1. Title Page

Protocol Title: Dietary linoleic acid regulation of omega-3 HUFA metabolism; satiety and body composition among overweight female subjects

Protocol Number: 11-AA-0028
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Principal Investigator

Name, Degree	Branch/Institute
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Total requested accrual
(0) Patients (separately describe patient groups)
(180) Volunteers

Project Uses Ionizing Radiation: ☐ No X☐ Yes (attach *RSC/RDSC documentation)*
☐ Medically-indicated only
X☐ Research-related only (DEXA scans)
☐ Both

IND/IDE X☐ No ☐Yes *(attach FDA documentation)*
Drug/Device/#_____
Sponsor: _____

Durable Power of Attorney X☐ No ☐ Yes
Multi-institutional Project X☐ No ☐ Yes
Institution_____ FWA #_____
Date of IRB approval_____ *(attach IRB documentation)*

Data and Safety Monitoring Board X☐ No ☐ Yes

Technology Transfer Agreement X☐ No ☐ Yes
Agreement type and number _____Expiration Date_____

Confidential Disclosure Agreement X☐ No ☐ Yes

Samples are being stored ☐ No X☐ Yes

3. Precis

3.1 Objective

To evaluate the effects of lowering the dietary nutrient linoleic acid (LA) as a controlled variable on: 1) tissue accretion of omega-6 and omega-3 highly unsaturated fatty acids (HUFA); 2) bioactive endocannabinoids derived from the omega-6 HUFA arachidonic acid (AA); 3) insulin sensitivity; 4) satiety and hunger; and 5) body composition. Modifications in dietary LA will be offset with reciprocal changes in the nutrient oleic acid (OA) to maintain equivalency of calories and nutrients.

3.2 Study Population

Up to 180 overweight, but otherwise healthy pre-menopausal women volunteers (aged 18 through 50) with body mass index (BMI) between 25 and 35 kg/m^2, will be enrolled.
With the anticipated dropout rates described in Section 7.4, we anticipate that 84 of these subjects will be randomized to study diets, and 64 subjects will complete the 12 week diet phase.

3.3.1 Three Group Comparative Analysis

A total of 84 subjects will be randomized to one of 3 healthy and nutritionally complete diets that differ only in the fatty acid composition of added visible oils. All foods will be provided through the NIH metabolic kitchen, with subjects visiting the kitchen daily Monday through Friday to obtain foods. Each Friday, subjects will carry home foods packed for their weekend meals. Baseline testing battery will include: 1) blood composition of omega-6 and omega-3 HUFA; 2) endocannabinoid, eicosanoid, and docosanoid derivatives of omega-6 and omega-3 HUFA; 3) insulin function assessment; 4) body composition assessment; 4) functional satiety assessment; and 5) self-reported measures of satiety, hunger and affective states. The full baseline testing battery will be repeated after 84 days of randomized treatment. An abbreviated testing battery will be performed after 28 and 56 days of randomized treatment. A subset of participants will undergo additional inpatient testing as described in Section 3.3.2. The remaining subjects will remain ambulatory throughout the study. Participants will be advised to continue their usual medical care and medications throughout the study.

3.3.2 36-week Continuation Sub Study

At completion of the 84 day (12 week) study, participants in the low LA group (Group B) will have the option of continuing their study diets for another 252 days (36 weeks). Continuing subjects will consume the same low LA (1 en%) diet. During the continuation phase, the abbreviated testing battery will be completed after 6, 12, 18, 24 and 30 weeks, and the full testing battery will be repeated after 36 weeks.

3.4 Outcome Measures

3.4A Primary Dependent Variables

The primary outcomes will be the proportion of omega-6 HUFA in total HUFA (%omega-6 in HUFA) and two endocannabinoids derived from omega-6 AA, 2-Arachidonoylglycerol (2-AG) and *N*-Arachidonoylethanolamine (Anandamide or AEA).

3.4B Secondary Dependent Variables

Secondary outcome measures will include blood concentrations of the omega-6 HUFA Arachidonic Acid (AA); the omega-3 HUFA Eicosapentaenoic Acid (EPA) and DHA; the rates of change in %omega-6 in HUFA, AA, EPA, DHA, 2-AG and AEA over time; adipose and muscle tissue fatty acid composition; endocrine hormones; gene expression profiles; psychometrics; functional satiety; 24-hr energy metabolism and substrate utilization; and body composition, as follows. Omega-6 and omega-3 HUFA

and total fat content will be measured in adipose and muscle tissue biopsies. Other endocannabinoid, eicosanoid and docosanoid derivatives of omega-6 and omega-3 HUFA will be measured in plasma via mass spectrometry.
Psychometric outcomes will include self-reported measures of satiety and affective states. Functional satiety will be assessed via buffet-style food array testing. Body composition outcomes will include DEXA, 3T MRI, anthropometric measurements and bioelectrical impedance. Genotyping will also be performed to assess for interactions between selected gene variants and effects of the intervention.

4. Introduction/ Scientific Rationale

4.1 Role of endocannabinoids in craving and satiety

Numerous crucial regulatory activities have been attributed to endocannabinoids, and their contribution to a host of seemingly unrelated chronic conditions is a matter of intense inquiry (1, 2). The endocannabinoid system includes the two endogenous ligands 2-arachidonoylglycerol (2-AG) and *N*-arachidonoylethanolamine (Anadamide or AEA) and two cannabinoid receptors (CB-1 and CB-2). CB-1 receptors are located primarily in the brain, but also in the GI tract, adrenal glands, liver, adipose tissue, and skeletal muscle (3). Excessive activity of the endocannabinoid system has recently emerged as having a central role in promoting obesity through at least three organ systems (4, 5). 1) Centrally, excessive 2-AG stimulates the CB-1 receptor, which increases hunger-induced increases in food intake, and decrease in satiety. 2) In liver, CB-1 stimulation increases *de novo* hepatic lipogenesis through activation of the fatty acid biosynthetic pathway. 3) In adipose tissue, CB-1 expression has been implicated in the control of adiponectin secretion and lipoprotein lipase activity.

Amid growing evidence that endocannabinoid signaling is up-regulated in obesity, pharmaceutical companies began developing CB-1 receptor antagonists. In 2006, the European Medicines Agency approved rimonabant, a CB-1 receptor inverse agonist, following impressive clinical trial results in both obese and diabetic patients. Specifically, placebo-controlled trials demonstrated considerable weight-loss (nearly 15 lbs), and significant improvements in: 1) waist circumference; 2) HDL-cholesterol; 3) triglycerides; 4) inflammatory markers; 5) blood pressure; 6) fasting glucose; and 7) fasting insulin (6, 7). In 2008, however, rimonabant was pulled from the market due to serious psychiatric side effects, including suicide (8). Because full pharmacological blockade of the CB-1 receptor is effective in treating obesity and related metabolic derangements, a safer alternative to pharmaceuticals for diminishing endocannabinoid hyperactivity may have widespread beneficial public health implications related to obesity and metabolic syndrome. Given the fact that endocannabinoids have important physiological functions, partial CB-1 receptor blockade may be preferable to full inhibition.

In a similar manner to food, endocannabinoid hyperactivity has been linked to impairment of satiety responses for alcohol (4, 9) and other substances of abuse (10). Impairment of satiety responses may be due to the shared downstream effects on signaling pathways (cAMP/PKA) regulated by CB-1 receptors and D2 dopamine receptors in the ventral tegmental area and striatum (11, 12). Thus, we posit that excessive endocannabinoid activity, and the ensuing neuroadaptations in the brain reward system, manifest phenotypically as deficits in satiety regulation for foods and substances of abuse, hepatic steatosis and excessive adipose accumulation. In this study we will not assess substances of abuse but will assess satiety for foods, and relevant metabolic changes.

4.2 HUFA as precursors to endocannabinoids

Endocannabinoids are not stored in cellular vesicles but rather are formed enzymatically from membrane phosopholipid-fatty acid precursors on demand (13). The two best characterized endocannabinoids, 2-AG and AEA, are both metabolic derivatives of a single fatty acid precursor, omega-6 arachidonic acid (AA).

Because humans cannot synthesize AA *de novo* (14), tissue phospholipid concentrations of AA (PL-AA) are dependent upon dietary intake of: 1) omega-6 AA (15); 2) the precursor to AA–linoleic acid (LA) (16); and 3) competing fatty acids such as omega-3 EPA and DHA (14, 15, 17). Therefore, by restricting dietary omega-6 fatty acids and/or boosting intake of competing omega-3 fatty acids, humans may be able to reduce PL-AA content, subsequent endocannabinoid synthesis from PL-AA, and the metabolic consequences of high 2-AG and AEA levels.

To test this hypothesis, we asked if selectively raising one omega-6 fatty acid in the rodent diet (while keeping total fat composition constant) would result in endocannabinoid hyperactivity and excess adiposity. We first asked whether the selective increase of linoleic acid (LA), from 1 % of energy (en%) to 8 en%, could elevate PL-AA in membranes and in turn also elevate endocannabinoid levels in liver and brain. To test if the proportion of PL-AA precursor pool was the critical variable, we also added 1 en% of energy as EPA and DHA to the 8 en% LA diet.

Results

Increasing dietary LA from 1 en% to 8 en% increased PL-AA (by 59%) and doubled liver 2-AG in the medium fat diet (see Table 1). Conversely, the addition of 1 en% as EPA and DHA to the 8 en% LA diet decreased PL-AA and halved liver 2-AG, indicating that regulation of the PL-AA in the phospholipid pool is a critical determinant for endocannabinoid production. A similar pattern was seen in the high fat diet. A comparable pattern of adverse effects of 8 en% LA was observed for feeding efficiency (defined as weight gained per kcal of food consumption), leptin levels, adiponectin levels (inverse pattern) and increased adiposity (see Fig. 1, differing letters indicate statistical differences at $p< 0.05$)

Table 1. Diet design, initiated in mothers, pups fed up to age 14 weeks, n=10 pups per group

	Medium fat (MF)			High fat (HF)		
	1 en% LA	**8 en% LA**	**8 en % LA + EPA/DHA**	**1 en% LA**	**8 en% LA**	**8 en% LA + EPA/DHA**
Fat en%	35	35	35	60	60	60
LA en%	1	8	8	1	8	8
ALA en%	1	1	1	1	1	1
EPA+DHA en%	-	-	1	-	-	1
MUFA en%	8	8	9	7	7	7
SFA en%	25	18	16	51	44	42
PL-AA	2.99+/- 1.26	4.74+/- 0.86	2.73+/-0.34	1.94+/-0.28	5.02+/-0.89	2.92+/-0.36
PL-EPA	0.85+/-0.33	0.13+/-0.02	0.87+/-0.25	1.07+/-0.19	0.14+/-0.02	1.17+-0.16
PL-DHA	4.73+/-2.02	2.87+/-0.50	3.49+/-0.65	3.74+/-0.50	3.07+/-0.59	4.70+/-0.66

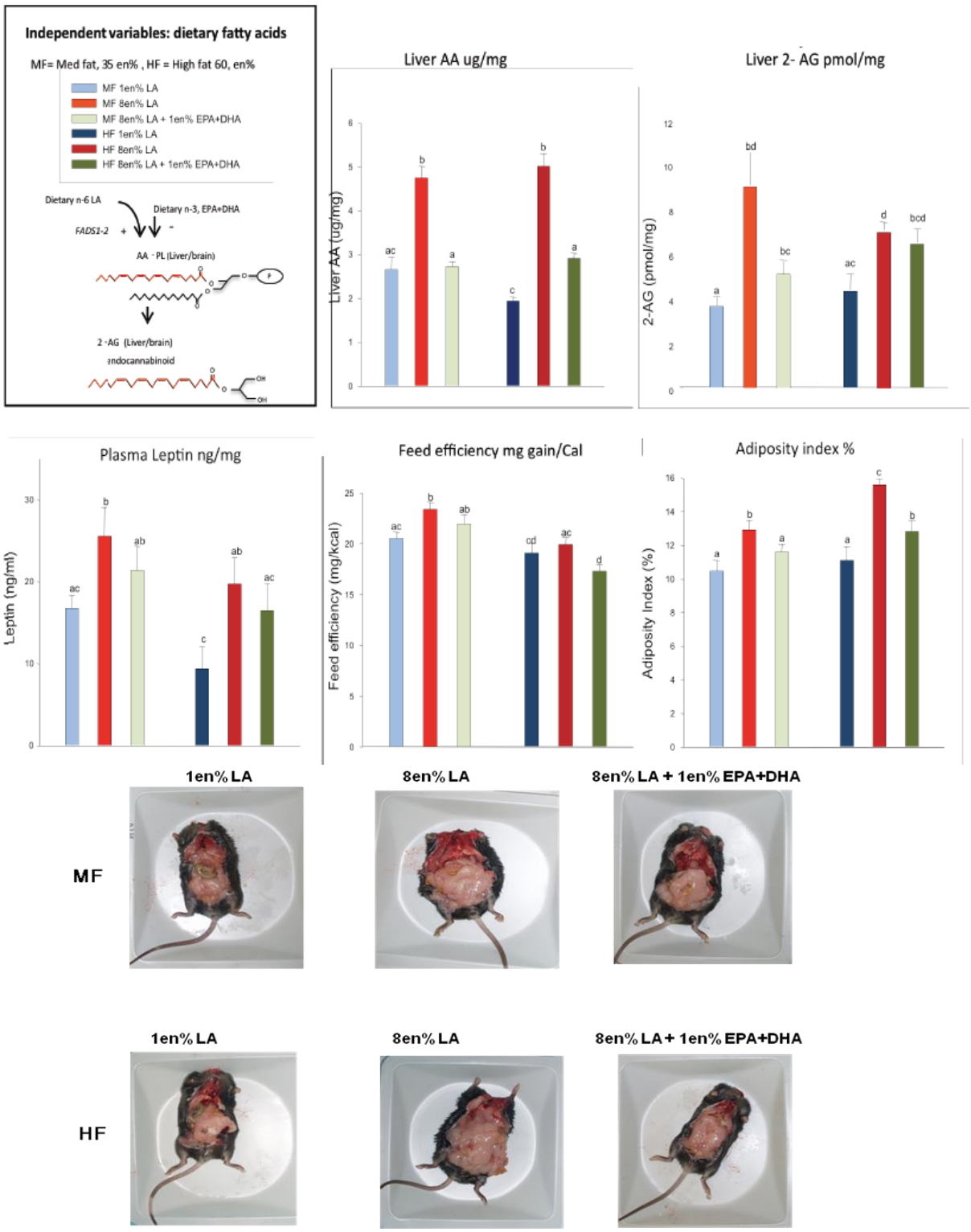
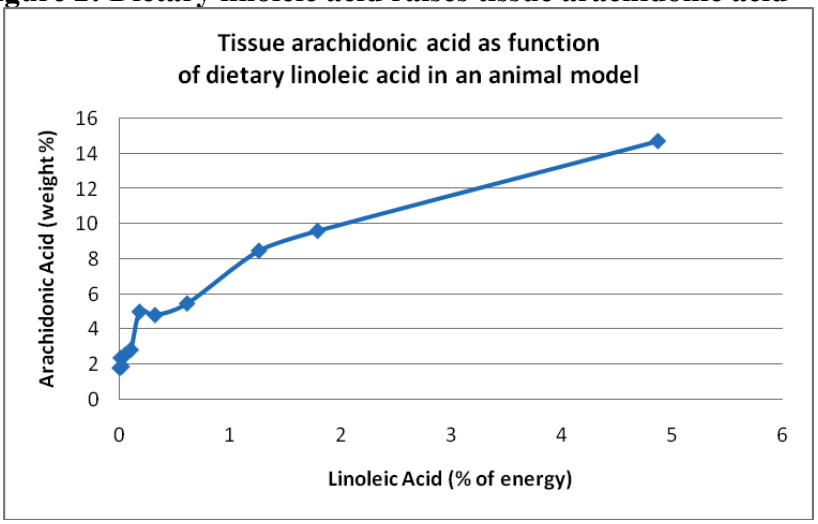


Figure 1: Dietary linoleic acid increased the omega-6 endocannabinoid precursor pool and induced endocannabinoid hyperactivity and obesity in rats.

In addition to raising PL-AA, the 8 en% LA diet reciprocally decreased both EPA (by 85%) and DHA (by 39%) in liver phospholipids compared to their 1 en% LA counterparts. Because PL-EPA and PL-DHA act as rival substrates with PL-AA for enzymatic conversion to omega-6 (or omega-3) endocannabinoid derivatives (18), these deficits in omega-3 EPA and DHA exacerbate omega-6 endocannabinoid hyperactivity.

Our results agree in principle with landmark rodent studies conducted nearly 50 years ago, which clearly demonstrated that tissue PL-AA concentrations increase progressively as dietary omega-6 LA increases up to 5 en%. In their classic 1963 study, Mohrhauer and Holman showed that when dietary LA ranged from 0 to 4.87 en% for 100 days, the accumulation of PL-AA in liver lipids ranged from 1.8 to 14.7% by weight (19). Similar robust variability in tissue omega-6 PL-AA content was seen in brain tissue (20) and erythrocytes (21) when dietary LA ranged between 0 and 5 en%. (See Fig. 2). However, further increases in dietary LA above 5 en% had comparatively minor effects on tissue PL-AA content (22), presumably because AA accumulation has already 'plateaued'.

Figure 2: Dietary linoleic acid raises tissue arachidonic acid



From Morhauer H 1963; 4;151

4.3 Translation from rats to humans

Do these principles apply to humans?

Like rodents, humans lack the enzymatic machinery to synthesize AA *de novo*. Thus, human tissue concentrations of PL-AA and other omega-6 HUFA are dependent upon dietary consumption of AA; its precursor–LA; and omega-3 acids that compete with omega-6 acids for enzymatic elongation, desaturation, acylation, and incorporation into human tissue phospholipids (17). Competition continues as omega-6 and omega-3 HUFA act as rival substrates for enzymatic cleavage and conversion into omega-6 and omega-3 endocannabinoids. Therefore, as in rodents, the reduction of dietary LA is a plausible strategy for decreasing tissue PL-AA and its endocannabinoid derivatives in *human* tissues.

Pilot data from the combined analysis of the first 20 participants in a randomized dietary trial testing the effects of a 12-week reduction of dietary LA, found that AA declined significantly, and EPA+DHA increased significantly, with or without added EPA+DHA (Table 2). These data indicate that 12 weeks is sufficient to detect significant changes in AA, EPA, and DHA. However, dynamic equilibrium in erythrocyte fatty acids was not reached between weeks 8 and 12 weeks.

Table 2. Means of erythrocyte fatty acids over the 12-week intervention, Chronic Daily Headache study, May 2010

	Baseline (n=20)		Week 4 (n = 20)		Week 8 (n=20)		Week 12 (n=18)	
Variable	Mean	95% CI	Mean	95% CI	Mean	95% CI	Mean	95% CI
Omega-3 index	4.4	3.9, 5.0	6.2	5.4, 7.1	7.0	5.9, 8.1	7.8	6.5, 9.0
Omega-6 in HUFA	76.3	74.5, 78.0	70.4	67.5, 73.2	67.4	63.7, 71.0	64.7	60.3, 69.0
DHA	4.0	3.5, 4.5	5.2	4.5, 5.8	5.7	4.9, 6.5	6.3	5.4, 7.3
EPA	0.46	0.39, 0.54	1.1	0.8, 1.3	1.3	0.95, 1.6	1.4	1.0, 1.8
AA	14.8	14.5, 15.2	14.4	13.7, 15.1	13.8	13.2, 14.5	13.7	12.9, 14.5
LA	11.5	10.8, 12.1	9.5	8.8, 10.2	9.3	8.6, 10.0	9.5	8.8, 10.3

The release of omega-6 and omega-3 fatty acids from adipose tissue stores is expected to prolong the time to necessary to reach dynamic equilibrium in plasma and erythrocyte phospholipids. Because adipose tissue fatty acids have an estimated half-life of 300 to 600 days (23-25), interventions substantially longer than our 12-week (84 day) diet phase are expected to produce even more substantial metabolic and clinical effects. Therefore, a substantially longer dietary trial is likely to be necessary to attain maximal metabolic effects, and to provide new knowledge about the potential confounding role of omega-6 and omega-3 fatty acids in adipose tissue stores. In conjunction with the preceding 84 day diet phase, subjects who complete the continuation phase will maintain study diets for 336 days, which will allow adequate time to assess the potential confounding role from the release of adipose tissue omega-6 and omega-3 fatty acids. The 36-week (252 day) duration of the continuation phase was therefore selected as a reasonable compromise between: 1) the desire to assess the confounding role of adipose stores of omega-6 and omega-3 fatty acids, and 2) the practicality of keeping volunteers on study diets for prolonged periods.

In conjunction with the plausible biological mechanisms described above, our rodent data and human pilot data provide proof of principle for our hypothesis that lowering dietary LA will raise tissue PL-EPA and PL-DHA, lower tissue PL-AA, attenuate excessive omega-6 endocannabinoid activity, enhance satiety and diminish excessive craving for pleasurable stimuli such as food or substances of abuse. The critical knowledge gap, and the logical next step, is to test our hypothesis in randomized controlled trial in humans.

5. Study objectives and hypotheses

5.1 Study objective

To evaluate the effects of modifying the dietary nutrient LA via controlled substitution for oleic acid (OA) on the blood omega-6 and omega-3 HUFA composition; endocannabinoids and other lipid mediators derived from omega-6 and omega-3 HUFA; and the rates of change in the omega-6 and omega-3 HUFA and their metabolic derivatives over time. We will also evaluate the effects of modifying dietary LA on insulin sensitivity; hunger and satiety; and body composition.

5.2 Specific hypotheses

5.2.1A Primary aims
To evaluate the effects of modifying the dietary nutrient LA on the %omega-6 in HUFA; as well as two endocannabinoids derived from omega-6 PL-AA, 2-AG and AEA.

5.2.1B Primary hypotheses
Lowering dietary intake of LA as a controlled variable from 8% to 1% of daily food energy will significantly decrease the %omega-6 in HUFA and the endocannabinoid derivatives of omega-6 AA, 2-AG and AEA.

5.2.2A Secondary aims
To assess the effects of modifying the dietary nutrient LA on the rates of change in omega-6 and omega-3 HUFA, 2-AG, and AEA over time; adipose and muscle tissue fatty acid content, relevant metabolic mediators measured in plasma, body composition, functional satiety, and psychometrics, as follows.
Metabolic outcomes will include other endocannabinoids, eicosanoids and docosanoids derived from omega-6 and omega-3 HUFA, 24-hr energy metabolism, and endocrine hormones related to obesity.
Adipose and muscle tissue outcomes will include tissue concentrations of omega-6 and omega-3 HUFA. Body composition outcomes will include DEXA, 3T MRI, anthropometric measurements and bioelectrical impedance.
Functional satiety will be assessed via (buffet-style) food array testing.
Psychometric outcomes will include self-reported measures of hunger, satiety, and affective states. Genotyping will also be performed to assess for interactions between selected gene variants and effects of the intervention.

5.2.2B Secondary hypotheses
Lowering dietary intake of LA as a controlled variable from 8 en% to 1 en% will result in:
- Increased omega-3 HUFA and reduced omega-6 HUFA in muscle and adipose tissue biopsies
- Reduced total fat content of adipose and muscle tissue biopsies
- Reciprocal increases in accretion of the omega-3 HUFA and their endocannabinoid, eicosanoid, and docosanoid derivatives.
- Favorable effects on insulin sensitivity and endocrine hormone function.
- Favorable effects on gene expression profiles.
- Favorable effects on hunger, satiety, and affective states.
- Increased functional satiety.
- Reduced adiposity and increased lean body mass.

6 Subjects

6.1 Description of study population
Subjects will be overweight, but otherwise healthy, pre-menopausal ambulatory volunteers aged 18 to 50.

6.2 Inclusion criteria
To be eligible for this research study, participants must:
1. Be between 18 and 50 years of age.
2. Have regular menstrual cycles.
3. Be willing to use an effective method of birth control such as hormonal contraception, intrauterine device, barrier methods combined with spermicide, or surgical sterilization.
4. Have a body mass index of 25 to 35 kg/m^2.
5. Be otherwise healthy as determined by history, medical examination and laboratory tests.
6. Be able to come to the NIH Clinical Center every weekday for 3 months.
7. Be able to understand the consent form, and provide informed written consent.

6.3 Exclusion criteria

Participants are not eligible for this research study if they:

1. Have been pregnant or have breast fed within the last 2 years.
2. Work, or have an immediate family member who works, with a study investigator. Immediate family is defined as a spouse, parent, child or sibling, whether biological or legally adopted.
3. Are HIV positive.
4. Have any current, unstable medical conditions including respiratory insufficiency requiring oxygen therapy; cardiac ischemia; cardiac or hepatic failure; or acute neurological illness as assessed by history and physical exam.
5. Have evidence of diseases that may influence metabolism (e.g. overt diabetes mellitus, thyroid disease, cancer)
6. Have any current or past history of eating disorders such as binge eating or anorexia nervosa.
7. Have had weight loss (bariatric) surgery.
8. Have gained or lost more than 3% of your current weight in the past 3 months.
9. Exercise strenuously, like running, swimming, or basketball, 4 or more times per week.
10. Are planning to begin a strenuous exercise program in the next 4 months.
11. Take supplements that contain omega-6 or omega-3 fatty acids (e.g. fish, cod liver, borage, evening primrose oils)
12. Have significant dietary limitations (e.g. multiple food allergies/intolerances, vegan diet) or special dietary requirements that are difficult to accommodate with study diets
13. Have significant claustrophobia that would preclude study tests.
14. Have a history of alcohol or substance abuse or dependence in the past 5 years.
15. Drink more than 2 alcoholic beverages per day, on average, or 6 drinks per sitting in the past year. Participants may drink up to one alcoholic beverage per day during the study.
16. Used marijuana, amphetamines, cocaine, or heroin even once over the last year. Participants may not use marijuana, amphetamines, cocaine, or heroin during the study.
17. Have smoked 2 or more cigarettes per week, on average, over the past year. Participants may not use tobacco products during the study.
18. Eat fish 3 or more times per week on average.
19. Have a known bleeding disorder.
20. Take medications or supplements that may interfere with this study by altering energy metabolism, nutrient absorption, or food intake. Regular use of the following compounds warrants exclusion: medications for the management of diabetes (Type 1 or 2), typical and atypical antipsychotics, thyroid medications, glucocorticoids, orlistat, decongestants, anti-histamines, and medications for cardiac conditions (e.g beta blockers), attention disorders (e.g. amphetamine derivatives), blood lipid disorders (e.g. statins, fibrates), and hypertension (e.g. thiazide diuretics), as well as certain anti-depressants (e.g. tricyclics), and supplements that impact energy metabolism, such as ephedrine.
21. Regularly take an anticoagulant medication, such as warfarin or aspirin.
22. Have a pacemaker, brain stimulator, or other implanted electrical device, permanent eyeliner, metallic prosthesis (including metal pins and rods, heart valves, and cochlear implants) that would preclude MRI scans.
23. Are currently participating in any other clinical research studies that include blood draws or other procedures.
24. Have a serum FSH level greater than 20 units per liter.

See Appendix 6: Eligibility Checklist

7. Study Design and Methods:

7.1 Study overview and design

This randomized controlled, 3-arm dietary trial aims to assess the rate and extent of changes in the composition of omega-6 and omega-3 HUFA and their metabolic derivatives in response to marked dietary changes in the nutrient omega-6 LA, in ambulatory overweight premenopausal women. The main trial is divided into two phases: 1) a 4 week 'baseline phase', and 2) a 12 week 'diet phase'. There is also an optional 36 week 'continuation phase' for participants receiving diet "B", as discussed below. During the baseline phase, prior to randomization, eligible subjects will complete 7-day food records. Those who are compliant with the food records and meet other eligibility criteria will undergo a baseline testing battery that includes: 1) blood parameters; 2) muscle and adipose tissue biopsies; 3) body composition assessment; 4) functional satiety assessment; and 5) psychometric testing. Participants will then be randomized to one of 3 healthy and nutritionally complete diets with equivalent macronutrient composition differing only in fatty acid content.

After randomization, participants will receive *all* foods through the NIH Metabolic Kitchen for the duration of the 12-week diet phase (i.e. meal replacement). During the first 4 weeks, the iso-caloric 'weight maintenance' phase, participants will be provided with the same amounts of daily food calories as they consumed during the 7-day record collection. During the following 8 week 'ad libitum' phase (from 4 to 12 weeks), participants will receive the same diets but will be allowed to eat as many servings of study foods as they desire. After completion of the 12 week diet phase, the full testing battery will be repeated.

At completion of the 12 week diet phase, a subset of participants (those receiving diet 'B') will have the option of continuing their study diets for an additional 36 weeks. During this 'continuation phase', the diets will be identical to their preceding diets. After 6, 12, 18, 24, and 30 weeks of the continuation phase, subjects will participate in follow-up testing sessions. At completion of the 36-week continuation phase, the complete baseline testing battery will be repeated.

Participants will remain ambulatory throughout the study. Subjects will visit the NIH Metabolic Kitchen daily Monday through Friday to obtain foods, and will be advised to continue their usual medical care and medications throughout the study.

7.2 Recruitment

Subjects will be recruited through the NIH Normal Volunteer Office, by word of mouth and through local advertisement (IRB approved flyers, newsletters, websites).

7.3 Screening Methods

7.3.1 Pre-screening

The NIH Patient Recruitment and Public Liaison Office will receive inquiries from interested study subjects. Pre-screening by this office will exclude patients who cannot regularly attend the NIH Metabolic Unit to acquire study foods, and those with significant claustrophobia that will preclude the imaging tests that are required to participate in the full protocol. All others will be contacted by the protocol team to review exclusion criteria. Eligible subjects will be invited to Clinical Research Center for a screening visit.

7.3.2 Screening visit (Baseline Phase Visit 1)

Patients will be invited to come to the OP-9 (Outpatient 9) Multiple Medical Specialties Clinic to obtain informed consent by a member of the investigative team. A limited physical exam with current weight and evaluation of venous access will be performed. A thorough medication history will be obtained with special attention given to drugs that may confound the interpretation of metabolic testing such as psychotropic agents, antiretroviral agents, sympathomimetic amines or beta blockers. An

electrocardiogram and baseline blood work (electrolytes, chronic liver disease work-up, renal function, vitamins, thyroid function, HgbA1C, HIV test), a serum pregnancy test and a urine test to screen for illicit drugs will be obtained. Instructions for completing detailed 7-day food records will be given to participants by a member of the Nutrition Dept staff.

7.4 Study Design

We anticipate that 84 subjects will need to complete the baseline phase and be randomized, with an anticipated dropout of about 20 subjects during the diet phase, in order for 64 subjects to complete the 12-week diet phase. Based on previous NIH Clinical Center dietary protocols with a similar baseline phase, we anticipate that 30 to 55% of enrolled subjects will not complete the baseline phase due to lack of attendance, non-compliance with 7-day food records, and/or discovery of an underlying exclusionary condition during the baseline phase history, physical examination or laboratory testing. Therefore, *up to*180 subjects will be enrolled in the baseline phase so that a sufficient number of subjects (64) will complete the diet phase.

After completing the baseline phase, 84 subjects will be randomized to 1 of 3 diets (Table 3), to be maintained for 12 consecutive weeks. We anticipate that about 20% of subjects will dropout during the diet phase. Therefore 64 subjects are expected to complete the diet phase.

Table 3: Fatty Acids in 3 Study Diets

Diet Group	omega-6 LA*	omega-3 ALA	omega-6 AA	omega-3 HUFA	%omega-6 in HUFA**
A. Orange	8.00	0.40	0.045	0.045	80
B. Blue	1.00	0.40	0.045	0.045	50
C. Green	1.00	0.40	0.045	0.81	20

*Fatty acid intake as percentages of daily food energy (en%).

** Predicted percentage of omega-6 HUFA in total omega-6 and omega-3 HUFA, via the Lands empirical equation, as detailed in Appendix 2

Please see Section 7.1: Study overview and design and Appendix 1: Study Design Flow Diagram.

7.5 Study Procedures

The main study lasts 16 weeks and is divided into 2 phases: 1) a 4 week ‘baseline phase’, and 2) a 12 week ‘diet phase’. There is also an optional 36 week ‘continuation phase’ for participants receiving diet “B”.

During the 4 week baseline phase participants will visit the NIH outpatient clinics three times as shown in Table 3 below. Visit 1 is a screening visit lasting about 1 hour. During Visit 2 laboratory tests will be reviewed, and participants will fill out questionnaires, which will take about 2 hours. At Visit 3, participants will receive about 6 hours of testing and will be randomly assigned to 1 of 3 study diets.

During the 12 week diet phase participants will eat only the study foods that are supplied by the NIH metabolic kitchen. They will visit the NIH outpatient metabolic unit every morning for breakfast (Monday-Friday) for all 12 weeks of the diet phase. Each of these 60 visits will last about 20 minutes, for a total of 20 hours. They will fill out the Comprehensive Psychopathological Rating Scale-Self-Affective (CPRS) questionnaire (see Section 7.6.6) after breakfast once every 2 weeks, which takes about 10

minutes to complete. Once every 4 weeks after breakfast, on Diet Day 28 and 56, they will have additional testing for about 1.5 hours; on Diet Day 84 they will have additional testing for about 6 hours as shown in Table 4 below. During the optional 9 month (36 week) continuation phase participants continue to eat only the study foods supplied by the metabolic kitchen. They will continue to visit the NIH outpatient metabolic unit every morning for breakfast (Monday-Friday) for all 36 weeks of the continuation phase. Each of these 180 visits will last about 20 minutes, for a total of 60 hours. Participants will fill out the CPRS questionnaire after breakfast once every 2 weeks, which requires about 10 minutes to complete. Once every 6 weeks after breakfast, participants will undergo additional testing for 3 to 6 hours as shown in Table 5 below.

All study procedures are for research purposes only.

Baseline Phase
The sequence of procedures in the baseline phase is given in Table 4. All time-points are targets, and may be adjusted based on practical considerations.

Table 4: Procedures during the Baseline Phase
Baseline Phase Testing Visits (4 week phase, Days 1-28)

Procedure	**Visit 1** (Day 1)	**Visit 2** (Day 14)	**Visit 3** (Day 21-28)
Blood Draw	▲		▲
Saliva DNA testing	▲		
Urine Drug Screen	▲		
Urine Laboratory Testing	▲		▲
7-day Food Records	▲	▲	
Medical Exam and Interview	▲	▲	
Eating Questionnaires		▲	
Mood Questionnaires		▲	
Weight, Anthropometrics and Vital Signs	▲	▲	▲
Electrocardiogram (EKG)	▲		
DEXA Scan			▲
Muscle and Adipose Tissue Biopsies			▲
Buffet Meal Test			▲

Baseline phase: 1st Visit
The first visit of the baseline phase is described in detail in Section 7.3.2 Screening Visit.

Baseline phase: 2nd Visit
Participants will return for an outpatient visit about 14 days after Baseline Visit 1. The 7-day food records and the results of the laboratory testing will be reviewed. Questionnaires to evaluate eating, satiety and mood will be administered (see Section 7.6.6). Those who remain eligible will be scheduled for a 3rd baseline visit.

Baseline phase: 3rd Visit

Participants will return for an outpatient visit about 7 to 14 days after the 2nd Visit. If their weight is stable, participants will have additional tests, which will require about 6 hours (Testing procedures are listed in Table 4).
Participants will receive a schedule for the remaining study visits and tests. Participants will then be randomized to 1 of 3 diets as described in Section 7.6.2.

Study Diets
The 3 study diets differ only in the amounts of fatty acids that they contain, as described in Sections 8.2. The diets are designed by NIH Clinical center dietitians to be nutritionally complete. All foods will be provided by the NIH metabolic kitchen. Participants are required to visit the NIH Clinical Center each morning, Monday through Friday, to eat breakfast and to pick up study foods, and to return uneaten food to the kitchen. Participants are also asked to keep a daily log of food and beverage intake.

We will strive for 100% adherence to study diets and daily visit schedules, but recognize based on previous experience and reviews of literature that this may not be practical or achievable in an ambulatory setting for this length of time. Therefore, to maximize adherence participants are provided with a day of emergency meals to eat during missed days whenever possible. They will also receive group-specific food-based guidance from the nutrition team designed to mimic their diet group if they are unable to pick up study diets for more than one day. Subjects will record/report any non-study foods or beverages throughout the study and this will be analyzed as part of the study diet. Finally, we will measure adherence to study diets using two proxies: (1) the LA content of circulating plasma measured at baseline and 4, 8, and 12 weeks of diet exposure; and (2) the number of days they did not pick up study meals. In a posthoc/exploratory manner, we will test for effect modification of the diet effects based on adherence. If effect modification is evident, we will present the results of the adherent subgroup as a posthoc analysis.
Diet Phase
The sequence of procedures in the Diet Phase is given in Table 5. All time-points are targets, and may be adjusted based on practical considerations.

Table 5: Procedures during the Diet Phase
Diet Phase Testing Visits (12 week phase, Diet Days 1-84)

Procedure	**Visit 1** (Day 28)	**Visit 2** (Day 56)	**Visit 3** (Day 84)
Blood draw	▲	▲	▲
Urine Pregnancy Test	▲	▲	▲
Mood Questionnaires	▲	▲	▲
Weight, Anthropometrics and Vital Signs	▲	▲	▲
DEXA Scan	▲	▲	▲
MRI Scan			▲
Muscle and Adipose Tissue Biopsies			▲
Buffet Meal Test	▲		▲

During the 12 week diet phase participants will visit the NIH outpatient metabolic unit every morning for breakfast (Monday-Friday). Each of these 60 meal visits will last about 20 minutes, for a total of 60 hours. Participants will complete the CPRS questionnaire after breakfast once every 2 weeks, which takes about 10 minutes to complete.

On 2 separate occasions after breakfast, once every 4 weeks on Days 28 and 56, participants will have a testing session lasting about 1.5 hours (Testing procedures are listed in Table 5). Twelve weeks after beginning the diet phase (Day 84), participants will have testing that lasts about 6 hours. They will have a shorter 2 hour testing session 4 and 8 weeks after beginning the diet phase.

Optional Continuation Phase
Those participants who were assigned to either diet "A" or diet "C" will have completed their participation after the 12-week diet phase of the study. Those who were assigned to diet "B" will be given the option of continuing a study diet for an additional 9 months (36 weeks).

The sequence of procedures in the Continuation Phase is given in Table 6. All time-points are targets, and may be adjusted based on practical considerations.

Table 6: Procedures during the Continuation Phase
Diet Continuation Testing Phase Visits (36 week phase, Diet Days 85-336)

Procedure	**Visit 1** Day 126	**Visit 2** Day 168	**Visit 3** Day 210	**Visit 4** Day 252	**Visit 5** Day 294	**Visit 6** Day 336
Blood draw	▲	▲	▲	▲	▲	▲
Urine Pregnancy Test		▲		▲		▲
Mood Questionnaires	▲	▲	▲	▲	▲	▲
Weight, Anthropometrics and Vital Signs	▲	▲	▲	▲	▲	▲
DEXA Scan		▲		▲		▲
MRI Scan						▲
Muscle and Adipose Tissue Biopsies						▲
Buffet Meal Test						▲

During the 36-week continuation phase, participants will continue to receive the study diet "B" that they received in the 12 week diet phase as shown in Appendix 1: Study Design Flow Diagram.
As in the main diet phase, participants will continue to visit the NIH outpatient metabolic unit every morning for about 20 minutes for breakfast (Monday-Friday). Each of these 180 visits will last about 20 minutes, for a total of 60 hours. Participants will fill out the CPRS questionnaire for about 10 minutes after breakfast once every 2 weeks.

On 5 separate occasions after breakfast, at 6, 12, 18, 24 and 30 weeks into the continuation phase, participants will have a testing session lasting about 1.5 hours. At 36 weeks into the continuation phase participants will have a testing session lasting about 6 hours (Testing procedures are listed in Table 6).

7.6 Study Procedure Specifics

7.6.1 Food Records
Food records will be used to assess recent and usual intake of kilocalories, macro- and micronutrients as well as assess usual eating patterns. The information will be used to plan the caloric content of the diet to be served from

metabolic kitchen. The food record will be analyzed using the Nutrition Data System for Research (NDS-R), developed by the Nutrition Coordinating Center, University of Minnesota, Minneapolis, MN.

7.6.2 Randomization and Masking

At completion of the pre-intervention phase, participants will be randomized to 1 of the 3 treatment groups by computer. This process eliminates experimenter bias in group assignment. The computer program uses a random numbers generator and is programmed to ensure appropriate numbers of subjects in each arm of the study within a permuted variable block size of 3-4 patients. The computer program generates a treatment assignment associated with each eligible subject's name and study ID number and documents these in an uneditable form with a date stamp. The research dietitian will have password-protected access to the computer-generated treatment assignments. The dietitian will use this information to assign each participant to one of the 3 treatment arms, which will be designated by a letter and color (e.g. Diet A: Orange). Because the 3 diets differ only in the fatty acid content of added visible oils, which are clear and can be distinguished only by nutritional analysis, the participants will remain blinded to their treatment. The research dietitian, by necessity, needs to know which group the patient is assigned in order to provide appropriate foods. No other research or clinical investigators will be told which arm the participants have been assigned.

7.6.3 Specimen collection for biomarker testing

Blood Collection

To assess the blood compositions of omega-6 and omega-3 fatty acids and their metabolic derivatives, fasting venous blood will be collected at 4 timepoints in the main study (Baseline Visit 3; Diet Phase Visits 1, 2 and 3). Blood will be collected from participants in the optional continuation phase at 6 additional time points (see Table 5: Continuation Phase Visits). Blood will be immediately processed and stored at -80 degrees C. See Table in Appendix 2 for a list of fatty acids and their metabolic mediators that will be assessed.

Genotyping

This project will collect DNA samples from the saliva of well phenotyped overweight subjects for genotyping. Genotyping will be performed on all study participants. Single gene mutations/polymorphisms of interest include: 1) those involved in fatty acid metabolism; 2) those implicated in the pathogenesis of obesity; 3) those implicated in the pathogenesis of medical and psychiatric conditions associated with obesity; 4) those linked to certain behavioral characteristics relevant to obesity and obesity-related comorbidities. On the consent form, participants can designate whether or not the DNA samples can also be stored for future research projects.

Muscle and adipose tissue biopsies and tissue procurement and post-biopsy pain assessment

Fat and muscle biopsies will be obtained before and after the dietary interventions, by the study physicians and/or nurse practitioner. In accordance with established NIDDK practices, biopsies will be performed on the Metabolic Unit or the adjacent 5SW day hospital, as described below.

Subcutaneous adipose tissue (3-5 g) will be removed from the abdominal region by aspiration with a 16-gauge needle under local anesthesia (e.g. 2% xylocaine).

Muscle biopsies (100-250 mg) will be obtained under local anesthesia from the vastus lateralis, as follows. The patient will be prepped and draped in sterile fashion. The muscle biopsy procedure involves numbing a nickel-sized portion of the skin of the thigh with a local anesthetic and making a small incision, approximately 1/4 inch. The biopsy needle is then inserted via the incision through adipose tissue and muscle fascia into the muscle. A small sample of muscle tissue is removed. The incision will then be closed with steri-strips and covered with a dressing. If the muscle is too deep to be reach, the muscle biopsy will not be attempted.

The biopsy tissue specimens will be separated into two aliquots for muscle and three aliquots for adipose tissue for assessment of fatty acid composition and other biochemical assays, frozen in dry ice and stored at -80ºC for future studies.
Post-biopsy pain will be evaluated using the standard Clinical Center Numeric Rating Scale (Score range 0-10) before and after the biopsy, and twice daily (upon awakening and at lunch time) for 3 additional days.

7.6.4 Insulin Sensitivity
Homeostasis Model of Assessment – Insulin Resistance (HOMA-IR)
To assess insulin sensitivity, fasting blood glucose and insulin will be collected at each testing visit, for calculation of the HOMA-IR, a validated biomarker for estimating insulin sensitivity.

7.6.5 Functional Satiety assessment
Ad-libitum Buffet Meal
Participants will be fed a standard breakfast meal containing 15% of energy needs (calculated using the Mifflin St. Joer equation with an activity factor of 1.5). Between the breakfast and buffet meal participants will be asked to fill out Visual Analogue Scales asking questions about hunger and satiety at 30-60 minute intervals. Six hours following the standard breakfast the patient will be placed in a food laboratory and presented with a buffet meal. They will be instructed to "eat until they are full." The buffet meal will contain foods that will be weighed pre and post consumption in a metabolic kitchen so that the precise amounts of foods consumed can be calculated.

Buffet Meal Screening
Food Preference Questionnaire:
At screening patients will fill out a 50 item food preference questionnaire with buffet items embedded within it. If the patient reports dislike of $\geq$ 50% of the food items on the buffet, they will be excluded from participation.

7.6.6 Psychometric assessments
Questionnaires to Evaluate Eating Behavior and Satiety
WALI- Weight and Lifestyle Inventory: This is a comprehensive questionnaire encompassing the subject's weight and health history. It includes sections on self-reported eating and physical activity patterns. Eating disorders such as binge eating, night eating syndrome and other abnormal eating patterns can be evaluated with this assessment. Estimated completion time is 60-90 minutes.

Three Factor Eating Questionnaire: An instrument developed by Stunkard and Messick to measure dietary restraint, disinhibition and hunger. Consists of 51 questions related to these 3 factors of human eating behavior. Estimated completion time is 20-30 minutes.

Questionnaires to Evaluate Mood
POMS- Profile of Mood States: The POMS assessment provides a rapid, economical method of assessing transient, fluctuating active mood states. The POMS can be readministered as frequently as every week. The scale contains 65 items, which subjects rate on the Likert scale (0 to 4). A summation of scores for tension, depression, anxiety, fatigue, and confusion are subtracted from the vigor score to yield an overall description of subject's mood state. Estimated completion time is 10-15 minutes.

FS- Flourishing Scale: This scale consists of 8 questions related to subject's feelings about their personal accomplishments in life. Subjects rate each item on a numerical scale. High scores represent people who feel

they are leading thriving, resourceful and capable lives, and thus is a measure of positive affect. Estimated completion time is 5-15 minutes.

PANAS- Positive and Negative Affect Schedule: This assessment is a self-rated affect scale which distills emotionally descriptive words into either positive or negative mood valences. The subject gives a numerical rating to each emotionally descriptive word given in the list of words. Positive and Negative affectivity are constructed by summing the ratings for these words. The test can be repeatedly administered with valid results; even within a single day. Estimated completion time is 10-15 minutes.

CPRS-S-A- Comprehensive Pyschopathological Rating Scale-Self-Affective: Contains 19 questions condensed and rephrased from the full CPRS for self-implementation to quickly assess the subject's mood and anxiety level. It can be repeatedly administered, up to every three days. It will be administered weekly in this study. Estimated completion time 5-15 minutes.

7.6.7 Body Composition

Anthropometric measurements and bioelectrical impedance

Height, weight, and circumferences will be obtained using standardized procedures. Bioelectric impedance spectroscopy (BIS, Xitron 4200, Xitron Technologies, San Diego, CA) testing will be used to assess fluid status (ECW, ICW, TBW). Subjects are measured while lying supine on a nonconductive surface. Bioelectric resistance and reactance values are measured after induction of electrical signals with a maximum current of 800 μA at multiple frequencies.

Dual Energy X-ray Absorptiometry (DEXA) scan

DEXA (iDXA, GE Medicalsystems, Madison WI) will be used to measure body composition. This technique has found extensive clinical and research applications in addition to the assessment of bone mineralization status. With this technique, one can determine total and regional body fat and fat-free masses and can estimate appendicular muscle. DEXA produces photons at two different energy levels, 40 and 70 KeV. The photons pass through tissues and attenuate at rates related to elemental composition. Bone mineral, with highly attenuating calcium and phosphorous, is readily distinguished from soft tissues. The different elemental profiles of fat and bone-mineral free lean allows for the analysis of soft tissue fat content, so that bone mineral, fat, and bone mineral fat-free lean components may be resolved. The DEXA has a slight radiation associated (an effective dose of about 1 mrem total exposure to the tissue/scan, approximately equal to one day of background exposure). Half-body scans may need to be performed for subjects that extend beyond the scanning area (26, 27).

Assessment of visceral, liver and cardiac adipose (3T MRI)

Overweight and obesity are associated with fat accumulation in the abdominal cavity (visceral fat) and other important sites such as the liver (28, 29) and muscle, referred to collectively as "ectopic fat" (30). Magnetic resonance imaging (MRI) is used to quantify triglyceride content in the abdominal cavity, liver and heart. Two techniques will be employed to measure ectopic fat: proton MR spectroscopy (MRS) (29) and multi-echo imaging using the 3.0 T magnet (31).

7.6.8 Assessment of Dietary Adherence

Adherence to study diets will be assessed via multiple methods. The fatty acid compositions of all study foods will be determined and all study foods that are dispensed to study subjects will be recorded. Participants will be asked to return any foods they do not eat. They will be instructed to record any consumption of outside foods in a food log, including the specific food type (name brand if possible) and serving size. Missed breakfast and food pick-up visits will be recorded. In addition, blood concentration of LA will be measured every 4 weeks during the diet phase, and every 6 weeks during the continuation phase.

7.6.9 Follow-up/ termination procedures

There is no required follow-up for this study after completion of the experimental protocol. Participants will be advised to continue receiving their usual medical care throughout the duration of the study. With patient consent, clinically-relevant information obtained through participation in the protocol may be shared with participants and appropriate health care providers.

7.6.10 Radiation exposure

DEXA scans will be performed solely for research purposes. Please see sections 8.7 and 8.9 for risks.

7.6.11 Medications and devices requiring IND/IDE

No medications or devices requiring IND/IDE will be used in this study.

7.6.12 Storage of research samples and data

Since many potentially important markers and genes relevant to obesity and related comorbidities are unknown at the present time, all research samples including DNA will be coded for storage in refrigerators and freezers in a locked laboratory. Retention of codes to identify the sources of research samples is necessary to perform correlations between genotype and phenotype, and between dietary intervention and outcomes. The plan is to store the samples until they are analyzed. These samples will be stored indefinitely. The IRB will be notified in the event these samples are accidentally destroyed, lost or are anonymized. Tissue banking of DNA and tissue specimens will only include participants who specifically provide consent for future studies.
Some clinically relevant research data will be stored indefinitely in the medical record and will be accessible to the patient for review by others of their choosing (doctors, insurance companies etc.) after executing a release of information. The validated psychometric instruments used in this protocol have been converted to an electronic web-based securedata entry platform administered by the NIAAA. . The security of the data management system f is curated by the NIAAA in agreement with NIH data security policies. Most data generated in this study will be stored and maintained in the NIAAA clinical database, which is password protected and secure.

Use of research samples and data

Currently, the research use of samples is outlined in the protocol. Stored materials will be used for future evaluation of metabolic mediators derived from fatty acids, as well as metabolic mediators implicated in obesity and related medical and psychiatric comorbidities as the field evolves.

Collaborations on stored tissue and blood samples

Any collaborations requiring transfer of patient material will be done in a coded fashion after informing the IRB and obtaining necessary assurances from the outside institution. The protocol will be amended and IRB approval will be sought when such collaborations are established.

8 Risks and Discomforts

8.1 Procedures with No Medical Risk and Minimal Discomfort

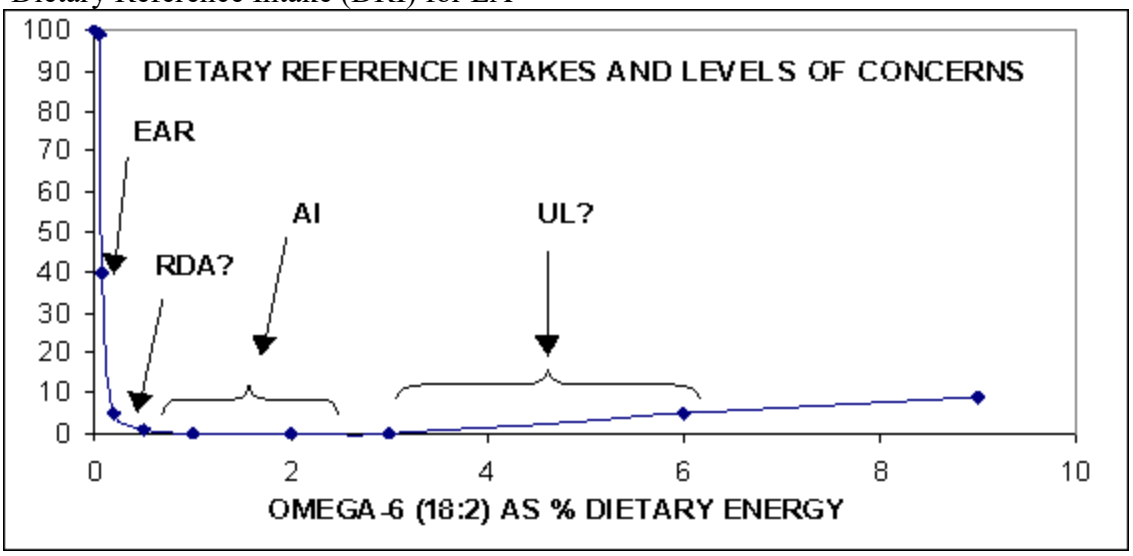
The history and physical exam, body measurements, bioelectrical impedance, vital signs, electrocardiogram, buffet meal test, and questionnaires to evaluate eating history and quality of life have no medical risk and minimal discomfort.

8.2 Study Diets

We will provide healthy and nutritionally complete diets to all intervention groups. The diets will differ in their fatty acid composition. In general, there is minimal risk associated with adverse events as fatty acids in edible form are GRAS (generally recognized as safe) by federal regulatory agencies.

The controlled dietary variable to be changed in this study is linoleic acid (LA), a common nutrient in foods. The minimal dietary requirement for LA to prevent signs and symptoms of deficiency, as reviewed and developed by William EM Lands, PhD is between 0.05% and 0.50% of energy (see Figure below, available at http://efaeducation.nih.gov/sig/dri.html). Furthermore, several modern populations consume less than 1 % of energy from LA with no apparent ill-effects (32). The American Heart Association recently advised that it is safe to consume "at least 5 to 10% of energy" from LA (33). In our study diets, LA intakes will range from 1.0 % to 8.0 % of energy. At these ranges, there is little concern for risk as they are well above the minimal dietary requirement and in ranges that are commonly consumed by modern humans.

Dietary Reference Intake (DRI) for LA



Some published articles with information related to these four categories are listed below.

EAR-Unwanted dermal signs were present in 100% infants receiving 0.04 % energy as linoleate, but in only 40% infants receiving 0.07 % energy as linoleate and not in any receiving 1.3 %. The EAR may be near 0.06 % of energy (34); **RDA**-Thousands of healthy infants were raised with diets containing omega-6 as 0.4 to 0.9 % energy, and calculation showed that probably less than 0.5 % energy was needed to prevent deficiency symptoms in infants. This level may meet requirements for 97% to 98% of infants (35, 36); **AI**- Although not rigorously determined, intakes from 1 % to 3 % of daily energy as omega-6 seem adequate (37); **UL**-Excessive omega-6 eicosanoid actions and thrombotic deaths seem less frequent with balanced omega-3/omega-6 intakes (38). **Quintiles of dietary intake of LA**-in the USA have the following means: 2.9; 4.4; 5.5; 6.9; 10.3 en% (39).

A subset of participants will also receive 1 to 2 grams of omega-3 EPA and DHA from fatty fish. There is a theoretical increase in bleeding times with omega-3 fatty acid intake. However, human supplement trials, including trials with considerably higher doses of omega-3 fatty acids, have not found increased bleeding risk (40, 41), with or without concurrent use of antithrombotic medications. We will use doses from foods that well within the usual daily intakes of populations with regular fish intake, such as Japan

and Iceland (42, 43). Therefore, there is little concern for increased risk due to increased omega-3 fatty acid intake.

It is possible that participants could have an allergic response to study foods. During the screening visit, participants will provide information on known dietary allergens and the diets will be adapted accordingly, or if necessary the participants will be excluded. Participants will also be advised to alert the research staff as soon as possible if they experience symptoms such as rash, hives, wheezing, or other signs of food allergy.

With any change in dietary intake there is potential for gastrointestinal distress in human subjects. Subjects will be asked to report signs of distress. Participants will be advised to use appropriate medications and/or digestive aids (H2 blockers, bean-o, etc) as necessary.

This study also requires daily workday visits to NIH for 12 weeks. It requires that participants only eat the food we provide. They may find these requirements inconvenient or burdensome. They may not like the study foods. Each of the study diets is nutritionally complete so it is unlikely that eating the diet will compromise health.

8.3 Specimen Collection for biomarker testing

Blood Collection

Participants may have some discomfort and bruising at the site of needle entry. There is a very small risk of fainting. Infection in the area of the needle insertion is rare.

The amount of blood drawn from participants shall not exceed the specified limit of 550 ml (or 10.5 mL/kg) for any 8-week period for adult volunteers, provided in "Guidelines for Limits of Blood Drawn for Research Purposes in the Clinical Center" (M95-9 (rev.) 5 June 2009).

Since participants will have BMI between 25-35, the limit of 550 mL per 8-week period will apply. During the main study, which includes the baseline phase and 12-week diet phase, we will draw no more than 1 cup of blood (1 cup = 8 fl oz = 235 mL) for research purposes at any one time and no more than 3 cups during the entire main study. We will draw no more than 2 cups (470 mL) of blood during any 8-week period.

For participants in the optional 36-week continuation phase, we will draw no more than 3 additional cups of blood for research purposes, and no more than 1.5 cups (353 mL) during any 8-week period.

Genetic Testing

The genetic testing that will be done as part of this study is done for research purposes only. It will not provide any information about participants' health or ancestry. It is our policy to not provide the results of such genetic testing.

8.4 Muscle and adipose tissue biopsies and tissue procurement

The major risks/discomforts include pain, bruising, infection, and scarring. The procedures will be performed under sterile technique to minimize the chances of infection. Local anesthetic will be used to minimize pain. Ice will be applied to the site immediately after the procedure to limit bruising, swelling and tenderness. After subcutaneous tissue biopsy, patients will be monitored by a nurse. Post-biopsy the incision site will be cleaned and closed with adhesive wound closures and covered with gauze and translucent dressing tape. Study participants will be instructed to report to the clinical staff any changes at

the biopsy site including bleeding, secretion, erythema, pain, and signs and symptoms of infection. Study participants will be instructed to self-monitor the incision site after discharge from the Clinical Center.

8.5 Questionnaires to Assess Mood

Some subjects may find the questions or tests boring or frustrating. They may feel uncomfortable discussing their mood and personality with the study staff. They will be instructed to answer the questions as well as and as honestly as they can. There are no direct physical risks associated with these tests. It is possible that important information may be revealed about the subject that in some cases may benefit from medical intervention. In this case, the patient will be advised to share this information with their doctor for follow-up. Furthermore, with the patient's consent, results from these tests will be forwarded to their physician of choice for follow-up.

8.6 Body Composition

Dual Energy X-ray Absorptiometry (DEXA) SCAN

DEXA will be used to measure body composition using x-rays to non-invasively assess skeletal and soft tissue density. The procedure involves low-level radiation exposure. The expected exposure for a scan is (0.001 rem) or the equivalent of less than one day's exposure to natural background radiation. For more information see Radiation in Section 8.9.

Magnetic Resonance Imaging (MRI) assessment of visceral, liver and cardiac fat

People are at risk for injury from the MRI magnet if they have pacemakers or other electrical devices, brain stimulators, some types of dental implants, aneurysm clips (metal clips on the wall of a large artery), metallic prostheses (including metal pins and rods, heart valves, and cochlear implants), permanent eyeliner, implanted delivery pump, or shrapnel fragments. Welders and metalworkers are also at risk for injury because of possible small metal fragments in the eye of which they may be unaware. Subjects will be screened for these conditions before having any scan, and if any are found, the subject will not receive an MRI scan. It is not known if MRI is completely safe for a developing fetus. Therefore, all women of childbearing potential will have a pregnancy test performed no more than 24 hours before each MRI scan. The scan will not be done if the pregnancy test is positive.

People with fear of confined spaces may become anxious during an MRI. Those with back problems may have back pain or discomfort from lying in the scanner. The noise from the scanner is loud enough to damage hearing, especially in people who already have hearing loss. Everyone having a research MRI scan will be fitted with hearing protection. Subjects will be asked to complete an MRI screening form for each MRI scan.

8.7 Radiation from DEXA Scans

This research study involves exposure to radiation from the 4 DEXA scans in the main study and 3 additional DEXA scans in the optional continuation phase. This radiation exposure is not required for medical care and is for research purposes only. The amount of radiation the subject will receive in the main study is 0.004 rem, and in continuation phase is an additional 0.003 rem, which are below the guideline of 5 rem per year allowed for research subjects by the NIH Radiation Safety Committee. The average person in the United States receives a radiation exposure of 0.3 rem per year from natural sources, such as the sun, outer space, and the earth's air and soil. If a subject would like more information about radiation a copy of the pamphlet An Introduction to Radiation for NIH Research Subjects will be provided.

While there is no direct evidence that the amount of exposure from participating in this study is harmful, there is indirect evidence it may not be completely safe. There may be a very slight increase in the risk of cancer.

Participants will be instructed to tell the doctor if they have had any radiation exposure in the past year, either from other research studies or from medical tests or care, so the research staff can make sure that the subject will not receive too much radiation. Radiation exposure includes x-rays taken in radiology departments, cardiac catheterization, and fluoroscopy as well as nuclear medicine scans in which radioactive materials were injected into the body.

If a subject is pregnant or breast feeding, she may not participate in this research study. It is best to avoid radiation exposure to unborn or nursing children since they are more sensitive to radiation than adults.

9 Subject Monitoring, adverse event grading and withdrawal

9.1 Parameters to be monitored

Participants will be closely monitored for adverse reactions by medically qualified staff during the invasive procedures; including the tissue biopsy procedures, the blood sample collection. Appropriate medical care and treatment will be provided if necessary. Study participants will have daily contact with the metabolic kitchen staff during weekdays and will be encouraged to report any potential adverse reactions to study foods.

During the diet phase and continuation phase, the CPRS will be administered to monitor participants' mood. It is possible that important information may be revealed about the subject that in some cases may benefit from medical intervention. In this case, the patient will be advised to share this information with their doctor for follow-up. Furthermore, with the patient's consent, results from these tests will be forwarded to their physician of choice for follow-up.

Participants will be withdrawn from the study if they do not follow the instructions outlined in the protocol and informed consent form. Non-compliance will be set as a threshold of deviation from the provided diet and will be determined by medically qualified staff. Participants may also, at any time, choose to discontinue their participation from the study.

10. Outcome Measures

10.1 Primary Outcome Measures

- Plasma phospholipid fatty acid composition
- Plasma endocannabinoids derived from PL-AA

10.2 Secondary Outcome Measures

- Muscle and adipose tissue fatty acid composition
- Metabolic derivatives of omega-3 HUFA and omega-6 HUFA in plasma
- Pre- and postprandial endocrine hormones
- 24-hr energy metabolism and substrate utilization
- Self-reported eating behavior, satiety, and affective states
- Functional satiety assessment with buffet-style food array
- Anthropomorphic measures and DEXA scan
- 3T MRI for body composition

11. Statistical Analyses

11.1 Overview

This study is a three-arm dietary intervention trial with a baseline measurement and with repeated measures of the outcome variables over time. The primary aim is to measure the pre- post- changes in our primary outcome variables: %omega-6 in HUFA, 2-AG and AEA. In secondary Aim 1, we will measure the rates of change in our primary outcome variables. Exploratory aims will be directed towards the extent and rates of change in our secondary outcome variables (see Section 10.2) in response to dietary interventions.

11.2 Analysis of Data

Fatty acid concentration data will be examined for homogeneity of variance and normality of distribution, and if necessary transformed to meet this criterion. A probability of 0.05 or lower will be considered significant.

Primary Aim: The sample size was selected to provide adequate power (0.8) to test our Primary Aim, as described in section 11.3. In this Aim we plan to assess the extent of changes in %omega-6 in HUFA; 2-AG and AEA; after 84 days of targeted dietary interventions. To achieve this aim we will treat the data coming from a four-group (control and two treatment groups), pre-post study design. There will be a measurement for all the individuals in the study at randomization and at 84 days. The outcome will be the measurement at 84 days.

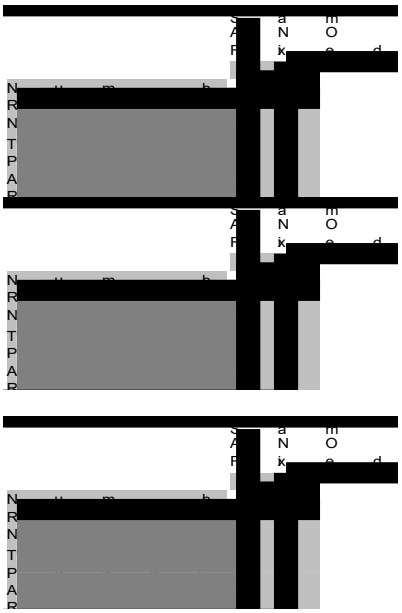
Secondary Aim 1: To assess the rate of changes in %omega-6 in HUFA; PL-AA; 2-AG and AEA; PL-EPA; and PL-DHA, we will make use of the repeated measures of the outcome variables measured at baseline, and 28, 56 and 84 days into the diet phase. The goal is to compare the rates of change in these outcome variables over time in the three groups. It is expected that the rates of change will be fastest in Group C (the low LA plus omega-3 HUFA diet group).

Exploratory Aims: Additional exploratory analyses will be performed to further examine the metabolic and clinical effects the of dietary interventions by: 1) assessing the extent and rates of change of the *other* metabolic mediators and clinical outcome measures, as detailed in Section 10; 2) examining the association between the changes in blood concentrations of fatty acid precursors (e.g. PL-AA) and their metabolic derivatives (e.g. 2-AG); 3) examining the association between changes in precursor fatty acids and clinical outcome measures; and 4) examining the association between changes in the metabolic derivatives of precursor fatty acids and clinical outcome measures.

11.3 Estimation of Power and Sample Size

We will use one-way analysis of variance to compare the proportion of %omega-6 in HUFA measured at 12 weeks among three groups. Assuming that the %omega-6 in HUFA will be, on average, 0.8, 0.5 and 0.2 in the three groups at the end of the 12 weeks (as predicted by Lands' empirical equation and the nutrient intake targets), we calculated required sample size at three values of standard deviation: the maximum value of 0.5, at 0.4 and 0.3. These values translate to root mean square standardized effect is calculated as 0.6, 0.75, and 1.0. To detect these effect sizes, at a power of 80% and an alpha of 0.05, we will need either 15 individuals per group (s = 0.5), 10 (s = 0.4), or 6 (s = 0.3).

Fig. 6. Sample Size and Power Curve



12 Human Subjects protection

12.1 Regulatory and Ethical Considerations

The study will be conducted in accordance with Good Clinical Practice (GCP), 21 CFR Parts 50 and 56 and all applicable regulatory requirements.

Pre-menopausal women, post-menopausal women, and men are known to have major differences in their metabolism of omega-6 and omega-3 fatty acids (44-48). Specifically, pre-menopausal women efficiently convert dietary omega-6 and omega-3 18-carbon precursors (omega-6 LA and omega-3 alpha-linoleic acid) into omega-6 AA and omega-3 DHA, which compete for incorporation into tissue membranes (44, 46, 49, 50). Critically omega-6 AA is the immediate metabolic precursor to endocannabinoids (13), the primary outcome of the study. This efficient conversion is due to the effects of estrogen on liver enzymes (46, 49). Because endocannabinoids regulate hunger, satiety and metabolism, we expect pre-menopausal women to uniquely benefit from lowering dietary LA. By contrast, men and post-menopausal women are inefficient converters of omega-6 and omega-3 18-carbon precursors to AA and DHA (14, 44, 46). Because low LA diets are not expected to efficiently reduce tissue concentrations of AA, or its endocannabinoid derivatives in post-menopausal women and men, they are considerably less likely to benefit from lowering dietary LA.

12.2 Equitable Inclusion

The study population will consist of non-pregnant, non-lactating female subjects. Subjects that fulfill the study criteria will be included regardless of race, ethnicity, socioeconomic, religious, political or similar factors. Based on open recruitment from the NIH federal employee population, the expected racial composition of the study population will be 20.3% African-American, 2.8% Hispanic, 15.9% Asian or Pacific Islander, 0.6% Native American and 60.4% White (based on Office of Personnel Management Demographic Statistics available at: https://www.opm.gov/policy-data-oversight/data-analysis-documentation/federal-employment-reports/demographics/2010/table1mw.pdf).
Only English speaking volunteers will be included in this study because several of the psychometric instruments (e.g. POMS, PANAS, FS, CPRS) have not been validated in other languages.

12.3 Inclusion of children or other vulnerable subjects
No children will participate in this protocol because children metabolize fatty acids differently than adults, and weight gain is not necessarily undesirable in this growing population.

12.4 Justification of sensitive procedures
The controlled dietary variables to be modified in this study are linoleic acid and oleic acid, which are common nutrients in foods. There is minimal risk associated with adverse events as fatty acids in edible form are GRAS (generally recognized as safe) by federal regulatory agencies.

Procedures that may cause some discomfort to subjects:

1) *Blood sampling:* Trained medical personnel will carry out the blood draws which may cause some discomfort to the subject.
2) *DEXA scan:* Trained medical personnel will perform the scan to reduce discomfort and risk to the subject. The expected exposure for a scan is (0.001 rem) or less than one day's exposure to natural background radiation.
3) *Psychiatric and Neuropyschological Assessment measures:* Research team personnel trained in the administration of psychometric measures will assist the subject in answering survey questions in the vicinity of trained medical personnel. Any confusion or unexpected response will be taken into consideration for follow-up by the trained medical personnel.
4) *Tissue Biopsy:* Muscle and adipose tissue biopsies will be performed by trained medical personnel. Subject will be monitored for signs of undue discomfort . Medication and appropriate palliative care will be provided if necessary.
5) *3T MRI Scan:* Trained personnel will perform the scan to reduce discomfort and risk to the subject. In the 3T scanner, it is possible that the patient may experience peripheral nerve stimulation resulting in a sensation of mild twitching, warmth or vibration. Medical staff will monitor the subjects and administer appropriate care if necessary.
6) *Storage of Private Health Information in a Research Database:* Clinical information from each subject will be stored in a retrievable database at NIH. This information will remain confidential and protected like the medical record of the subject. Information with identifiers will not be released to any investigators or agencies/companies without the explicit consent of the subject.
7) *Storage of Patient Samples:* Coded blood and tissue samples without patient names or personal identifiers will be stored in secured freezers at 5625 Fishers Lane, Room 3N-01 or in the NIAAA Clinical Core lab freezers in the 1-SE area of the clinical center. Samples will be processed upon completion of the study and destroyed when analysis is complete.

Reasons for Sensitive Procedures:

1) *Blood sampling:* Procedure is necessary to find the tissue fatty acids concentrations and [eCBs]. Previous work in this area has been performed successfully in rodents, but has not been translated to humans in a clinical trial.
2) *DEXA Scan:* Accurate determination of body composition is essential for determining the contribution of dietary fatty acids on body composition.
3) *Psychiatric and Neuropsychological Assessment measures:* These assessments are critical for determining each subject's eating habits, mood, and propensity for addictive behavior at each stage in the dietary intervention.
4) *Tissue Biopsy*: Muscle and adipose tissue biopsies are needed to determine local and specific tissue compositional changes due to the changed LA composition of the diets.

5) *3T MRI Scan:* Non-invasive imaging for body composition is necessary to avoid the risks of deep tissue sampling of subjects for visceral fat and liver composition information.
6) *Storage of Private Health Information in a Research Database:* The storage of clinical information in retrievable databases is necessary to facilitate collaborative input and analysis. This information will remain confidential and protected like the medical record of the subject.
7) *Storage of Patient Samples:* The storage of clinical samples is necessary for collaboration and assurance of similar analysis conditions across all of the study samples.

Notes on Secure Data and Sample Storage:
Data will be collected and entered on form sets labeled with a unique coded identifier that is not related to the patient's name or any other personal identifier (Soc Security number, NIH employee number, etc). Data will be kept in a locked cabinet or in secure computer files with access only for the Principal Investigator (PI) and Associate Investigators (AIs) involved with the study. When entered into the electronic databases, information will be under the CRC and NIH network regular access control. In addition, any information allowing the identification of the participants will be removed. The only possibility to reconstruct the link between database records and information will be through the unique identifier. The translation key will be stored in a locked cabinet or secure computer file with access available only to the PI and AIs named in this protocol document.
Blood and tissue samples will be destroyed after all of the analyses are completed. Research data will be stored for seven years following the study and destroyed according to NIH guidelines.

12.5 Safeguards for vulnerable populations
No vulnerable populations are recruited into this study. Although NIH employees may participate, study investigators and their superiors, subordinates and immediate family members (adult children, spouses, parents, siblings) will be excluded. Non-pregnant, non-lactating females will be recruited; but pregnancy testing will be provided for these females before sensitive procedures if deemed reasonable and/or necessary.

12.6 Qualifications of Investigators
The Laboratory of Membrane Biochemistry and Biophysics (LMBB), Section of Nutritional Neuroscience (SNN) program at NIAAA is an internationally respected laboratory in the area of fatty acid metabolism and nutritional neuroscience. The central focus of the SNN is to examine the impact of deficiencies in long chain omega-3 fatty acids on adverse neurodevelopmental and psychiatric outcomes. This section translates basic biochemical and biophysical fatty acid and lipid results into clinical applications.
The first in vivo studies of the essential fatty acid (EFA) metabolism in alcoholics and smokers have been performed within the SNN. Highly sensitive and selective methodology has been developed within our research group allowing for the safe and non-invasive assessment of EFA metabolism in vivo. Thus, arguably the worldwide lead in the metabolomics of EFA has been gained at the SNN.

Christopher Ramsden, MD is a Lieutenant Commander, Commissioned Corps of the USPHS for LMBB/SNN at NIAAA. Dr. Ramsden is a licensed and board certified physician with current credentials at the NIH Clinical Center. He completed a transitional medicine internship and a clinical medical residency training in Physical Medicine and Rehabilitation (PM&R). He has research experience at the Rehabilitation Institute of Chicago-Northwestern Feinberg School of Medicine, and as a postdoctoral research fellow at UNC-Chapel Hill. He received a grant from the Mayday Fund to develop and implement dietary interventions for 70 patients with treatment-resistant Chronic Daily Headache at UNC-

Chapel Hill. Dr. Ramsden is Adjunct Research Faculty in the Department of PM&R at UNC, where the translational project is ongoing. The low omega-6 diets and low omega-6 plus high omega-3 diets employed in the UNC study serve as a basis for our study interventions. ***Dr. Ramsden may obtain consent.***

CAPT Joseph Hibbeln, MD, USPHS is the Acting Chief of the LMBB/SNN at NIAAA. Dr. Hibbeln has participated in extensive international collaborative clinical trials of omega-3 fatty acids for the prevention of suicide, postpartum depression, and violence. He is a primary collaborator in the Avon Longitudinal Study of Parents and Children, Bristol, UK, examining the residual effect of nutritional insufficiencies in pregnancy in childhood neurodevelopmental outcomes and relevant gene-nutrient interactions. Dr. Hibbeln was one of the first investigators to draw attention to the importance of omega-3 fatty acids in psychiatric disorders. Dr. Hibbeln conceived and implemented the rodent study that provided pilot data for this project.
Dr. Hibbeln may obtain consent.

Amber Courville, PhD, RD is a Registered Dietitian with a doctorate in nutrition. She is a dietitian for the NIH clinical center nutrition dept and researcher in the metabolic unit of the NIDDK at the NIH clinical center. Dr. Courville has clinical research experience in measuring satiety and hunger in the context of the endocannabinoid metabolome, and in conducting clinical trials with variable intakes of fatty acids. Her expertise includes the development of relevant research paradigms for study of many facets of human appetite. ***Dr. Courville may obtain consent.***

Kong Y. Chen, PhD is an experienced metabolic researcher at the Clinical Endocrine Metabolism unit of the NIDDK. He joined the NIDDK in 2006 after serving as Director of the Energy Balance Core Lab and Human Metabolism and Exercise Physiology Lab at Vanderbilt University. Dr. Chen has focused in the measurements of energy metabolism using whole-room indirect calorimeters (metabolic chambers). Dr. Chen and his lab are also investigating the physiological regulations of energy metabolism in lean and obese individuals, as well as improving the technologies for measuring body composition and physical activity. ***Dr. Chen may obtain consent.***

Nancy Diazgranados, M.D., M.Sc., is a Staff Clinician and Deputy Clinical Director, Office of the Clinical Director (OCD), NIAAA. She received her Doctoral Degree in Medicine and Surgery from the Pontificia Universidad Javeriana in Bogota, Colombia in 2001. She completed her Psychiatry residency at Albert Einstein Medical Center and a Master in Science Degree in Pharmacology at Thomas Jefferson University in 2007. In 2008 she became a diplomat of the American Board of Psychiatry and Neurology. She continued her training as a Post-Doctoral Clinical Research Fellow in the Intramural Program at NIMH. In 2013 she joined NIAAA. Dr. Diazgranados will serve as an Associate Investigator. She will evaluate participants and provide clinical care as required. She is experienced with conducting the Informed Consent process and has completed the NIMH, HSPU Informed Consent training. ***Dr. Diazgranados may obtain consent for this study.***

Tonette Vinson, C.R.N.P., OCN, is an Adult Nurse Practitioner in NIAAA. She obtained her Masters of Science in Nursing degree from The University of Pennsylvania in 2003. Tonette will serve as an Associate Investigator. She will evaluate participants and provide clinical care as required. She will aid in data collection for the study. She is experienced with conducting

the Informed Consent process and has completed the NIMH, HSPU Informed Consent training. ***Ms. Vinson may obtain consent for the study.***

Yvonne Horneffer, M.S.N., CRNP, is a board certified Adult Nurse Practitioner. She obtained her Master of Science in Nursing in 2001 from George Mason University in collaboration with the George Washington University School of Medicine. Prior to joining NIAAA Yvonne was an Associate Investigator at the National Cancer Institute's early drug development program from 2006 to 2016 conducting Phase 0 and Phase 1 clinical trials. Yvonne is also a Certified Nephrology Nurse who served as an Associate Investigator at NIDDK's kidney and pancreas transplant program from 2004 to early 2006. With prior background in nursing management and surgical nursing, in her spare time, Yvonne organizes, plans, and leads a team of volunteer surgeons and other health care providers on an annual surgical mission to the Philippines. She will evaluate participants and provide clinical care as required. She will aid in data collection for the study. She is experienced with conducting the Informed Consent process and has completed the NIMH, HSPU Informed Consent training. ***Ms. Horneffer may obtain consent for the study***

Yuhong Lin, PhD is a staff scientist in the LMBB/SNN at NIAAA. Dr. Lin has a doctorate in Medical Science from Shanghai Jiao Tong University School of Medicine was also a former Associate Professor in the Department of Experimental Nuclear Medicine there. Dr. Lin has 20+ years of experience in analytical biochemistry and 10 years of specialized experience in the gas chromatography-mass spectrometry of fatty acids and their metabolites; including multiple stable isotope techniques. ***Dr. Lin will not obtain consent.***

Laura Musse, CRNP, MS is a Special Volunteer Researcher in the Clinical Endocrine Metabolism unit of the NIDDK at the NIH Clinical Center. She is a Certified Nurse Practioner with procedural experience performing bone marrow biopsies, and has been trained in our fat and in muscle tissue biopsy procedures by Drs. Louis Simchowitz and Asem Ali. ***Ms. Musse may obtain consent.***

Sharon Majchrzak-Hong, MS is a chemist in the LMBB/SNN at NIAAA. She has 12 years experience in the field of lipid research; including chemical and data analysis from both animal laboratory experiments and human clinical studies. Her previous non-research experience in education includes handling confidential legal documents. ***Ms. Majchrzak-Hong will not obtain consent.***

Beth MacIntosh, MPH, RD, is a Research Nutritionist at the UNC-Chapel Hill Clinical Translational Research Center. She has experience in developing palatable dietary menus, meal plans and recipes with varying levels of essential fatty acids. Along with Dr. Ramsden, she designed and implemented low omega-6 and low omega-6 plus high omega-3 diets for an ongoing study in patients with Chronic Daily Headache. ***Ms. MacIntosh will not obtain consent.***

D5-LNA Infusion Amendment Collaborators

Stanley I. Rapoport, M.D., is Chief of the BPMS NIA, who has published extensively in the areas of lipid metabolism and brain function in health and disease. He received his MD degree from Harvard Medical School,

and is clinically credentialed at the NIH Clinical Center. The laboratory that he directs has developed and validated the infusion method and analysis in rodents. These methods form a major basis for the Infusion Amendment employed in this human protocol. He is involved in the design of the protocol, and its conduct, and in data analysis and writing up results.
***Dr. Rapoport will not obtain consent*.**

13. Benefits

This study will provide generalizable knowledge on the impacts of dietary fatty acids on endocannabinoid synthesis, satiety, eating behavior, and obesity. This is an experimental, unproven approach that may not prove beneficial to study participants.

14. Classification of Risk

- No direct benefit, but likely to yield generalizable knowledge for preventative nutrition
- More than minimal risk
- The risks are reasonable in relation to anticipated benefits. We will discover critical new information on how modifying LA in the diet affects metabolism, satiety and body composition. We will also gain valuable insights the plausibility of future safe dietary interventions for the treatment and prevention of obesity as alternatives to risky surgical procedures and pharmaceutical products.

15. Consent documents and process

15.1 Who will obtain Consent

A copy of the consent document may be mailed or emailed to potential participants for their review prior to the screening visit. The informed consent process will include a face to face meeting with the study investigators that are designated to obtain consent in Section 12 of this protocol. The training and qualifications of non- MD and RN investigators who will obtain consent are as follows: Amber Courville, RD, PhD is a registered dietitian with a doctorate degree in nutrition. Amber has expertise specifically related to medical procedures done under our protocol, and currently obtains consent on several protocols that have the same outcome measures and medical procedures. Kong Chen, PhD has expertise specifically related to medical procedures done under our protocol, and currently obtains consent on several protocols that have the same outcome measures and medical procedures.

16. Data Safety and Monitoring & 17. Adverse Event Reporting

16.1 Data Safety and Monitoring Board

The main dietary intervention protocol uses standard clinical and laboratory procedures to study the effects of modifying dietary LA on body composition and response. Since the research procedures are to be performed in adult subjects and such procedures involve minor increase over minimal risk, the collection, monitoring and analysis of adverse events will be the responsibility of the Principal Investigator and the investigative team. No DSMB will be convened.

16.2 Data Safety and Monitoring Plan

Elements of the Data Safety and Monitoring Plan

(1) Monitoring mechanism:

The Principal Investigator and the investigative team will monitor the protocol participant daily meal visit data for signs of participant distress and signs of dietary non-compliance.

(2) Frequency:
The Principal Investigator will meet with study staff weekly to discuss participant well-being and study progress.

(3) Stop or change rules:
The study will be stopped when 64 participants finish the diet phase. In the unlikely situation that multiple participants experience adverse events resulting from the consumption of the study foods, these events will be reported to the IRB and the study will be evaluated for continuation if these events are determined to be serious and life-threatening. Participants will be removed from the diet at the recommendation of the nutrition team or if they display signs of a serious adverse event or non-compliance as evaluated by the Principal Investigator, Lead Associate Investigator(s) or nutrition or medical staff.

(4) Advanced Plans:
No interim or futility analyses are planned. The study requires more cohort to reach the estimated sample size of 64 participants.

(5) Information to be monitored:
a. Attendance at daily meal visits as recorded in participant medical record;
b. Daily vital signs as recorded in participant medical record;
c. CPRS to monitor participant mood as stated on page 26.

(6) Communication:
A record of participants removed from the diet phase will be reported to the Addictions IRB at the time of annual review. Serious adverse events will be reported according to the required NIH Clinical Center guidelines as stated in section 17 on the next page.

16.3 Data Monitoring for Quality Assurance
Data monitoring for quality assurance will be the responsibility of the Principal Investigator and the investigative team.

(1) Monitoring mechanism:
The Principal Investigator and the investigative team will monitor the protocol participant questionnaire data and participant binder data.

(2) Frequency:
As stated above, paper questionnaire data and other information in the participant binders will be reviewed bimonthly.

(3) Information to be monitored:
PANAS, POMS and FS psychometric data for each participant will be evaluated for self-report form completeness and status of data entry. Location and status of participant biospecimens will also be tracked and recorded in study sample spreadsheet logs. Status of imaging data will also be noted.

(4) Communication:

Missing psychometric data will be reported to the Principal Investigator and summarized in a report to the IRB at the time of annual review. A summary record of participants who refuse participation in the biopsies and MRI scan will also be reported to the IRB at the time of annual review.

17. Adverse Event Reporting

Adverse events
In the course of a dietary study, adverse events may occur. Unanticipated adverse events that occur as a direct result of research studies initiated at the NIH, but not related to the natural history of the underlying disease will be summarized at the time of the annual review.
Serious Adverse events
A serious adverse event is any adverse experience that:
1. Results in death;
2. Is life threatening;
3. Results in hospitalization or prolongs hospitalization;
4. Results in persistent or significant disability/incapacity;
5. Results in congenital anomaly/birth defect; or
6. Results in a condition, which in the judgment of the investigator represents a significant hazard.

Serious adverse events that are possibly, probably or definitely related to participation in research studies performed at the NIH Clinical Center will be reported. However, serious adverse events (other than death) that are clearly and unambiguously due to extraneous causes (accidental, environmental or coincidental events) will not be reported.
All serious adverse events will be reported using the NIH Serious Adverse Event Report Form to the NIAAA CNS IRB, and NIAAA Clinical Director as soon as possible, but no later than seven days of death or life threatening serious adverse event or within fifteen days after the occurrence of all other forms of serious adverse events.

18. Alternatives to participation or alternative therapies

Subjects are healthy volunteers and are not recruited for the treatment of a medical condition.
Participants are counseled to continue their usual medical care for the duration of the study.

19. Confidentiality

19.1 Medical records
This protocol is covered by a Certificate of Confidentiality (CoC) issued by the US Department of Health and Human Services (US:DHHS), National Institutes of Health (NIH). This information will remain confidential and protected according to NIH guidelines. Some clinically relevant research data will be stored indefinitely in the medical record and will be accessible to the patient for review by others of their choosing (doctors, insurance companies etc.) after executing a release of information.

19.2 Research Data

Data will be collected and entered on form sets labeled with a unique coded identifier that is not related to the patient's name or any other personal identifier (Soc Security number, NIH employee number, etc). Data will be kept in a locked cabinet or in secure computer files with access only for the Principal Investigator (PI) and Associate Investigators (AIs) involved with the study. When entered into the electronic databases, information will be under the CRC and NIH network regular access control. In addition, any information allowing the identification of the participants will be removed. The only possibility to reconstruct the link between database records and information will be through the unique identifier. The translation key will be stored in a locked cabinet or secure computer file with access available only to the PI and AIs named in this protocol document.

The validated psychometric instruments used in this protocol have been converted to an electronic web-based secure data entry platform administered by the NIAAA. The security of the data management system is curated by the NIAAA in agreement with NIH data security policies.
Most data generated in this study will be stored and maintained in the NIAAA clinical database which is password protected and secure.

19.3 Sample Storage

Coded blood and tissue samples without patient names or personal identifiers will be stored in secured freezers at 5625 Fishers Lane, Room 3N-01 or in the NIAAA Clinical Core lab freezers in the 1-SE area of the clinical center. Samples will be processed upon completion of the study and destroyed when analysis is complete. Any additional collaborations requiring transfer of patient material will be done in a coded fashion after informing the IRB and obtaining necessary assurances from the outside institution. The protocol will be amended and IRB approval will be sought when such collaborations are established.

20. Conflict of Interest/Technology Transfer

NIH guidelines on conflict of interest have been distributed to all investigators. There are no conflicts of interest to report. Technology transfer is not applicable to this protocol.

21. Compensation

Remuneration

Remuneration for participation in this study is based on existing NIH guidelines based on inconvenience, to offset potential loss of earnings and travel expenses. The amount paid will be pro-rated based upon participation, and does not include the free foods that will be provided.

For the baseline phase, participants will be compensated a maximum of 250 dollars.

Baseline Phase Visits	Compensation
Visit 1	50
Visit 2	50
Visit 3	150
Maximum Baseline Phase Compensation	**250.00**

For the diet phase, participants will be compensated a maximum of 450 dollars.

Diet Phase	Compensation
Attendance at Daily Meals	150

Visit 1	50
Visit 2	50
Visit 3	200
Maximum Diet Phase Compensation	**450.00**

For the continuation phase, participants will be compensated a maximum of 960 dollars.

Continuation Phase	**Compensation**
Attendance at Daily Meals	360
Visit 1	50
Visit 2	50
Visit 3	50
Visit 4	50
Visit 5	50
Visit 6	350
Total Compensation for Continuation Phase	**960.00**

S1. Deuterated alpha linolenic acid (d5-LNA) Infusion Substudy

S2. Precis

Objective

To quantify hepatic synthesis-secretion rates of long-chain omega-3 polyunsaturated fatty acids (omega-3 LCPUFAs) from circulating unesterified alpha-linolenic acid (LNA) in humans consuming a typical US diet. To evaluate the effects of lowering the dietary nutrient linoleic acid (LA) as a controlled variable on omega-3 LCPUFA synthesis-secretion rates. Modifications in dietary LA will be offset with reciprocal changes in the nutrient oleic acid (OA) to maintain equivalency of calories and nutrients.

Study Population

A subset of up to 24 participants who are enrolled in the main protocol NIAAA-11-AA-0028 'Dietary linoleic acid regulation of omega-3 PUFA metabolism; satiety and body composition among overweight female subjects', will be invited to participate in this Infusion Substudy. Participants must be in the final two weeks of the main protocol (11-AA-0028) intervention phase in order to participate in the Infusion Substudy.

Infusion Substudy Overview

Participation in the Infusion Substudy requires one approximately 22-hour hospital admission visit, including an 8-hour infusion of d5-LNA with serial blood draws, in addition to the visits of the primary protocol. This additional visit will occur during the final two weeks of the 12-week diet phase, i.e., after 10-12 weeks of exposure to the study diets. As in the main protocol, participants will be advised to continue their usual medical care and medications throughout the study.

Outcome Measures

Primary Dependent Variables

The primary outcome will be the hepatic synthesis-secretion rate of docosahexaenoic acid (DHA) from infused d5-LNA.

S3. Scientific Rationale for Infusion Substudy

Potential importance of DHA and its metabolites in addictive disorders

DHA (22:6n-3) is the most abundant polyunsaturated fatty acid in the human brain and is particularly abundant in synaptic phospholipids. DHA has unique chemical and biophysical properties (51), and its docosanoid and endocannabinoid metabolites favorably modulate an array of biochemical processes including neurogenesis (52, 53) and neuro-inflammation (54). Deficits in DHA and its bioactive metabolites are proposed to contribute to suboptimal neurological function, increased relapse vulnerability and aggression in cocaine addicts and other substance abusers (55-58).

Can dietary linoleic acid lowering increase hepatic synthesis-secretion rates and tissue accumulation of n-3 DHA?

It is generally believed that synthesis of omega-3 DHA from dietary LNA is not sufficient to maintain adequate DHA in humans (59). However the long-term synthesis of DHA from LNA has not yet been evaluated at 'low' LA intakes that are consistent with evolutionary and historical US intakes, which are expected to result in increased DHA synthesis and esterification into tissue phospholipids. High omega-6

LA (18:2 n-6) consumption may reduce the omega-3 EPA and DHA content of human tissues by: (1) impairing enzymatic conversion of omega-3 LNA (18:3n-3) to 18:4n-3 (the precursor to EPA) by competing for the active site of D6-desaturase (60); (2) impairing enzymatic conversion of 24:5n-3 to 24:6n-3 (61), the precursor to DHA (22:6n-3) by competing for the active site of D6-desaturase in the endoplasmic reticulum; and (3) competing with omega-3 EPA and DHA for esterification into the sn-2 position of phospholipids (62)(**Fig. 7**: Omega-3 HUFA biosynthetic pathway).

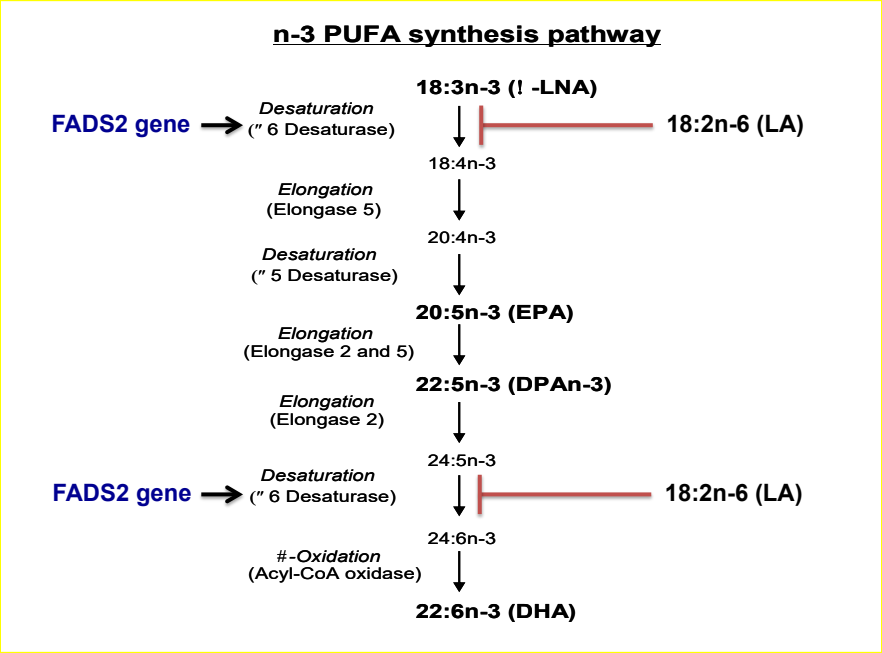


Fig. 7: Omega-3 HUFA Biosynthetic Pathway
D-6 desaturase is rate limiting for enzymatic conversion of the omega-3 precursor LNA (18:3n-3) to the omega-3 LCPUFA products EPA (20:5n-3) and DHA (22:6n-3). Red lines indicate that omega-6 LA (18:2n-6) competes with 18:3n-3 and 24:5n-3 for the active site of D-6 desaturase, impairing conversion of LNA to EPA and DHA. LA also competes for incorporation into tissue phospholipids (not shown). Variants in theFADS2 gene, which codes for the D-6 desaturase enzyme, also modify the efficiency of conversion from LNA to EPA and DHA.

A randomized trial that compared the effects of an infant formula with high LA (6.7 en%) with a low LA infant formula (1.7 en%) on infant tissue omega-3 EPA and DHA supports the proposition that high-LA intake decreases tissue omega-3 long-chain PUFA status. Infants in the low-LA group, compared with infants who received the high-LA formula, had 228% higher EPA (1.28 weight% compared with 0.39 weight%, respectively) and 29% higher DHA (4.48 weight% compared with 3.47 weight%, respectively) in erythrocyte membrane phospholipids. Importantly, however, these infant formulas did not contain the preformed omega-6 and omega-3 LCPUFAs present in adult omnivorous diets. Because dietary HUFA alter enzymatic conversion of LNA to EPA and DHA (via feedback inhibition) (63), it is not yet clear what extent dietary LA lowering increases the accumulation of EPA and DHA in normative human omnivorous diets.

Advantages of hepatic synthesis secretion rates of EPA and DHA versus fatty acid composition data
Assessment of the EPA and DHA composition of blood and other tissues provides a useful snapshot for assessing present omega-3 status. However, fatty acid compositional data do not allow for characterization of rates of synthesis-secretion of individual fatty acids from their precursors. Compositional data are therefore of limited use in for quantifying the dose of dietary LNA necessary to deliver a given dose EPA and DHA to human tissues. Stanley Rapoport et al. in the National Institute of Aging developed and validated a stable isotope LNA infusion methodology in a rodent model to ascertain

the synthesis-secretion rates of EPA and DHA (64, 65). In this collaborative amendment we intend to translate their methodology to our human trial to assess whether the omega-6 LA content of human diet is a major determinant of whole-body (mainly liver) synthesis-secretion rates of EPA and DHA from labeled LNA. Ascertainment of synthesis-secretion rates will allow for more accurate quantitative estimation of amounts of dietary LNA necessary to maintain optimal EPA and DHA, and determination of whether dietary LA lowering affects these estimates. These data may ultimately prove useful in the formulation of dietary guidelines, and the human infusion methods piloted here can be adapted to evaluate the effects of other variables (i.e., nutrients, drugs, alcohol abuse) on LNA conversion to EPA and DHA.

Is the relationship between dietary linoleic acid lowering and liver synthesis-secretion of omega-3 EPA and DHA modified by variants in the FADS1 and FADS2 genes?
Increases in synthesis-secretion rates of omega-3 EPA and DHA in response to controlled dietary LA lowering maybe altered by allele variants in the FADS gene locus (which code for rate limiting enzymatic conversion of LNA into EPA and DHA) (Figure 7). The 'fast converter' FADS allele variant indicates substantial conversion of the omega-3 precursor LNA to EPA and DHA. By contrast, the 'slow converter' allele variant indicates minimal conversion of the omega-3 precursor LNA to EPA and DHA. Homozygosity for the 'slow converter' allele may confer neuro-developmental and other risks when accompanied by suboptimal dietary intakes of seafood sources of preformed EPA and DHA(66). However, associations between alleles and adverse neuro-developmental outcomes have thus far been evaluated only in populations with uniformly high LA intakes (considerably higher than historical and evolutionary diets without added seed oils). Comparison of the 8 en% and 1 en% LA dietary interventions in this amendment will allow for evaluation of whether increases in whole-body synthesis-secretion rates of EPA and DHA after dietary LA lowering are dependent upon FADS gene variants.

S4. Study Objectives and Hypotheses (d5-LNA Infusion Amendment)

Study objectives
To demonstrate feasibility for characterizing the hepatic synthesis-secretion rates of DHA and other omega-3 HUFA from infused d5-LNA in humans. To evaluate the effects of lowering the dietary nutrient LA via controlled replacement with OA on DHA synthesis-secretion rates.

Specific hypotheses

Primary aims
To demonstrate feasibility for characterizing the human hepatic synthesis-secretion rates of DHA using a d5-LNA infusion methodology adapted from an NIH rodent model.
To assess increases in hepatic synthesis-rates for DHA from d5-LNA in response to controlled lowering of dietary omega-6 LA from 8 en% to 1 en%.

Primary hypotheses
Hepatic synthesis-secretion rates for DHA from infused d5-LNA can be successfully quantified in humans.
Lowering dietary intake of LA as a controlled variable from 8 en% to 1 en% will significantly increase synthesis-secretion rates for DHA and other omega-3 LCPUFAs.

S5. Subjects

Description of study population

Only participants that are currently enrolled in the main protocol 11-AA-0028, and nearing completion of 12-week intervention phase will be invited to participate in the Infusion Substudy. In order to enroll in the Infusion Substudy, subjects must continue to meet all eligibility criteria for the main protocol in order to enroll (see inclusion and exclusion criteria in Section 6 (pages 12-13) and Appendix 7).

S6. Study Design and Methods – Infusion Substudy

Recruitment

Participants who were randomized to either diet 'A' or diet 'B' and are in their final two weeks of the 12-week diet phase will be invited to participate in the Infusion Substudy. The study dietitian, the only investigator who is unblinded to treatment allocation, will do an initial screen of participant in diet 'A' or diet 'B' who may be eligible to participate. However, enrollment in the Infusion Substudy will require a new informed consent administered by the physicians and/or nurse practitioner specified in Section S11 (page 49).

Overview of Infusion Substudy Design and Methods

After at least 10 consecutive weeks of exposure to study diets, participants randomized to diet 'A' or diet 'B' (Table 7, Figure 8) will be invited to participate in the d5-LNA Infusion Substudy. No matching procedure will be employed. A maximum of 12 subjects in each group will participate.

Table 7: Fatty Acids of 2 Study Diets in the Infusion Amendment

Diet Group	omega-6 LA*	omega-3 LNA	omega-6 AA	omega-3 EPA+DHA
A. Orange	8.00	0.40	0.045	0.045
B. Blue	1.00	0.40	0.045	0.045

Fatty acid intakes as percentages of daily food energy (en%). LA indicates linoleic acid; LNA indicates alpha-linolenic acid; AA indicates arachidonic acid; EPA indicates eicosapentaenoic acid; DHA indicates docosahexaenoic acid. *Indicates that LA will be modified as a controlled variable via isocaloric substitution for oleic acid.

Participation requires a new consent specific to the Infusion Protocol and one additional visit lasting approximately 22-hours, including:

(1) Admission to the 5SW Metabolic Unit;
(2) Insertion of two intravenous catheters;
(3) Overnight fast;
(4) 8-hour infusion of d5-LNA with serial blood sampling.
(5) Completion of craving/hunger questionnaires.

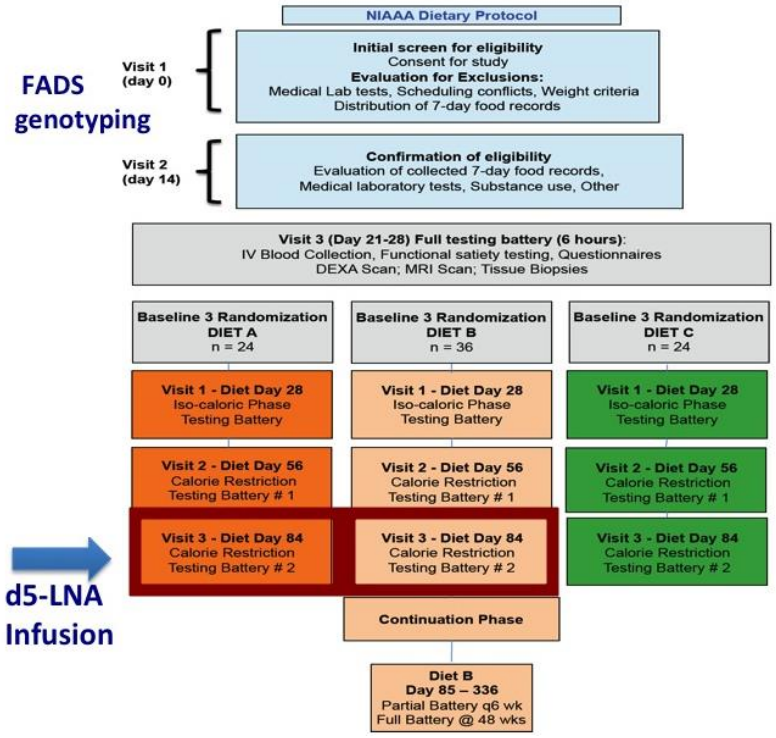


Fig. 8. Infusion Substudy Flow Diagram: Participants in Diet Group A (8 en% as LA) and Diet group B (1 en% as LA) are invited to participate in the Infusion Substudy. Participation requires one additional 22-hour study visit after 10-12 consecutive weeks of consuming study diets. Saliva is collected for FADS genotyping in the baseline phase of the main protocol. LA indicates omega-6 linoleic acid; d5-LNA indicates deuterated omega-3 alpha-linolenic acid.

Characteristics of Stable Isotope d5-LNA Infusion

Non-radioactive stable isotope-labeled alpha-linolenic acid (17,17,18,18,18-D5, 98%) (d5-LNA) in potassium salt will be prepared by Cambridge Isotope Laboratory; the product will be tested and verified as sterile and non-pyrogenic by Cambridge before delivery to NIH. The NIH Pharmacy will then mix the d5-LNA potassium salt into sterile water and then reconstitute with 25% human albumin, and then verify the identity, purity, and dose, sterility and non-pyrogenicity of these infusion materials. These procedures are performed in order to ensure that the infusion materials meet NIH Pharmacy standards for human infusion. George Grimes (Chief of the Pharmaceutical Development Section) is our Clinical Center Pharmacy contact (GGrimes@cc.nih.gov 301.496.1031).

A total dosage of d5-LNA of approximately 19.2 μmol per kg of body weight will be infused intravenously through a 0.2 micron filter at a constant rate for up to 8 hours during the feasibility stage. This initial dose estimate was calculated based on published human infusion trials (68) using deuterated palmitate and the endogenous steady state concentration of LNA in plasma (23) for an obese subject from the main protocol with body weight of 95kg. The half-life of unesterified infused fatty acids in human plasma is about 2 minutes, so that a steady state plasma concentration should be reached in about 10 minutes (5 half-lives). After the feasibility stage, we will adjust infusion rate, dose and duration.

Estimation of Plasma Volume

DEXA scans performed as part of the main protocol (11-AA-0028) will be used to estimate lean body mass for normalization of body fluid volumes as described by Boer. These estimates for plasma volume

will be used in calculating the infusion amount and rate, and in interpreting results as described in Section S.9.

Time points for Blood Collection and Fatty Acid Analysis
Timed venous blood samples will be collected from the start of d5-LNA infusion until 480 min (8 hours) to determine plasma levels of esterified and unesterified labeled fatty acids. Sampling will be at -10, -5, 0, 5, 10, 20, 40, 60, 80, 100, 120, 150, 180, 210, 240, 300, 360, 420, and 480 minutes (19 time points). A total blood volume of 80 mL will be collected. Blood samples will be used to determine the plasma concentrations over time of endogenous unesterified omega-6 and omega-3 fatty acids (nmol/ml) and d5-labeled unesterified and esterified omega-3 fatty acids (nmol/ml) and endocannabinoids with GC and GC-MS. Collected blood will be centrifuged at 2000g for 10 min, and plasma will be transferred to another tube, and kept at -80°C until assay. After lipid analysis of esterified and unesterified labeled and unlabeled fatty acids, hepatic synthesis-secretion rates will be calculated for EPA, DHA and other omega-3 fatty acids as described in Section S.9.

Genotyping
DNA is collected from sputum for analysis of FADS gene variants as part of the main protocol.

Infusion Substudy Procedure Specifics

Infusion Substudy Timeline
All study procedures are for research purposes only. The sequence of procedures is given in Table 8. All time-points are targets, and may be adjusted based on practical considerations.

Table 8: Timeline for Infusion Visit

Procedure	Time
Admission to 5/7SW Metabolic Unit	1800
Standardized research meal	2300
Craving/Hunger Visual Analog Scale (VAS)	2400 (at completion of research meal)
Begin 8-hour overnight fast	2400
Insertion of two IV lines: Antecubital (infusion) Heated hand (sample collection)	0630
Sample collection begins	0745
Repeat administration of craving/hunger VAS (0805, 1000, 1200, 1600)	0805
d5-LNA Infusion begins	0800
End Infusion/post-infusion meal	1600

Inpatient Admission with standard meal and overnight fast
Participants will be admitted to the 5-SW Metabolic Inpatient Unit the evening before the 8-hour d5-LNA infusion, and consume dinner. Their vital signs, weight, and height will be measured. Participants will consume a standardized 'meal' at 2300, and then begin an overnight fast at 2400. At 0630 the following morning, one catheter will be inserted into a forearm vein to administer stable isotopically-labeled tracers and a second catheter

will be inserted retrograde into the contralateral hand, which will be warmed with a thermostatically controlled box to obtain arterialized blood samples. The study will begin at 0745. At 0800, constant infusion of d5-LNA bound to 25% albumin will be started and maintained for up to 8 hours. To assess whole-body synthesis-secretion rates of EPA and DHA, fasting blood will be collected before (at -10, -5, 0 minute time points) and during (at 5, 10, 20, 40, 60, 80, 100, 120, 150, 180, 210, 240, 300, 360, 420, 480 minute time points) the d5-LNA Infusion. Participants will be instructed to rest 30 minutes prior to, and during the full 8-hour Infusion period. Participants can ambulate to the restroom as needed. At the conclusion of the Infusion, participants will be provided with a post-fasting meal consistent with their study diet.

Visual Analog Scale to assess craving and hunger
Upon completion of the standardized research meal (between 2300 and 2400), participants will complete a brief VAS scale to assess their craving and hunger for food. The same VAS will be administered after 8, 10, 12 and 16 hours of fasting (Infusion day times of 0805, 1000, 1200, and 1600).

Follow-up/ termination procedures
There is no required follow-up for this study after completion of the experimental protocol. Participants will be advised to continue receiving their usual medical care throughout the duration of the study. With patient consent, clinically-relevant information obtained through participation in the protocol may be shared with participants and appropriate health care providers.

Radiation exposure
There is no radiation exposure from participation in the Infusion Substudy.

Medications and devices requiring IND/IDE
No medications or devices requiring IND/IDE will be used in this study.
It is important to note that only a trace amount of d5-LNA is provided in the infusion materials. This labeled d5-LNA is a tiny fraction of the non-deuterated LNA pool present in human plasma and tissues. There is a precedent for using intravenous infusion of (non-radioactive) deuterated stable isotopes of fatty acids in human clinical trials. Bernard V. Miller III, MD (NIDDK, Co-I on this Infusion Amendment), has performed over 100 stable isotope tracer infusion procedures in human clinical trials in Washington University School of Medicine-St. Louis, Missouri. A recently published manuscript (68) describing these methods is attached to our amendment submission memorandum.

In 2008, The FDA Division of Metabolism and Endocrinology Products expressed the following policy regarding the use of stable isotopes in investigational metabolic studies: If a substance would not otherwise require submission of an IND, then that substance enriched with a stable isotope will not require an IND. If the isotope enrichment is for metabolic tracer studies employing drugs or non-pharmacological substances, such as glucose and individual amino acids, for which there are adequate data demonstrating their safety and if the substances are used within the limits for which the data are adequate, no IND will be required. However, oversight by an IRB and obtaining informed patient consent are recommended.

Storage of research samples and data
Please see section 7.6.12 for the main protocol procedures for storage and use of research samples and data, and collaborations on stored research samples and data. The same procedures apply to this Substudy.

S7. Risks and Discomforts – Infusion Substudy

Allergic Responses

It is possible that participants could have an allergic response to infusion materials. During the Infusion Substudy informed consent visit, and again during the infusion visit (prior to infusion), participants will provide information on known allergies and if necessary the participants will be excluded. Participants will also be advised to alert the research staff as soon as possible if they experience symptoms such as rash, hives, wheezing, or other signs of allergy or distress. A physician or nurse practitioner study investigator will be present at the bedside for the first two hours, and clinically available throughout the remainder of the infusion.

Infection
It is possible, but highly unlikely, that participants could be exposed to an infectious agent present in the human albumin or another component of the infusion materials. In order to minimize the risk of infection, the NIH pharmacy will perform pyrogenicity and sterility testing to certify that infusion materials meet NIH standards for human infusion. In addition, a 0.2 micron filter will be used to administer the infusion materials at the bedside.

Heat-related Injury
In order to collect arterialized blood from a hand vein, participants' hands will be heated to between 45 and 55°C. Participants will be advised to alert research staff if they experience a pain or a burning sensation. It is possible, but unlikely, that participants could experience a burn.

IV Insertion for Infusion and Specimen Collection
Participants may have some discomfort and bruising at the site of IV insertion for the infusion and serial blood collection. Infection in the area of the needle insertion is rare. There is a very small risk of fainting.

Fasting for the Infusion
Participants will undergo a 16-hour fast beginning at midnight (2400) the day before the infusion and ending at the conclusion of the infusion (1600). Participants will receive IV fluids with electrolytes throughout the infusion and a meal at the conclusion of the infusion.

S8. Subject Monitoring, adverse event grading and withdrawal – Infusion Substudy

Parameters to be monitored
Participants will be closely monitored for adverse reactions by medically qualified staff during the invasive procedures. The participants' vital signs will be closely monitored during the Infusion Procedure testing for any signs of subject distress. Appropriate medical care and treatment will be provided if necessary.
Participants will be withdrawn from the Infusion Substudy if they do not follow the instructions outlined in the protocol and informed consent form. Participants may also, at any time, choose to discontinue their participation from the Substudy.

S9. Outcome Measures (Infusion Substudy)

Primary Outcome Measure
Synthesis-secretion rates for omega-3 DHA with d5-LNA infusion, after 12-weeks of exposure to diets containing either 8 en% or 1 en% as omega-6 LA.

Human d5-LNA Infusion Model (adapted from Rapoport rodent model (64,65))**.**

The objective of this study is to quantify conversion rates of circulating unesterified plasma LNA into longer chain omega-3 fatty acids. It is assumed that most of this conversion will take place in the liver.

The idea is to infuse labeled d5-LNA, henceforth designated as LNA*, intravenously at a constant rate for up 8 hours, and to sample blood plasma (through a second intravenous catheter) periodically for measuring esterified and unesterified plasma concentrations of LNA* and omega-3 LCPUFAs (18:4n-3*, 20:4n-3*, 20:5n-3* (EPA), 22:6n-3* (DPA), 22:5n-3*, 24:6n-3*, and 22:6n-3* (DHA). Additionally, DEXA scan data from the primary protocol (11-AA-0028) will be used to estimate plasma volume V_{plasma}. Measurements also will be made of unesterified unlabeled LNA, DPA, EPA and DHA concentrations in plasma, to calculate actual conversion rates, as future studies may look at rates that may depend on these concentrations altered in disease or dietary states.

Initially, a constant infusion rate of approximately 2.2 umol/min will be established and infusion time will be 8 hours, for a total labeled infusion amount of 350 mg. Since the half-life of injected unesterified fatty acid is about 2 minutes in human plasma, a steady state concentration LNA* will be established within approximately 10 minutes after infusion is in initiated. Duration and rate can be adjusted as necessary to properly determine the enrichment of the tracer and fatty acid kinetics.

Equations

Assuming linearity, the net rate of synthesis of a labeled omega-3 LCPUFA (e.g. DHA*) within secreted VLDLs or other lipoproteins from labeled LNA* is given as:

$$\frac{V_{plasma}\,dC_{DHA*}}{dt} = \vec{k}C_{a\text{-}LNA*}$$

The rate of disappearance of DHA* from plasma is given as:

$$\overleftarrow{k}C_{DHA*}$$

where concentrations C are nmol (or other units)/ml plasma, t is time after infusion has begun, k-appearance and k-disappearance are synthesis coefficients in units of time^{-1}, and V_{plasma} is plasma volume (estimated via DEXA scan). Because there is no isotope effect on enzyme kinetics, rate coefficients k-appearance and k-disappearance are valid for the unlabeled PUFAs as well.

The change in quantity of DHA* in plasma at any time after infusion begins equals:

$$\frac{V_{plasma}\,dC_{DHA*}}{dt} = \vec{k}C_{a\text{-}LNA*} - \overleftarrow{k}C_{DHA*}$$

DHA can exist in two forms in plasma, esterified in lipoproteins and unesterified (after release from lipoproteins) and release of labeled unesterified fatty acid takes about 30 min to become evident in rat plasma after gavage (71). Because oxidative metabolism is 2.5 times faster in rats than in humans (69), it is expected that the delay may be up to 60-90 min. Additionally, during intravenous infusion of *LNA, esterified labeled n-3 PUFAs appear in plasma only after 30 min, and it is expected that in the present protocol they will also appear with a prolonged delay, between 60 and 90 min. In rat studies, the duration of intravenous infusion for 2 h has proven satisfactory (64, 65). For these reasons, we will start initially in humans with an infusion time of 8 hours, but may alter the duration depend on preliminary results.

However, the half-lives of LNA and DHA esterified in plasma lipoprotein (VLDL, LDL, HDL) in humans are 1 hour and 20 hours, respectively (70, 71). This means that after a delay in reaching steady-state rates of conversion of α-LNA to DHA and shorter chain n-3 fatty acid precursors into plasma lipoproteins, the initial slope of increased activity will essentially give the conversion coefficient k-

appearance. Any unesterified DHA* or other labeled unesterified omega-3 fatty acids that is released over time will have a half-life in plasma of about 2 minutes, and this will be taken into account as well.

In this study, we will use an infusion schedule to raise the concentration of d5-LNA as a step function, and will plot $V_{plasma}C_{DHA*}$ against time. The initial slope relating the $V_{plasma}C_{DHA*}$ against time after a delay will approximate the synthesis coefficient k* for DHA*; similar coefficients can be obtained for the shorter chain labeled n-3 fatty acids.

For more explicit analysis, as time proceeds, we will fit equation 4 to the data after inserting a time delay using a curve fitting procedure, to obtain $\vec{k}$ for the different n-3 products. $\vec{k}$ is the coefficient of conversion by whole body per given concentration of plasma LNA, in units of nmol/time, e.g. (nmol/ml, concentration) per unit time multiplied by ml (volume). This rate can be compared to our estimated rates of liver synthesis at the steady state, $\vec{k}$, in rats following the intravenous infusion of LNA* $\vec{k}$. is the plasma unesterified concentration of LNA.

The concentration curves representing LNA* and DHA* should look like Figure 9:

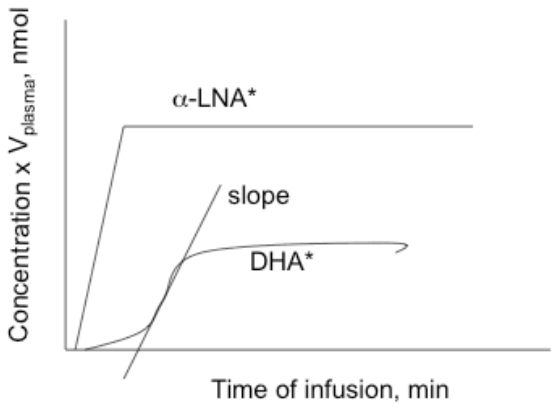


Fig. 9. Graphical depiction of concentration curves for d5-LNA and DHA.

S10. Statistical Analyses (Infusion Substudy)

Overview

This Infusion Substudy first demonstrates feasibility for characterizing the hepatic synthesis-secretion rates of DHA from infused d5-LNA in humans, and then compares synthesis-secretion rates of omega-3 DHA in two diet groups that differ only in dietary omega-6 LA content.

Analysis of Data

Primary Aim 1: In the feasibility portion of this study we will define the dose, rate and duration of the d5-LNA infusion necessary to characterize synthesis-secretion rates of DHA from infused d5-LNA. Based on our previous experiences using this methodology in a rodent model, we anticipate that these data can be obtained from the first 4 infusion participants.

Primary Aim 2:

In the diet group comparison, we will compare synthesis-secretion rates for omega-3 DHA from d5-LNA in response to controlled dietary omega-6 LA lowering from 8 en% to 1 en%. The synthesis-secretion

rate of labeled serial concentration data for labeled DHA will be compared in the two diet groups using a t-test. A probability of 0.05 or lower will be considered significant.
The FADS genotype will be measured as part of the main protocol. This will allow us to assess whether the "low converter" FADS genotype is a modifying variable, or to rule it out as a confounder.

Estimation of Sample Size
We estimate that 4 to 12 individuals in each group are needed to provide adequate power (0.8) to test Primary Aim 2, as shown in Figure 10.

Estimation of Power and Sample Size
We will use a t-test to compare the synthesis-secretion rates and coefficients measured at 4 weeks in the two groups (A and B). Assuming a 20-30% standard deviation in the synthesis-secretion rates based on our prior kinetic work in humans (74) and rodents (64) and a 67% difference in expected change in kinetics consistent with Pawlosky et al (74) and prior measured changes in DHA concentrations, we calculated required sample size(s) (Fig. 10). To detect these effect sizes, at a power of 80% and an alpha of 0.05, we will need 4-12 individuals per group.

Fig. 10. Sample Sizes for Endogenous DHA Synthesis Amendment

	Sample Size Calculation (Spreadsheet1) Two Means, t-Test, Ind. Samples H0: Mu1 = Mu2
	Value
Population Mean Mu1	14.1000
Population Mean Mu2	23.5470
Population S.D. (Sigma)	7.7620
Standardized Effect (Es)	-1.2171
Type I Error Rate (Alpha)	0.0500
Critical Value of t	2.0739
Power Goal	0.8000
Actual Power for Required N	0.8130
Required N (per group)	12.0000

	Sample Size Calculation (Spreac Two Means, t-Test, Ind. Sample H0: Mu1 = Mu2
	Value
Population Mean Mu1	14.1000
Population Mean Mu2	23.5470
Population S.D. (Sigma)	6.4684
Standardized Effect (Es)	-1.4605
Type I Error Rate (Alpha)	0.0500
Critical Value of t	2.1199
Power Goal	0.8000
Actual Power for Required N	0.8284
Required N (per group)	9.0000

	Sample Size Calculation (Spreadsheet1) Two Means, t-Test, Ind. Samples H0: Mu1 = Mu2
	Value
Population Mean Mu1	14.1000
Population Mean Mu2	23.5470
Population S.D. (Sigma)	3.7647
Standardized Effect (Es)	-2.5094
Type I Error Rate (Alpha)	0.0500
Critical Value of t	2.4469
Power Goal	0.8000
Actual Power for Required N	0.8386
Required N (per group)	4.0000

S11. Human Subjects protection – Infusion Substudy

For human subjects protection issues that apply to both the main protocol and the Infusion Substudy, please see Section 12 (Page 38) of the main protocol. Additional issues that apply to the Infusion Substudy are addressed in the following sections.

Justification of sensitive procedures
Procedures that may cause some discomfort to subjects:
Prolonged fast for d5-LNA Infusion: Participants are required to fast for up to 16 continuous hours. Participants will be provided with IV fluids but are likely to experience some hunger during the 8-hour infusion. They will be provided with a meal immediately after infusion.
IV insertion for d5-LNA Infusion: Trained medical personnel will carry out the insertion of two IV catheters, which may cause some discomfort to the subject.
Heated hand: Each participant's non-dominant hand will be placed in a heated box for approximately 4 hours during the infusion procedure to obtain "arterialized" blood samples for the proper determination of stable isotope tracer enrichment. The temperature inside the box will be set to between 44 to 54˚C (between 110 and 129˚F). A controller will maintain the temperature within ±2 degrees of the selected temperature. No significant adverse effects (including burning) have been reported using this equipment for extended time periods during stable isotope tracer infusions studies.
Blood sampling: Trained medical personnel will carry out the blood draws, which may cause some discomfort to the subject.
Storage of Patient Samples: Coded blood and tissue samples without patient names or personal identifiers will be stored in secured freezers at 5625 Fishers Lane, Room 3N-01 or in the NIAAA Clinical Core lab freezers in the 1-SE area of the clinical center. Samples will be processed upon completion of the study and destroyed when analysis is complete.

Reasons for Sensitive Procedures:

Prolonged fast for Infusion: Continuation of the typical 8-hour overnight fast for an additional 8 hours (the maximum duration of the infusion) is necessary in order to minimize the introduction of uncontrolled dietary variables that may alter the measured synthesis-secretion of omega-3 LCPUFAs (i.e. consumption of unlabeled LNA, LA and other competing fatty acids through diet).
IV insertion and blood sampling for Infusion: These IV catheters will be used for the Infusion and serial blood sampling, allowing for collection of blood on 19 occasions without serial blood draws. Blood sampling is necessary to determine the concentrations and labeled and unlabeled fatty acids for assessment of synthesis-secretion rates of omega-3 LCPUFAs. Previous work in this area has been performed successfully in rodents, but has not been translated to humans in a clinical trial.
Heated hand: The heated hand vein technique is used to obtain blood samples that are "arterialized", meaning they are more like arterial blood than venous blood. Using the heated hand vein technique is standard procedure used during stable isotope tracer infusion studies to ensure proper assessment of substrate kinetics. The heated hand vein technique avoids the use of more invasive procedures such as an arterial line.
Blood sampling: Procedure is necessary to assess the concentrations of labeled and unlabeled fatty acids in order to calculate hepatic synthesis-secretion rates for omega-3 DHA. Previous work in this area has been performed successfully in rodents, but has not been translated to humans in a clinical trial.
Storage of Patient Samples: The storage of clinical samples is necessary for collaboration and assurance of similar analysis conditions across all of the study samples.

Qualifications of Investigators – Infusion Substudy

Introduction
Enrollment in the Infusion Substudy will require a new informed consent, obtained via a face-to-face meeting with Dr. Ramsden and/or Laura Musse, CRNP, MS.

The Brain Physiology and Metabolism Section (BPMS) of the National Institute on Aging (NIA), of which Stanley I. Rapoport is Chief, is collaborating on the d5-LNA Infusion Amendment. The BPMS conducts basic and clinical research related to factors that regulation brain function and structure in health and disease. One focus is to identify the influence of dietary composition and liver metabolism of polyunsaturated fatty acids (PUFAs). Methods are developed to image regional brain metabolism of omega-6 AA and omega-3 DHA in unanesthetized animals and in humans (with positron emission tomography), and to quantify the ability of the liver to synthesize these PUFAs from their precursors LA and LNA, respectively, in vivo. The BPMS has developed the infusion methodology and analysis in rodents, which is a basis of this clinical amendment.

D5-LNA Infusion Amendment Collaborators

Stanley I. Rapoport, M.D., is Chief of the BPMS NIA, who has published extensively in the areas of lipid metabolism and brain function in health and disease. He received his MD degree from Harvard Medical School, and is clinically credentialed at the NIH Clinical Center. The laboratory that he directs has developed and validated the infusion method and analysis in rodents. These methods form a major basis for the Infusion Amendment employed in this human protocol. He is involved in the design of the protocol, and its conduct, and in data analysis and writing up results.
***Dr. Rapoport will not obtain consent*.**

S12. Benefits

This Infusion Substudy will provide generalizable knowledge on the normal DHA synthesis-secretion rates in humans, and the impact of dietary fatty acids on hepatic synthesis-secretion of DHA. This experimental procedure has no anticipated benefits for study participants.

S13. Classification of Risk

- No direct benefit, but likely to yield generalizable scientific knowledge
- More than minimal risk
- The risks are reasonable in relation to anticipated benefits. We will obtain important insights into normal DHA synthesis-secretion rates in humans, and learn whether modifying dietary LA alters synthesis-secretion rates of DHA. Both findings could ultimately have important implications for the prevention and/or adjunctive management of conditions including addictive disorders.

S14. Consent documents and process

Who will obtain Consent

The informed consent process for the Infusion Substudy will include a face-to-face meeting with Dr. Ramsden, Dr. Miller and/or Laura Musse, CRNP, MS.

S15. Data Safety and Monitoring & S16. Adverse Event Reporting

S15.1 Data Safety and Monitoring Board

The Infusion Substudy is two-arm study with an 8-hour intravenous infusion of a non-radioactive nutrient in a buffered solution with human albumin. Since the research procedures are to be performed in adult subjects and such procedures involve minor increase over minimal risk, the collection, monitoring and analysis of adverse events will be the responsibility of the Principal Investigator and the investigative team. No DSMB will be convened.

S15.2 Data Safety and Monitoring Plan

Elements of the Data Safety and Monitoring Plan

(1) Monitoring mechanism:
The Principal Investigator and/or medically qualified staff will monitor the infusion protocol participant vital signs data for signs of participant distress as stated on page 44.

(2) Frequency:
The Principal Investigator and/or medically qualified staff will be present at the beginning of the infusion and will stay on the unit for the first 2 hours of the procedure. Medically qualified staff will check the participant vital sign status at least hourly after these first 2 hours.

(3) Stop or change rules:
Participants may choose to stop the infusion procedure at any time. Participants will be reminded to report any signs of distress or discomfort to medically qualified staff

members. Medically qualified staff will determine if any signs of participant distress are present and are severe enough to abort the infusion procedure.

(4) Advanced Plans:

As stated on page 46, this study is designed with the first primary aim as a feasibility study and the data will be reviewed after the first 4 participants.

(5) Information to be monitored for participant safety:

Participant vital signs (as stated above)

(6) Communication:

A record of participants removed from the infusion substudy will be reported to the Addictions IRB at the time of annual review. Serious adverse events will be reported according to the required NIH guidelines as stated in section S16 on the next page.

S15.3 Data Monitoring for Quality Assurance

Data monitoring for quality assurance will be the responsibility of the Principal Investigator and the investigative team.

(1) Monitoring mechanism:
The Principal Investigator and the investigative team will monitor the protocol participant free and esterified fatty acid data.

(2) Frequency:
After each infusion, the blood samples will be analyzed for deuterated fatty acid concentration and the results will be reviewed by the Principal Investigator and the investigative team.

(3) Information to be monitored:
Deuterated fatty acid concentration will be monitored for appropriateness of infusion dose, rate and sampling schedule

(4) Communication:

Infusion substudy progress will be reported to the Addictions IRB at the time of annual review in the form of a summary report. This summary report will include the numbers of recruited, and enrolled participants in this substudy. It will also include a brief data report noting any need for adjustments in the infusion procedure or sampling schedule.

S16. Adverse Event Reporting

Adverse events

In the course of a dietary study and infusion amendment, adverse events may occur. Unanticipated adverse events that occur as a direct result of research studies initiated at the NIH, but not related to the natural history of the underlying disease will be summarized at the time of the annual review.

Serious Adverse events

A serious adverse event is any adverse experience that:

1. Results in death;
2. Is life threatening;
3. Results in hospitalization or prolongs hospitalization;
4. Results in persistent or significant disability/incapacity;
5. Results in congenital anomaly/birth defect; or

6. Results in a condition, which in the judgment of the investigator represents a significant hazard.

Serious adverse events that are possibly, probably or definitely related to participation in research studies performed at the NIH Clinical Center will be reported. However, serious adverse events (other than death) that are clearly and unambiguously due to extraneous causes (accidental, environmental or coincidental events) will not be reported.
All serious adverse events will be reported using the NIH Serious Adverse Event Report Form to the NIAAA CNS IRB, and NIAAA Clinical Director as soon as possible, but no later than seven days of death or life threatening serious adverse event or within fifteen days after the occurrence of all other forms of serious adverse events.

S17. Alternatives to participation or alternative therapies

Subjects are healthy volunteers and are not recruited for the treatment of a medical condition.
Participants are counseled to continue their usual medical care for the duration of the study.

S18. Confidentiality

Medical records
This information will remain confidential and protected according to NIH guidelines. Some clinically relevant research data will be stored indefinitely in the medical record and will be accessible to the patient for review by others of their choosing (doctors, insurance companies etc.) after executing a release of information.

Research Data

Data will be collected and entered on form sets labeled with a unique coded identifier that is not related to the patient's name or any other personal identifier (Soc Security number, NIH employee number, etc). Data will be kept in a locked cabinet or in secure computer files with access only for the Principal Investigator (PI) and Associate Investigators (AIs) involved with the study. When entered into the electronic databases, information will be under the CRC and NIH network regular access control. In addition, any information allowing the identification of the participants will be removed. The only possibility to reconstruct the link between database records and information will be through the unique identifier. The translation key will be stored in a locked cabinet or secure computer file with access available only to the PI and AIs named in this protocol document.

The validated psychometric instruments used in this protocol have been converted to an electronic web-based secure data entry platform) administered by the NIAAA. The security of the data management system is curated by the NIAAA IT department in agreement with NIH data security policies.
Most data generated in this study will be stored and maintained in the NIAAA clinical database which is password protected and secure.

Sample Storage
Coded blood and tissue samples without patient names or personal identifiers will be stored in secured freezers at 5625 Fishers Lane, Room 3N-01 or in the NIAAA Clinical Core lab freezers in the 1-SE area of the clinical center. Samples will be processed upon completion of the study and destroyed when analysis is complete. Any additional collaborations requiring transfer of patient material will be done in a coded fashion after informing the IRB and obtaining necessary assurances from the outside institution. The protocol will be amended and IRB approval will be sought when such collaborations are established.

S19. Conflict of Interest/Technology Transfer

NIH guidelines on conflict of interest have been distributed to all investigators. There are no conflicts of interest to report. Technology transfer is not applicable to this Infusion Substudy.

S20. Compensation

Remuneration for Infusion Substudy

Remuneration for participation in the Infusion Amendment is based on existing NIH guidelines based on inconvenience, to offset potential loss of earnings and travel expenses. Since participation involves one visit, the amount paid will not be pro-rated based upon participation. For the completion of all aspects Infusion Amendment Visit, participants will be compensated a maximum of **250 dollars**.

Note: References for the Infusion Substudy are in blue text in the Bibliography Section.

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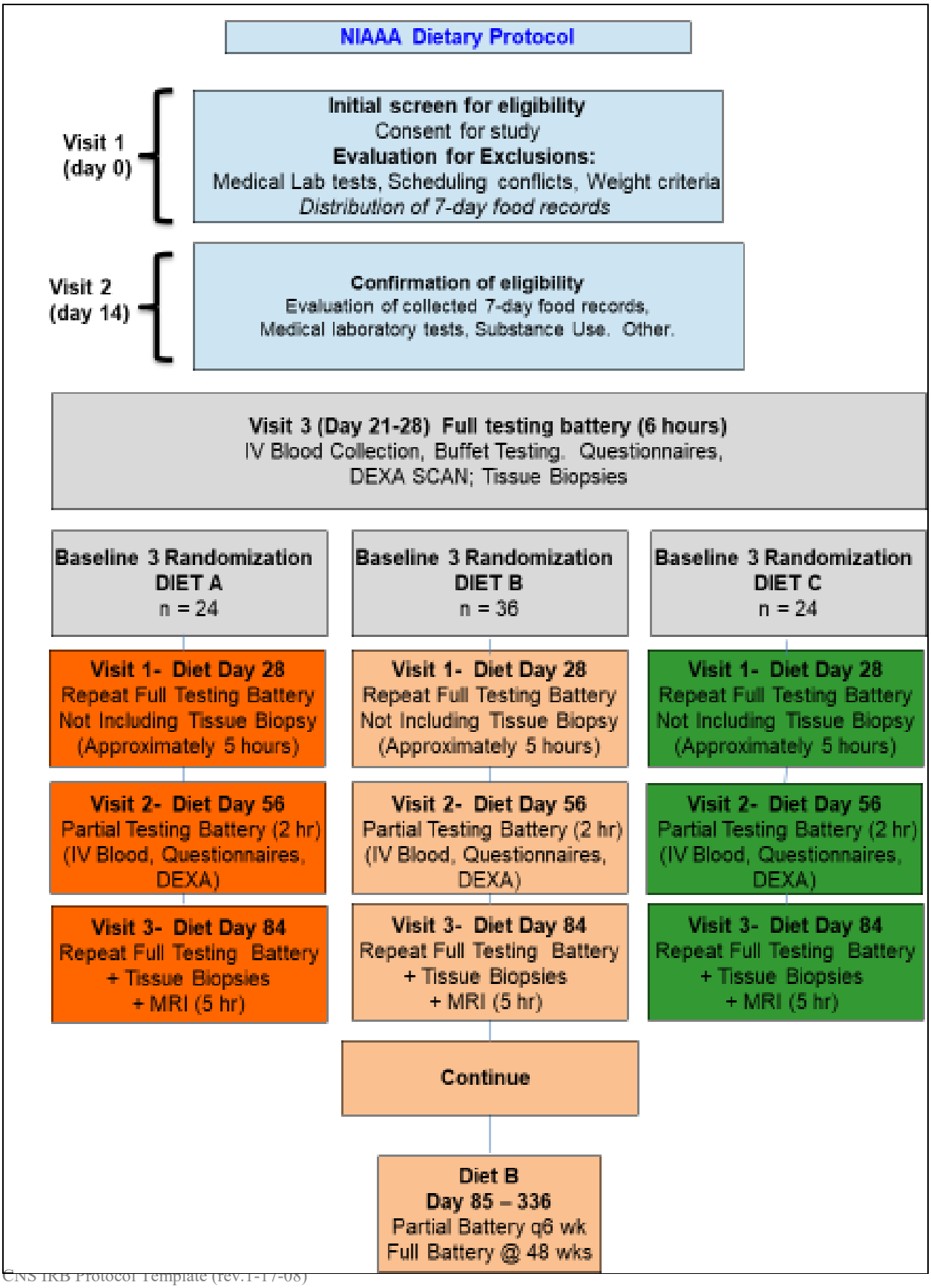
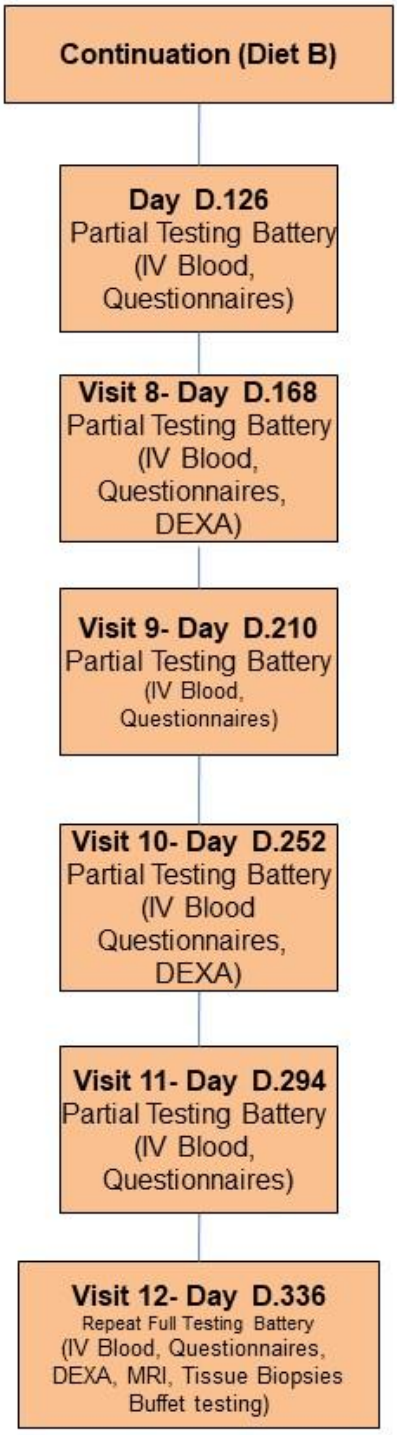
23. Appendices

Appendix 1: Study Flow Diagram


NIAAA Dietary Protocol
Visit 1 (day 0)
Initial screen for eligibility
Consent for study
Evaluation for Exclusions:
Medical Lab tests, Scheduling conflicts, Weight criteria
Distribution of 7-day food records
Visit 2 (day 14)
Confirmation of eligibility
Evaluation of collected 7-day food records,
Medical laboratory tests, Substance Use. Other.
Visit 3 (Day 21-28) Full testing battery (6 hours)
IV Blood Collection, Buffet Testing. Questionnaires,
DEXA SCAN; Tissue Biopsies
Baseline 3 Randomization
DIET A
n = 24
Baseline 3 Randomization
DIET B
n = 36
Baseline 3 Randomization
DIET C
n = 24
Visit 1- Diet Day 28
Repeat Full Testing Battery
Not Including Tissue Biopsy
(Approximately 5 hours)
Visit 1- Diet Day 28
Repeat Full Testing Battery
Not Including Tissue Biopsy
(Approximately 5 hours)
Visit 1- Diet Day 28
Repeat Full Testing Battery
Not Including Tissue Biopsy
(Approximately 5 hours)
Visit 2- Diet Day 56
Partial Testing Battery (2 hr)
(IV Blood, Questionnaires, DEXA)
Visit 2- Diet Day 56
Partial Testing Battery (2 hr)
(IV Blood, Questionnaires, DEXA)
Visit 2- Diet Day 56
Partial Testing Battery (2 hr)
(IV Blood, Questionnaires, DEXA)
Visit 3- Diet Day 84
Repeat Full Testing Battery
+ Tissue Biopsies
+ MRI (5 hr)
Visit 3- Diet Day 84
Repeat Full Testing Battery
+ Tissue Biopsies
+ MRI (5 hr)
Visit 3- Diet Day 84
Repeat Full Testing Battery
+ Tissue Biopsies
+ MRI (5 hr)
Continue
Diet B
Day 85 – 336
Partial Battery q6 wk
Full Battery @ 48 wks


Continuation (Diet B)
Day D.126
Partial Testing Battery
(IV Blood,
Questionnaires)
Visit 8- Day D.168
Partial Testing Battery
(IV Blood,
Questionnaires,
DEXA)
Visit 9- Day D.210
Partial Testing Battery
(IV Blood,
Questionnaires)
Visit 10- Day D.252
Partial Testing Battery
(IV Blood
Questionnaires,
DEXA)
Visit 11- Day D.294
Partial Testing Battery
(IV Blood,
Questionnaires)
Visit 12- Day D.336
Repeat Full Testing Battery
(IV Blood, Questionnaires,
DEXA, MRI, Tissue Biopsies
Buffet testing)

Appendix 2: Lands Equation for Predicting Percentage of omega-6 in HUFA (*%omega-6 in HUFA*)

In a series of human experiments, Lands et al. assessed the relationships between 18, 20, and 22 carbon omega-6 and omega-3 fatty acid composition of diets, within typical U.S. dietary ranges, and percentages of omega-6 and omega-3 HUFA in total HUFA (17). Within these ranges, tissue accumulation of omega-6 and omega-3 HUFA were surprisingly consistent with the principles that Mohrhauer and Holman observed in rats. By combining human and animal data, Lands developed empirical equations relating fatty acid composition of diet to tissue HUFA content.

Lands' equation for tissue HUFA maintenance as a function of diet:

$$\%\omega\text{-}6 \text{ in HUFA} = \frac{100}{1+\frac{HC_6}{en\%H6}\left(1+\frac{en\%H3}{HC_3}\right)} + \frac{100}{1+\frac{PC_6}{en\%P6}+\left(1+\frac{en\%P3}{PC_3}+\frac{en\%H3}{HI_3}+\frac{en\%0}{C_0}+\frac{en\%P6}{Ks}\right)}$$

where HC_3=3.0, HC_6= 0.70, PC_3=0.0555, PC_6=0.0441, HI_3=0.005, HI_6=0.040, C_0=5.0, K_s=0.175.

The Lands equation accurately predicts mean population omega-6 and omega-3 HUFA proportions in populations with radically different dietary omega-6 and omega-3, supporting validity of the equations. Mean tissue percentages of omega-6 in total HUFA (*%*omega-6 *in HUFA*) varied widely, from less than 30% to more than 80% of total HUFA, in populations consuming dissimilar diets.

TABLE 1. Mean *%ω-6 in HUFA* in worldwide populations

Location	Mean *%ω-6 in HUFA*
Ohio, U.S.A	87
Detroit, U.S.A	82
Quebec (Urban)	78
Japanese Americans	70
Southern Spain	60
Japan (Urban)	47
Quebec (Inuit)	44
Japan (Rural)	34
Greenland	32

In a recent study, the *%*omega-6 *in HUFA* measured in whole blood correlated closely with liver, heart, and brain HUFA status in autopsied rats and pigs (75). In this respect, *%*omega-6 *in HUFA* outperformed other HUFA biomarkers including erythrocyte EPA+DHA, which did not correlate with EPA+DHA content of any of these tissues (75). Thus, the demonstration that the empirical equation of Lands accurately predicts changes in *%*omega-6 *in HUFA* in blood following major reductions in dietary intakes of LA would have important implications for any condition affected by membrane AA content, or any disease in which AA-derived mediators have been implicated, no matter which tissues are involved in the disease process.

Appendix 3: Omega-6 (omega-6) and omega-3 Fatty Acids and their Metabolic Derivatives

omega-6 Fatty Acids	**Formula**
LA	18:2 omega-6
AA	20:4 omega-6
DTA	22:4 omega-6
DPA (omega-6)	22:5 omega-6
%n6 in HUFA (Lands equation)	100* [(Total omega-6 HUFA)/ (Total omega-6 and omega-3 HUFA)]
omega-3 Fatty Acids	
ALA	18:3 omega-3
EPA	20:5 omega-3
DPA (omega-3)	22:5 omega-3
DHA	22:6 omega-3
omega-3 Index	EPA + DHA
	Precursor
omega-6 AA derived Endocannabinoids	
2-AG	PL-AA
AEA	PL-AA
omega-3 EPA/DHA derived Endocannabinoids	
2-DHG	PL-DHA
2-EPG	PL-EPA
DHEA	PL-DHA
EPEA	PL-EPA
Other Endocannabinoids	
OEA	PL-Oleic acid (18:1 n-9)
PEA	PL-Palmitic acid (16:0)
omega-3 Docosanoids and Resolvins	
10s, 17s – diDHA	DHA
NPD1	DHA
RvD1	DHA
RvD2	DHA
RvE1	EPA
omega-6 AA derived Eicosanoids	
PGE2	AA
LTB4	AA
TXB2	AA

Key: LA linoleic acid; AA arachidonic acid; DTA docosatetraenoic acid; DPA docosapentaenoic acid; ALA alpha-linolenic acid; EPA eicosapentaenoic acid; DHA docosahexaenoic acid; 2-AG 2-arachidonoyl-glycerol; AEA anandamide; 2-DHG 2-docosahexaenoyl-glycerol; 2-EPG 2-eicosahexaenoyl-glycerol; DHEA docosahexaenoyl-ethanolamide; EPEA eicosapentaenoyl-ethanolamide; OEA oleoyl-ethanolamide; PEA palmitoyl-ethanolamide; NP neuroprotectin; Rv resolvin; PG prostaglandin; LT leukotriene; TX thromboxane

Appendix 4: Psychometric Questionnaires (pdfs)

Appendix 5: Eligibility Checklist – Main Protocol

INCLUSION CRITERIA	
Yes No	

☐	☐	Female gender
☐	☐	18 to 50 years of age
☐	☐	BMI between 25 and 35 kg/m^2
☐	☐	Use of effective contraception with regular menstrual cycles
☐	☐	FSH less than 20
☐	☐	ECG that is clinically unremarkable
☐	☐	Lab tests that are within normal limits or clinically unremarkable
☐	☐	Able to come to NIH Clinical Center every weekday for 3 months
☐	☐	Negative urine drug screen
☐	☐	Menstruating
☐	☐	Negative serum pregnancy test (b-HCG)
EXCLUSION CRITERIA		
Yes	No	
☐	☐	Major medical problems (CNS, cardiovascular, respiratory, GI, hepatic, renal): ☐ Major organ or body system dysfunction (Specify: _____) ☐ Endocrine disorders (Specify: _____) ☐ Other (Specify: _____)
☐	☐	Study investigator or their superior, subordinate, or immediate family member
☐	☐	Positive HIV test
☐	☐	Significant Dietary Limitations (Multiple Food Allergies/intolerances, vegan diet) or special dietary requirements that are difficult to accommodate
☐	☐	Past or present history of Eating Disorder (including binge eating) or psychiatric disease (Specify: _____)
☐	☐	Pregnancy and/or Lactation within the last 2 years
☐	☐	Have had bariatric surgery
☐	☐	Have gained or lost more than 3% of your current weight in the past 3 months
☐	☐	Seafood consumption more than 3 x per week
☐	☐	Regular use of supplements that contain omega-6 or omega-3 fatty acids (e.g. fish, cod liver oil, borage or evening primrose oil)
☐	☐	History of alcohol dependence or abuse in the past 5 years, or current regular use of alcohol, defined as more than 2 drinks per day or 6 drinks per sitting
☐	☐	Smoker (more than 2 cigarettes/week in the past year)
☐	☐	History of substance dependence or abuse in the past 5 years
☐	☐	Have a known bleeding disorder, or regularly use anti-coagulant medications
☐	☐	Regular use of prescription or OTC medications or supplements known to alter energy expenditure, affect nutrient absorption or alter food intake. [Examples include medications for diabetes (type I or II), antipsychotics, tricyclic anti-depressants, thyroid medications, cholesterol medications, anti-hypertensives, beta-blockers, orlistat, glucocorticoids, decongestants, antihistamines, ephedrine, methylphenidate, L-dopa] Others (Specify: _____)

Appendix 7: Eligibility Checklist – d5-LNA Infusion Amendment

INCLUSION CRITERIA		
Yes	No	
☐	☐	Met all eligibility criteria for enrollment in protocol 11-AA-0028
☐	☐	Presently enrolled and nearing completion of 12-week diet phase of 11-AA-0028
☐	☐	Negative pregnancy test
EXCLUSION CRITERIA		
Yes	No	
☐	☐	History of allergic or other adverse reaction to infusion components