

Study Protocol and Statistical Analysis Plan

C.1. Study Design

We will use a randomized single blind controlled trial design to address proposed aims. Specifically, the Center for Clinical Investigation (CCI) at Brigham and Women's Hospital will recruit adults aged 30+ years for participation in this study. In order to be eligible, a participant must be diagnosed with 1) type 2 diabetes at least one year prior to enrollment, 2) have elevated serum triglycerides (150-400 mg/dl), 3) a RHI ≤ 2.0 ¹³¹, and 4) be on statin treatment for at least six months at the time of enrollment. Additional eligibility criteria include a) ability to travel to the study site at Brigham and Women's Hospital for three study visits, the last two being spaced by 12 weeks, b) ability to provide informed consent, and c) willingness to abstain from fish oil and other forms of omega-3 supplements during the study period. Exclusion criteria include a) current eating disorders, b) pregnancy (using urine test for all premenopausal women of childbearing age without surgical sterilization), c) allergy to EPA or other omega-3 fatty acids, d) inability to provide informed consent, e) history or prevalent diagnosis of cancer, asthma, kidney insufficiency, stroke, or seizures, f) inability to provide blood specimens, g) current use of fish oil, >2 servings of fish per week, or omega-3 fatty acid supplements, and h) intention to move out of greater Boston area within one year. Complete list of inclusion and exclusion criteria is provided in Table 2.

Table 2. Inclusion and exclusion criteria

A. Inclusion criteria

- Age 30-75 years
- Hypertriglyceridemia (150-400 mg/dl)
- Statin use for at least six months at the time of screening
- Type 2 diabetes treated with diet and/or oral hypoglycemic agents diagnosed 1+ year from first visit
- Ability to provide informed consent and provide blood samples
- Willingness to abstain from fish oil, EPA, over the counter niacin, and other omega-3 fatty acid supplements during the study period (12 weeks)
- Ability to travel to the study site at Brigham and Women's Hospital for 3 study visits
- RHI of ≤ 2.0

B. Exclusion criteria

- Eating disorder or heavy drinkers (>2 standard drinks/d: a drink= 1.5 oz liquor, 4oz wine, or 12 oz beer)
- Treatment with chronic prescription pharmacotherapy for metabolic or cardiovascular disease management or risk factor modification (e.g., antihypertensive medications) that has not been stable for ≥ 4 weeks prior to screening
- Pregnant or lactating women
- Statin use <6 months at the time of screening
- Allergy to EPA, fish oil, shellfish, or other omega-3 fatty acids
- Current use of insulin, cyclophosphamide, estrogen, fibrates, niacin, hormone replacement therapy, testosterone, oral contraceptives, growth hormones, insulin-like growth factor-1, and other systemic steroids.
- Inability to provide informed consent or blood samples
- History or prevalent diagnosis of cancer, asthma, kidney insufficiency, stroke, seizures, allergic disorders, liver disease, or congestive heart failure
- Diagnosis of diabetes < 1 year prior to enrollment

- ❑ Intention to move out of greater Boston area within one year
 - ❑ Current use of omega-3 supplements, fish oil, or ≥ 2 servings of fish per week
 - ❑ Bleeding disorder or uncontrolled endocrine (i.e., thyroid) or metabolic disorders
 - ❑ Treatment with blood thinning drugs (i.e. warfarin and clopidogrel)
 - ❑ Major surgical operation 3 months before or after screening
 - ❑ Organ transplantation
 - ❑ Current participation in another trial or plan to do so during the study
 - ❑ Inability to give informed consent or to travel to the study center at Brigham and Women's Hospital
 - ❑ RHI of >2.0
 - ❑ Triglycerides <150 mg/dl or >400 mg/dl
 - ❑ Body mass index of $40+$ kg/m²
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C.2. Recruitment of Study Subjects

Upon receiving IRB approval from Brigham and Women's Hospital (BWH), potential participants will be recruited via usual advertisement techniques, including flyers posted on campus, media advertisement, and use of Partners Biobank (a letter co-signed by Partners Biobank PI and Dr. Djousse with a card and a prepaid return address will be used). Each flyer will contain pertinent information summarizing the study protocol and objectives and contact information for study staff. Interested participants will contact the study coordinator, who will conduct a telephone screening for eligibility. Once a potential study subject has been determined to be eligible via telephone, he/she will be provided with detailed information about the study by the PI/study coordinator. The potential participant will then review the study materials before determining if he/she will voluntarily participate in the study. If yes, he/she will call the investigative team for an appointment for the first visit (screening) at the CCI, Brigham and Women's Hospital, Boston, MA. The goal would be to recruit and enroll 30 people with prevalent DM, triglycerides between 150 and 400 mg/dl, and current statin use. We will build in margin for 10% attrition and recruit 33 subjects overall. Details on inclusion and exclusion criteria are provided in **Table 2**.

C.3. Randomization Scheme

Randomization will occur during the second study visit at the CCI for those who meet eligibility criteria. In this pilot study, 30 subjects will be randomized with equal probability to EPA intervention (n=15) or no EPA (no drug) (n=15) stratified according to gender. Permuted blocks (block size of 2) will be used to achieve groups of equal size¹³². We will create blocks within each gender and each ethnicity (Caucasian, African- American, and others) to assure balance distribution of gender and ethnicity between the two groups.

C.4. Study Visits

Visit # 1: During this visit, the PI will explain the study objectives, study protocol, and sequences of events and answer any remaining questions that the subject may have about the study. If after such conversation with the PI, the subject is still willing to freely participate in the study, then he/she will sign the informed consent prior to the beginning of the screening process. Specifically, subjects will obtain overview of the study and the PI will complete endothelial function assessment using EndoPat2000 device. Subjects with RHI of 2.0 or less will be eligible and will be asked to provide blood sample for triglyceride measurement. First visit will end with the collection of blood specimen and subjects will be told that the coordinator will notify them via telephone of the results for further eligibility. Only subjects with triglycerides between 150 and 400 mg/dl will be eligible to be invited to the second study visit.

Visit # 2: Eligible subjects identified during visit # 1 will be asked to the CCI fasting but will be allowed to take their usual morning medications. Subjects will be instructed to start fasting at 8PM of the night before the study visit. Pregnancy test will be done to rule out pregnancy among pre-menopausal women without history of surgical sterilization. For subjects randomized to Vascepa, women who can become pregnant during the study will be asked to use a contraceptive method (i.e., hormone, intrauterine device, barrier, or abstinence) during the study and six months after the final visit. During the second visit, we will collect baseline blood samples (20 ml) for measurement of biomarkers (hsCRP, ET-1, and oxLDL); other relevant biomarkers (i.e., TNF α , NTproBNP, apoC3, apoA5, galectin 3, free fatty acids, relevant single nucleotide polymorphisms, and fatty acids will be measured at a later time upon securing new funding on stored blood specimens). Resting blood pressure and pulse will be measured (2x) after 15 minutes of rest and information on age, sex, race, education, height, weight will be collected. Subjects will also complete a Willett food frequency questionnaire to assess their baseline diet. Digital pulse amplitude measurements (see C.6. below) will be done. A dedicated research assistant (RA) will discuss the randomization scheme provided by the statistician with the subjects. The RA will not be blinded to the treatment assignment. Otherwise, PI, study coordinator, statistician, and other staff at the lab measuring biomarkers will all be blinded. Subjects randomized to Vascepa will know that they are taking the drug containing omega-3, since the drug is not disguised and there is no placebo (open label trial). The RA will advise subjects not randomized to Vascepa (control subjects) to continue their usual habits. Each subject randomized to EPA will receive a 12-week supply of study EPA from the BWH pharmacy with the instructions to take 4 g/d. Each subject will receive a study diary to record potential adverse effects during the next 12 weeks. Enrolled subjects will be contacted by telephone once a month by the coordinator to query about potential adverse effects and remind them of the upcoming final visit.

Visit # 3: Subjects will be invited to come to the CCI fasting (starting from 8PM of the night before the visit) during the last visit, which will be 12 weeks after the second visit. Subjects will be allowed to take their morning medications prior to the visit. During the last visit, we will also complete count of any leftover pills among subjects randomized to EPA and repeat all study procedures conducted during the second visit.

C.5. Phlebotomy and Blood Processing at the CCI

Standard methods for phlebotomy and blood processing will be used. Red blood cells and plasma will be stored in 1 ml container at -170 degree freezer until assay measurements. All assays will be performed by a qualified laboratory selected based on quality of their work and competitive pricing bids (i.e., Dr. Tsai's laboratory at the University of Minnesota or Dr. Lichtenstein lab at Tufts University, Boston).

C.6. Assessment of Digital Pulse Amplitude

Study subjects will be asked to refrain from caffeine-containing beverages or tobacco consumption 12 hours prior to the measurement of endothelial function. Digital pulse amplitude will be measured in the fasting state (as outlined above) with a fingertip peripheral arterial tonometry (PAT) device (Endo-PAT2000, Itamar Medical, Caesarea, Israel) in a supine position after a minimum of 20 minute rest in a quiet room with controlled temperature (20-23 degree Celsius). Details on digital pulse amplitude measurements have been published elsewhere^{131,133,134}. Briefly, the PAT device contains a pneumatic plethysmograph, which applies a uniform pressure to the surface of the distal index finger, thereby allowing measurement of pulse volume changes in the finger. Baseline pulse amplitude will be measured from each finger tip for about 5 minutes. The arterial flow will then be interrupted for 5 minutes with a cuff placed

on a proximal forearm with an occlusion pressure equal to the highest value of 200 mm Hg or the subject's systolic blood pressure plus 60 mm Hg. The contralateral arm (dominant arm) will serve as control. Lack of residual pulsatility will be monitored throughout the occlusion period. Pulse amplitude will be recorded electronically in the test and control fingers and analyzed by a computerized and automated algorithm that provides the average pulse amplitude for each 30-second interval after forearm cuff deflation up to 4 minutes (Itamar Medical). The change from the baseline measurement will be expressed as the reactive hyperemia index (RHI), which in part reflects vasodilator function of the digital microcirculation¹³⁵. Previous studies have demonstrated that RHI is to a large part dependent on nitric oxide bioavailability¹³⁶.

For statistical analysis, we will calculate the pulse amplitude response to hyperemia for each 30-second interval as a ratio of the post-deflation pulse amplitude to the baseline pulse amplitude as described previously¹³³. The RHI will be computed by dividing the ratio obtained on the test side (side with blood pressure cuff) over the ratio obtained on the control finger. Since the peak of the RHI has been shown to occur between 90 and 120 seconds after the cuff release¹³³, we will use the average of RHIs obtained at 90 and 120 seconds post deflation for analysis. The reproducibility of this technique has been described previously^{134,137-139}. RHI is correlated with traditional brachial ultrasound and CVD risk factors^{133,140}. Low RHI has been associated with CAD events¹⁴¹⁻¹⁴³ and improve CAD prediction beyond traditional risk factors¹⁴³.

In order to preserve blinding of key personnel, the PI will complete the assessment of endothelial function and an independent research assistant will share the assignment group with the study subject once all activities of study visit # 2 have been completed and PI and other staff have left the CCI. The RA will instruct the subjects assigned to Vascepa on how to obtain the study drug at the BWH research pharmacy. Furthermore, during the third visit, the RA will see all study subjects before any blinded staff and collect Vascepa bottle with remaining capsules when applicable. This way, the possibility that the PI or study staff inadvertently sees the Vascepa bottle is minimized. Subjects will be reminded not to disclose their group status to study staff other than the RA assigning the group.

C.7. Laboratory Assays

In a random sample of 5 subjects in each arm, we will measure fatty acid profile at visit # 2 and visit # 3 to assess compliance with EPA intervention. The fatty acid profile will be measured in plasma using the method previously described by Cao et al¹⁴⁴. For the extraction of plasma phospholipid fatty acids, 0.3 mL of plasma is mixed with 0.7 volume of 0.9% saline. Then, 50 μ L of 2 g/L 17:0 standard (diheptadecanoyl phosphatidylcholine) is added to monitor the extraction efficiency. Lipids are extracted from plasma with a mixture of chloroform:methanol (2:1, v/v), and cholesterol, triglycerides and phospholipid subclasses are separated on a silica thin-layer chromatography plate in a solvent mixture of petroleum ether, diethyl ether, and glacial acetic acid (80:20:1, v/v/v). The band of phospholipids is harvested for the formation of methyl esters. Fatty acid methyl esters are prepared with 1.5 mL of 14% boron trifluoride in methanol, incubated at 80°C for 90 minutes, and extracted with petroleum ether. The final product is dissolved in heptane and injected onto a capillary Varian CP7420 100-m column with a Hewlett Packard 5890 gas chromatograph (GC) equipped with a HP6890A autosampler. The GC is configured for a single capillary column with a flame ionization detector and interfaced with HP chemstation software. Fatty acid methyl esters from 12:0 through 24:1n9 are separated, identified and expressed as percent of total fatty acids. The following coefficients of variation were obtained on 20 blind duplicates: linoleic acid = 2.6%; ALA= 2.4%; arachidonic acid = 2.4%; EPA= 3.3%; docosapentaenoic acid (DPA) = 2.9% and DHA= 2.7% at Dr. Tsai's laboratory at the University of Minnesota (one of our collaborative laboratories).

Plasma hsCRP will be measured by Sandwich enzyme linked immunosorbent assay (ELISA)¹⁴⁵. Plasma oxidized LDL and plasma ET-1 will be measured using a commercially available sandwich-enzyme immunoassay kit (R & D);

All samples will be labeled with the date of collection, subject ID, and visit #. Since blood samples will be processed and stored in subzero freezer by the CCI lab personnel, PI and study staff as well as lab personnel measuring assays will be blinded from the drug assignment.

A summary of major steps of the study is provided below (**Table 3**).

Table 3. Time points for activities during the study period

Activities	Study Week		
	-4 to -1	0	12
Prescreening via telephone	x		
Measurement of triglycerides	x		
Final screening visit	x		
Random allocation to EPA or no EPA and study diary		x	
PAT measurements (endothelial function) and phlebotomy	x	x	x
Demographics, height, weight, medical history, food questionnaire		x	x
Blood pressure and biomarkers (OxLDL, ET-1, and hsCRP)		x	x

C.8. Data Management

Our team has developed a sophisticated computer system to create and maintain data sets. Out-of-range, internally inconsistent, and unclear data will be reviewed and corrected as necessary. Data collected during each visit will be double-entered into the database system. All data will undergo additional within-form and across-time checks to verify accuracy. This database will be maintained on a UNIX server that is backed up nightly, ensuring at least two current copies at all times. Data entry is performed by two different study staff with many years of experience in data processing. The two generated files will be compared. Any errors in coding or keying will be corrected promptly. Access to the network is password-protected to assure confidentiality.

D. Approach to Achieve Proposed Aims

D.1. Main Aim: **Effects of EPA on endothelial function in adults with diabetes and hypertriglyceridemia**

Initially, descriptive statistics such as the minimum, maximum, median, mean and standard deviation for each continuous variable and frequency table for each categorical variable will be used to summarize the data as well as detect outliers, data entry mistakes, and missing values. Exploratory graphical techniques such as Boxplots, Histograms, Quantile-Quantile plots, and Stem and Leaf plots will be used to further examine these data. The normality of the distribution of primary outcome of interest will be examined with a normal probability plot. The differences of baseline characteristics between EPA and placebo groups will be assessed using two sample t-tests, Chi-square tests or other non-parametric tests as appropriate. The primary analysis will be a comparison of the change in RHI from baseline to post-intervention between the 2 groups of subjects randomized to either 4g/d of EPA or no drug using a two sample t test or Wilcoxon rank-sum test. Analyses will follow the intention-to-treat principle although compliance adjusted analysis will be explored where applicable.

In addition, we will develop a general linear model including treatment and gender as the main effects, and the interaction term between treatment and gender. We will also examine

confounding by age, gender, blood pressure, smoking, exercise, body mass index, and alcohol consumption. However, due to limited sample size of this pilot study, we will evaluate each covariate separately, and the final adjusted model will include no more than 5 predictors.

We expect an increase in RHI in the EPA group compared with the control group. The main goal is to estimate this effect size in order to properly design the future confirmatory trial. A lack of statistical significance in this pilot study could be due to several possibilities: 1) four grams per day of EPA do not influence endothelial function in this population; 2) unbalanced distribution of other determinants of digital pulse amplitude that we were unable to control in this pilot study biased the findings; a future larger study would address this issue; 3) lack of effect may be attributable to inadequate power to detect a smaller improvement in endothelial function; such concern will not be an issue in the main trial with adequate statistical power. We will calculate nutrients and energy intake from food questionnaires and assess any change in dietary patterns during the study period (by comparing post- and pre-nutrients). All analyses will be completed by blinded staff and unblinding will only occur when primary and secondary analyses are completed.

D.2. Secondary Aim: **Effects of EPA on CVD biomarkers in subjects with DM and hypertriglyceridemia**

Same approach as in the primary aim will be used for the secondary aim. Briefly, the distribution of each outcome variable will be examined and Box-Cox transformations may be applied. A two sample t-test or Willcoxon rank sum test will be used to compare each outcome between treatment and control groups. A multiple linear regression model will be used for adjusted analysis. Due to the small sample size, potential confounding factors will be assessed separately and may be included in the final model when applicable.

D.3. Statistical Power

This is a pilot trial in which we will generate preliminary data to test the effects of EPA on endothelial function in DM subjects. The purpose of this study is to generate exploratory and hypothesis generating data, and most importantly, to estimate the effect size with standard error in order to determine the appropriate sample size with sufficient power for the future confirmatory trial. Assuming a standard deviation of 0.4 and 15 subjects per arm, we will have 80% power to detect an effect size of 1.05.