Protocol # 15-H-0173
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CLINICAL RESEARCH PROJECT

Protocol Title: Comparison of EPA- and DHA-rich fish oils on lipoprotein metabolism in adults

Abbreviated Title: EPA and DHA Supplements on PCSK9 levels

Identifying Words: Lipoproteins, NMR, LDL-C, HDL-C, Triglycerides, PCSK9, Cholesterol

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Subjects in study:

Number	Sex	Age Range
200 (goal is 21 evaluable subjects) * evaluable subjects are subjects that have completed the full time line of the study (Figure 4), have collected stool specimens and thru the analysis of red cell membrane omega-3 fatty acids (RCM) are found to have taken and absorbed the supplement, so they can be analyzed for the effect on PCSK9 levels*	M/F	≥18

Product Uses Ionizing Radiation: No
Project Uses IND/IDE: Yes
Project Uses "Durable Power of Attorney": No
Off Site: No

Multi-Institutional Project: No
Tech Transfer: Yes

Précis

This is a novel randomized crossover, double-blinded pilot study that aims to investigate the effects of different omega-3 fatty acids, namely EPA and DHA, on lipoprotein metabolism. Subjects will be unblinded for performance of measurements after they complete the study. Subjects will receive EPA or DHA supplements for approximately 6 weeks with a wash out period of 8 weeks between the two arms of the study. The study consists of 4 outpatient visits when laboratory or research samples and CAVI tests will be performed. A 7-day food diary, pill count, and red cell membrane n-3 levels will be monitored to assess compliance.

Serum cholesterol is transported by lipoproteins, such as VLDL, LDL and HDL, which vary in their relationship to cardiovascular risk. LDL is proatherogenic, whereas HDL is cardio-protective. Fish oil supplementation, such as EPA and DHA, has been shown to reduce triglycerides. EPA supplementation has also been shown to lower LDL-C, whereas DHA can raise both LDL-C and HDL-C. These differential effects on lipoproteins may alter the cardiovascular protection afforded by fish oil supplementation. This study will test the hypothesis that EPA and DHA may differ in their LDL-C lowering ability because of differences in how they modulate plasma PCSK9 levels, which is a major determinant of LDL-C levels. In addition, we will assess other parameters related to lipoprotein composition and function that may impact the cardioprotective effect of EPA and DHA. Other reported beneficial effects of omega-3 fatty acid supplementation, such as decreased platelet coaguability, markers of inflammation and changes in gut microbiota, will also be monitored.

Table of Content

1. Objectives

The overall objective is to elucidate the differential changes that EPA- and DHA-rich oils induce on lipoproteins and lipoprotein metabolism. In particular, we will focus on the effect of EPA and DHA on plasma levels of PCSK9 as well as LDL-C levels. This will be first accomplished by performing a small pilot study to assist in the design of a potential future larger clinical trial.

2. Introduction

A 2007 survey done by the National Center for Health Statistics and NCCAM on the use of complementary and alternative medicine (CAM) in US determined that omega-3 fish oil supplements were the most commonly used non-vitamin/non-mineral natural product consumed by adults, and the second most commonly consumed supplement by children[1]. Among the proportion of survey participants who had used natural products in the last 30 days, 37% of adults and 31% of children had taken an omega-3 supplement for health reasons. Increasing evidence has indicated multiple health benefit of fish oil intake, including its ability to prevent heart disease, inflammation, dyslipidemia and diabetes via multiple mechanisms[2]. The beneficial effects of fish oils appear to be due to long-chain omega-3 fatty acids, namely eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which are similar in structure but different in their carbon backbone length and degree of unsaturation. EPA has 20 carbon atoms and 5 double bonds (20:5), whereas DHA has a longer chain of 22 carbon atoms and 6 double bonds (22:6). Their precursors, shorter-chain omega-3 fatty acids, primarily alpha-linolenic acid (18:3), are abundant in the diet, but only a very small percent of these fatty acids are converted to the longer chain EPA and even less to DHA (Fig. 1)[3]. The interconversion of DHA and EPA is also a very inefficient process, thus plasma levels of EPA and DHA are primarily derived from dietary sources, such as fish.

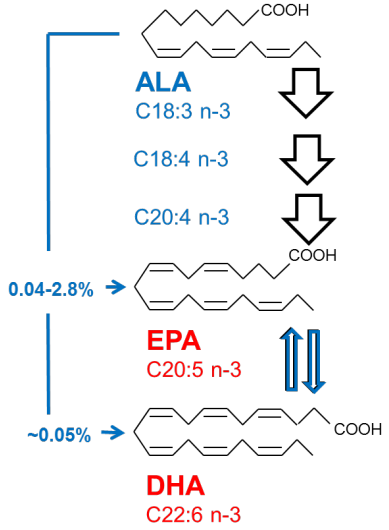


Fig. 1 Metabolic pathway of omega-3 fatty acids

The intake of total omega-3 fatty acids in the United States is relatively low at ~1.6 g/d (~0.7% of energy intake). Of this, alpha-linolenic acid accounts for ~1.4 g/d, and only 0.1 to 0.2 g/d comes from EPA and DHA[4]. A variety of governmental, scientific, and international organizations have established recommended target or minimum consumption levels of fish and omega-3 fatty acids. For long-chain omega-3 fatty acids, most guidelines are for combined EPA+DHA and are based on primary prevention of cardiovascular disease in the general population[5-8]. Generally, these dietary guidelines converge on a recommended minimum consumption of 250–500 mg/d of combined EPA+DHA, and even higher doses for the secondary prevention of cardiovascular disease. For example, American Heart Association (AHA) recommends ~1 g/d of combined EPA+DHA for patients with documented CHD, and 2-4 g/d of combined EPA+DHA for patients with elevated triglycerides. However, most guidelines do not make separate specific recommendations about EPA versus DHA consumption.

Fish oils can substantially vary in the amounts and ratios of EPA and DHA that they contain. For example, tuna oil naturally contains more DHA than EPA (Table 1). In contrast, blue-fin fish, such as anchovy and sardine, contain more EPA than DHA. Since most commercial fish oil/omega-3 supplements are generally purified and concentrated from different marine sources, which have different EPA to DHA ratios, widely different ranges of EPA/DHA are commercially available, but most typically range between 0.7 to 1.4 (Table 2).

Table 1. Main fatty acid composition of different types of fish oil

Fatty acids (%)	**Sardine oil**	**Tuna oil**
C16:0	6.7	16.5
C16:1	8.9	4.8
C18:0	0.7	4.5
C18:1	5.8	16.5
C18:2 n-6	1.1	1.3
C18:3 n-3	0.9	0.5
C20:1	0.8	2.2
C20:4 n-6	1.2	2
C20:5 n-3 (EPA)	28.5	6.4
C22:1	0.1	0.4
C22:5 n-3	2.7	1.5
C22:6 n-3 (DHA)	11.8	24

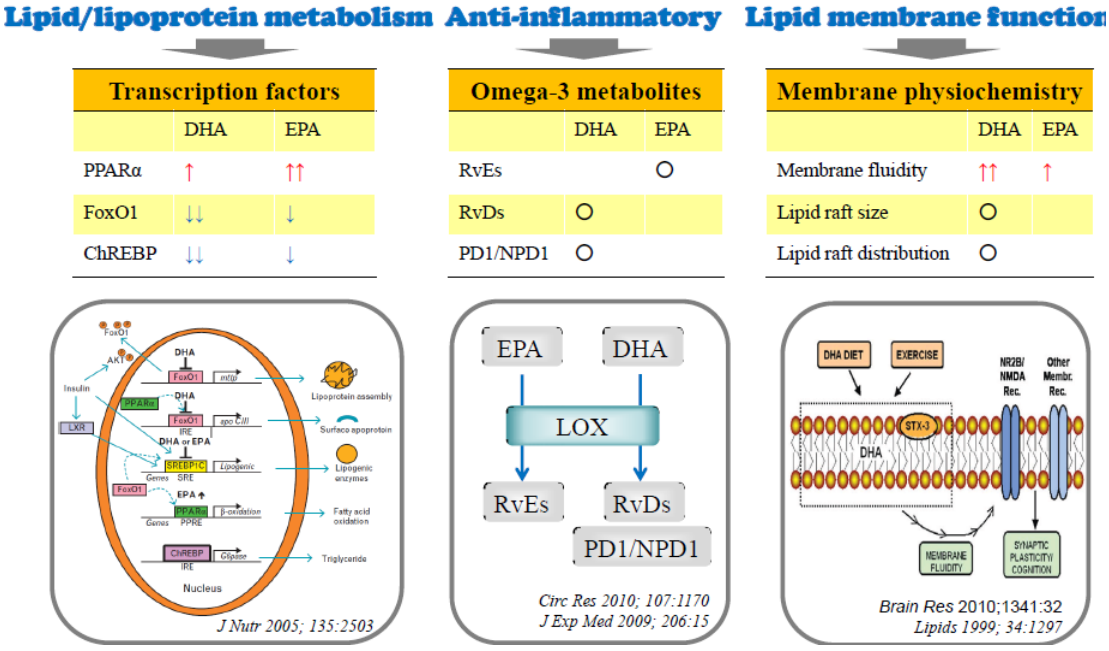
Table 2. EPA/DHA ratios in various market-available fish oil supplements

Omega-3 supplement Product	EPA : DHA ratio
Barlean's Cod liver oil	0.7
Jarrow Maxt DHA liquid	0.7
Barlean's Omega Swirl	1
Carlson's Medomega	1
Omega Cure	1
OmegaMaine Omega-3	1.1
Nordic Natural Ultimate Omega	1.4
Garden of Life	1.4
Nordic Natural Omega-3	1.5
Nutra Sea Ascenta Balanced	1.5
Olympian Lab Omega-3 Liquid	1.5
DaVinci Labs of Vermont Liquid Omega with D3	1.5
Metagenics Liquid	1.5
Opti-OmegaQ Dr. Sinatra's	1.5
PFO Pure Fish Oil Health from the Sun	1.5
Twinlab Emulsified Super	1.5
Nordic Naturals Omega-3 fish oils	1.5
Carlson's The Very Finest Fish Oil	1.6
Eskimo Liquid	1.6
Dr. Sears Zone Omega Rx	2
Natural Factors, Dr.Murray's Rx Omega-3 Factors	2
Nutri-Supreme Research Omega-3	2
Sealogix Omega-3 Liquid	2
MegaRed Omega-3 KrillOil	2.1
Ocean Blue Profession Omega-3	2.3

The mechanisms involved in the shared and different physiological effects between EPA and DHA are not fully understood[9]. For example, EPA and DHA have shared and distinct effects on nuclear receptors and transcription factors, metabolites, and lipid membrane function (Fig. 2). In general, long-chain omega-3 fatty acids are natural ligands of several nuclear receptors that regulate gene expression, including PPAR, hepatic nuclear factor, liver X receptors, and retinoid X receptors. They also alter expression of transcription factors, such as sterol regulatory element binding-protein and carbohydrate response element binding-protein (ChREBP)[10], which may contribute to the physiologic effects of omega-3 fatty acids, e.g., on lipid metabolism and inflammation. EPA, however, is a more potent agonist of PPAR-alpha than DHA, whereas DHA appears to more strongly regulate Hepatocyte Nuclear Factor 4-alpha (HNF-4-alpha), Forkhead box protein O1 (FoxO1), and ChREBP[11]. Both EPA and DHA also give rise to resolvins, a newly described class of specialized pro-resolving mediator

(SPM) that protects against prolonged inflammation and tissue injury in several animal models, including peritonitis and ischemia-reperfusion[12]. However, only DHA is metabolized to other classes of SPM, such as neuroprotectin-D1 and maresins, which may confer the unique inflammation-resolving properties of DHA[13,14]. In animal models, neuroprotectin-D1 promotes neuronal cell survival and may contribute to protective effects of DHA against ischemic stroke[15]. In addition, although both EPA and DHA alter lipid membrane properties, DHA appears to be especially effective in altering lipid membrane structure and function due to its longer hydrocarbon chain length and higher degree of unsaturation[16]. For example, in rat aortic endothelial cells, DHA incorporation increased membrane fluidity more so than EPA[17]. In cultured mouse lymphocytes, DHA but not EPA modified the distribution and size of lipid rafts, specialized lipid domains in cellular membranes with major regulatory roles on protein function and signaling events[18].

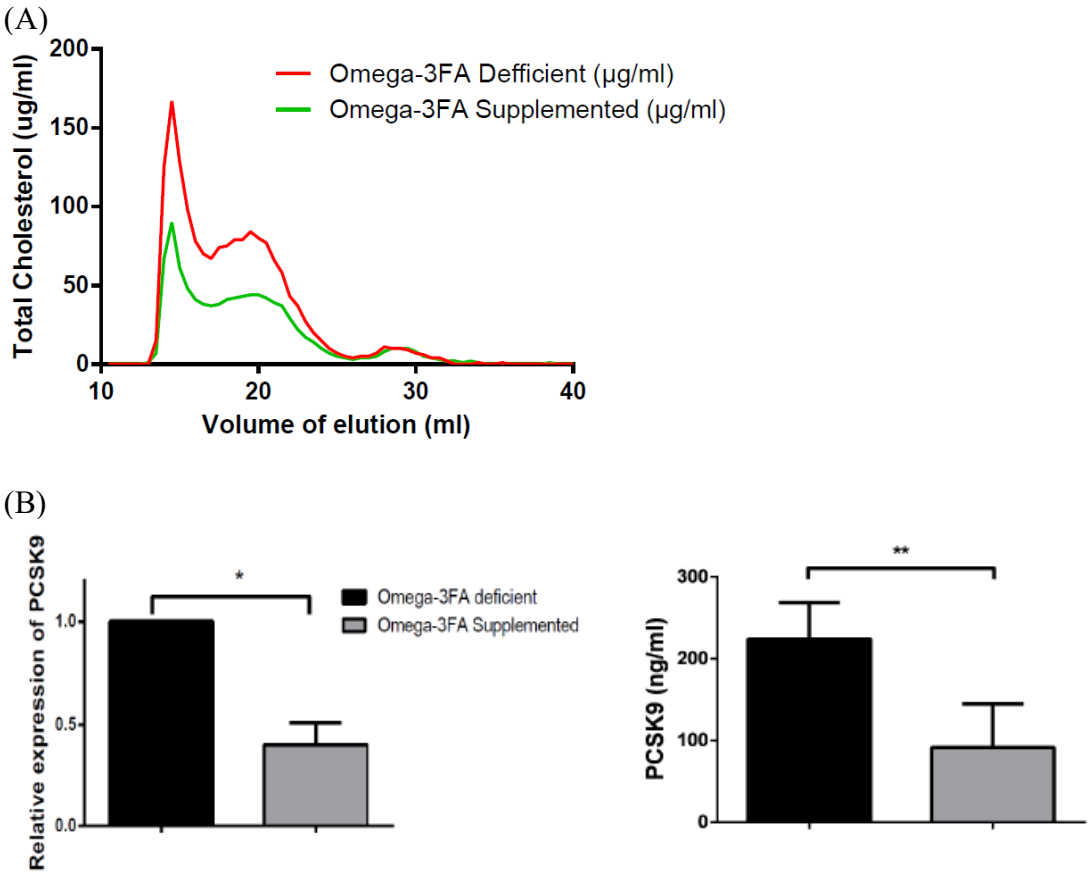
Fig. 2 Differences between EPA and DHA at molecular levels



Several investigations have evaluated the combined and separate effects of EPA and DHA supplementation on both TG-lowering and LDL-C raising. In a meta-analysis of 21 studies, it was concluded that both EPA and DHA decreased triglyceride levels, primarily by promoting fatty-acid catabolism via peroxisomal beta-oxidation, and also by inhibiting lipogenesis in the liver, and accelerating clearance of TG from the plasma[19,20]. However, subjects treated with DHA, but not EPA, showed increases in LDL-C, as well as in HDL-C (Fig. 3) [21-25]. Mori et al. reported that 4 g of purified DHA, but not EPA, increased LDL particle size in mildly hyperlipidemic men. Furthermore, EPA and DHA had a different influence on HDL size subfractions[23]. The mechanism for the differential effect of EPA and DHA on LDL-C and other lipoprotein changes is not fully understood nor whether these differences in lipoprotein metabolism relate to cardiovascular disease.

Recently, proprotein convertase subtilisin/kexin type 9 (PCSK9), a plasma protein produced in the liver and the intestine, has been identified as a major determinant of LDL-C levels and is now a promising therapeutic target [26,27]. PCSK9 binds to the extracellular domain of the hepatic LDL-receptor causing its internalization and degradation, which leads to increased plasma levels of LDL-C. New LDL-C lowering therapies are being developed using monoclonal antibodies against PCSK9 to interfere with this process[28]. We recently observed in apoE-knockout mice on a high fat diet treated for 13-weeks with the human equivalent to 4g/day of omega-3 fatty acids with an EPA:DHA ratio-1.5, a significant decrease in LDL-C and VLDL-C compared to untreated apoE-knockout mice on the same diet (Fig. 3A). Both hepatic mRNA levels of PCSK9 and plasma PCSK9 protein levels were significantly decreased in mice receiving the omega-3 fatty acid supplement (Fig. 3B), which could possibly account for the observed beneficial changes in the serum lipoproteins.

Fig. 3. Effect of Omega-3 fatty acid on plasma cholesterol levels (A), and PCSK9 mRNA expression in liver (B; the left panel) and plasma levels (B; the right panel) in apoE-/- mice.



In this clinical trial, we plan to examine the effect of omega-3 fatty acid supplements enriched in either EPA of DHA on lipid and lipoprotein metabolism. In particular, we will

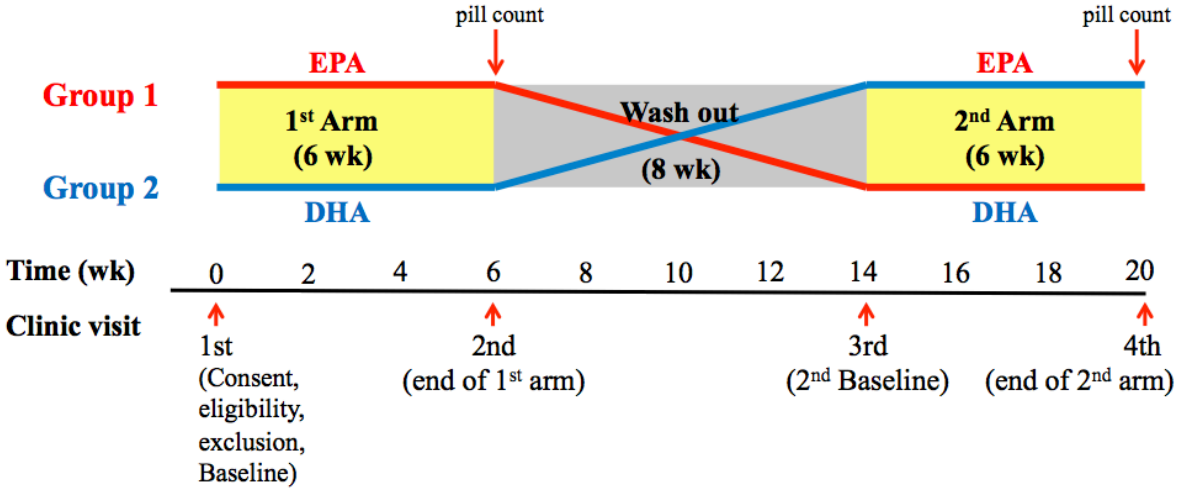
examine their effect of LDL-C and LDL subfraction distribution and whether these differences can be attributed to changes in plasma PCSK9 levels. In addition, we will perform a detailed composition and functional analysis of lipoproteins on subjects treated with the EPA versus DHA enriched fish oil supplements. Given the widespread use of fish oils in the general population and the recent concern about their possible lack of effectiveness for cardiovascular disease protection[29,30], a more detailed understanding on their effect on lipoprotein metabolism is needed. This may ultimately lead to new insights into the optimum ratio of omega-3 fatty acids to use in fish oil supplements for cardiovascular health protection.

3. Study Design and Methods

3.1 Overview

The study will be a prospective double-blinded randomized crossover study (Fig. 4). During the first visit, subjects will be screened by an exclusion/inclusion questionnaire and baseline clinical laboratory testing. If not excluded, they may also be assessed for vascular compliance by Cardio-ankle vascular index (CAVI) testing[31,32] and the nutritionist will obtain a 7-day food diary. Eligible subjects will be randomized by the NIH pharmacist to take either EPA- or DHA-rich fish oil capsules equivalent to 3g of EPA or DHA for 6 ± 1 week in the first arm of the study and will return for a follow up. After a wash-out period of 8 ± 2 weeks, after a 3rd visit, they will receive the other fish oil supplement for 6 ± 1 week in the second arm of the study. During the fourth and final visit, at the end of the second arm, they will repeat clinical laboratory and may repeat CAVI testing. The participant compliance will be monitored by a seven-day food diary questionnaire, and pill counts at each return visit. In addition, fatty acid composition of red blood cells will be assessed by GC analysis[33]. Subjects may be contacted by phone or email at least once during each period to monitor compliance and any adverse events. Subjects should also consent to be contacted for a future study. After completion of the second arm, results will be unblinded for each patient.

Fig. 4. Study design



3.2 Visits

There will be 4 visits for this study. The return visits can be delayed or anticipated up to two weeks, if there are scheduling problems. The first study day will be the screening and baseline followed by three additional visits coinciding with study landmarks. The study will be discussed in detail with interested subjects. Any procedures needed to assess eligibility (i.e. blood laboratory tests, pregnancy test) will be performed after obtaining informed consent. Prior to the first visit a telephone call may be used to give general information about the study, to explain the exclusion/inclusion criteria and the tests that will be performed. This will not be considered a screening. In addition, phone calls may be used during the study to clarify any questions and to support compliance to the study. Any adverse event will be collected and reported as per item 12 of this proposal.

Visit 1: At the first visit (screening and baseline), the subjects will be consented and will be screened by an exclusion/inclusion questionnaire, baseline laboratory tests and a pregnancy test for females. In addition, 10 mL of blood and a stool sample (optional) will be stored for research tests. The subject will have vital signs and Body Mass Index measured, and will undergo a history and physical examination. If not excluded, they will receive a diet and exercise assessment to determine their nutritional status, a physical examination and a Cardio-Ankle Vascular Index (CAVI) test may be performed. Once eligibility is confirmed, subjects will be randomized by the NIH pharmacist, will receive a 6-week (or up to 7 weeks, if necessary) supply of the first dietary supplement (DHA or EPA), will be instructed to take 4 tablets, 3 times a day, after meals, for 6 weeks (12 tablets a day) and will be scheduled to return for a second visit.

Re-screening Visit as applicable: Repeat laboratory values, including baseline research blood, may be needed to reassess eligibility within 40 days of a screening failure. If a subject

is found ineligible, during or after the first visit, the 10 mL of research blood will be discarded.

Visit 2: Six weeks after the first visit (+/- 1 week) the subject will return for their second visit. They will receive laboratory tests and a pregnancy test for female. In addition, 10 mL of blood and a stool sample (optional) will be stored for research tests. The subject will have vital signs and Body Mass Index measured, will undergo a brief physical examination, receive a diet and exercise assessment to determine their nutritional status, and a Cardio-Ankle Vascular Index (CAVI) test may be performed. A pill count will also be performed to evaluate compliance. At the end of the 2nd visit, the subjects will be instructed to discontinue supplementation for 8 weeks.

Visit 3: The third visit will occur 14 weeks (+/- 1 week) after starting supplementation. At this visit, the subjects will receive laboratory tests and a pregnancy test for female. In addition, 10 mL of blood and a stool sample (optional) will be stored for research tests. The subject will have vital signs and Body Mass Index measured, will undergo a brief physical examination, may receive a diet and exercise assessment to determine their nutritional status, and a Cardio-Ankle Vascular Index (CAVI) test may be performed. At the end of the 3rd visit, subjects will receive a 6 week supply (or up to 7 weeks, if necessary) of the second dietary supplement (EPA or DHA) and will be instructed to take 4 tablets, 3 times a day, after meals, for 6 weeks (12 tablets a day).

Visit 4: The fourth, and final, visit will occur 6 weeks (+/- 1 week) after starting the second supplementation. At this visit, the subjects will receive laboratory tests and a pregnancy test for female. In addition, 10 mL of blood and a stool sample (optional) will be stored for research tests. The subject will have vital signs and Body Mass Index measured, will undergo a brief physical examination, may receive a diet and exercise assessment to determine their nutritional status, and a Cardio-Ankle Vascular Index (CAVI) test may be performed. A pill count will also be performed to evaluate compliance. At the end of the 4th visit, the subjects will be instructed to discontinue supplementation.

Subjects will be encouraged to complete the seven day food record prior to the return visits, in order to facilitate the nutritional and exercise assessment. However, if the subject does not complete, or only partially completed the seven day food record, the nutritional and exercise assessment will still take place and it will determine any changes in life-style.

The supplement may be dispensed in full or in 2 different prescriptions.

4. Subject Accrual

Participants will be recruited via flyer and/or recruitment advertisement placed in the NIH Record, the NHLBI Recruitment website, the Clinical Center News and by email or listserv.

4.1 Inclusion Criteria

- Male and female participants 18 years of age or above.
- Subject must be healthy, with no known history of cardiovascular disease.
- Subject understands protocol and provides written, informed consent in addition to a willingness to comply with specified follow-up evaluations.

4.2 Exclusion Criteria

- Pregnancy, planned pregnancy (within the study period) or women currently breastfeeding.
- Subjects with weight changes greater than 20% over the past 3 months.
- Subjects planning a significant change in diet or exercise levels.
- Subjects already consuming more than 1.5 g per day of EPA/DHA in any form.
- Subjects taking supplements or medications that affect lipoproteins for at least the past six weeks including fish oil supplements, bile-acid sequestrants, plant sterol supplements, fibrates, statins or Niacin.
- Subjects diagnosed with cancer or IBD, or that have taken diarrhea inhibitors, laxatives or prebiotics in the week before stool sampling (optional), or antibiotics within 3 months before sampling.
- Subjects taking daily aspirin or other anti-platelet or anti-coagulants agents (Plavix).
- History of prostate Cancer
- Subjects with known bleeding disorders (for example, Hemophilia)
- Known sensitivity or allergy to fish, shellfish or omega-3 fatty acids supplements
- Subjects with chronic diarrhea, gastric bypass or lap-band procedures, ostomies, bowel motility problems, or other conditions that could affect intestinal fat absorption
- Subjects with any acute and life-threatening condition, such as prior sudden cardiac arrest, acute myocardial infarction (last three months), stroke, embolism
- Liver enzymes (AST or ALT) levels above 3x upper limit of normal
- Subjects initiating new medications or patients on multiple medications may also be excluded according to investigator discretion
- Subjects previously diagnosed with cardiac dysrhythmia
- Subjects with clinically diagnosed hepatic disease (including but not limited to auto immune disease, hepatitis and cirrhosis)
- Anticipated surgery during the study period
- Blood donation in the last 2 weeks or planned blood donation during the study
- Subjects requiring regular transfusions for any reason
- Subjects may also be excluded for any reason that may compromise their safety or the accuracy of research data.

- Subjects being treated with tamoxifen, estrogens, or progestins that have not been stable for >4 weeks.
- Subjects that TSH levels are greater than1.5xULN or clinical evidence of hypothyroidism

5. Supplements

Supplements will be stored, dispensed and disposed by the NIH CC pharmacy. The supplements that will be studied will be provided by Nissui Ominogenki ® as EPA-rich fish oil and DHA-rich fish oil, omega-3 acid triglycerides. The supplements will be supplied as a liquid-filled gel capsule for oral administration. Each capsule of EPA-rich fish oil contains at minimum 255 mg of EPA and 110 mg of DHA from fish oils (EPA to DHA ratio: 2.3). Each capsule of DHA-rich fish oil contains at minimum 274 mg of DHA and 74 mg of EPA from fish oils (EPA to DHA ratio: 0.27). The participants will be instructed to take study drug containing approximately either 3g per day of EPA or 3g per day of DHA in three divided doses (4 capsules, 3 times a day after meals). With each regimen, the total dosage of EPA plus DHA will be approximately 4g per day. The dosing is comparable with earlier clinical trials[21-25]. They will also be advised to take the supplements provided immediately after meals, as they are better absorbed with fat.

Product Name: EPA-rich fish oil and DHA-rich fish oil

Supply: **Nippon Suisan Kaisha Ltd.**
Product Description: Obtained from Nippon Suisan Kaisha, Ltd.
Storage: Room temperature at 25°C (77°F); excursions permitted to 15° to 30°C (59° to 86°F)

Route of Administration: Oral

Preparation: Manufactured by:

Nippon Suisan Kaisha, Ltd.

2-6-2 Otemachi Chiyoda-ku. Tokyo 100-8686

Ingredients:

Active: GMP produced and packaged omega-3 –fatty acid triglyceride, EPA and DHA.

Inactive: delta-tocopherol, processed starch, glycerolglycerin, carrageenan, gelatin, and vitamin E

Toxicities: None Known. The toxicity of EPA- or DHA-rich fish oil supplement has not been studied in patients with renal or hepatic impairment.

Drug Interactions: Clinical studies have not been done to thoroughly examine the effect of EPA- or DHA-rich fish oil supplement with anticoagulants. Patients receiving these fish oil supplements and an anticoagulant or other drug affecting coagulation should be monitored periodically (e.g., aspirin, NSAIDS, warfarin, coumarin).

Stability*:* EPA- or DHA-rich fish oil supplement will be maintained and distributed as directed by the supplement manufacturer to maximize maintenance and the quality of active and inactive ingredients in the supplement. Lots will be dated 3 years from expiration. Capsules are not to be frozen.

Off-label use: Since EPA-rich fish oil and DHA-rich fish oil will be used as a dietary supplement and intended only to affect the structure or function of the body and not for a therapeutic purpose a IND is not needed. Considering the clinical investigation is designed to study the relationship between a dietary supplement's effect on normal structure or function in humans or to characterize the mechanism by which a dietary supplement acts to maintain such structure or function this study would not need to be conducted under an IND. Under DSHEA, a dietary supplement is not considered a drug and is not subject to the premarket approval requirements for drugs if the intended use for which it is marketed is only to affect the structure or any function of the body (i.e., not intended to be used for a therapeutic purpose). Similarly, whether an IND is needed for a clinical investigation evaluating a dietary supplement is determined by the intent of the clinical investigation. If the clinical investigation is intended only to evaluate the dietary supplement's effect on the structure or function of the body, an IND is ***not*** required.

However, in order to use a reliable source of EPA-rich fish oil and DHA-rich fish oil, since, most U.S. over the counter available supplements are not guaranteed to have EPA/DHA concentration intended to use in this study, Nippon Suisan Kaisha, Ltd has generously agreed to provide reliable EPA-rich and DHA-rich fish oil for this study, which is the same product available in the Japanese market, under a clinical supply agreement with NHLBI. Because the products are manufactured outside of U.S., FDA guidelines express the requirement of an IND, while the USDA requires a parallel approval for the importation of the supplements.

6. Laboratory Methods

Up to 40 mL of fasting blood will be collected at each visit to perform the clinical and or research tests. Clinical tests that may be used are listed below and will be performed at the NIH Clinical Center Department of Laboratory Medicine on the second floor of building 10 in the Clinical Center.

Clinical laboratory tests:

- Liver function test (ALT, AST, bilirubin)
- Uric acid

• Creatinine
• Creatinine kinase (CK)
• Insulin
• Fasting glucose
• Pregnancy testing
• Fibrinogen
• PT, PTT
• Whole blood aggregation
• CBC
• PSA

Clinical lipid and lipoprotein related tests:
• Lipid Panel (Total cholesterol, LDL-C, HDL-C, Triglyceride)
• Lipoprotein Profile (NMR)
• ApoA-I
• ApoB
• Hs-CRP
• HbA1C
• Alpha-1-antitrypsin

The PCSK9 test will be used to measure as the primary endpoint. In addition, we may also conduct some or all of the following research tests: PHA stimulation whole blood, LC-MS-based proteomics, GC-MS-based lipidomics, specific proteins/enzymes related to lipoprotein metabolism (CETP, PLTP, PON, LCAT, LPL), ApoC-II, apoC-III, apoA-V, cholesterol efflux studies, ex-vivo lipolysis, fatty acid analysis of red blood cells, plasma and buffy coat, leptin, adipoleptin, RNA expression by microarray, flow cytometry phenotyping of white blood cells, measurement of plasma, other lipid and lipoprotein metabolism related markers cytokines and Molecular analysis of gut Microbiota.

Standard of care laboratory tests and procedures not listed above may be requested and will not be used for this research but will only be used to evaluate or elucidate the patients' health.

7. Sample and Data Storage and Confidentiality

Intended use: Samples and data collected under this protocol may be used to study changes in lipoproteins values and characteristics, coagulation function, blood glucose, gut Microbiota and inflammatory markers, or any other information leading to analysis that may help reduce cardiovascular risk. Some information may be entered directly into spreadsheets and should be consider source data.

Storage: Samples will be stored at -80C freezers on the 5th floor of building 10 in the 5D corridor, following current NIH sample storage guidelines. Access to research samples will

be limited, using a locked freezer. Samples and data will be stored, using codes assigned by the investigators or their designee(s). Only the members of the research team will have access to the samples and data. Data will be collected during scheduled visits. Data will be kept on the NHLBI P drive, accessible through password-protected computers only available for the study investigators and the study statistician. Research samples will be in accordance with NHLBI DIR Biospecimen policy. Data will also be stored in locked file cabinets in locked offices in NIH Clinical Center. Primary and final analyzed data will have identifiers so that research data can be attributed to an individual participant.

At the completion of the protocol (termination), the IRB and FDA will be notified that the trial has finished.

8. Monitoring of Subjects and Criteria for Withdrawal of Subjects

8.1 Stopping Rules for Subjects

- A single episode of severe gastrointestinal discomfort that in consultation with the physician is severe enough to discontinue the protocol
- Moderate gastrointestinal symptoms that persists for more than 5 consecutive days relating to the supplementation and as determined by contact with the investigator
- Pregnancy and breastfeeding
- Anaphylactic reaction due to consumption of the fish oil supplement
- New onset atrial fibrillation/flutter
- Major bleeding episode
- Subjects taking less than 75% of the fish oil supplement
- Any other severe symptoms related to the treatment and as determined by contact with the physician.

Subjects who are found to be pregnant or wish to breastfeed during the study will automatically be withdrawn. A pregnancy screening will be done for all female volunteers at the first visit and subjects will be excluded from the study if pregnant. It is not known whether the omega-3 supplements are secreted in breast milk. It is therefore not advisable for any breastfeeding woman to participate in this study. Adverse effects of omega-3 supplements may include atrial fibrillation. Patients that develop symptoms of atrial fibrillation (palpitations, rapid heart rate, fainting, shortness of breath, etc.) will be instructed to visit the emergency room and seek treatment immediately. In order to prevent any future events, participants with atrial fibrillation will be withdrawn from the study. Individuals that develop an allergy to fish or shellfish may experience an anaphylactic reaction from the treatment and will also be required to visit the emergency room immediately and also withdraw from the study.

9. Analysis of the Study

The primary outcome measurement of this study is plasma PCSK9 levels. As secondary outcome measurements, changes in triglycerides, change in LDL-C, lipoprotein particle composition and size, HDL cholesterol efflux capacity and changes in gut Microbiota. Each subject will serve as their own control and will not be randomized into separate treatment groups.

9.1 Sample Size Determination

Sample Size Determination and Methods

Sample size determination is based on change in PCSK9 protein concentration between the baseline and follow-up measurements. First, changes in PCSK9 values will be transformed if necessary to approximate a normal distribution. Using these transformed change values, a normal approximation will be used to estimate the mean and standard deviation.

Secondary analysis will investigate the effects of age, gender, BMI, and other factors on change in PCSK9 values by using a linear model. Similar analysis for the secondary outcomes of changes in lipid concentrations will also be conducted.

Sample Size

We have no way to estimate the standard deviation, σ, of change in PCSK9 protein concentration in humans. One of the goals of this pilot study is to obtain such an estimate. Our estimate of standard deviation will be our sample standard deviation, s. A normal approximation gives

$$\frac{(n-1)s^2}{\sigma^2} \sim {\chi^2}_{n-1}$$

with a (1-β)x100% upper bound of

$$\sigma \leq \frac{\sqrt{n-1}\, s}{\sqrt{{\chi^2}_{n-1}(\beta)}}$$

where ${\chi^2}_{n-1}(\beta)$ is the β quantile of the chi-squared distribution with n-1 degrees of freedom.

With 21 evaluable subjects*, an 80% upper bound (β=.2) on σ is 1.2s. Thus we would be able to conservatively estimate σ for use in planning a main study.

* evaluable subjects are subjects that have completed the full time line of the study (Figure 4), have collected stool specimens and thru the analysis of red cell membrane omega-3 fatty acids (RCM) are found to have taken and absorbed the supplement, so they can be analyzed for the effect on PCSK9 levels*

Primary endpoint: change in plasma PCSK9 level, compared to baseline, after 6 weeks of EPA- or DHA-rich fish oil supplementation.

9.2 Statistical Analysis

Descriptive statistics will be calculated for all variables. A Box-Cox transformation will be fit and used to transform the change from baseline values to approximate normality. The upper bound for σ will be estimated as shown above. Analysis of the transformed change from baseline values will compare the mean changes while accounting for period and group effects.

10. Human Subject Protection

10.1 Rationale for Subject Selection

Subjects of all genders will be considered for inclusion in this study. There will be no racial, ethnic, or gender discrimination. Cognitively impaired and institutionalized persons will not participate in this study. Criteria for exclusion or withdrawal from the study are based on the presence of other disease processes that may interfere with the interpretation of our results or situations that may be harmful to the health of subjects.

10.2 Rationale for the Exclusion of Children

Participant older than 18 will be considered for inclusion in this study. There is not sufficient supporting data to establish an upper limit or safe intake of fish oil/omega-3 fatty acids, however, the FDA has ruled that intakes of up to 3 g/d of marine omega-3 fatty acids are generally recognized as safe for inclusion in the diet[34].

10.3 Rationale for the Exclusion of Pregnant Women

Subjects must not be pregnant or actively seeking pregnancy in order to participate in this study. Pregnancy may introduce unpredictable effects on lipoprotein metabolism and influence the results of the study. The specific effects of pregnancy in this context may be the subject of a separate study. Some form of contraception must be used by subjects while enrolled. Contraception use will be determined by a questionnaire given to the subjects at time of enrollment.

10.4 Inclusion of NIH Staff

NIH staff (employees, NIH contractors, special volunteers, guest researchers, and trainees) may voluntarily participate in this protocol.

- If the individual requesting to participate in the protocol is a co-worker, the consent from the NIH staff member (co-worker) will not be obtained by the staff member's direct supervisor but by another research staff member approved for obtaining informed consent who is not a co-worker.

- Neither participation nor refusal to participate as a subject in this protocol will have an effect, either beneficial or adverse, on the participant's employment or position at NIH.
- The consenting staff member will make the NIH Information Sheet on Staff Research Participation available to staff members who are considering enrolling in research. (SOP 14F, Appendix C Appendix A of the protocol)
- Employee subjects' privacy and confidentiality will be respected by protocol and consenting staff the same as for all subjects participating in research protocols. However, all subjects will be made aware that there are limits to these protections.
- Recruitment, enrollment and compensation of NIH employee subjects will be consistent with the Guidelines for the Inclusion of Staff in NIH Intramural Research Studies (December 2015) (SOP 14F, Appendix A) and NIH Policy Manual Chapter 2300-630-3,"Leave Policy for NIH Employees Participating in NIH Medical Research Studies" (HRPP SOP 14F, Appendix B).

10.5 Evaluation of Benefits and Risks/Discomforts

Benefits: There are no direct benefits to the patient. However, omega-3 supplements have been shown to decrease triglyceride levels in several clinical trials. These benefits may lead to a decreased risk of future cardiovascular events. Routine clinical laboratory testing and disclosure of results will be available to all subjects. Subjects are also entitled to being notified of results from the clinical cardio-ankle vascular measurement.

Risks/Benefit Analysis:
As of June 25, 2019, this study is now closed to new subject accrual and continues in data analysis only and the level of risk is minimal.

Risk (45 CFR 46.102 (h)(i)): The research involves no more than minimal risk to subjects and no prospect of direct benefit to individual subjects, but likely to yield generalizable knowledge about the condition or disorder under study.

Study Agent:
EPA- or DHA-rich fish oil, commercially available fish oil supplements have minimum risk to subjects.

Generally, adverse effects of omega-3 fatty acid supplement may include atrial fibrillation of new onset and increased propensity for bleeding. Drug interactions may exist between individuals receiving Aspirin or clopidogrel (Plavix).

The most common side effects of fish oil include taste perversion, and eructation (belching). Participants will be notified of these effects in the informed consent document. Any subject exhibiting symptoms of this condition including palpitations, rapid heart rate, or shortness of breath will instructed to go to the emergency room immediately and be withdrawn from the study. Individuals with an unknown allergy to fish or shellfish not disclosed at screening may experience an anaphylactic reaction, which will also be instructed to go immediately to an emergency room and will require withdrawal from the study. In rare cases, fish oil supplement can trigger allergic reactions. Symptoms may include difficulty breathing, swelling of the face, fever, or flu-like symptoms and a skin rash. Some studies

reported consumption of omega-3-fatty acids is associated with a prolongation of bleeding time. However, the prolongation of bleeding time has not been shown to surpass normal limits and did not result in clinically significant bleeding episodes[35,36]. Any patient currently treated with an anticoagulant will be excluded from this study.

Blood Draw: Subjects may feel lightheaded or dizzy after having blood drawn. There may be pain at the vein puncture site and a slight risk of bruising. To minimize this risk, the routine blood-drawing protocol will be followed and pressure will be applied to the area.

The protocol will follow the NIH Clinical Center MAS policy M95-9 guidelines for limits of blood drawn for research purpose in the Clinical Center. For adults, no more than 10.5 mL/kg or 550 mL, whichever is smaller, will be drawn for research purposes over any 8-week period.

Cardio-Ankle Vascular Index (CAVI): Inflation of blood pressure cuffs may cause transient discomfort. Subjects with fragile skin may suffer minor trauma (as per ABIs, related to usual BP measurements). This procedure involves the use of a VaSera VS-1500N system; an FDA approved vascular screening system.

10.6 Protocol Consent Processes and Documents

Each subject will receive an oral and written explanation of the goals, procedures, and risks of this study. The original, signed informed consent document will be placed in the medical record, and the subject will receive a signed copy of the informed consent document. A copy will also be placed in the shadow chart. A member of the protocol team will be available to answer questions about the study to be performed.

An individual who is an AI on the protocol but not a co-worker will obtain consent from NIH employees. NIH employees will be given the NIH Information Sheet on Employee Research Participation to help them understand the possible consequences of participation.

10.7 Patient Advocate

A patient's rights representative is available to patients on this protocol. The representative can be reached at 301-496-2626 and is located in Building 10. Patients may ask any questions about the study and may withdraw their consent at any time.

11. Conflict of Interest

There are no conflicts of interest with any financial organization regarding the material mentioned in this protocol.

12. Adverse Event Management

The following adverse event management guidelines are intended to ensure the safety of each patient while on the study. Adverse events will be attributed (unrelated, unlikely, possibly,

probably or definitively) to study medication and/ or disease and AEs will be graded by severity utilizing CTC version 4.0. A copy of the criteria can be downloaded at http://ctep.cancer.gov/protocolDevelopment/electronic_applications/ctc.htm.

All adverse events occurring during the study, including those observed by or reported to the research team, will be recorded.

An abnormal laboratory value or changes from an abnormal baseline value will be considered an AE if the laboratory abnormality is characterized by any of the following:

- Results in discontinuation from the study
- Is associated with clinical signs or symptoms
- Requires treatment or any other therapeutic intervention
- Is judged by the Investigator to be of significant clinical impact

If any abnormal laboratory result is considered clinically significant, the investigator will provide details about the action taken with respect to the test drug and about the patient's outcome.

Abnormal laboratory values not associated with clinical symptoms will be evaluated but will not be considered an AE.

The laboratory results will be monitored by healthcare professionals and documented if clinically significant by the MD or any AIs in the study.

Any and all serious adverse events relating to the acquisition of blood samples, such as laceration, dissection, thrombosis of an artery, especially if those events cause injury to the hand requiring surgical correction, will be reported verbally and in writing to the Clinical Director of both the NHLBI and the Clinical Center and the chair of the IRB. The verbal report will occur within 48 hours of the occurrence. The written report of the serious adverse event (e.g., death or life-threatening adverse event) will be reported within 7 days. For all other serious adverse events relating to the acquisition of blood samples, the written report will be within 15 days.

The PI will report SAEs to the Sponsor according to the requirements of 21 CFR 312.64(b) and as agreed upon with the sponsor. The Sponsor (or designee) will determine the reportability of the event to the FDA and IND safety report will be submitted to the FDA as required as either a IND Safety Report or Annual report.

12.1 IND Annual Report

A summary of all SAEs, non-serious AEs, and other events will be recorded and submitted to the Sponsor and FDA in annual progress reports (21 CFR 312.64(b)). Annual progress reports will be submitted within 60 days after the anniversary date of the IND.

Events will be submitted to Dr. Marcelo Amar, IND Sponsor at:
Marcelo Amar, M.D.
9000 Rockville Pike, Building 10
Rm CRC 8N228, NHLBI
Bethesda, MD 20892

13. NIH Intramural IRB and NHLBI CD Reporting

13.1 Reports to the IRB

The PI or designee will refer to HRPP Policy 801 "Reporting Research Events" and HRPP 802 "Non-compliance in Human Subject Research" to determine IRB reporting requirements and timelines.

13.2 Reports to the CD

The PI or designee will refer to NHLBI DIR guidelines to determine CD reporting requirements and timelines.

14. Data and Safety Monitoring Plan

Based on clinical trials, the omega-3 fish oil supplements have been well tolerated and are unlikely to increase morbidity and mortality in subjects meeting the inclusion criteria of the study.

Protocol Monitoring

As per ICH-GCP 5.18 and 21 CFR 312.50 clinical protocols are required to be adequately monitored by the study sponsor. The monitoring of this study will be conducted by Clinical Research Associates (CRAs)/Monitors employed by an independent firm and working under an agreement with NHLBI to monitor aspects of the study in accordance with the appropriate regulations and the approved protocol. The objectives of a monitoring visit will be: 1) to verify the existence of signed informed consent form (ICF) and documentation of the ICF process for each monitored subject; 2) to verify the prompt and accurate recording of all monitored data points, and prompt reporting of all SAEs; 3) to compare abstracted information with individual subjects' records and source documents (subject's charts, laboratory analyses and test results, physicians' progress notes, nurses' notes, and any other relevant original subject information); and 4) to help ensure investigators are in compliance with the protocol. The monitors also will inspect the clinical site regulatory files to ensure that regulatory requirements (Office for Human Research Protections-OHRP), FDA and applicable guidelines (ICH-GCP) are being followed. During the monitoring visits, the

investigator (and/or designee) and other study personnel will be available to discuss the study progress and monitoring visit.

The investigator (and/or designee) will make study documents (e.g., consent forms and pertinent hospital or clinical records readily available for inspection by the local IRB, the FDA, the site monitors, and the NHLBI staff for confirmation of the study data.

Safety Monitoring

Principal Investigator: Accrual and safety data will be monitored by the PI. The protocol will be continuously evaluated for any unusual or unpredicted complications with the aim of detecting and preventing unacceptable increase in morbidity and mortality over and above that anticipated from niacin.

NHLBI IRB

Accrual and safety data will be monitored and reviewed annually by the Institutional Review Board (IRB). Prior to implementation of this study, the protocol and the proposed patient informed consent document will be reviewed and approved by the properly constituted IRB operating according to the 45 CFR 46 Code of Federal regulations. This committee must approve all amendments to the protocol or informed consent document, and conduct continuing annual review so long as the protocol is open to accrual or follow up of subjects.

FDA: An annual progress report, any amendments to the protocol, and any change in the status of the protocol will be forwarded to the FDA in compliance with 21CFR 312.33

15. Compensation

Compensation will be provided to the subjects for their time and inconvenience of participating on this protocol based on the values listed below:

Procedures	Inconvenience Unit	$	Frequency	Total $$
Outpatient Visit (first hour)	2	$20	Up to 5	$100
Outpatient Visit (additional hours, up to 4 hours)	1(per hour)	$10	Up to 5	$200
Physical Examination	1	$10	Up to 5	$50
Screening Blood Draw	1	$10	Up to 2	$20
Research Blood Draw	1	$10	4	$40
Diet & Exercise Assessment	1	$10	3	$30
Stool collection (optional)	3	$30	4	$120
CAVI	2	$20	3	$60
Total potential compensation:				$620

Subjects will receive partial compensation (one third payment) if they participate up to at least the second outpatient visit but will receive full compensation only after completing the entire study.
Compensation of NIH staff will be consistent with the Guidelines for the Inclusion of Staff in NIH Intramural Research Studies (December 2015) (SOP 14F, Appendix A) and NIH Policy Manual Chapter 2300-630-,"Leave Policy for NIH Employees Participating in NIH Medical Research Studies" (HRPP SOP 14F, Appendix B).

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17. APPENDIX A: NIH INFORMATION SHEET ON STAFF RESEARCH PARTICIPATION (DECEMBER 2015)

As an NIH employee, contractor, Special Volunteer, Guest Researcher, or trainee, you may participate in intramural research studies unless it is prohibited by your Institute or Center (IC), or if you are excluded by the criteria of the protocol in which you want to enroll. The inclusion of NIH staff in a particular protocol must also be approved by the IRB. You may be motivated by altruism, a commitment to research in your own or related fields, or want access to clinical trials of potential direct therapeutic benefit. When deciding, you should make an informed decision about participation. This information sheet offers some points to consider for NIH staff who are considering research participation at NIH.

First, similar to any individual who is considering research participation, you should seek adequate information about the study purpose, what is required of you in terms of procedures, interventions and time, and the potential risks and benefits of participation. For more information, see the NIH Clinical Center's public website "Are Clinical Studies for You?" at http://www.cc.nih.gov/participate/studies.shtml.

When you are thinking about participation in a research study that is being conducted by your supervisor, or others with whom you work closely in your laboratory, branch, or unit, you should consider some additional factors:

A. **Possible bias:** Are you confident that you can be unbiased about reporting answers, side effects, or other information that could influence the study outcome or risk to you?

B. **Confidentiality:** Are you comfortable sharing your medical history (including, for example, mental health history or STDs) and your social history (e.g. substance use) with study investigators who may be your coworkers, or with the possibility of them discovering something about your health during the study (e.g. pregnancy status or a new diagnosis)? Although every effort will be made to protect your information and keep it private and confidential, your information will be available in medical records and it will be available to authorized users outside of the study team, both in an identifiable and unidentified manner.

C. **Pressure**: Do you perceive any pressure or expectations from your supervisor or colleagues regarding participation? Could that pressure influence your decision or make it difficult for you to choose whether or not to participate? Remember that it is your choice whether or not to participate and that your decision to participate or not should not have an effect, either beneficial or adverse, on your position at NIH.

D. **Time and Compensation:** Can you take time off from work to complete the study requirements or participate solely during non-duty hours? Can you receive compensation for your participation in this study? Will your supervisor give you permission to participate

during work hours? See the NIH Policy Manual 2300- 630-3 Leave Policy for NIH Employees Participating in NIH Medical Research Studies.

E. **Consent Process:** Is the person obtaining your consent for the study your supervisor, a subordinate, or co-worker? If so, is there an independent person monitoring the consent process? If the study PI is a supervisor and intends to obtain consent from you, an independent person (e.g., through Bioethics or the NIMH Human Subjects Protections Unit [HSPU], or others as approved by the IRB) must monitor the consent process. If the person obtaining consent from you is a co-worker then an independent person (e.g., through Bioethics or the NIMH HSPU, or others as approved by the IRB) may be required to monitor the consent process, as determined by the IRB for the specific study.

If you are thinking of enrolling as a subject at the NIH Clinical Center and you have any questions or concerns, please contact the Office of Human Subjects Research Protections (OHSRP) at 301-402-3444 and/ or the Patient Representative if you are thinking of enrolling as a subject at the NIH Clinical Center on 301-496-2626. If you are at a NIH site outside the Clinical Center then please contact local site leadership.