of the given substance in each species. Building on this concept, it is feasible that a mathematical model describing the expected influence of dietary n-3 fatty acids on the same endpoints in different species can be derived. Below, we introduce a simple mathematical model for the standardization of supplementation that allows for direct comparison of biological endpoints between rodents and humans. We have put forth a rudimentary, easy to use, estimation of this model with the knowledge that a more precise estimation in the future may be necessary.

TABLE 1-5

Review of supplemental doses of omega-3 fatty acids given in recent rodent studies.

Study	ALA	EPA	DHA	HED[1]		
	-------- *energy %* --------			*grams*		
				ALA	*EPA*	*DHA*
Cognault et al.(259)	16.8			37.3		
Massiera et al. (260)	5.7			12.7		
Miyazawa et al. (261)	3.6			8		
Mori et al. (262)	23.2			51.6		
Morise et al. (263)	13.8			30.7		
Takemura et al.(264)	11.4			25.3		
Saito et al. (265)	8.2	7.8	7.6	18.2	17.3	16.9
Oarada et al.(266)	16.1	5.1	3.3	35.8	11.3	7.3
Ide, et al.(111)	16.7	2.9	8.6	37.1	6.4	19.1
Kim et al.(267)	10.6	1.2	6.2	23.5	2.7	13.8
Takahashi et al. (268)	23.9	3.8	12.4	53.1	8.4	27.6
Moison et al.(269)		6.9	4.0		15.3	8.9
		10.2	7.5		22.7	16.7
Choi-Kwon et al. (270)		3.5	2.3		7.8	5.1
Joshi et al. (271)		2.4	1.7		5.3	3.7
Akisu et al. (272)		3.4	2.3		7.6	5.1
Oarada et al. (273)		5.4	5.4		12	12
Watanabe et al. (274)		5.4	5.4		12	12
Wang et al. (275)			9.7			21.6
Cha et al. (276)			2.6			5.8
			6.5			14.4

[1] Human Equivalent Dose based on energy% of a 2000 calorie diet.

Significance of omega-3 fatty acid form

The media as well as medical professionals often propagate a blurred distinction between the expected attributes of ALA and those of LCn3, EPA, and DHA. N-3 fatty acids are lumped together as a unified entity and even prescribed almost interchangeably. The benefits reviewed above show that there is a much greater research base supporting a beneficial effect of LCn3 intake than for ALA. We have attempted to further clarify this difference in the context of an experimental design with background long chain fatty acids and supplemental doses that better represent human intake. This comparison is necessary to demonstrate the individual effects of ALA and LCn3 on tissue accumulation, conversion to metabolites, and influence on pertinent endpoints.

1.4 Summary

Jon R. Held summarizes the purpose of animal model research as follows, "The whole history of biomedical research tells us the countless benefits to humans obtainable by asking the right questions of the right animal model (237)." We believe that the validity of n-3 fatty acid research in the rodent model extends only as far as the design of the experiment. Supplemental studies in animals are by nature wrought with many complex associations between the diet of man and the murine diet. In order for the "right question" and the "right animal model" to yield meaningful answers, methodological changes are necessary in the way that n-3 fatty acid research in rodents is performed.

1.5 Research Objective

1. To develop a hypothetical diet model for the study of n-3 fatty acids in rodents that allows for better comparison of mouse and human endpoints.
2. To clarify the relative impact of n-3 fatty acid form and amount on measurable biological endpoints.

Specific aims

1. To test the use of energy% as a model for the addition of fatty acids to rodent diets with comparison to archival human data.
2. To compare the effect of ALA and EPA on plasma and erythrocyte phospholipid fatty acid content when these fatty acids are given at equivalent supplemental levels in the context of a background diet that contains long chain PUFA (n-3 and n-6).

2 RELEVANT DOSING FOR ANIMAL MODELS: A SCALING APPROACH

2.1 Introduction

The design and implementation of dietary research with animal models requires thoughtful examination of interspecies variation. In order for data achieved from these studies to be used for meaningful extrapolation, levels of supplemental nutrients must be a reasonable representation of what can be consumed by humans. Are we considering these interspecies differences when we design dietary studies, or has the use of animal models become so widespread that we have overlooked the need for calculated extrapolation? If dietary animal studies are to have true predictive value for anticipated results in humans, we must stretch beyond the scope of nutrition and adopt an integrative perception of animal modeling that considers the discoveries of parallel disciplines.

A natural starting point for this integration is the study of basal metabolic rate and its relationship to bodyweight in mammals. The analysis of metabolic rate has been one of the most prevalent examples of interspecies comparison over the last century. In 1932, Max Kleiber, well known pioneer in the field of animal energetics, discovered that over a large range of animal size (from rats to oxen) linear regression of the logs of weight and metabolic rate produced a ¾ exponent (277-279) based on the following equation:

$$Y = ax^b$$

Where Y is metabolic rate in kcal, x is bodyweight in kilograms, a is the proportionality constant, and b is the scaling exponent and the slope of the regression line depicting this relationship (280). Kleiber's discovery differed from a long held reliance on ⅔, which was the anticipated exponent from geometric evaluation of surface area and volume alone (281, 282). Shortly after Kleiber's discovery, Brody et al. (283), added more mammals to Kleiber's analysis and deduced the same scaling exponent, ¾ , for animals ranging in size from a mouse to an elephant, yielding the often cited "mouse to elephant curve". However, ¾ scaling was not without its dissenters. For a variety of reasons, several researchers have held firm to ⅔ scaling (284-288).

In the past decade, there have been many interesting attempts to expand our understanding of why Kleiber's ¾ scaling may exist in nature. West et al. (289-293), have presented several papers detailing the physical constraints of "hierarchical branching networks" within organisms that dictate a ¼ (or multiple thereof, including ¾) scaling between many biological variables and bodyweight. This theory was tested in mammalian species, birds, plants, unicellular organisms, sub-cellular fractions (mitochondria), and enzymes; and the conclusion was made that metabolism is confined to the dimensions of circulation and circulation is dictated by the same fractal geometric confines from one species to another. Other views supporting ¾ scaling include:

- A "multiple cause model of allometry"(294) that takes into account every ATP utilizing and generating construct within the biological system that

contributes to metabolic rate (Ex: organ size, hormonal state, temperature, pressure, ecological parameters, etc…) (295-297)

- An ecological perspective (298), in which the relationship between metabolic rate and body size is examined based on the influence of geographical region of evolution.
- The use of geographical food productivity and abundance (299) to determine the influence of food availability in different regions on metabolic rate.

Figure 2-1 summarizes some of the major theories used to explain the scaling of metabolic rate between animal species and how this fits into the scheme of dietary intake.

Three-quarter power scaling in nature is an example of how comparative physiology can be used to guide the design of animal model research, and particularly, the dosing of supplemental components in dietary studies using animal models. Currently, no clear standard of practice drives the derivation of nutrient doses in dietary animal model research. Doses are conceived based on various methods such as: comparison of weights between animals and humans, duplication of doses from similar studies, and in some cases, dosing values derived merely to maximize the potential for positive results without conscious interspecies evaluation. Therefore, we set out to establish a mathematical model that could be used as a guideline for choosing levels of n-3 PUFA in rodent diets that would facilitate reasonable extrapolation to humans. In order to be useful,

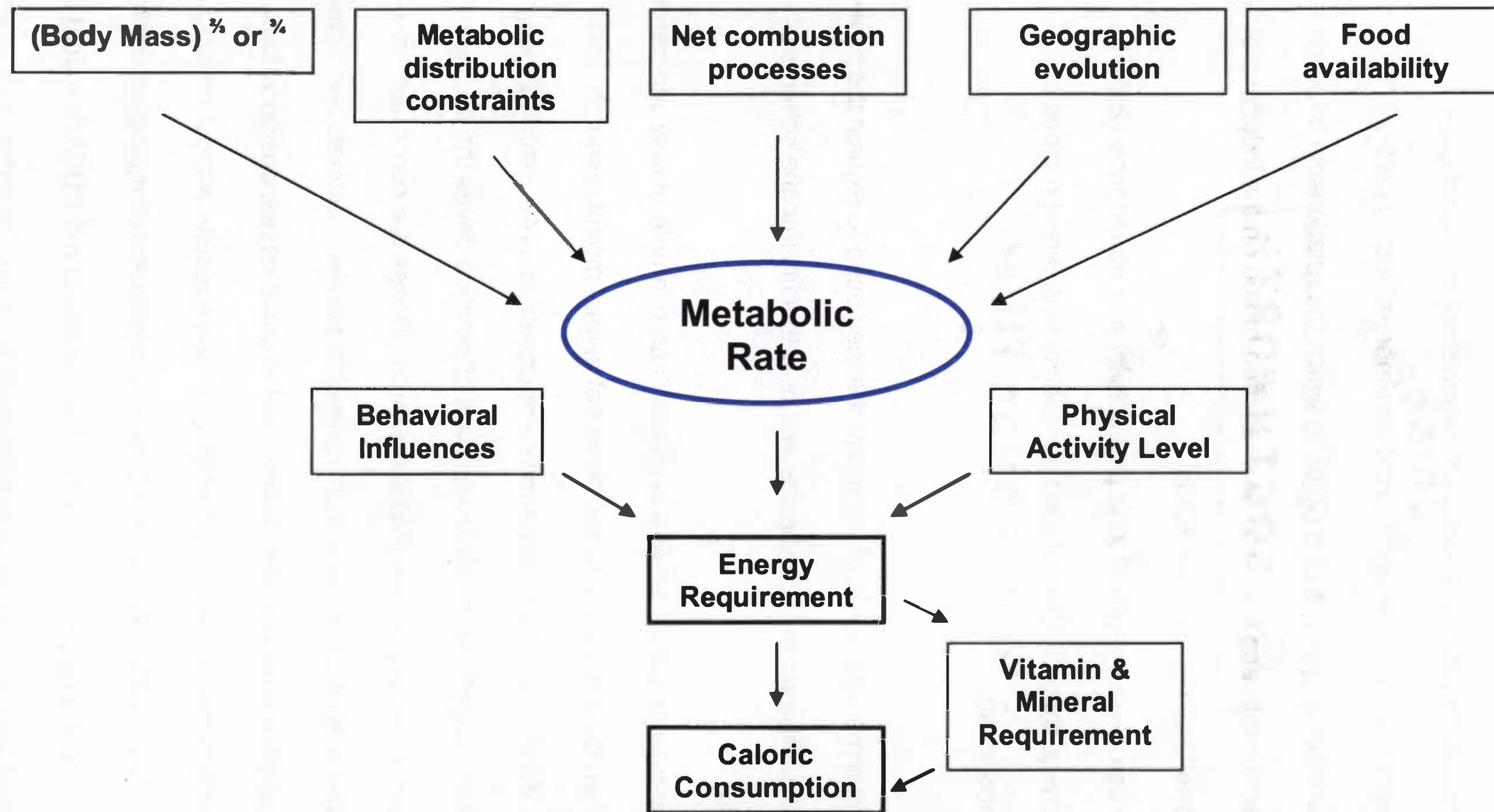


Figure 2-1: Interconnection of metabolic rate scaling theories and effect on nutrient requirement and intake.

this model should: linearize the relationship between human and rodent data; be applicable to the general range of n-3 fatty acid studies; be tested on the basis of its ability to yield similar biological effects in animal models and humans; and most importantly, be simple to use.

Because supplemental components in dietary studies either have caloric value or are dependent on the difference in metabolic rate between species, we suggest that a model based on consideration of daily energy consumption in rodents and humans may offer a means of dose standardization. The comparison of daily energy consumption between two organisms (ex: between mouse and man) should be an indirect picture of the differences in digestion, absorption, energy expended in activity, metabolic rate, and nutrient needs between the two organisms, particularly if they are in energy balance with no net weight gain or loss. Supplementation based on energy consumption is supported by Rucker et al., who suggest, *"… when making interspecies comparisons from a nutrition perspective, the strongest case is made when a measure of metabolic body size or food intake, rather than bodyweight is used to extrapolate the dosages required for a given response"* (300). Rucker also points to the inherent similarity between caloric needs and water-soluble vitamin requirements in homeotherms (301). Since vitamin requirements and caloric intake are under similar biological controls, a "human equivalent dose" model should be able to predict both the supplementation levels in animals that would match human intake and should also be able to predict human vitamin requirements from known animal vitamin requirements. Vitamin and mineral

intake recommendations have been made for both rodents and humans, and loosely represent perceived requirements. These known recommendations offer a means to test the predictability of scaling theories between rodents and humans.

2.2 Methods

Several published works have established the nutrient needs of humans (302-306) and mice (307, 308). We used this information as a means to test the ability of the following hypothetical models to predict the Dietary Reference Intakes for humans from mouse requirements when used as a mathematical conversion factor:

A. Bodyweight (in kilograms)

B. [Bodyweight (Kg)]^ ¾

C. Daily energy consumption (% energy).

Derivation of the above scaling factors for mouse to human comparison is detailed in **Table 2-1**. Briefly, human weight (Kg), [weight (Kg)] ^ ¾, and daily calorie intake was divided by the same data for an average mature mouse to produce a conversion factor based on each hypothetical scaling model (**A-C**). Established murine vitamin and mineral intake recommendations were multiplied by each conversion factor and compared to the Dietary Reference Intakes of each nutrient for humans. The levels of linoleic acid (LA) and α-linolenic (ALA) acid present in the typical daily intake of rodent diet were also used to test the

TABLE 2-1

Derivation of conversion factors

	Mouse[1]	Human	Human / Mouse
Bodyweight (Kg)	0.025	70	2800
[Bodyweight (Kg)]^ ¾	0.063	24.2	384.8
Daily caloric intake	14.4	2000	138.89

[1] Based on a C57BL/6J adult mouse, consuming 4gms/day AIN93M diet (307).

predictive value of each factor to estimate human adequate intake (AI) of LA and ALA.

2.3 Results

Table 2-2 details the use of each conversion factor to estimate the Dietary Reference Intakes for vitamins, minerals, and fatty acids (LA and ALA) from known mouse nutrient requirements (307). In comparison to the factors based on weight (Kg) and [weight (Kg)] ¾, the factor for energy% best predicted the DRIs for 12 of 12 vitamins and 9 of 12 minerals. The minerals in which energy% was not the best scaling predictor were sodium, potassium, and chloride. The estimations based on energy% were also the best predictor for the AI of LA and ALA in humans.

TABLE 2-2

Scaling of daily nutrient requirements for mice to human Dietary Reference Intakes [1,2]

Nutrient	Daily Requirement	Scaling Factors				
		Body Weight (Kg)[3]	[Body weight (Kg)]^.75 [3]	% Energy[4]	RDA/AI[5]	
VITAMINS						
Thiamin	0.0214	59.9	8.2	3.0	1.2	mg
Riboflavin	0.024	67.2	9.2	3.3	1.3	mg
Vitamin B6	0.0233	65.2	9.0	3.2	1.3	mg
Niacin	0.12	336	46	17	16	mg
Folate	8	22,400	3079	1111	400	µg
Biotin	0.8	2240	308	111	30	µg
Vitamin E	0.201	563	77	28	15	mg[6]
Vitamin D	3	8400	1155	417	5	µg
Vitamin A	4.8	13440	1847	667	900	µg[7]
Vitamin K	3	8400	1155	417	120	µg
Vitamin B12	0.1	280	38	14	2.4	µg
Pantothenate	0.0586	164	23	8	5	mg
MINERALS						
Calcium	19.99	55972	7695	2776	1000	mg
Phosphorus	7.98	22344	3072	1108	700	mg
Potassium	14.40	40310	5542	2000	4700	mg
Sodium	4.11	11505	1582	571	1500	mg
Chloride	6.29	17614	2421	874	2300	mg
Magnesium	2.026	5673	780	281	400	mg
Chromium	8	22,400	3079	1111	35	µg
Copper	24.2	67, 620	9,296	3,354	900	µg
Iodine	0.83	2,325	320	115	150	µg
Iron	0.14	392	54	19	8	mg
Selenium	0.6	1,680	231	83	55	µg
Zinc	0.12	337	46	17	11	mg

TABLE 2-2

Continued

Nutrient	Daily Requirement	Scaling Factors			RDA/AI[5]	
		Body Weight (Kg)[3]	[Body weight (Kg)]$^{.75}$ [3]	% Energy[4]		
FATTY ACIDS						
α-linolenic acid	0.011	30.8	4.2	1.6	1.6	gm (302)
Linoleic acid	0.086	242	33	12	15	gm (302)

[1] Source: (302-306, 308)
[2] Based on 4 grams (average daily intake) of AIN93M purified rodent diet.
[3] Based on a 70 Kg human and 25 gram mouse.
[4] Based on 14.4 calories (average daily mouse intake based on 4 grams AIN93M diet) (307) and 2000 calorie human diet.
[5] Based on 30 year old male
[6] α-tocopherol
[7] µg retinol

2.4 Discussion

Selection of appropriate doses for supplemented nutrients in animal studies is a common challenge faced by nutrition researchers. Extrapolation of animal model data to humans necessitates the use of supplemental doses that are both biologically relevant and reasonable for human intake. However, there are currently no standards of practice guiding researchers in the addition of dietary components in animal studies other than precedent. In the words of Yates, et al., *"We need principles of data extrapolation to justify transforming data from one system (species) into data about another, different system. We need to know what scaling principles can guide the designs of our experimental protocols"* (309). The tentative search for a "human equivalent dose" lies at a complex intersection of diverse fields of insight, from comparative physiology and evolutionary biology, to bioenergetics and theoretical mathematics. It is from this intersection, with consideration and input from this diversity, that we present a simplified approach to relevant nutrient dosing in animal studies.

Dietary recommendations for animals are formulated to meet daily dietary requirements and to avoid un-utilized excesses. Diets are formulated to provide levels of nutrients to support growth, reproduction, lactation and maintain health. As such, micronutrient and amino acid compositions of these diets are well characterized and defined. Any deviation from recommended nutrient levels requires appropriate justification. This is not the case with dietary fats. While the lipid content has been standardized in rodent diets to provide either corn oil at 5% (w/w) (eg., AIN76A) (239) or soybean oil at 4% or 7% (w/w) (AIN93M or

AIN93G, respectively) (307), liberties are taken with fatty acid compositions that appear to lack reasonable justification; such as replacing all the corn oil in a diet with pure coconut oil to simulate a high saturated fat diet. This is particularly true with studies evaluating the dietary effects of n-3 PUFA. For example, a random search of recent literature reveals the large variation of levels of n-3 PUFA that are provided in rodent diets, primarily at the expense of corn oil. These intakes, in the form of ALA, EPA and/or DHA, ranged from 2.6-40 energy% (**Table 1-5**). This is in comparison with typical human intakes of 0.5 and <0.09 energy% for ALA and EPA+DHA, respectively (6-8). Will these levels produce biologically relevant data if they are incompatible with human consumption? While the uses of PUFA are tailored for the objectives of each study, it seems reasonable that guidelines framing appropriate levels are needed, with justification warranted when unusual deviations from these guidelines occur.

Recently, Rucker and Storm (300) discussed approaches that have been used to extrapolate nutrient intakes to that of humans. Their paper plotted the $\log_{10}$ of body weight (Kg) against the $\log_{10}$ of nutrient intake (mg) in 5 different species, including humans. In doing so, metabolic differences of species were taken into account and the relationship of nutrient intake versus body weight for each of the species became linear. Similarly, we are interested in establishing a mathematical model that provides the foundation for allometric scaling of dietary PUFA intake between rodent and humans.

Evaluation of several different conversion factors lends credence to a dosing model based on energy% as an appropriate factor for dietary components

between species. Though energy % is not a precise match for every nutrient, overall, it produces a better match to human nutrient recommendations than factors based on bodyweight (Kg) or [bodyweight]^ ¾. Precise mathematical modeling of the relationship between an animal's nutrient needs and the DRIs is beyond the scope of this paper, however, we believe that allometric scaling based on an energy % factor produces a better model for derivation of a "human equivalent dose" than other methods currently employed. In order to further test the predictive value of energy% dosing in mice and humans, we have also tested this hypothesis in the form of a pharmacodynamic experiment with omega-3 fatty acid supplementation and hematological endpoints in both species (Chapter 3).

2.5 Conclusions

We suggest that adding dietary components on the basis of energy % is an efficient way to narrow the gap between human intake and rodent intake. It is our hope that the presentation of this model will stimulate necessary thought and debate concerning the supplemental levels that are currently used in dietary studies. Ideally, dietary studies of the future will represent a true integration of theoretical comparative physiology and nutrition science. The issues raised here are only the beginning.

3 HUMAN EQUIVALENT DOSE MODELING FOR OMEGA-3 FATTY ACID SUPPLEMENTATION IN C57BL/6J MICE

3.1 Abstract

The rodent model is often used to study the impact of dietary n-3 fatty acids on a variety of biological endpoints, and the results of these studies have been used to explain anticipated effects of n-3 fatty acid intake in humans. However, supplemental levels of n-3 fatty acids that are commonly used in rodent studies do not represent reasonable human intake, by comparison. Currently there is no standard method for the addition of n-3 fatty acids to rodent diets. We tested a mathematical model for dosing supplemental levels of α-linolenic acid (ALA) and eicosapentaenoic acid (EPA) to rodent diets on the basis of energy%. C57Bl/6J mice were fed a background diet that modeled typical Western intake in both macronutrient and fatty acid composition. Three levels of ALA and EPA (0.3, 0.8, and 1.4 energy%) were supplemented to either a normal-ALA control diet (0.6 energy% ALA) or a high-ALA control diet (1.2 energy% ALA). Plasma and erythrocyte phospholipid fatty acid changes were determined and compared to archival human n-3 fatty acid supplementation studies reporting the same tissue endpoints. In mice, supplemental EPA had a greater effect than supplemental ALA on both plasma and erythrocyte phospholipid EPA. Docosahexaenoic acid (DHA) levels in mice were only minimally changed by either ALA or EPA supplementation. Use of the high- ALA control diet resulted in attenuated phospholipid fatty acid changes in both tissues compared to the

normal-ALA control diet for both supplemented fatty acids. At each supplemented dose of ALA or EPA, changes in murine plasma or erythrocyte phospholipid EPA exceeded changes observed in the same human tissues by 2-4 fold when compared to equivalent human supplemental doses in energy%. Tissue changes observed using the high-ALA control diet better modeled the results observed in humans at the same supplemental energy% for both ALA and EPA in plasma and erythrocyte phospholipids. This is the first study to use pharmacodynamic modeling to compare the effect of supplemental n-3 doses on mouse and human endpoints. The addition of n-3 fatty acids to rodent diets on the basis of energy% represents a reasonable improvement to current dosing strategies. This data is useful, both as a guideline for n-3 fatty acid dosing in rodent studies and as a reference point for future calculated refinements in dosing.

3.2 Introduction

Recently, we suggested the use of energy% as a model for lipid dosing in dietary research using the rodent model. Comparison of rodent nutrient requirements and human intake recommendations reveal that an energy% factor has better predictive value for estimating human requirements from animal requirements than either weight (Kg) or (weight (Kg))$^{3/4}$ (Chapter 2). The current study is designed to further test the applicability of an energy% model based on the comparison of common biological endpoints in mice and humans. Energy% is used as a tool to formulate a “Western” background diet as well as

supplemental doses of two common n-3 fatty acids: α-linolenic acid (18:3n-3, ALA) and eicosapentaenoic acid (20:5n-3, EPA). By capturing corresponding mouse and human endpoints and standardizing the supplementation values on the basis of energy%, we offer a touchstone for anticipated effects at these doses and a template for further investigation in the quest for a "human equivalent dose".

3.3 Materials and Methods

Animals

Male, C57BL/6J mice (The Jackson Laboratory, Bar Harbor, ME) were used in the following experiments. All mice were received at 35-42 days of age and housed 5 per cage. Animals were given free access to food and water and were maintained in a climate controlled facility with programmed light and dark cycle. Mice were checked daily for health and general well-being. An Animal Use Protocol was filed and approved by the University of Tennessee Institutional Animal Care and Use Committee (IACUC).

Diets

All diets were prepared by Research Diets (New Brunswick, NJ) (**Tables 3-1** and **3-2**). The fatty acid ethyl esters were purchased from Nu-Chek Prep (Elysian, MN). Aliquots of diet were bagged separately and stored at -20° C

TABLE 3-1

Diet Composition - High ALA study

Diet Component	Control	ALA-1	ALA-2	ALA-3	EPA-1	EPA-2	EPA-3
	g/kg						
Casein	230	230	231	232	230	231	232
L-Cystine	3.4	3.4	3.4	3.4	34	3.4	3.4
Cornstarch	266	264	259	253	264	259	253
Maltodextrin 10	86	86	86	86	86	86	86
Sucrose	115	115	115	115	115	115	115
Cellulose	57	57	57	57	57	57	57
Cocoa Butter	43	43	43	43	43	43	43
Linseed Oil	5.2	5.2	5.2	5.2	5.2	5.2	5.2
Palm Oil	60	60	60	60	60	60	60
Safflower Oil	33	33	33	33	33	33	33
Sunflower, Trisun[1]	31	31	31	31	31	31	31
18:3 n-3 ethyl ester[2]	3.18	4.72	7.16	10.43	3.19	3.20	3.21
20:4 n-6 ethyl ester[2]	0.40	0.40	0.40	0.40	0.40	0.40	0.40
20:5 n-3 ethyl ester[2]	0.17	0.17	0.17	0.17	1.72	4.25	7.18
22:6 n-3 ethyl ester[2]	0.29	0.29	0.29	0.29	0.29	0.29	0.29
Mineral Mix S10026[3]	11.5	11.5	11.5	11.5	11.5	11.5	11.5
Dicalcium Phosphate	15	15	15	15	15	15	15
Calcium Carbonate	6.3	6.3	6.3	6.3	6.3	6.3	6.3
Potassium Citrate	19	19	19	19	19	19	19
Vitamin Mix V13401[4]	11.5	11.5	11.5	11.5	11.5	11.5	11.5
Choline Bitartrate	2.3	2.3	2.3	2.3	2.3	2.3	2.3
α-Vitamin E Acetate[5]	0.15	0.15	0.15	0.15	0.15	0.15	0.15
Tertiary-butyl hydroquinone	0.03	0.03	0.03	0.03	0.03	0.03	0.03

[1] High-Oleic acid sunflower oil, Humko Oil Products (Humboldt, TN).
[2] 18:3 n-3, 20:4 n-6, 20:5 n-3, and 22:6 n-3 ethyl esters supplied by Nu-Chek Prep (Elysian, MN).
[3] Components per Kg Mineral Mix: NaCl, 259 g; magnesium oxide, 41.9 g, $MgSO_4 \cdot 7 H_2O$, 257.6 g; chromium potassium sulfate $\cdot 12 H_2O$, 1.925 g; cupric carbonate, 1.05 g; sodium fluoride, 0.2 g; potassium iodate, 0.035 g; ferric citrate, 21.0 g; manganous carbonate, 12.25 g; ammonium molybdate, 0.3 g; sodium selenite, 0.035 g; zinc carbonate, 5.6 g; sucrose, 399.105 g (Research Diets, New Brunswick, NJ).
[4] Components per Kg Vitamin Mix: vitamin A palmitate (500,000 IU/gm), 0.8 g; cholecalciferol (100,000 IU/gm), 1.0 g; menadione sodium bisulfite, 0.08 g; biotin (1.0%), 2.0 g; cyanocobalamin (0.1%), 1.0 g; folic acid, 0.2 g; nicotinic acid, 3.0 g; calcium pantothenate, 1.6 g; pyridoxine- HCl, 0.7 g; riboflavin, 0.6 g; thiamin HCl, 0.6 g; sucrose, 988.42 g (Research Diets, New Brunswick, NJ).
[5] 500 IU/gm

TABLE 3-2

Diet Composition-Normal ALA study

Diet Component	Control	ALA-1	ALA-2	ALA-3	EPA-1	EPA-2	EPA-3
	g/kg						
Casein	170	170	170	170	170	170	170
L-Cystine	3	3	3	3	3	3	3
Cornstarch	336	336	336	336	336	336	336
Maltodextrin 10	85	85	85	85	85	85	85
Sucrose	114	114	114	114	114	114	114
Cellulose	57	57	57	57	57	57	57
Cocoa Butter	40	40	40	40	40	40	40
Linseed Oil	5	5	5	5	5	5	5
Palm Oil	56	56	56	56	56	56	56
Safflower Oil	31	31	31	31	31	31	31
Sunflower, Trisun[1]	29	29	29	29	29	29	29
Total ethyl esters[2]	7.5	7.4	7.4	7.4	7.5	7.5	7.5
18:1 n-9 ethyl ester	6.7	5.2	3.3	0	5.2	3.3	0
18:3 n-3 ethyl ester	0	1.4	3.3	6.6	0	0	0
20:4 n-6 ethyl ester	0.4	0.4	0.4	0.4	0.4	0.4	0.4
20:5 n-3 ethyl ester	0.17	0.17	0.17	0.17	1.6	3.5	6.8
22:6 n-3 ethyl ester	0.27	0.27	0.27	0.27	0.27	0.27	0.27
Mineral Mix S10026[3]	11	11	11	11	11	11	11
Dicalcium Phosphate	15	15	15	15	15	15	15
Calcium Carbonate	6	6	6	6	6	6	6
Potassium Citrate	19	19	19	19	19	19	19

TABLE 3-2

Continued

Diet Component	Control	ALA-1	ALA-2	ALA-3	EPA-1	EPA-2	EPA-3
	g/kg						
Vitamin Mix V13401[4]	11	11	11	11	11	11	11
Choline Bitartrate	2	2	2	2	2	2	2
α -Vitamin E Acetate[5]	0.15	0.15	0.15	0.15	0.15	0.15	0.15
Tertiary-butyl hydroquinone	0.03	0.03	0.03	0.03	0.03	0.03	0.03

[1] High-Oleic acid sunflower oil, Humko Oil Products (Humboldt, TN).
[2] All ethyl esters supplied by Nu-Chek Prep (Elysian, MN).
[3] Components per Kg Mineral Mix: NaCl, 259 g; magnesium oxide, 41.9 g, $MgSO_4 \cdot 7 H_2O$, 257.6 g; chromium potassium sulfate $\cdot 12 H_2O$, 1.925 g; cupric carbonate, 1.05 g; sodium fluoride, 0.2 g; potassium iodate, 0.035 g; ferric citrate, 21.0 g; manganous carbonate, 12.25 g; ammonium molybdate, 0.3 g; sodium selenite, 0.035 g; zinc carbonate, 5.6 g; sucrose, 399.105 g. (Research Diets, New Brunswick, NJ).
[4] Components per Kg Vitamin Mix: vitamin A palmitate (500,000 IU/gm), 0.8 g; cholecalciferol (100,000 IU/gm), 1.0 g; menadione sodium bisulfite, 0.08 g; biotin (1.0%), 2.0 g; cyanocobalamin (0.1%), 1.0 g; folic acid, 0.2 g; nicotinic acid, 3.0 g; calcium pantothenate, 1.6 g; pyridoxine- HCl, 0.7 g; riboflavin, 0.6 g; thiamin HCl, 0.6 g; sucrose, 988.42 g. (Research Diets, New Brunswick, NJ).
[5] 500 IU/gm

under an atmosphere of nitrogen to minimize fatty acid oxidation. Animals were provided fresh diet each day.

Background diet

This research employed two diets as the background diet, the US17 diet (310) (Research Diets, New Brunswick, NJ; diet # D12486B) and a modified version of the US17 diet (Research Diets, New Brunswick, NJ; diet # D05010801). The US17 diet was originally designed to mimic the Western diet with regards to lipid and fatty acid composition (saturated, monounsaturated, and polyunsaturated fatty acids). The PUFA content was limited to linoleic acid (LA) and α-linolenic acid (ALA) to allow for the addition and evaluation of other dietary PUFA (310). One of the experiments used the US17 diet as the background diet (**High ALA, H-ALA**); however, for the other study (**Normal ALA, N-ALA**) the macronutrient distribution was revisited and these values were adjusted to better reflect the protein and carbohydrate content of the Western diet (based on % kilocalories) (**Table 3-3**) (311) . The protein content was adjusted from 21 energy% (original US17 formulation) to 16 energy%, and the carbohydrate content was changed from 43.2 energy% to 50 energy%. The background lipid content was retained at ~34 energy%. While we had no intention of modifying the protein and carbohydrate content of the background diet between the studies, it was brought to our attention that the protein content of the original US17 diet was high following the first experiment. Although we have no data to suggest

TABLE 3-3

Comparison of High ALA and Normal ALA control diets with typical Western consumption

Diet Component	High-ALA Diet	Normal-ALA Diet	HED[1] H-ALA	HED N-ALA	Typical Intake
	---------------- *energy %*	----------------	--------- *gms*	----------	
Carbohydrate	43.2	50			50.0 – 52.6 en % (311)
Protein	21	16			14.6 – 14.9 en % (311)
Fat:	35.8	34			32.1 – 32.3 en % (311)
MUFA	14.3	15			14 en % (89, 312)
SFA	12.9	11.6			11 en % (311)
PUFA	8.6	7.4			7 en % (89, 312)
Fatty Acids:					
18:2 n-6	7.4[2]	6.9[2]	16.4	15.3	13-17 gms (89, 312)
18:3 n-3 (ALA)	1.2	0.6	2.6	1.3	1.1-1.8 gms (6, 7, 312)
20:4 n-6 (AA)	0.08	0.08	0.177	0.177	~ 170 mg (89, 313)
20:5 n-3 (EPA)	0.034	0.034	0.075	0.075	100-200 mg,
22:6 n-3 (DHA)	0.058	0.054	0.128	0.120	*EPA + DHA* (6, 7)

[1] Human Equivalent Dose based on energy % equivalent in a 2000 calorie diet.
[2] Source:(314)and personal communication: Angela Gajda, Research Diets, New Brunswick, N.J.

otherwise, a search of the literature did not reveal any evidence that the tissue phospholipid fatty acid composition would be significantly affected by these changes. Nevertheless, this should be considered when evaluating the results.

Because the typical Western diet contains small amounts of arachidonic acid (AA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) (in addition to LA and ALA), each of these fatty acids (AA, EPA, and DHA) was added to all diets at a dose that modeled typical Western consumption (based on caloric density) (**Table 3-3**) (6, 7, 313, 315).

Control diets

Two dietary studies (**Normal ALA** and **High ALA**) were designed to test the effects of increasing supplemental amounts of dietary ALA or EPA on changes in plasma and erythrocyte phospholipid PUFA content. The **Normal ALA (N- ALA)** study employed a control diet containing ALA at a level of 0.6% of energy, whereas the **High ALA (H-ALA)** study used a control diet containing ALA at a level of 1.2 % of energy (**Table 3-3**). These levels are equivalent to 1.3 g/d and 2.6 g/d when extrapolated based on caloric density (2000 kcal/d). According to the DRIs, the AI for ALA is 1.1 g/d for women and 1.6 g/d for men (302); which is equivalent to 0.5% -0.7% energy. The LA content of the diets was designed to be kept constant at ~7 % of energy.

Experimental diets

Experimental diets for both studies were formulated using the control diet as a base for supplemented fatty acids. Ethyl esters of ALA or EPA at levels of approximately 0.15%, 0.4%, and 0.7% (w/w), which is equivalent to 0.3%, 0.8%, and 1.4 energy%, respectively, were added to both control diets (**Table 3-4**). Supplemental doses were low enough that changes in total fat content between treatment groups would not be expected to confound results attributed to supplemental fatty acids. However, in the **Normal-ALA** study, fatty acids were supplemented such that any changes in tissue PUFA phospholipid content could only be attributed to the supplemented n-3 PUFA, by adding supplemented ethyl esters at the expense of neutral fatty acid oleic acid (18:1 n-9). The upper supplemented dose represented by groups ALA-3 and EPA-3 is equivalent to the maximum levels of long chain n-3 PUFA recommended by the U.S. Food and Drug Administration (3 g/d) (11) (**Table 3-4**). The fatty acid compositions of the experimental diets in the **Normal-ALA** study and the **High-ALA** study are presented in **Tables 3-5 and 3-6**.

Experimental design

For both studies, mice were randomized into respective diet groups upon arrival, by weight. Mice were fed a standard pre-experimental powdered diet mix for acclimatization to powdered meals. Mice were then fed treatment diets for 20-21 days ad libitum. At the end of the diet treatment period mice were fasted

TABLE 3-4

Treatment groups and supplemental doses in High and Normal ALA studies

Treatment Group	High-ALA			Normal-ALA		
	Supplemental ALA or EPA	Supplemental ALA or EPA	HED[1]	Supplemental ALA or EPA	Supplemental ALA or EPA	HED
	wt %	*energy %*	*gms*	*wt %*	*energy %*	*gms*
ALA-1	0.15	0.3	0.67	0.15	0.3	0.67
ALA-2	0.40	0.80	1.78	0.33	0.66	1.47
ALA-3	0.73	1.45	3.22	0.66	1.32	2.93
EPA-1	0.15	0.3	0.67	0.15	0.3	0.67
EPA-2	0.41	0.82	1.82	0.33	0.66	1.47
EPA-3	0.69	1.39	3.10	0.66	1.32	2.93

[1] Human Equivalent Dose (HED) based on energy% equivalence in a 2000 calorie human diet.

TABLE 3-5

Fatty acid composition of diets used in the High-ALA study[1]

Fatty Acid	Control	ALA-1	ALA-2	ALA-3	EPA-1	EPA-2	EPA-3
	mole %						
10:0	0.07	ND	0.07	ND	ND	ND	ND
12:0	1.23	1.06	1.15	1.06	1.19	1.19	1.02
14:0	1.13	1.16	1.13	1.09	1.20	1.19	1.11
16:0	23.95	24.21	24.01	23.59	24.49	24.12	23.10
16:1	0.17	0.17	0.17	0.16	0.17	0.17	0.16
18:0	11.48	11.45	11.12	11.06	11.06	11.08	11.21
18:1 (n-9)	38.43	37.75	37.04	36.70	38.17	37.74	36.58
18:1 (n-7)	0.57	0.54	0.55	0.54	0.57	0.56	0.55
18:2 (n-6)	18.29	18.19	17.95	17.61	17.94	17.66	17.79
18:3 (n-3)	3.32	4.14	5.50	6.90	3.20	3.18	3.39
20:0	0.46	0.46	0.45	0.45	0.45	0.46	0.45
20:1	0.13	0.13	0.13	0.13	0.13	0.13	0.12
20:4 (n-6)	0.19	0.19	0.19	0.18	0.19	0.19	0.19
20:5 (n-3)	0.09	0.09	0.10	0.09	0.79	1.88	3.84
22:0	0.22	0.22	0.21	0.21	0.21	0.21	0.22
22.5 (n-3)	0.12	0.11	0.11	0.11	0.11	0.11	0.11
22:6 (n-3)	0.13	0.13	0.13	0.13	0.13	0.12	0.13

TABLE 3-5

Continued

Fatty Acid	Control	ALA-1	ALA-2	ALA-3	EPA-1	EPA-2	EPA-3
	mole %						
Total saturated FA	38.55	38.55	38.14	37.45	38.60	38.25	37.10
Total MUFA	39.30	38.59	37.89	37.53	39.04	38.60	37.42
Total PUFA	22.16	22.85	23.97	25.02	22.36	23.16	25.48
Total (n-6)	18.49	18.38	18.14	17.79	18.13	17.86	18.00
Total (n-3)	3.67	4.47	5.83	7.23	4.23	5.30	7.48
P:S ratio	0.57	0.59	0.63	0.67	0.57	0.60	0.68
(n-3):(n-6) ratio	0.20	0.24	0.32	0.41	0.23	0.30	0.42

[1] Powder diets were processed using a modified Bligh and Dyer fatty acid analysis (316) (see full description in methods section).
[2] Abbreviations: ND- not detected.

TABLE 3-6

Fatty acid composition of diets used in the Normal-ALA study[1]

Fatty Acid	Control	ALA-1	ALA-2	ALA-3	EPA-1	EPA-2	EPA-3
				mole %			
10:0	0.15	0.07	0.11	0.13	0.12	0.13	ND
12:0	1.94	1.73	1.43	1.61	1.79	1.66	1.74
14:0	1.26	1.26	1.20	1.28	1.23	1.24	1.17
16:0	22.81	23.01	22.61	23.06	22.87	22.89	22.48
16:1	0.16	0.16	0.15	0.16	0.15	0.16	0.15
18:0	10.84	11.16	11.36	11.20	11.25	11.32	11.45
18:1 (n-9)	41.25	40.35	39.67	37.51	40.36	39.45	37.68
18:1 (n-7)	0.61	0.59	0.60	0.60	0.59	0.57	0.60
18:2 (n-6)	17.84	17.71	17.84	17.66	17.67	17.59	17.74
18:3 (n-3)	1.73	2.55	3.54	5.41	1.71	1.71	1.72
20:0	0.45	0.46	0.48	0.46	0.47	0.47	0.49
20:1	0.14	0.14	0.14	0.14	0.14	0.14	0.14
20:4 (n-6)	0.21	0.21	0.21	0.21	0.21	0.22	0.24
20:5 (n-3)	0.10	0.10	0.11	0.09	0.91	1.95	3.87
22:0	0.22	0.22	0.24	0.22	0.23	0.23	0.24
22.5 (n-3)	0.13	0.13	0.14	0.13	0.13	0.13	0.14
22:6 (n-3)	0.17	0.15	0.16	0.15	0.15	0.15	0.16

TABLE 3-6

Continued

Fatty Acid	Control	ALA-1	ALA-2	ALA-3	EPA-1	EPA-2	EPA-3
	mole %						
Total saturated FA	37.67	37.91	37.44	37.96	37.97	37.93	37.56
Total MUFA	42.15	41.24	40.56	38.40	41.24	40.32	38.57
Total PUFA	20.18	20.86	21.99	23.64	20.78	21.75	23.87
Total (n-6)	18.05	17.92	18.05	17.87	17.88	17.81	17.98
Total (n-3)	2.14	2.94	3.94	5.77	2.90	3.94	5.89
P:S ratio	0.54	0.55	0.59	0.62	0.55	0.57	0.64
(n-3):(n-6) ratio	0.12	0.16	0.22	0.32	0.16	0.22	0.33

[1] Powder diets were processed using a modified Bligh and Dyer fatty acid analysis (316) (see full description in methods section).

overnight and killed via carbon dioxide asphyxiation, followed by blood and tissue sample collection.

Blood collection

Animals were exsanguinated via cardiac puncture using standard anticoagulant (dried lithium heparin syringes (Portex brand, Smiths Medical, Keene, NH) or 3.8% sodium citrate). Collected blood was transferred to microcentrifuge tubes and inverted. For each experiment, anticoagulated blood was centrifuged at 660 x g for 4 minutes at room temperature (317). Platelet rich plasma (PRP) was removed and used for plasma fatty acid analysis. Remaining packed erythrocytes were suspended in 1 ml 0.9% saline, vortexed, and used for erythrocyte fatty acid analysis.

Plasma and erythrocyte phospholipid fatty acid analysis

Fatty acids were extracted from plasma and erythrocytes using a modified Bligh and Dyer analysis (316). Briefly, total lipid extracts were obtained using a 1:2 (v/v) chloroform/methanol mixture and 1,2-diheptadecanoyl-sn-glycero-3-phosphorylcholine added initially as an internal standard. The lipid soluble layer was separated twice using saturated sodium chloride solution and chloroform and the lower layer was removed. Removed lipid extracts were dried under an atmosphere of nitrogen. Dried total lipid was resuspended in a small volume of chloroform and separated into respective phospholipids, neutral lipids, and free fatty acids on High Performance Thin-Layer Chromatography plates (silica gel

60, Merck, Darmstadt, Germany). An 8:1 (v/v) chloroform/methanol solvent system was used for HPLC separation. The phospholipid band was scraped from the origin and saponified with 0.5 mol/L sodium hydroxide in methanol for 15 minutes at 86°C. Fatty acids were then methylated using 14% boron trifluoride in methanol for 10 minutes at 86°C. Following acidification with 0.7 mol/L hydrochloric acid in methanol, fatty acid methyl esters were extracted twice with hexane, dried under nitrogen, resuspended in a small volume of hexane, and injected onto a Hewlett Packard model 5890 series II gas chromatograph (Palo Alto, CA) with flame ionization detector. Fatty acids were separated using a DB 23, 33m, .25mm diameter capillary glass column packed with fused silica (J & W Scientific, Folsom, CA). Column temperature was programmed to rise from 160° C at a rate of 3.5 degrees per minute up to a final temperature of 250°C. Hydrogen was used as the carrier gas. Fatty acid peaks were identified by comparison to known fatty acid standards (Nu-Chek Prep, Elysian, MN) and peak area of internal standard 17:0 was used to determine mole percent of sample peaks.

Identification and selection of clinical trials

This research was designed to evaluate the impact of dietary n-3 PUFA on fatty acid changes in mice and humans in comparable tissues. While searching for human data for comparison, the following keywords were used individually and in various combinations: plasma phospholipids, erythrocyte phospholipids,

human, omega-3, n-3, fatty acids, ALA, α-linolenic acid, EPA, eicosapentaenoic acid, DHA, docosahexaenoic acid, supplementation, and intervention. The search strategy was not designed to be exhaustive, but to provide sufficient data revealing the overall landscape and general pattern of the effects of dietary n-3 PUFA in humans for comparison to murine effects. Additional sources were identified from cited references and review articles. Initial searches yielded ~60 studies reporting plasma phospholipid changes and ~25 studies reporting erythrocyte phospholipid changes were analyzed. Papers included in the study had to report baseline tissue fatty acid compositions and changes in the tissue post intervention. Dietary interventions ranged from 1 week to 12 months with most interventions lasting between 1-3 months. The typical human study involved omega-3 supplementation given in addition to a general diet. Supplementation of omega-3 fatty acids could be in form of ethyl esters, n-3 PUFA containing capsules, vegetable oils (i.e. flaxseed oil, soybean, canola, etc..) fish and fish oil, so long as the supplemented n-3 PUFA intakes could be determined. All blood samples for fatty acid analysis had to be collected following a fast. Both international and domestic studies were included. Studies were not weighted on the basis of participant number. Exclusion criteria included invasive interventions (such as surgery), subjects less than 18 years of age, and women who were pregnant. After review and exclusion based on the above factors, roughly 40 studies remained that were used for comparison to murine data.

Presentation of the human data

The human data are presented in ascending order along the x-axis by increasing energy% of the specified supplemented fatty acid, with the y-axis representing the percent change in a specified plasma or erythrocyte phospholipid fatty acid as indicated. Difference in pre and post tissue measures were used to calculate percent change from original values.

Statistical analyses

Phospholipid fatty acid content in plasma and erythrocytes was compared across treatment groups using one-way analysis of variance (ANOVA), followed by Fisher's least significant difference post-hoc test to determine significant differences between groups. Data was considered significant at $p < 0.05$. Statistical analysis was performed with SPSS software (Chicago, IL).

3.4 Results

Analysis of intake and weights

Weekly group weight means (in grams) were consistent between groups and between studies (**Figure A-1**). Average daily food intake was also consistent between groups (**Figure A-2**).

Changes in phospholipid fatty acids

At the end of the experimental period, fatty acid composition was determined in plasma and erythrocyte phospholipids for both studies (**N-ALA** and **H-ALA**). Differences in fatty acid composition between experimental groups and control groups were determined for each PUFA of interest and plotted as percent change. Murine data was compared to data from archival human clinical trials in comparable tissues. For each human study, changes in plasma/serum or erythrocyte phospholipid fatty acids from baseline to post intervention were determined and, likewise, were plotted as percent change. For graphical representation, murine and human data representing change in the same tissue and fatty acid were combined and ordered along the x-axis based on ascending amounts of supplemented omega-3 fatty acid (in energy%).

Effects of ALA supplementation on changes in plasma and erythrocyte phospholipid fatty acids

Plasma phospholipid EPA content in **N-ALA** mice supplemented with increasing amounts of ALA responded in a dose dependent manner (**Table 3-7**). The content of EPA in plasma was relatively low in the control group (0.1 mol %) and increased 2, 5, and 8 fold following ALA supplementation of 0.3%, 0.66%, and 1.32% of energy, respectively. DHA was modestly higher by 17% and 21% in the ALA -2 and ALA -3 supplemented groups, respectively. Similar trends were observed with changes in EPA in erythrocyte phospholipids; however, significant differences were only observed in the in the highest ALA

TABLE 3-7

Plasma phospholipid fatty acid composition by treatment group in the Normal ALA study[1]

Fatty Acid	Control	ALA-1	ALA-2	ALA-3	EPA-1	EPA-2	EPA-3
n =	5	4	5	5	5	4	5
	mole % of total fatty acids						
14:0	0.3 ± 0.1	0.2 ± 0.0	0.4 ± 0.1	0.5 ± 0.1	0.4 ± 0.1	0.4 ± 0.2	0.3 ± 0.2
16:0	27.8 ± 0.7^{ab}	26.6 ± 0.6^{a}	28.7 ± 0.2^{bc}	28.8 ± 0.4^{bc}	29.6 ± 0.6^{c}	29.7 ± 0.7^{c}	28.9 ± 0.5^{c}
16:1	0.4 ± 0.1	0.3 ± 0.1	0.7 ± 0.2	0.9 ± 0.2	0.8 ± 0.1	0.7 ± 0.2	0.7 ± 0.3
18:0	17.9 ± 1.2^{b}	18.3 ± 0.6^{b}	14.5 ± 0.5^{a}	14.6 ± 0.6^{a}	13.9 ± 0.5^{a}	14.5 ± 0.6^{a}	15.2 ± 0.7^{a}
18:1 (n-9)	12.6 ± 0.7	12.8 ± 0.5	11.7 ± 0.5	12.1 ± 0.7	12.2 ± 0.7	11.7 ± 0.7	11.8 ± 0.9
18:1 (n-7)	0.8 ± 0.0	0.8 ± 0.2	0.8 ± 0.0	0.9 ± 0.1	0.7 ± 0.1	0.8 ± 0.0	0.8 ± 0.0
18:2 (n-6)	17.9 ± 0.5^{a}	21.3 ± 0.9^{b}	18.6 ± 0.5^{a}	18.4 ± 0.7^{a}	19.1 ± 0.3^{a}	18.3 ± 0.1^{a}	18.5 ± 0.3^{a}
18:3 (n-6)	0.3 ± 0.1^{cd}	0.2 ± 0.0^{ab}	0.3 ± 0.0^{cd}	0.3 ± 0.1^{bc}	0.4 ± 0.0^{d}	0.3 ± 0.0^{bcd}	0.2 ± 0.1^{a}
18:3 (n-3)	0.1 ± 0.0^{a}	0.1 ± 0.0^{a}	0.3 ± 0.0^{b}	0.4 ± 0.1^{b}	0.1 ± 0.1^{a}	0.1 ± 0.1^{a}	0.1 ± 0.1^{a}
20:0	< 0.1	0.2 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.1	0.1 ± 0.1
20:1	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1
20:2	0.1 ± 0.0^{ab}	0.2 ± 0.1^{bc}	0.2 ± 0.0^{bc}	0.2 ± 0.0^{c}	0.1 ± 0.1^{ab}	0.1 ± 0.1^{ab}	< 0.1^{a}
20:3 (n-6)	1.1 ± 0.0^{a}	1.7 ± 0.1^{b}	1.4 ± 0.0^{a}	1.8 ± 0.1^{b}	1.2 ± 0.1^{a}	1.4 ± 0.1^{a}	1.3 ± 0.1^{a}
20:4 (n-6)	12.8 ± 0.6^{cd}	9.7 ± 0.8^{ab}	12.9 ± 0.2^{d}	10.8 ± 0.4^{b}	11.3 ± 0.7^{bc}	10.4 ± 0.6^{b}	8.2 ± 0.4^{a}
20:5 (n-3)	0.1 ± 0.1^{a}	0.2 ± 0.0^{b}	0.5 ± 0.0^{bc}	0.8 ± 0.0^{cd}	0.9 ± 0.0^{d}	1.7 ± 0.2^{e}	3.2 ± 0.2^{f}
22:4 (n-6)	0.1 ± 0.0^{a}	0.2 ± 0.1^{b}	0.1 ± 0.0^{a}	0.1 ± 0.0^{a}	ND	ND	ND
22.5 (n-6)	0.2 ± 0.1^{b}	0.3 ± 0.2^{b}	< 0.1^{a}	< 0.1^{a}	ND	ND	ND
22:5 (n-3)	0.2 ± 0.1^{a}	0.3 ± 0.1^{ab}	0.4 ± 0.0^{b}	0.5 ± 0.0^{c}	0.6 ± 0.0^{c}	0.9 ± 0.1^{d}	1.3 ± 0.0^{e}
22:6 (n-3)	7.1 ± 0.2^{a}	6.4 ± 0.3^{a}	8.3 ± 0.1^{b}	8.6 ± 0.1^{b}	8.8 ± 0.3^{b}	9.0 ± 0.5^{b}	9.3 ± 0.4^{b}
Total (n-3)	7.6 ± 0.3^{a}	7.3 ± 0.3^{a}	9.6 ± 0.1^{b}	10.4 ± 0.1^{b}	10.3 ± 0.3^{b}	11.7 ± 0.4^{c}	13.9 ± 0.4^{d}
Total (n-6)	32.4 ± 0.6^{cd}	33.3 ± 0.7^{de}	33.3 ± 0.5^{de}	31.3 ± 0.7^{bc}	31.9 ± 0.6^{bcd}	30.3 ± 0.7^{b}	28.2 ± 0.7^{a}
(n-3):(n-6) ratio	0.2 ± 0.0^{a}	0.2 ± 0.0^{a}	0.3 ± 0.0^{b}	0.3 ± 0.0^{c}	0.3 ± 0.0^{c}	0.4 ± 0.0^{d}	0.5 ± 0.0^{e}

[1] Results are means ± SEM. Difference in letter superscripts within the same row indicate statistical significance, $p < .05$.
[2] Abbreviations: ALA- alpha- linolenic acid, EPA- eicosapentaenoic acid, ND- not detected

supplemented group (ALA- 3) (**Table 3-8**). As observed in plasma, DHA levels in erythrocytes were modestly higher in the ALA-2 and ALA-3 supplemented groups by 13% and 11%, respectively.

When the level of ALA in the background diet was doubled (1.2% energy) (**H-ALA**), effects on tissue n-3 PUFA content were attenuated (compared to the **N-ALA** study) following progressive supplementation of ALA (**Table 3-9** and **Table 3-10**). In plasma phospholipids, the changes in EPA levels were only 2.0 and 2.7 fold higher in the ALA-2 and ALA-3 supplemented groups, while there were no significant changes in DHA.

Comparison of changes in mouse and human phospholipid fatty acids following ALA supplementation

When compared to archival human data, the changes observed in the mouse phospholipid EPA were greater than human changes when the supplemented doses in the **N-ALA** groups were matched to human data by caloric density of supplemented fatty acid (**Figure 3-1** and **Table 3-11**). However, the responses observed in the mouse diets containing the higher background levels of ALA (**H-ALA** groups) appear to be better correlated with the human data with respect to changes in EPA levels in both plasma and erythrocyte phospholipids. ALA had a marginal effect on DHA change in plasma/serum phospholipids in both mice and humans except for one study, Ghafoorunissa et al.(318), however, it appeared that this was due to low initial

TABLE 3-8

Erythrocyte phospholipid fatty acid composition by treatment group in the Normal ALA study[1]

Fatty Acid	Control	ALA-1	ALA-2	ALA-3	EPA-1	EPA-2	EPA-3
n =	5	4	5	5	5	5	5
	mole % of total fatty acids						
14:0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.2 ± 0.1
16:0	30.2 ± 0.1^{ab}	30.0 ± 0.1^{ab}	29.6 ± 0.3^{a}	29.6 ± 0.2^{a}	29.9 ± 0.2^{ab}	30.6 ± 0.5^{bc}	31.3 ± 0.6^{c}
16:1	0.4 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.3 ± 0.0	0.3 ± 0.1
18:0	11.8 ± 0.3	12.0 ± 0.0	11.4 ± 0.1	11.8 ± 0.4	12.7 ± 1.2	12.2 ± 0.1	12.0 ± 0.3
18:1 (n-9)	14.7 ± 0.2	15.0 ± 0.0	14.6 ± 0.2	14.7 ± 0.2	16.0 ± 1.4	15.1 ± 0.1	15.0 ± 0.2
18:1 (n-7)	0.9 ± 0.1	0.9 ± 0.0	0.9 ± 0.0	0.9 ± 0.0	0.9 ± 0.1	0.8 ± 0.0	0.8 ± 0.0
18:2 (n-6)	10.6 ± 0.7	11.2 ± 0.2	11.0 ± 0.6	12.3 ± 0.1	12.5 ± 0.8	11.2 ± 0.3	11.1 ± 0.2
18:3 (n-6)	0.1 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.1 ± 0.0
18:3 (n-3)	ND	0.1 ± 0.0^{a}	0.2 ± 0.0^{b}	0.3 ± 0.0^{c}	ND	ND	ND
20:0	0.1 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.1 ± 0.0
20:1	0.3 ± 0.0^{b}	0.3 ± 0.0^{b}	0.3 ± 0.0^{b}	0.3 ± 0.0^{b}	0.4 ± 0.0^{b}	0.3 ± 0.0^{b}	0.2 ± 0.1^{a}
20:2	0.3 ± 0.0^{b}	0.3 ± 0.0^{b}	0.3 ± 0.0^{b}	0.3 ± 0.0^{b}	0.3 ± 0.0^{b}	0.3 ± 0.0^{b}	0.2 ± 0.1^{a}
20:3 (n-6)	1.3 ± 0.1^{c}	1.4 ± 0.1^{c}	1.2 ± 0.1^{c}	1.3 ± 0.0^{c}	1.3 ± 0.1^{c}	1.1 ± 0.0^{b}	1.0 ± 0.0^{a}
20:4 (n-6)	18.0 ± 0.8^{d}	16.6 ± 0.3^{cd}	17.6 ± 0.8^{d}	15.7 ± 0.3^{c}	16.2 ± 0.8^{c}	13.2 ± 0.4^{b}	11.4 ± 0.3^{a}
20:5 (n-3)	0.3 ± 0.0^{a}	0.4 ± 0.0^{a}	0.5 ± 0.1^{a}	0.9 ± 0.0^{b}	1.6 ± 0.1^{c}	2.4 ± 0.1^{d}	4.5 ± 0.2^{e}
22:4 (n-6)	1.6 ± 0.1^{e}	1.4 ± 0.0^{de}	1.4 ± 0.1^{d}	1.2 ± 0.0^{c}	1.3 ± 0.1^{c}	1.0 ± 0.1^{b}	0.9 ± 0.0^{a}
22:5 (n-6)	0.8 ± 0.1^{d}	0.7 ± 0.0^{cd}	0.7 ± 0.1^{cd}	0.5 ± 0.0^{bc}	0.5 ± 0.0^{abc}	0.4 ± 0.0^{ab}	0.3 ± 0.1^{a}
22:5 (n-3)	0.6 ± 0.1^{a}	0.8 ± 0.0^{ab}	0.9 ± 0.1^{b}	1.1 ± 0.0^{c}	1.4 ± 0.1^{c}	1.7 ± 0.1^{d}	2.4 ± 0.1^{e}
22:6 (n-3)	7.5 ± 0.2^{a}	7.9 ± 0.3^{ab}	8.5 ± 0.3^{bc}	8.3 ± 0.1^{bc}	9.1 ± 0.5^{bc}	8.7 ± 0.2^{c}	8.2 ± 0.2^{bc}
Total (n-3)	8.4 ± 0.3^{a}	9.2 ± 0.2^{a}	10.1 ± 0.4^{b}	10.6 ± 0.2^{bc}	12.1 ± 0.7^{c}	12.8 ± 0.3^{d}	15.1 ± 0.5^{e}
Total (n-6)	32.4 ± 0.5^{e}	31.5 ± 0.2^{de}	32.1 ± 0.3^{de}	31.1 ± 0.4^{d}	32.1 ± 1.8^{c}	27.1 ± 0.3^{b}	24.8 ± 0.5^{a}
(n-3):(n-6) ratio	0.3 ± 0.0^{a}	0.3 ± 0.0^{b}	0.3 ± 0.0^{bc}	0.3 ± 0.0^{c}	0.4 ± 0.0^{d}	0.5 ± 0.0	0.6 ± 0.0^{e}

[1] Results are means ± SEM. Different letter superscripts within the same row indicate statistical significance, $p < .05$.
[2] Abbreviations: ALA- alpha- linolenic acid, EPA- eicosapentaenoic acid, ND- not detected.

TABLE 3-9

Plasma phospholipid fatty acid composition by treatment group in the High ALA study[1]

Fatty Acid	Control	ALA-1	ALA-2	ALA-3	EPA-1	EPA-2	EPA-3
n =	5	5	5	5	5	5	5
	mole % of total fatty acids						
14:0	0.2 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.4 ± 0.1	0.1 ± 0.1	0.4 ± 0.2	0.2 ± 0.1
16:0	30.5 ± 0.1	30.9 ± 0.5	30.6 ± 0.5	30.6 ± 0.4	31.1 ± 0.4	31.2 ± 0.2	31.2 ± 0.4
16:1	0.3 ± 0.0	0.4 ± 0.0	0.3 ± 0.1	0.4 ± 0.0	0.4 ± 0.0	0.3 ± 0.1	0.4 ± 0.0
18:0	16.1 ± 0.1	15.5 ± 0.4	16.1 ± 0.2	15.5 ± 0.3	15.2 ± 0.3	15.2 ± 0.2	15.8 ± 0.3
18:1 (n-9)	10.4 ± 0.2	9.7 ± 0.1	9.8 ± 0.3	9.8 ± 0.4	10.2 ± 0.2	9.9 ± 0.3	9.9 ± 0.3
18:1 (n-7)	0.6 ± 0.0^{cd}	0.6 ± 0.0^{ab}	0.6 ± 0.0^{bcd}	0.6 ± 0.0^{ab}	0.7 ± 0.0^{d}	0.6 ± 0.0^{bc}	0.5 ± 0.0^{a}
18:2 (n-6)	19.4 ± 0.2	17.9 ± 0.5	19.1 ± 0.8	19.4 ± 0.4	18.7 ± 0.5	19.3 ± 0.4	18.8 ± 0.2
18:3 (n-6)	0.4 ± 0.0	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.0	0.3 ± 0.0	0.1 ± 0.1
18:3 (n-3)	ND	0.1 ± 0.1^{a}	0.1 ± 0.1^{a}	0.4 ± 0.0^{b}	0.1 ± 0.1^{a}	0.1 ± 0.1^{a}	ND
20:2	ND	ND	< 0.1	ND	ND	ND	ND
20:3 (n-6)	1.0 ± 0.0	1.0 ± 0.1	1.1 ± 0.1	1.1 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.0
20:4 (n-6)	11.8 ± 0.2^{de}	12.7 ± 0.3^{e}	10.9 ± 0.5^{cd}	10.7 ± 0.2^{c}	11.1 ± 0.1^{cd}	9.5 ± 0.4^{b}	8.1 ± 0.3^{a}
20:5 (n-3)	0.3 ± 0.0^{a}	0.4 ± 0.0^{ab}	0.6 ± 0.0^{bc}	0.8 ± 0.0^{cd}	0.9 ± 0.0^{d}	1.7 ± 0.1^{e}	3.2 ± 0.2^{f}
22:5 (n-3)	0.4 ± 0.0^{a}	0.5 ± 0.0^{a}	0.5 ± 0.0^{b}	0.6 ± 0.0^{b}	0.6 ± 0.0^{b}	0.9 ± 0.0^{c}	1.1 ± 0.1^{d}
22:6 (n-3)	8.5 ± 0.1	9.9 ± 0.4	9.6 ± 0.6	9.4 ± 0.2	9.7 ± 0.3	9.7 ± 0.3	9.8 ± 0.2
Total (n-3)	9.2 ± 0.1^{a}	10.8 ± 0.4^{b}	10.9 ± 0.6^{b}	11.2 ± 0.2^{b}	11.4 ± 0.3^{bc}	12.3 ± 0.3^{c}	14.1 ± 0.1^{d}
Total (n-6)	32.6 ± 0.1^{d}	31.8 ± 0.4^{cd}	31.4 ± 0.7^{bcd}	31.5 ± 0.4^{cd}	31.1 ± 0.4^{bc}	30.1 ± 0.5^{b}	28.0 ± 0.4^{a}
(n-3):(n-6) ratio	0.3 ± 0.0^{a}	0.3 ± 0.0^{b}	0.4 ± 0.0^{b}	0.4 ± 0.0^{b}	0.4 ± 0.0^{bc}	0.4 ± 0.0^{c}	0.5 ± 0.0^{d}

[1] Results are means ± SEM. Different alphabetic superscripts denote statistical significance within each fatty acid, $p < .05$.
[2] Abbreviations: ALA- alpha- linolenic acid, EPA- eicosapentaenoic acid, ND- not detected.

TABLE 3-10

Erythrocyte phospholipid fatty acid composition by treatment group in the High ALA study[1]

Fatty Acid	Control	ALA-1	ALA-2	ALA-3	EPA-1	EPA-2	EPA-3
n =	5	5	5	5	5	5	5
	mole % of total fatty acids						
14:0	0.1 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.1	0.2 ± 0.0	0.2 ± 0.0
16:0	32.0 ± 0.1^{ab}	31.6 ± 0.2^{a}	32.2 ± 0.2^{b}	32.1 ± 0.2^{b}	32.1 ± 0.1^{b}	32.2 ± 0.2^{b}	33.0 ± 0.1^{c}
16:1	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0
18:0	12.3 ± 0.0	12.5 ± 0.1	12.3 ± 0.1	12.2 ± 0.1	12.2 ± 0.1	12.3 ± 0.1	12.3 ± 0.1
18:1 (n-9)	14.2 ± 0.1^{bc}	13.8 ± 0.0	14.1 ± 0.1^{ab}	14.1 ± 0.1^{ab}	14.3 ± 0.2^{bc}	14.4 ± 0.1^{bc}	14.6 ± 0.1^{c}
18:1 (n-7)	0.9 ± 0.0^{b}	0.9 ± 0.0^{ab}	0.9 ± 0.0^{b}	0.9 ± 0.0^{b}	0.9 ± 0.0^{b}	0.9 ± 0.0^{ab}	0.8 ± 0.0^{a}
18:2 (n-6)	11.0 ± 0.1	10.9 ± 0.3	11.3 ± 0.3	11.5 ± 0.1	11.0 ± 0.3	11.1 ± 0.2	10.4 ± 0.1
18:3 (n-6)	0.1 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
18:3 (n-3)	0.1 ± 0.0^{a}	0.2 ± 0.0^{a}	0.3 ± 0.0^{c}	0.3 ± 0.0^{b}	0.2 ± 0.0^{a}	0.1 ± 0.0^{a}	0.1 ± 0.0^{a}
20:1	0.3 ± 0.0^{b}	0.3 ± 0.0^{b}	0.3 ± 0.0^{b}	0.3 ± 0.0^{c}	0.3 ± 0.0^{b}	0.3 ± 0.0^{a}	0.3 ± 0.0^{a}
20:2	0.3 ± 0.0^{e}	0.3 ± 0.0^{d}	0.3 ± 0.0^{cd}	0.3 ± 0.0^{de}	0.3 ± 0.0^{b}	0.3 ± 0.0^{c}	0.3 ± 0.0^{a}
20:3 (n-6)	1.3 ± 0.0^{c}	1.3 ± 0.0^{c}	1.3 ± 0.0^{c}	1.3 ± 0.0^{c}	1.2 ± 0.0^{c}	1.1 ± 0.0^{b}	1.0 ± 0.0^{a}
20:4 (n-6)	15.2 ± 0.2^{d}	15.5 ± 0.2^{d}	14.0 ± 0.2^{c}	13.8 ± 0.2^{c}	13.8 ± 0.2^{c}	12.1 ± 0.2^{b}	9.8 ± 0.0^{a}
20:5 (n-3)	0.5 ± 0.0^{a}	0.6 ± 0.0^{a}	0.8 ± 0.0^{b}	1.0 ± 0.0^{b}	1.5 ± 0.0^{c}	2.8 ± 0.0^{d}	4.8 ± 0.2^{e}
22:4 (n-6)	1.2 ± 0.0^{d}	1.2 ± 0.0^{d}	1.0 ± 0.0^{c}	1.0 ± 0.0^{c}	1.0 ± 0.0^{c}	0.8 ± 0.0^{b}	0.7 ± 0.0^{a}
22:5 (n-6)	0.5 ± 0.0^{f}	0.4 ± 0.0^{e}	0.3 ± 0.0^{c}	0.3 ± 0.0^{cd}	0.3 ± 0.0^{d}	0.2 ± 0.0^{b}	0.2 ± 0.0^{a}
22:5 (n-3)	1.2 ± 0.0^{a}	1.2 ± 0.0^{a}	1.4 ± 0.0^{c}	1.5 ± 0.0^{b}	1.6 ± 0.0^{c}	2.0 ± 0.0^{d}	2.6 ± 0.1^{e}
22:6 (n-3)	8.2 ± 0.0^{a}	8.6 ± 0.1^{b}	8.7 ± 0.2^{b}	8.8 ± 0.1^{b}	8.6 ± 0.1^{b}	8.6 ± 0.1^{b}	8.5 ± 0.1^{ab}
Total (n-3)	10.0 ± 0.1^{a}	10.5 ± 0.1^{b}	11.2 ± 0.2^{d}	11.6 ± 0.1^{c}	11.8 ± 0.1^{d}	13.6 ± 0.2^{e}	16.0 ± 0.2^{f}
Total (n-6)	29.3 ± 0.1^{e}	29.4 ± 0.1^{e}	28.1 ± 0.2^{cd}	27.9 ± 0.2^{d}	27.5 ± 0.1^{c}	25.5 ± 0.3^{b}	22.2 ± 0.2^{a}
(n-3):(n-6) ratio	0.3 ± 0.0^{a}	0.4 ± 0.0^{a}	0.4 ± 0.0^{bc}	0.4 ± 0.0^{b}	0.4 ± 0.0^{c}	0.5 ± 0.0^{d}	0.7 ± 0.2^{e}

[1] Results are means ± SEM. Different alphabetic superscripts denote statistical significance within each fatty acid, $p < .05$.
[2] Abbreviations: ALA- alpha- linolenic acid, EPA- eicosapentaenoic acid, ND- not detected.

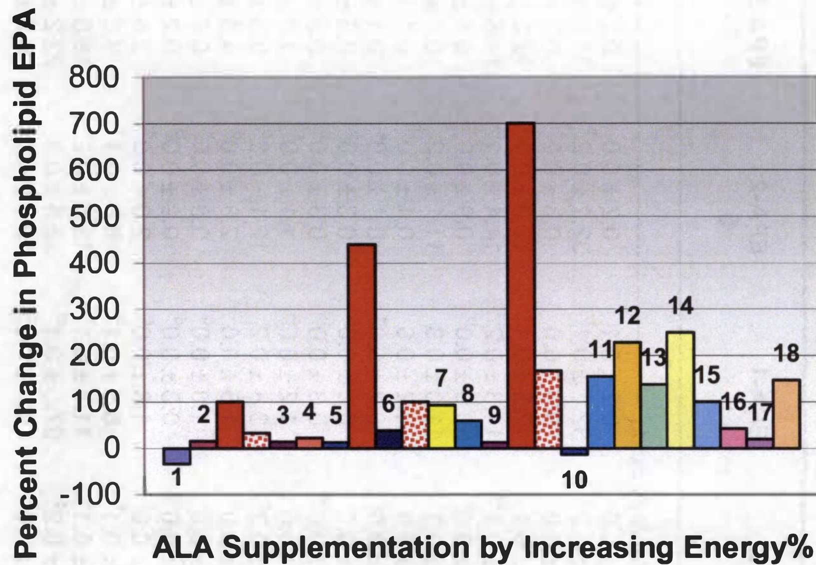


Figure 3-1: Percent change in plasma/serum phospholipid EPA by increasing ALA supplementation in energy%. Murine data is represented by red bars (**N-ALA**) or red speckled bars (**H-ALA**), with human studies intermingled horizontally. Numerical superscripts correspond to human study descriptions in **Table 3-11**.

TABLE 3-11

Human ALA supplementation studies used in ***Figure 3-1***

Vertical bar in Figure 3-1	Author	Supplemental Energy% ALA	Citation
1	Sinclair	0.2	(319)
2, 6	Seppanen-Laakso	0.22, 0.7	(320)
3, 4	James	0.31, 0.62	(321)
5	Thies	0.65	(322)
7, 11	Finnegan	1.15, 2.89	(323)
8	Wallace	1.2	(324)
9, 14	Li	1.21, 5.5	(325)
10	Valsta	1.6	(326)
12	Mest	4	(327)
13	Mantzioris	4.9	(328)
15	Cunnane	6	(329)
16, 17	Singer[1]	13.6	(330)
18	Beitz	14.7	(331)

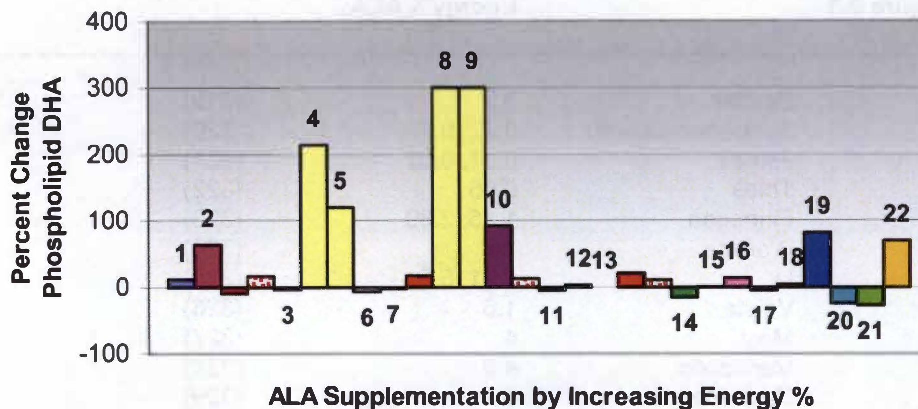
[1] Supplemental values were the same with 16 representing normolipidemics and 17 representing hyperlipidemics.

DHA levels, thus the potential for increase may have been greater than other human studies (**Figure 3-2** and **Table 3-12**).

Effects of EPA supplementation on changes in plasma and erythrocyte phospholipid fatty acids

Plasma phospholipid EPA content in **N- ALA** mice supplemented with increasing amounts of EPA changed significantly in a dose dependent manner (**Table 3-7**). The content of EPA in plasma phospholipids was increased 9, 17, and 32 fold following EPA supplementation of 0.3%, 0.66%, and 1.32% of energy, respectively. The changes observed in DHA in the lowest EPA supplemented group (EPA-1) were equivalent to the changes observed in the

A.



B.

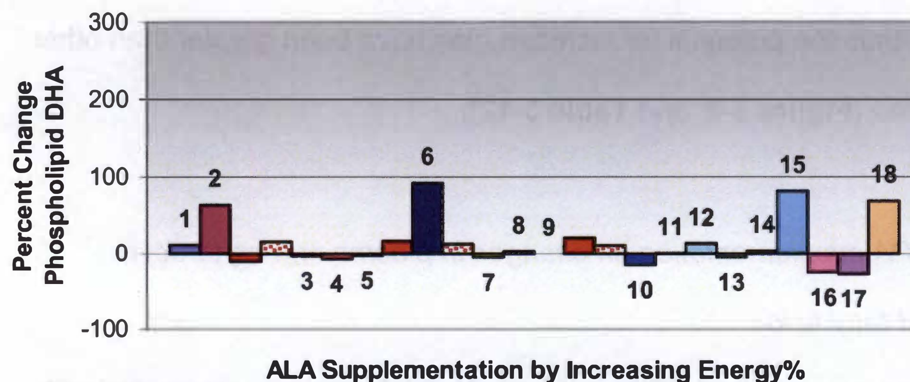


Figure 3-2: Percent change in plasma/serum phospholipid DHA by increasing ALA supplementation in energy%: (**A**) Ghafoorunissa (318) in yellow and (**B**) Excluding Ghafoorunissa, for comparison. Murine data is represented by red bars (**N-ALA**) or red speckled bars (**H-ALA**), with human studies intermingled horizontally. Numerical superscripts correspond to human study descriptions in **Table 3-12** (A and B).

TABLE 3-12

Human ALA supplementation studies used in Figure 3-2

DHA studies	Vertical bar in Figure 3-2	Author	Supplemental Energy% ALA	Citation
A)	1	Sinclair	0.2	(319)
	2, 10	Seppanen-Laakso	0.22, 0.7	(320)
	3, 6	James	0.31, 0.62	(321)
	4, 5, *8, 9*	Ghafoorunissa	0.6, *0.7*	(318)
	7	Thies	0.65	(322)
	11, 15	Finnegan	1.15, 2.89	(323)
	12	Wallace	1.2	(324)
	13, 17	Li	1.21, 5.5	(325)
	14	Valsta	1.6	(326)
	16	Mantzioris	4.9	(328)
	18	Cunnane	6	(329)
	19	Mest	7.4	(327)
	20, 21	Singer[1]	13.6	(330)
	22	Beitz	14.7	(331)
B)	1	Sinclair	0.2	(319)
	2, 6	Seppanen-Laakso	0.22, 0.7	(320)
	3, 4	James	0.31, 0.62	(321)
	5	Thies	0.65	(322)
	7, 11	Finnegan	1.15, 2.89	(323)
	8	Wallace	1.2	(324)
	9, 13	Li	1.21, 5.5	(325)
	10	Valsta	1.6	(326)
	12	Mantzioris	4.9	(328)
	14	Cunnane	6	(329)
	15	Mest	7.4	(327)
	16, 17	Singer[1]	13.6	(330)
	18	Beitz	14.7	(331)

[1] Supplemental values were the same with 20A and 16B representing normolipidemics and 21A and 17B representing hyperlipidemics.

highest ALA group (ALA-3), with no further increase despite increasing EPA supplementation 2 and 4 fold (EPA-2, and EPA-3, respectively). Similar trends were observed with changes in EPA in erythrocytes (**Table 3-8**), with dose dependent increases in phospholipid EPA. As observed with the plasma phospholipid fraction, changes in erythrocyte DHA levels were significantly higher in the EPA-1 group, but no additional changes were observed in the higher supplemented EPA groups (EPA-2 and EPA-3).

When the levels of ALA in the background diet doubled (1.2% energy) (**H-ALA**), the changes in tissue EPA content were attenuated in comparison to changes in the **N-ALA** study (**Tables 3-7** and **3-8** versus **Tables 3-9** and **3-10**). However, tissue levels (in absolute mol%) were comparable following supplementation irrespective of the background diet (**Table 3-8** compared to **Table 3-10,** and **Table 3-7** compared to **Table 3-9**). Interestingly, no changes in plasma phospholipid DHA levels were observed with increasing dietary levels of EPA when compared to the control group (**Table 3-9**). Changes in erythrocyte phospholipid DHA levels in the lowest supplemented group increased only 5% with no further changes despite increasing levels of EPA in the diet 2 and 4 fold (**Table 3-10**).

Comparison of changes in mouse and human phospholipid fatty acids following EPA supplementation

Comparing our results using pure EPA-ethyl esters to human studies in which a combination of EPA+DHA (in the form of fish or fish oil) is the common

treatment modality posed a challenge. However, we identified 5 studies with human subjects that supplemented only EPA and 6 human studies that supplemented only DHA. The impact of both the EPA only and DHA only studies on plasma phospholipid EPA (**Figure 3-3**) and DHA (**Figure 3-4**) were plotted together. Results from these studies indicate that EPA is increased when only EPA is supplemented, but to a lesser extent when only DHA is given. Results also show that DHA is increased when only DHA is given, but to a lesser extent when only EPA is given. We used these observations as a guide when determining how to order human studies in which combination EPA + DHA were supplemented. When tissue EPA changes were examined, we chose to order the studies by increasing content of EPA (rather than total n-3 content). Likewise, when DHA changes were examined, we chose to order the studies by content of DHA (rather than total n-3 content).

Percent change in plasma phospholipid EPA in both mouse and human supplementation studies is shown in **Figure 3-5**. Studies are arranged in ascending order of the EPA portion of long chain omega-3 fatty acid supplementation (**Table 3-14**). When compared to human data, the changes observed in the mouse phospholipid EPA levels were dramatically higher when the supplemented doses in the **N-ALA** groups were matched by caloric density. However, the responses observed in the mouse diets containing the higher background levels of ALA (**H-ALA** groups) appear to be better correlated with the human data with respect to changes in EPA in both plasma and erythrocyte phospholipids (**Figures 3-5 and 3-6**).

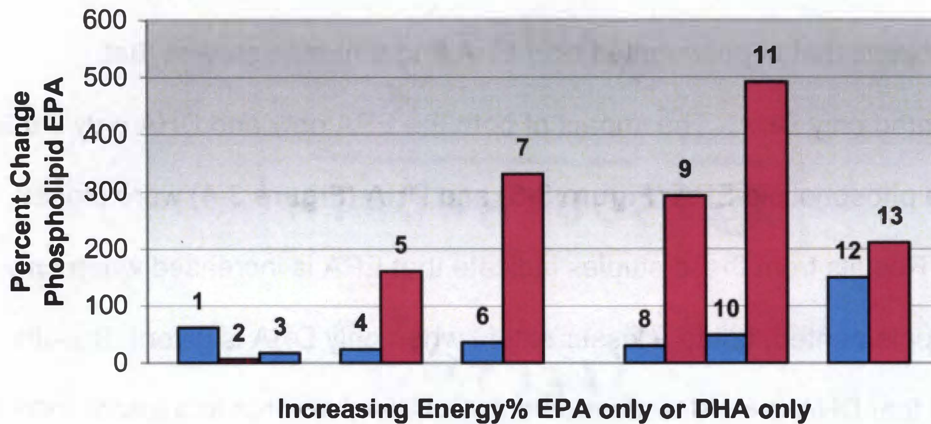


Figure 3-3: Impact of supplementation with only EPA (pink bars) or only DHA (aqua bars) on percent change in plasma/serum phospholipid EPA. Numerical superscripts correspond to human study descriptions in **Table 3-13**.

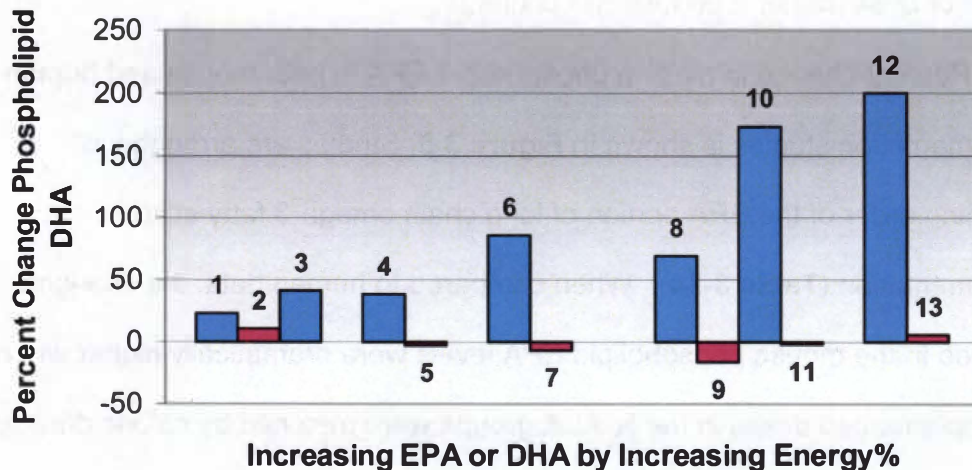


Figure 3-4: Impact of supplementation with only EPA (pink bars) or only DHA (aqua bars) on percent change in plasma/serum phospholipid DHA. Numerical superscripts correspond to human study descriptions in **Table 3-13**.

TABLE 3-13

Human supplementation studies used in ***Figure** **3-3*** *and* ***3-4***

Fatty acid	Bars in Figs. 3-3, 3-4	Author	Supplemental Energy% ALA	Citation
DHA only	1,3	Jensen	0.06, 0.09	(332)
	4	Thies	0.23	(322)
	6	Vidgren	0.6	(333)
	8	Grimsgaard	1.3	(334)
	10	Mori	1.5	(335)
	12	Buckley	2.2	(336)
EPA only	2	Driss	0.07	(337)
	5,7	James	0.31, 0.62	(321)
	9	Grimsgaard	1.4	(334)
	11	Mori	1.6	(335)
	13	Buckley	2.2	(336)

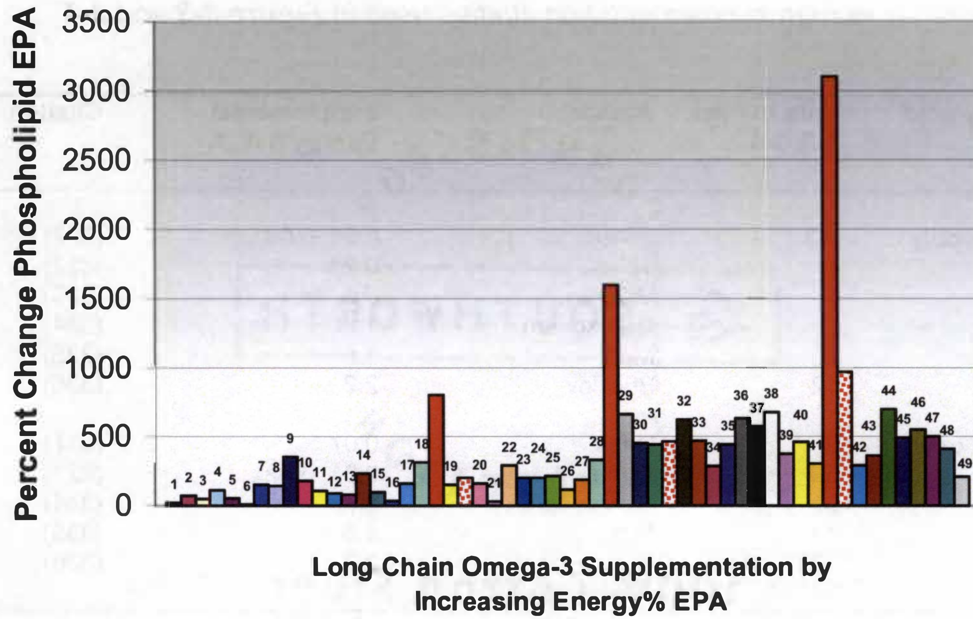


Figure 3-5: Percent change in plasma/serum phospholipid EPA by increasing EPA content of total long chain n-3 supplementation (in energy%). Murine data is represented by red bars (**N-ALA**) or red speckled bars (**H-ALA**), with human studies intermingled horizontally. Numerical superscripts correspond to human study descriptions in **Table 3-14**.

TABLE 3-14

*Human supplementation studies used in **Figure 3-5***

Vertical bar in Figure 3-5	Author	Supplemental Energy% LCPn3	Citation
		Total (EPA, DHA, DPA)	
1	Jensen	0.12 (0.02, 0.1)	(332)
2	Finnegan	0.09 (0.03, 0.06)	(323)
3	Engstrom	0.13 (0.05, 0.08)	(338)
4	Wallace	0.15 (0.05, 0.1)	(324)
5	Sinclair	0.12 (0.06,0.01, 0.05)	(339)
6	Driss	0.07 (EPA only)	(337)
7	Ghafoorunissa	0.13 (0.08, 0.05)	(318)
8	Wallace	0.33 (0.1, 0.23)	(324)
9	Vidgren	0.48 (0.11, 0.32, 0.05)	(333)
10	Sinclair	0.64 (0.12, 0.52)	(319)
11	Vidgren	0.41 (0.14, 0.24, 0.03)	(333)
12	Finnegan	0.4 (0.15, 0.25)	(323)
13	Vognild	0.53 (0.19, 0.24, 0.1)	(340)
14	Wallace	0.7 (0.2, 0.5)	(324)
15	Engstrom	0.4 (0.2, 0.2)	(338)
16	Vognild	0.65 (0.22, 0.33, 0.1)	(340)
17	Thies	0.32 (0.23, 0.09)	(322)
18	Hodge	0.51 (0.27, 0.20, 0.04)	(341)
19	Sinclair	0.94 (0.3, 0.64)	(339)
20	James	0.31 (EPA only)	(321)
21	Vognild	0.81 (0.33, 0.45, 0.03)	(340)
22	Sanders	1.24 (0.34, 0.9)	(342)
23	Von Schacky	0.95 (0.38, 0.57)	(343)
24	Kew	3.01 (0.38, 2.2, 0.43)	(344)
25	Blonk	0.7 (0.4, 0.3)	(345)
26	Vognild	1.24 (0.45, 0.66, 0.13)	(340)
27	Vognild	1.37 (0.54, 0.77, 0.06)	(340)
28	James	0.62 (EPA only)	(345)
29	Cerbone	1.66 (0.7, 0.96)	(346)
30	Von Schacky	1.92 (0.78, 1.14)	(343)
31	Blonk	1.3 (0.8, 0.5)	(345)
32	Singer	2.29 (0.89, 1.4)	(330)
33	Laidlaw	1.8 (1.0, 0.8)	(347)
34	Gronn	2.1 (1.0, 0.9, 0.2)	(348)
35	Stark	1.8 (1.08, 0.72)	(349)
36	Mantzioris	1.5 (1.1, 0.4)	(328)
37	Mantzioris	1.5 (1.1, 0.4)	(328)
38	Gronn	2.4 (1.1, 1.1, 0.2)	(348)
39	Gibney	1.7 (1.2, 0.5)	(350)
40	Stark	2.0 (1.2, 0.8)	(351)
41	Gronn	2.6 (1.2, 1.1, 0.3)	(348)

TABLE 3-14

Continued

Vertical bar in Figure 3-5	Author	Supplemental Energy% LCPn3	Citation
		Total (EPA, DHA, DPA)	
42	Grimsgaard	1.4 (EPA only)	(334)
43	Blonk	2.5 (1.5, 1.0)	(345)
44	Von Schacky	3.82 (1.56, 2.29)	(343)
45	Mori	1.6 (EPA only)	(335)
46	Engstrom	3.32 (2.0, 1.32)	(352)
47	Engstrom	3.32 (2.0, 1.32)	(352)
48	Kew	2.5 (2.1, 0.33, 0.07)	(344)
49	Buckley	2.2 (EPA only)	(336)

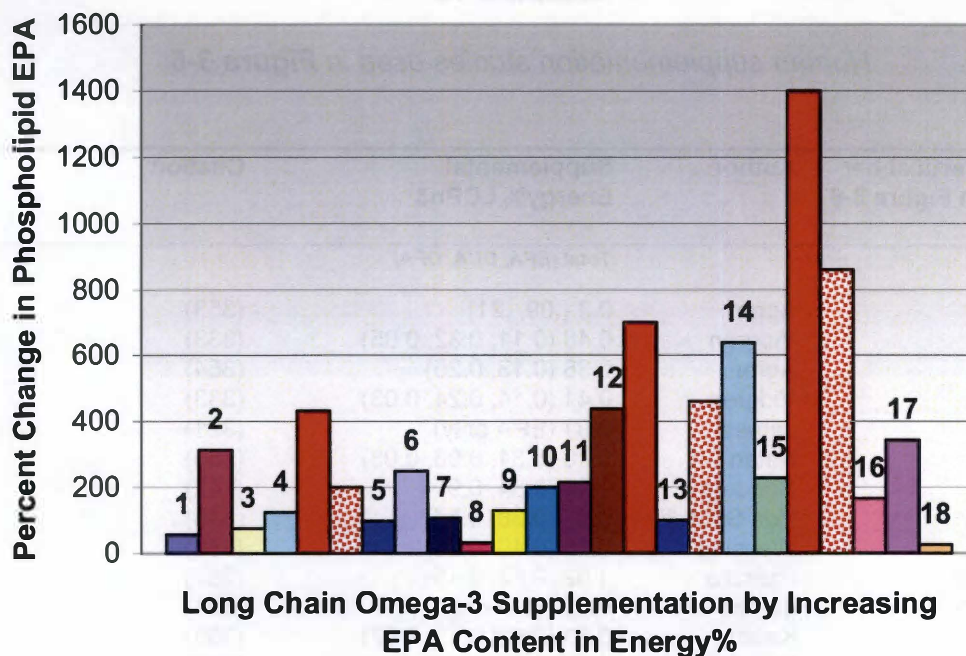


Figure 3-6: Percent change in erythrocyte phospholipid EPA by increasing EPA content of total long chain n-3 supplementation (in energy%). Murine data is represented by red bars (**N-ALA**) or red speckled bars (**H-ALA**), with human studies intermingled horizontally. Numerical superscripts correspond to human study descriptions in **Table 3-15**.

TABLE 3-15

Human supplementation studies used in ***Figure 3-6***

Vertical bar in Figure 3-6	Author	Supplemental Energy% LCPn3	Citation
		Total (EPA, DHA, DPA)	
1	Agren	0.3 (.09, .21)	(353)
2	Vidgren	0.48 (0.11, 0.32, 0.05)	(333)
3	Agren	0.38 (0.13, 0.25)	(354)
4	Vidgren	0.41 (0.14, 0.24, 0.03)	(333)
5	James	0.31 (EPA only)	(321)
6	Katan	0.40 (0.31, 0.06, 0.03)	(355)
7	Sanders	1.24 (0.34, 0.9)	(342)
8	Von Schacky	0.95 (0.38, 0.57)	(343)
9	Wensing	0.75 (0.49, 0.26)	(356)
10	Palozza	1.02 (0.57, 0.45)	(357)
11	James	0.62 (EPA only)	(321)
12	Katan	0.83 (0.63, 0.13, 0.07)	(355)
13	Von Schacky	1.92 (0.78, 1.14)	(343)
14	Katan	1.23 (0.94, 0.19, 0.01)	(355)
15	Palozza	2.08 (1.10, 0.98)	(357)
16	Von Schacky	3.85 (1.56, 2.29)	(343)
17	Palozza	3.14 (1.67, 1.47)	(357)
18	Hagve	2.6 (1.7, 0.9)	(358)

3.5 Discussion

In 1932, Max Kleiber, pioneer in the field of animal energetics, presented an equation that simplified the understanding of interspecies differences in metabolic rate: $Y= ax^{3/4}$, where Y is the metabolic rate in kcal, x is bodyweight in kilograms, a is the proportionality constant, and ¾ is the scaling exponent and the slope of the regression line depicting this relationship (277-279). Further characterization and development of interspecies comparison as it relates to nutrient intake was present by Rucker (300, 301) and Storm (300), who emphasized the need for attention to interspecies metabolic rate differences when comparing vitamin and mineral requirements between species. The current study is a product of these questions in light of n-3 fatty acid research using the rodent model. Historic attempts to linearize and explain interspecies differences are applicable not only to metabolic rate and vitamin andmineral requirements, but other nutrient components as well. Here, we have tested a simple, hypothetical dosing model for adding supplemental n-3 fatty acids to rodent diets on the basis of energy %. This model was created based on theoretical conclusions of interspecies comparisons previously presented (Chapter 2).

The driving force of this evaluation is a calculated attempt to achieve supplemental doses in the rodent model that offer better predictive value for human endpoints. Research examining the relationship between n-3 fatty acid supplementation and cancer outcomes is one example where better predictive value is needed. Currently, there exists a disparity between the outcomes

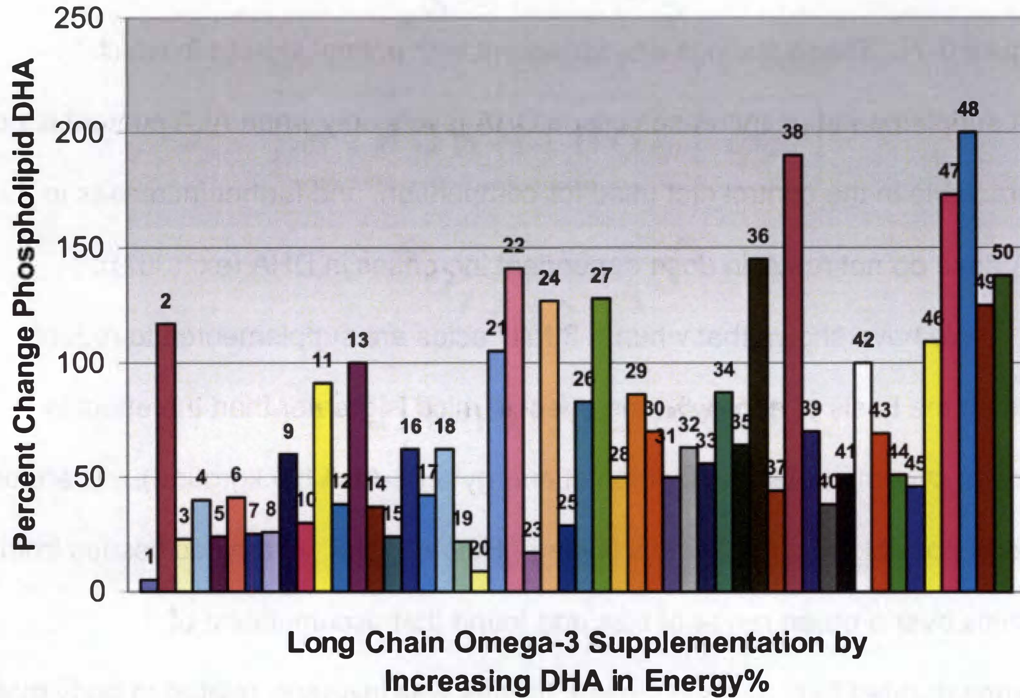
achieved in human clinical trials with n-3 fatty acids and cell culture or animal model studies in cancer research (95). For example, while animal studies support a role for n-3 fatty acid effect on colorectal cancer, some human studies imply an association (165-167), while others do not (359, 360). We believe that one explanation for this inconsistency may be the difference in the amounts of n-3 fatty acids that are supplemented in animal model experiments versus the doses that are given in clinical trials. For example, a literature search of recent animal model studies supplementing n-3 fatty acids reveals that when the animal doses are translated to human doses on the basis of energy %, n-3 supplementation ranged from 6-90 gms/day (259-276, 361). Doses from the majority of these studies would not be compatible with reasonable daily human consumption, and therefore, results observed from these studies may not be reproducible in humans.

In the current study, we designed background diets that contained long chain PUFA (both n-3 and n-6) that are present in the typical human diet in order to compare murine results to archival human trial data. We also used supplemental doses that were a reasonable representation of intake that could be achieved in humans (670 mg – 3.2 gms/d on the basis of energy%, **Table 3-4**). We found that in C57BL/6J mice at these supplemental levels, both ALA and EPA significantly increased EPA in both plasma and erythrocytes, with EPA supplementation having a greater effect on phospholipid EPA than ALA supplementation. DHA, on the other hand, was less influenced by either ALA or EPA supplementation and generally increased only when DHA was

supplemented (**Figures 3-2** and **3-4**). In addition, it was found that DHA did not increase interminably in response to increasing supplemental DHA doses (**Figure 3-7**). These findings are consistent with animal studies in which ALA supplementation increases tissue DHA levels only when ALA content is not appreciable in the control diet used for comparison, and further increases in ALA dose do not result in dose dependent increases in DHA (ex: (362)).

We have shown that when n-3 fatty acids are supplemented to rodent diets on the basis of energy%, the effect in mice is greater than the effect in humans at comparable supplemental energy% (**N-ALA** background). There are several possible reasons for this finding. Hulbert, et al., examined tissues from animals over a broad range of size and found that accumulation of polyunsaturated fatty acids in several tissues was inversely related to body mass, suggesting that tissue from small animals (ex:rodents) would have a higher tissue unsaturation index than the same tissue from larger animals (ex:humans) (254). This may explain why the **H-ALA** background produced changes that better matched archival human changes than the **N-ALA** background (based on total tissue unsaturation). Differences in tissue fatty acid changes may also reflect a difference in the capacity for enzymatic conversion of ALA to long chain metabolites between rodents and humans (363). The discrepancy between rodent and human tissue changes may also suggest that doses derived on the basis of caloric density do not produce human equivalent effects. However, if these low supplemental doses produce changes that are greater than human

A.



B.

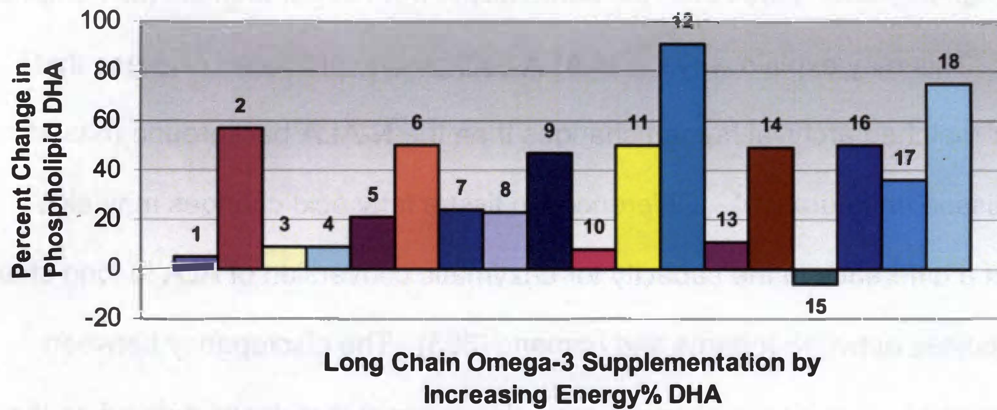


Figure 3-7: Percent change in plasma /serum (**A**), and erythrocyte (**B**) phospholipid DHA in human studies by increasing DHA content of total long chain n-3 supplementation (in energy%). Numerical superscripts correspond to human study descriptions in **Table 3-16**.

TABLE 3-16

*Human supplementation studies used in **Figure 3-7***

Vertical bar in Figure 3-7	Author	Supplemental Energy% LCPn3	Citation
		Total (EPA, DHA, DPA)	
1	Sinclair	0.12 (0.06,0.01, 0.05)	(339)
2	Ghafoorunissa	0.13 (0.08, 0.05)	(318)
3	Jensen	0.06 (DHA only)	(332)
4	Finnegan	0.09 (0.03, 0.06)	(323)
5	Engstrom	0.13 (0.05, 0.08)	(338)
6	Jensen	0.09 (DHA only)	(332)
7	Thies	0.32 (0.23, 0.09)	(322)
8	Jensen	0.12 (0.02, 0.1)	(332)
9	Wallace	0.15 (0.05, 0.1)	(324)
10	Engstrom	0.4 (0.2, 0.2)	(338)
11	Hodge	0.51 (0.27, 0.20, 0.04)	(341)
12	Thies	0.23 (DHA only)	(332)
13	Wallace	0.33 (0.1, 0.23)	(324)
14	Vidgren	0.41 (0.14, 0.24, 0.03)	(333)
15	Vognild	0.53 (0.19, 0.24, 0.1)	(340)
16	Finnegan	0.4 (0.15, 0.25)	(323)
17	Blonk	0.7 (0.4, 0.3)	(345)
18	Vidgren	0.48 (0.11, 0.32, 0.05)	(333)
19	Vognild	0.65 (0.22, 0.33, 0.1)	(340)
20	Kew	2.5 (2.1, 0.33, 0.07)	(344)
21	Mantzioris	1.5 (1.1, 0.4)	(328)
22	Mantzioris	1.5 (1.1, 0.4)	(328)
23	Vognild	0.81 (0.33, 0.45, 0.03)	(340)
24	Wallace	0.7 (0.2, 0.5)	(324)
25	Gibney	1.7 (1.2, 0.5)	(350)
26	Blonk	1.3 (0.8, 0.5)	(345)
27	Sinclair	0.64 (0.12, 0.52)	(319)
28	Von Schacky	0.95 (0.38, 0.57)	(343)
29	Vidgren	0.6 (DHA only)	(333)
30	Sinclair	0.94 (0.3, 0.64)	(339)
31	Vognild	1.24 (0.45, 0.66, 0.13)	(340)
32	Stark	1.8 (1.08, 0.72)	(349)
33	Vognild	1.37 (0.54, 0.77, 0.06)	(340)
34	Laidlaw	1.8 (1.0, 0.8)	(347)
35	Stark	2.0 (1.2, 0.8)	(351)
36	Sanders	1.24 (0.34, 0.9)	(342)
37	Gronn	2.1 (1.0, 0.9, 0.2)	(348)
38	Cerbone	1.66 (0.7, 0.96)	(346)
39	Blonk	2.5 (1.5, 1.0)	(345)
40	Gronn	2.4 (1.1, 1.1, 0.2)	(348)
41	Gronn	2.6 (1.2, 1.1, 0.3)	(348)
42	Von Schacky	1.92 (0.78, 1.14)	(343)

TABLE 3-16

Continued

Vertical bar in Figure 3-7	Author	Supplemental Energy% LCPn3	Citation
		Total (EPA, DHA, DPA)	
A (cont'd).			
43	Grimsgaard	1.3 (DHA only)	(334)
44	Engstrom	3.32 (2.0, 1.32)	(352)
45	Engstrom	3.32 (2.0, 1.32)	(352)
46	Singer	2.29 (0.89, 1.4)	(330)
47	Mori	1.5 (DHA only)	(335)
48	Buckley	2.2 (DHA only)	(336)
49	Kew	3.01 (0.38, 2.2, 0.43)	(344)
50	Von Schacky	3.82 (1.56, 2.29)	(343)
B.			
1	Katan	0.40 (0.31, 0.06, 0.03)	(355)
2	Payet	0.09 (0.008, 0.08)	(364)
3	Katan	0.83 (0.63, 0.13, 0.07)	(355)
4	Katan	1.23 (0.94, 0.19, 0.01)	(355)
5	Agren	0.3 (.09, .21)	(353)
6	Vidgren	0.41 (0.14, 0.24, 0.03)	(333)
7	Agren	0.38 (0.13, 0.25)	(354)
8	Wensing	0.75 (0.49, 0.26)	(356)
9	Vidgren	0.48 (0.11, 0.32, 0.05)	(333)
10	Palozza	1.02 (0.57, 0.45)	(357)
11	Von Schacky	0.95 (0.38, 0.57)	(343)
12	Vidgren	0.6 (DHA only)	(333)
13	Sanders	1.24 (0.34, 0.9)	(342)
14	Hagve	2.6 (1.7, 0.9)	(358)
15	Palozza	2.08 (1.10, 0.98)	(357)
16	Von Schacky	1.92 (0.78, 1.14)	(343)
17	Palozza	3.14 (1.67, 1.47)	(357)
18	Von Schacky	3.85 (1.56, 2.29)	(343)

changes in similar endpoints, we feel confident that the differences between mouse and human changes in this study are conservative compared to the differences that can be expected from supplemental n-3 studies in the general literature with even greater doses.

Dosing on the basis of energy% represents a reasonable method for the addition of n-3 fatty acids to rodent studies. Without a current standard for supplemental dosing strategies, this study is unique in that it sheds light on the gross over-estimation of human response that can be expected from animal studies in which large n-3 doses are supplemented. It is also unique in that mouse changes were shown to exceed human tissue changes even when long chain PUFA (n-3 and n-6) were present in the diet. This further suggests that typical rodent studies, in which long chain PUFA are not present in the control/background diet, can be expected to overestimate human endpoint effects.

3.6 Conclusions

While the use of energy% as a dosing model does not precisely predict changes in human endpoints, we feel that it establishes a guideline for dosing that considers interspecies variation inherent in dietary research using animal models. In order for rodent studies using supplemental n-3 doses to be meaningful, we must reevaluate how doses are selected. It is our hope that by offering this vivid comparison between effects in rodents and humans at known

supplemental levels, we are offering both a point of reference and a justification for change.

4 SUMMARY AND CONCLUSIONS

The establishment of a "human equivalent dose" model for n-3 fatty acid supplementation is necessary to validate both past and future fatty acid research using animal models. Human equivalent dosing offers a useful pre-clinical screening tool for establishing n-3 fatty acid effects in animal models prior to investment in larger and more expensive human clinical trials. However, attention to dosing "equivalence" is currently not standard in animal studies supplementing n-3 fatty acids. In the present study, we have presented a simple and unique model for supplementing n-3 fatty acids to rodent diets on the basis of energy%. We compared the effects of n-3 fatty acid supplementation on plasma and erythrocyte phospholipid fatty acids in C57BL/6J mice and archival human studies measuring the same endpoints. This allowed us to test the ability of energy% dosing to predict expected changes in humans. Comparison between changes in rodent and human tissues at the same energy% of supplemented ALA and EPA showed that murine changes in both plasma and erythrocyte phospholipid fatty acids exceeded changes observed in humans. In addition, increasing ALA content in the background diet produced changes in both tissues that were more comparable to results seen in humans. While energy% did not precisely predict changes in human endpoints, dosing based on energy% represents a decrease from supplemental levels that are typically used in rodent studies and therefore offers an improvement to common rodent supplemental n-3 fatty acid doses. We believe that energy% dosing offers a more conservative

estimate of human endpoint changes than doses that are currently in use. Future research is necessary to refine with precision a mathematical model that will more closely predict changes in human endpoints. It is our hope that by providing these comparisons we will encourage fatty acid dosing that is based on a calculated comparison between humans and animal models.

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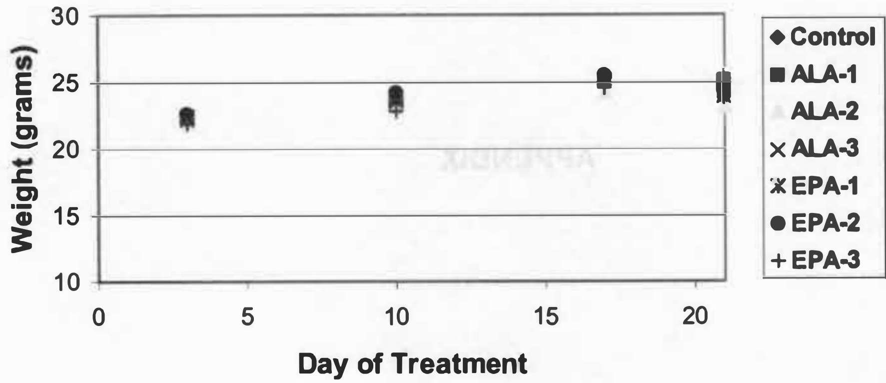
APPENDIX

A.



B.

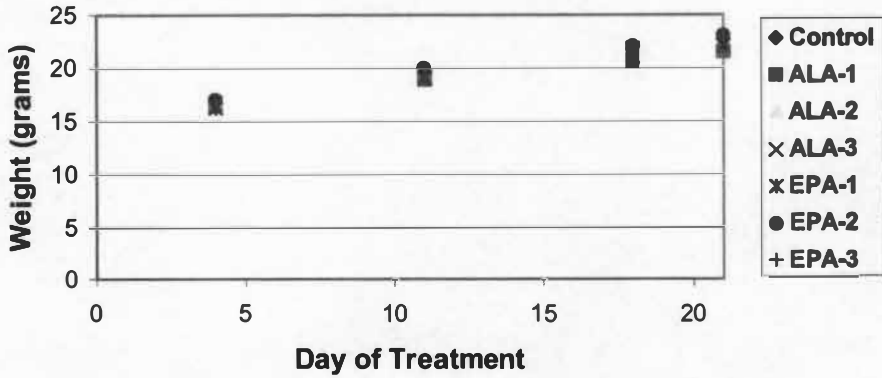
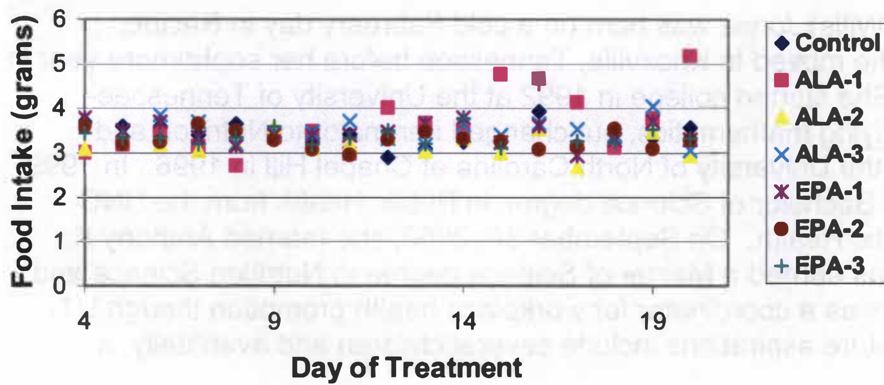


Figure A-1: Weekly weights and ending weight of mice in (a) **Normal-ALA** study and (b) **High-ALA** study. At the start of treatment period, mice in the **Normal- ALA** study were 17 days older than the mice in the **High ALA** study.

A.



B.

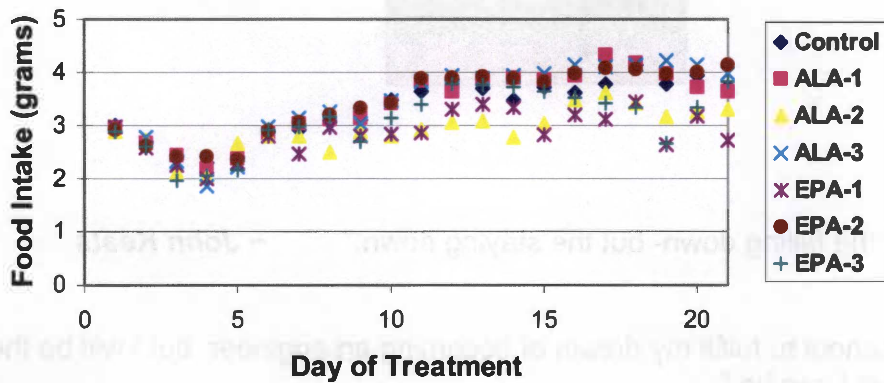


Figure A-2: Daily average food intake for mice in the (a) **Normal-ALA** study and (b) **High-ALA** study. At the start of treatment period, mice in the **Normal-ALA** study were 17 days older than the mice in the **High-ALA** study.

VITA

Laura (Wills) Jones was born on a cold February day in Racine, Wisconsin. She moved to Knoxville, Tennessee before her sophomore year in high school. She started college in 1992 at the University of Tennessee-Knoxville, studying mathematics, but changed her major to Nutrition and transferred to the University of North Carolina at Chapel Hill in 1996. In 1999, she finished a Bachelor of Science degree in Public Health from the UNC-School of Public Health. On September 30, 2000, she married Anthony K. Jones. She has earned a Master of Science degree in Nutrition Science and currently works as a coordinator for workplace health promotion though UT-Extension. Future aspirations include several children and eventually, a doctorate.

"Failure is not the falling down- but the staying down." ***~ John Keats***

"I can't go to school to fulfill my dream of becoming an engineer, but I will be the best printer that I can be."

~ Harry Wills (my grandfather)

"I have fought the good fight, I have finished the race, I have kept the faith."

II Timothy 4:7

"It is not that we think that we can do anything of lasting value by ourselves. Our *only* power and success come from God."

II Corinthians 3:5

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