CCCWFU 98113
Comprehensive Cancer Center of Wake Forest University
A Randomized, Placebo-Controlled Phase II Clinical Trial of Omega-3 PUFA Dietary Supplementation in Patients with Stage I-III Breast Carcinoma

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Participating Institution(s):	Wake Forest School of Medicine
Funding Sponsor:	American Institute for Cancer Research
Version Date:	03.24.15

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SCHEMA

Objectives:

- To determine if omega-3 dietary supplementation results in higher omega-3 PUFA levels in surgical specimens of normal and malignant breast tissue in women who took omega 3 tablets in comparison to those who took placebo.
- To determine if omega-3 dietary supplementation results in higher omega-3 PUFA levels in plasma and red blood cells in women who took omega 3 tablets in comparison to those who took placebo.
- To determine if omega-3 dietary supplementation affects the metabolites of omega-3 and omega-6 PUFA in surgical specimens of malignant and normal breast tissue in comparison to controls.
- To determine if women who take omega-3 dietary supplementation have less proliferation and greater apoptosis in malignant breast tissue in comparison to women who take placebo.

Subject Eligibility:

- Women scheduled to undergo surgical removal of newly diagnosed, histologically confirmed clinical stage I to III breast carcinoma and carcinoma in situ (including lobular carcinoma in situ and ductal carcinoma in situ).
- Tumor measurement of at least 1 centimeter on imaging or physical exam
- Age $\geq$18 years.
- Ability to understand and the willingness to sign a written IRB-approved informed consent document.
- No use of any NSAID or full-dose ASA-containing NSAID while taking study drug
- No use of omega-3 fatty acid supplements within 1 month of enrollment
- No patients with an allergy or known hypersensitivity to fish

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1.0 Introduction and Background

1.1 Dietary sources of fatty acids

Saturated and monounsaturated fatty acids (FA) can be synthesized by human cells and obtained from diet. By contrast, polyunsaturated fatty acids (PUFA) are essential FA that cannot be synthesized by mammals and must be obtained from diet. Thus the PUFA content of a tissue is dependent mainly on dietary intake. The shortest of the omega-6 series of PUFA is linoleic acid (LA, 18:2, n-6) which is abundant in corn, sunflower, safflower, olive and other vegetable oils. Its 18 carbon omega-3 counterpart, α-linolenic acid (ALA, 18:3, n-3), is also a plant product found in leafy vegetables such as kale, spinach, broccoli and Brussels sprouts as well as walnuts and seeds such as flax and mustard. Both LA and ALA are converted through a series of enzymes to longer chain PUFA: LA to arachidonic acid (AA, 20:4, n-6), and ALA to eicosapentaenoic acid (EPA, 20:5, n-3) and docosahexaenoic acid (DHA, 22:6, n-3). LA is the most abundant FA in Western diets with consumption in the US that is 10-fold that of ALA (reviewed in (1)). Cell culture and animal studies have shown that that a high intake of LA lowers the conversion of ALA to EPA (2) and that the major source of EPA and DHA in humans is the dietary intake of salt water fish (3).

1.2 Dietary n-3 PUFA and breast cancer

In human population studies, an inverse relationship has been observed between breast cancer incidence and calories from fish oil (4, 5). Nevertheless, current epidemiological literature on the association of marine PUFA and cancer remains controversial (6-8). One problem is that these studies have relied on data from self-reported dietary FA intakes or from estimates based on national consumption assessments that correlate poorly with direct measurements of FA in patient samples. The effect of n-3 PUFA depends on levels achieved in individuals and on the omega-3 PUFA content of the fish consumed. The EURAMIC study is one of the largest to use adipose tissue as a primary exposure measure for dietary fat intake (9). In this study, a lower ratio of n-6/n-3 PUFA was detected in adipose tissue of control compared to breast cancer subjects. A more recent study confirmed higher n-6 PUFA in adipose tissue of breast cancer patients compared to control individuals (10). However outstanding gaps in existing knowledge of the potential benefit of n-3 PUFA in modifying breast cancer risk remains the lack of actual measurement of the PUFA and PUFA metabolites in **malignant and normal breast** tissues and an understanding of how dietary PUFA metabolism may contribute to and be altered in the neoplastic state.

New data from the Women's Intervention Nutrition Study (WINS) indicates that the risk of breast cancer recurrence can be reduced by a consuming a low fat diet (11). However, no attention was paid to specific FA species in the diet or how this might impact the FA content of breast tissue. Two earlier studies, one conducted with Japanese (12), the other

with Finnish patients (13), identified increases in total PUFA in cancer compared to benign breast tissue. No such data are available for patients consuming "Western" diets. Finally, studies extant assume a tight or even 1:1 relation between PUFA in plasma, red blood cells (RBC), and breast cancer. Although many studies including our own in non-human primates (14) have demonstrated that plasma FA profiles reflect dietary intake, our preliminary studies contradict the notion that RBC and to a lesser extent plasma measurements reflect the milieu in breast tissue.

Mechanisms for modification of cancer risk by PUFA. Studies in rodents strongly support a promoting role of omega-6 PUFA and a protective role of omega-3 PUFA in breast cancer (15-21): the growth of primary as well as metastatic tumors is inhibited by omega-3 PUFA-rich diets and promoted by omega-6 PUFA-rich diets. Several studies have suggested a potential use for omega-3 PUFA as a nutritional adjuvant therapy. In athymic nude mice with human breast cancer xenografts, lung metastases were inhibited by dietary supplementation with omega-3 PUFA initiated before or after surgical removal of the primary tumors (22). Dietary omega-3 PUFA also increased the efficacy of chemotherapeutic agents, doxorubicin (23) and mitomycin C, (24) in inhibiting tumor growth. These studies strongly suggest potential benefits of omega-3 PUFA supplementation at all levels of breast cancer. Although several mechanisms have been suggested by animal and cell culture studies, the most often cited mode for omega-3 PUFA action is their ability to block metabolism of omega-6 PUFA, AA and LA (25-28). When AA and LA are released from cell membrane phospholipids, they are oxygenated by cyclooxygenases (COX)-1/2, 5-lipoxygenase (LOX), 12-LOX, and 15-LOX-1/2 to prostaglandin (PG)E_2, 5-hydroxy-eicosatetraenoate (HETE), 12-HETE, 15-HETE, and 13- hydroxy-octadecadienoic acid (HODE) (27, 29-32). These metabolites can promote breast cancer growth and the pathways making them may be overactive in breast cancer (27, 29-34). In contrast, omega-3 PUFA inhibit the release and metabolism of AA and LA to reduce formation of the omega-6 PUFA metabolites (27, 29-32, 35). Thus, the development and malignant behavior of breast cancer may reflect the overproduction of omega-6 PUFA-derived growth factors fueled by an omega-6 PUFA-rich diet and omega-3 PUFA-rich diets may inhibit this over-production. Our pilot data strongly support this concept.

Past studies on the effects of omega-6 PUFA metabolites and drugs that inhibit the oxygenases that make them have implicated the metabolites and oxygenases as growth factors. However, they varied widely as to which oxygenases and metabolites are critical to the proliferation and survival of cultured human breast cancer cells (29, 32, 35-37). Recent studies, including those in animals and humans, continue this disagreement. COX-2 and its PGE_2 product are elevated in mouse models of breast cancer; COX-2 over-expression induces breast tumors and *Cox-2* knockout suppresses these tumors (33, 34). Indeed, drugs that block COX-2 are associated with a reduced incidence of human breast cancer in human epidemiological studies (38) as well as mouse models of breast cancer (39-41). COX-1 may contribute to these effects or, in the absence of COX-2, mediate them (34, 35). On the other hand, 5-LOX inhibitors are particularly effective in

stopping the growth of cultured human breast cancer cells, and 5-LOX metabolites 5-HETE and 5-oxo-ETE (made from 5-HETE by a dehydrogenase; both act on cells through a common receptor, OXE (42)), are more effective than PGE_2 in stimulating cultured human breast cancer cells to proliferate (43-47). Furthermore, mRNA for 5-LOX and its activating protein (FLAP) are increased in human malignant as opposed to normal breast tissue and in node (+) compared to node (-) disease; FLAP message levels correlate negatively with overall and disease-free survival (48, 49). Studies focusing on the 12-LOX/12-HETE axis find 12-HETE is somewhat weaker than 5-HETE in stimulating human breast cancer cell proliferation (47) but forced expression of 12-LOX stimulates the proliferation of human breast cancer cells in vitro and in mice (50). 12-LOX-p message is over-expressed in malignant compared to normal human breast tissue and cell lines (32, 48, 51) and, when coupled with FLAP mRNA levels, provides a better prognostic indicator of overall and disease-free survival in breast cancer than FLAP mRNA alone (49). Studies of 15-LOX find that 15-HETE and its hydroperoxy precursor, 15-HpETE, may or may not inhibit the proliferation of breast cancer cells (46, 52) but mRNA and protein levels of their parent oxygenases, 15-LOX-1 and 15-LOX-2, are decreased in malignant compared to normal human breast tissue (48, 53). Patients with low levels of message and protein for these oxygenases, or low 15-LOX-1/15-LOX-2 message or protein ratios, have higher recurrence rates and shortened survivals (53). Thus, a 15-LOX, particularly 15-LOX-1, may suppress tumor growth. Nonetheless, human breast cancer cells metabolize LA to 13-HODE through the action of 15-LOX's (and COX-1/2) (54). 13-HODE is implicated in the proliferation, invasiveness, and metastatic behavior of breast cancer cells (54, 55).

It is fair to say that the animal and human breast cancer studies to date have: 1) assigned multiple and often contradictory roles to the oxygenases and omega-6 PUFA metabolites in human breast cancer; 2) omitted companion studies on normal breast tissue; 3) not addressed the role of diet in modifying the lipid milieu of breast tissue; and most importantly, 4) focused on one or a limited range of oxygenases without measuring the metabolites per se. Because of the difficulty in measuring the metabolites, this last failure represents a critical barrier to progress in understanding the role(s) of PUFA in breast cancer. Studies typically identified one or two omega-6 PUFA metabolites using high performance liquid chromatography (HPLC) or immunoassays in animals or cultured cells but generally have not examined humans. HPLC assays do not distinguish between closely eluting metabolites (e.g. PGE_2 versus PGE_3) nor detect metabolites much below 100 milligram levels. Immunoassays also do not distinguish omega-6 PUFA metabolites from their omega-3 PUFA counterparts and are not available for all the metabolites. Thus these methods are too insensitive and indiscriminant for human studies, particularly those analyzing the impact of omega-3 diets. In consequence, levels of the metabolites in malignant and normal breast tissue, the significance of their presence, and the impact of omega-3 PUFA diets on this presence are unknown. We have developed a multiple response monitoring-liquid chromatography-tandem mass spectroscopy (MRM-LC/MS/MS) method that measures pg levels of PUFA metabolites in tissues to answer these vital questions.

1.3 Rationale

We hypothesize that omega-3 PUFA dietary supplementation will lead to a decrease in the omega-6 PUFA metabolite milieu in malignant breast tissue and will be associated with favorable prognostic markers. We will randomize women prior to definitive breast surgery to two grams of omega-3 fatty acid supplements daily or a matching placebo. We will measure the levels of omega-6 PUFA, omega-3 PUFA, and certain of their metabolites in the malignant breast tissue removed at surgery and compare the two groups. Further, we hypothesize that a defined level of omega-3 PUFA dietary supplementation will: 1) "reset" the PUFA metabolic pathway in malignant breast cells to one associated with a better prognosis by lowering the tumor load of omega-6 PUFA metabolites, and 2) fuel the production of omega-3 PUFA metabolites with anti-growth activity. For comparative purposes, we will pair these measurements to those made in nearby normal breast tissue.

2.0 Objectives

2.1 To determine if omega-3 dietary supplementation results in higher PUFA levels in surgical specimens of normal and malignant breast tissue in women who took omega 3 tablets in comparison to those who took placebo.

2.2 To determine if omega-3 dietary supplementation results in higher PUFA levels in plasma and red blood cells in women who took omega 3 tablets in comparison to those who took placebo

2.3 To determine if omega-3 dietary supplementation affects the metabolites of omega-3 and omega-6 PUFA in surgical specimens of malignant and normal breast tissue in comparison to controls.

2.4 To determine if women who take omega-3 dietary supplementation have less proliferation and greater apoptosis in malignant breast tissue in comparison to women who take placebo.

3.0 Patient Selection

This clinical trial can fulfill its objective only if patients appropriate for this trial are enrolled. All relevant medical and other considerations should be taken into account when deciding whether this protocol is appropriate for a particular patient. Physicians should consider the risks and benefits of any therapy, and therefore only enroll patients for whom this treatment is appropriate.

3.1 Inclusion Criteria

3.1.1 Newly diagnosed stage I to III breast cancer and carcinoma in situ (including lobular carcinoma in situ [LCIS] and ductal carcinoma in situ [DCIS])
3.1.2 Breast surgery (lumpectomy or mastectomy) is planned for at least 7 days from the day of enrollment.
3.1.3 Age ≥18 years.
3.1.4 Ability to understand and the willingness to sign an IRB-approved written informed consent document.
3.1.5 Tumor measures at least 1 centimeter on imaging or physical exam

3.2 Exclusion Criteria

3.2.1 Any patient with surgery scheduled < 7days after biopsy.
3.2.2 Patients who are unable to refrain from the use of any NSAID or full-dose ASA-containing NSAID while taking study drug.
3.2.3 Patients who will receive neoadjuvant chemotherapy are not eligible.
3.2.4 Patients who are currently taking omega-3 fatty acids, as they are unable to be randomized to placebo.
3.2.5 Patients who have previously taken omega-3 fatty acid within 1 month prior to study enrollment
3.2.6 Patients with an allergy or known hypersensitivity to fish
3.2.7 Women who are pregnant or breastfeeding

3.3 Inclusion of Women and Minorities

Women and members of all races and ethnic groups are eligible for this trial.

4.0 Registration Procedures

All patients entered on any CCCWFU trial, whether treatment, companion, or cancer control trial, **must** be registered with the CCCWFU Protocol Registrar or entered into ORIS Screening Log within 24 hours of Informed Consent. Patients **must** be registered prior to the initiation of treatment.

In order to ensure prompt registration of your patient, please:
1. Complete the Eligibility Checklist (Appendix A)
2. Complete the Protocol Registration Form (Appendix A)
3. Alert the WFUHS registrar by phone, *and then* send the signed Informed Consent Form, Eligibility Checklist and Protocol Registration Form to the registrar, either by fax or e-mail.

Contact Information:

Protocol Registrar PHONE (336) 713-6767
Protocol Registrar FAX (336) 713-6772
Protocol Registrar E-MAIL (registra@wakehealth.edu)

*Protocol Registration is open from 8:30 AM - 4:00 PM, Monday-Friday.

4. Please fax/e-mail ALL eligibility source documents with registration. Patients **will not** be registered without all required supporting documents.

Note: If labs were performed at an outside institution, please provide a printout of the results. Please ensure that the most recent lab values are sent.

To complete the registration process, the Registrar will:

- assign a patient study number
- randomize the patient
- register the patient on the study

5.0 Treatment Plan

5.1 Study-Related Interventions

	Baseline	Intervention Period[d]	Day of Surgery
Informed consent	X		
Randomization	X		
Demographics	X		
Medical history	X		
Concurrent meds	X	X[a]	
Physical exam	X		
Vital signs	X		
Height, Weight, M^2	X		
Blood collection	X[e]		X[e]
Specimen Collection			X
Adverse event evaluation		X	X
Supplement Administration		X[c]	
Optional core biopsy for research purposes	X[b]		

a: Conducted via telephone for each patient on day 7 after enrollment.
b: If available from clinically-obtained specimens.
c: 2 grams of omega-3 PUFA or placebo PO daily during the intervention period.
d: The intervention period is defined as the day after enrollment until the day prior to surgery.
e: Whole blood; 4ml collected into EDTA tube

Patients will be presented with the study and, if interested, provide written informed consent during their standard-of-care office visit. In addition, the routine physical conducted as standard of care during this visit will be used to obtain patients' medical history, concurrent medications, and vital signs for the research study.

5.2 Omega-3 PUFA Supplementation

Participants will be randomized to receive either two grams of omega-3 PUFA or placebo daily until the day before surgery. Omega-3 PUFA is supplied as a one gram capsule (see section 8.0 for a full description of the product). Placebos will be identical to the omega-3 supplements in size and color and will contain 99% soybean oil and less than 1% each of natural vitamin E, natural lemon flavor, marine lipid, and rosemary extract. PUFA and placebo capsules will be certified toxin-free products, and PUFA capsules will have verified EPA and DHA levels.

Patients on both arms of the study will be instructed to take two capsules daily from the day after enrollment and the last two capsules the day prior to surgery.

The date that surgery is planned will be known at the time of randomization and an ample amount of the appropriate capsules will be supplied by the research nurse, plus four days' worth of extra capsules. If there is a delay in the planned surgery, the patient will be told to continue taking the capsules as directed until the day prior to the revised surgery date.

After one week of taking the capsules, the research nurse will call the patient to assess for adverse effects, self-reported compliance, confirm the surgery date, and address any other issues. In the event that the extra capsules are not sufficient to cover the remaining time until the scheduled date of surgery, additional capsules can be mailed to the patient's home if needed. If the patient remains on study beyond 7 days, then additional phone calls will occur at day 14 and day 21 (and every seven days thereafter) until the patient undergoes surgery. Appendix C should be used to document the information obtained during these phone calls.

5.3 General Concomitant Medication and Supportive Care Guidelines

The use of any NSAID or full-dose ASA-containing NSAID while taking study drug prohibited. Should the use of these agents be deemed necessary, the participant in question will be discontinued from the study. Participants will be asked to use acetaminophen if needed during the study.

All other medications and/or therapies during the intervention period will be performed as per standard of care, at the discretion of the participant's physician.

5.4 Duration of Subject Involvement

Subject participation extends from enrollment (at the clinic visit for the initial diagnosis) until the perioperative collection of study tissue specimens (usually 7-14 days). There will be no follow-up period, and the need for subject discontinuation is expected to be minimal. Nonetheless, participants will be withdrawn from the study in the event of any of the following:

- Intercurrent illness that prevents further administration of treatment,
- Patient decides to withdraw from the study,
- Patient develops the need to use of any NSAID or full-dose ASA-containing NSAID between biopsy and surgery, or
- General or specific changes in the patient's condition render the patient unacceptable for further treatment in the judgment of the investigator.

6.0 Adverse Events List and Reporting Requirements

6.1 Adverse Event List for Omega-3 Fatty Acids

Supplementation with omega-3 PUFAs is generally well-tolerated and safe. Theoretical concerns have been voiced regarding omega-3 PUFA supplementation aggravating bleeding; however, a recent study confirmed findings of prior smaller studies and failed to demonstrate any increased hemorrhagic risk across a variety of indices.(56)

Possible adverse events include nausea, burping with fishy aftertaste, or gastrointestinal problems.(57)

6.2 Adverse Event Characteristics

- **CTCAE term (AE description) and grade:** The descriptions and grading scales found in the revised NCI Common Terminology Criteria for Adverse Events (CTCAE) version 4.0 will be utilized for AE reporting. All appropriate treatment areas should have access to a copy of the CTCAE version 4.0. A copy of the CTCAE version 4.0 can be downloaded from the CTEP web site (http://ctep.cancer.gov).

- **'Expectedness'**: AEs can be 'Unexpected' or 'Expected' (see Section 7.1 above) for expedited reporting purposes only.

- **Attribution** of the AE:
  - Definite – The AE *is clearly related* to the study treatment.
  - Probable – The AE *is likely related* to the study treatment.
  - Possible – The AE *may be related* to the study treatment.
  - Unlikely – The AE *is doubtfully related* to the study treatment.
  - Unrelated – The AE *is clearly NOT related* to the study treatment.

6.3 STRC SAE Reporting Requirements

The Safety and Toxicity Reporting Committee (STRC) is responsible for reviewing SAEs for CCCWFU Institutional studies as outlined in Appendix B. STRC currently requires that all unexpected 4 and all grade 5 SAEs on these trials be reported to them for review. All Clinical Research Management (CRM) staff members assisting a Principal Investigator in investigating, documenting and reporting an SAE qualifying for STRC reporting are responsible for informing a clinical member of the STRC as well as the entire committee via the email notification procedure of the occurrence of an SAE.

6.4 WFUHS IRB AE Reporting Requirements

Any unanticipated problems involving risks to subjects or others and adverse events shall be promptly reported to the IRB, according to institutional policy. Reporting to the IRB is required regardless of the funding source, study sponsor, or whether the event involves an investigational or marketed drug, biologic or device. Reportable events are not limited to physical injury, but include psychological, economic and social harm. Reportable events may arise as a result of drugs, biological agents, devices, procedures or other interventions, or as a result of questionnaires, surveys, observations or other interactions with research subjects.

All members of the research team are responsible for the appropriate reporting to the IRB and other applicable parties of unanticipated problems involving risk to subjects or others. The Principal Investigator, however, is ultimately responsible for ensuring the prompt reporting of unanticipated problems involving risk to subjects or others to the IRB. The Principal Investigator is also responsible for ensuring that all reported unanticipated risks to subjects and others which they receive are reviewed to determine whether the report represents a change in the risks and/or benefits to study participants, and whether any changes in the informed consent, protocol or other study-related documents are required.

Any unanticipated problems involving risks to subjects or others occurring at a site where the study has been approved by the WFUHS IRB (internal events) must be reported to the WFUHS IRB within 7 calendar days of the investigator or other members of the study team becoming aware of the event.

Any unanticipated problems involving risks to subjects or others occurring at another site conducting the same study that has been approved by the WFUHS IRB (external events) must be reported to the WFUHS IRB within 7 calendar days of the investigator or other members of the study team becoming aware of the event.

Any event, incident, experience, or outcome that alters the risk versus potential benefit of the research and as a result warrants a substantive change in the research protocol or informed consent process/document in order to insure the safety, rights or welfare of research subjects.

7.0 Pharmaceutical Information

A list of the adverse events and potential risks associated with the investigational or commercial agents administered in this study can be found in Section 7.1.

7.1 Pharmaceutical Information for PUFA Supplement

Product description: The omega-3 supplements used in this study will be provided by Nordic Naturals. The specific omega-3 formulation to be used in this study is ProOmega®, a marine oil concentration containing omega-3 PUFA, eicosapentaenoic acid (EPA) and doxosahexaenoic acid (DHA), supplied in triglyceride form. Each 2 capsule dose contains 650 EPA / 450 DHA per 2 soft gels.

Route of administration: Patients will take two capsules by mouth per day.

7.2 Pharmaceutical Information for Placebo

Placebos used in this study will also be supplied by Nordic Naturals. Each capsule will be identical in size and shape to the PUFA supplements; however they will contain 99% soybean oil and less than 1% each of natural vitamin E, natural lemon flavor, marine lipid, and rosemary extract. Patients randomized to receive placebo will take two capsules by mouth per day.

8.0 Correlative/Special Studies

Between 5 and 100mg of tumor tissue and a similarly-sized section of adjacent normal tissue (obtained from sites equidistant from the site of prior fine needle aspiration) will be retrieved at surgery and immediately divided into sections for separate analyses of the metabolites and PUFA. For metabolites, a section is placed in 1 ml of tris-buffered saline (4 ºC, pH 7.4) containing 100 μM diethylenetriaminepentaacetic acid to chelate metals that attack alkenes, 80 μM of butylated hydroxytoluene to prevent oxidation, and 5 ng of each deuterated metabolite to serve as internal standards. For PUFA, a section will be suspended in ethanol (4 ºC) containing 80 μM butylated hydroxytoluene and 100 μM triphenyl-phosphine. Samples on ice will be transported to our lab. Metabolite samples will be extracted with hexane: ethyl acetate (1:1). Extracts will be blown dry under a stream of N_2, taken up in methanol, and stored under Argon at -80 ºC. PUFA samples will be stored at -80º under Argon in their original ethanol-inhibitor solution and extracted immediately before GC analysis.

All tissues will be coded with a unique identifier so that patient identities cannot be discerned.

Whole blood (4 ml) will be drawn into EDTA-containing tubes from patients both at baseline and immediately before surgery; placed on ice; adjusted to 100 μM in diethylenetriamine-pentaacetic acid and 80 μM butylated hydroxytoluene; transported on ice to our lab (Hanes 4032, contact Tiffany Walker-West at 336-716-3443 who will transport samples from OR to the lab); and centrifuged (1000g, 5 min, 4 º). RBC will be washed with tris-buffered saline (pH 7.4) and suspended in buffer containing 80 μM

butylated hydroxytoluene and 100 μM triphenylphosphine. RBC and plasma (1 ml) are stored at -80 ºC under Argon and extracted immediately before GC. In general, samples are placed in storage within 90 min of procurement.

Our Institution performs Mib1 on all patients undergoing breast cancer surgery. Tissue blocks will be obtained from the Department of Pathology archives for the preparation of slides for cleaved caspase-3 immunostaining in our laboratory.

For MRM-LC/MS/MS, breast tissues will be spiked with deuterated standards (5 ng) of each metabolite at the time of extraction except d_4-PGE_2 and d_8-5-HETE will be used as the internal standard for PGE_3 and 5-HEPE, respectively. We have synthesized d_5-17-OH-DHA for use as the internal standard for 17-OH-DHA. Extracts will be assayed for the daughter ion of each deuterated and non-deuterated species, all of which have unique LC elution times, M^{-1} masses, and/or M^{-1} breakdown ion masses (Table 1) relative to other monitored and unmonitored metabolites.

Table 1

Molecular weight of M^{-1} and daughter ions of species detected by MRM-LC/MS/MS

Species	M^{-1}	daughter ion
PGE_2	351	271
d_4-PGE_2	355	275
PGE_3	349	269
5-HETE	319	115
5-HEPE	317	115
d_8-5-HETE	327	115.8
15-HETE	319	218.8
d_8-15-HETE	327	225.7
12-HETE	319	179
d_8-12-HETE	327	184
13-HODE	295	194.7
d_4-13-HETE	299	197.7
17-HDHA	343	207.5
d_5-17-HDHA	348	200.85

Data are converted to pg/mg wet tissue weight and then corrected for processing losses by comparison to the recovery of their respective deuterated analogs. We validated the accuracy and precision of this method and determined the linearity of recoveries over 1 pg-100 ng of each metabolite in cultured mouse prostate cancer cells. Our MS system is a Waters Quatro II with a Z-spray interface and automated by a Spark Holland LC and a Reliance Autosampler and Conditioned Staker maintained at 4 ºC. We use a cone voltage of 35V and a capillary voltage of 2.4 kV for HETE, HODE, and DHA metabolites and 50V and 3.5 kV for PG metabolites. The LC system for the latter three types of metabolites is a Waters Corp YMC ODS-AQ 1.00 mm I.D.x100 mm length column eluted at 0.05 ml/min with 2 mM NH_4OAc, pH 8.0, as solvent A and MeOH as solvent B in the following gradients: 0 min, 70% B; 0-4 min to 90% B; 4-5 min, 90% B; 5-6 min to 70% B; 6-30 min, 70% B. The HETEs elute with complete baseline separation in this system. The LC system for PGs is a Phenomenex Luna Phenyl-Hexyl 1.00 mm I.D. x 150 mm length column eluted at 0.07 ml/min with H_2O as solvent A and CH_3CN, 0.1% formic acid, as solvent B in the following stepwise gradients: 0 min, injection; 0-6 min, 20% B; 6-6.1 min to 45% B; 6.1-7.1 min, 45% B; 7.1-7.2 min to 65% B; 7.2-9.2 min, 65% B; 9.2-9.3 min to 20% B; 9.3-15 min, 10 % B, 15 min.

We will study a total of 60 patients, analyzing the PUFA in their plasma before and after dietary supplementation, breast cancer, and normal breast tissue and the PUFA

metabolites in their breast cancer and normal breast tissue as described in Table 2. In some patients we may have the opportunity to collect a specimen at study enrollment for research purposes only. In that small subset we will have an additional core biopsy available for research purposes. We will evaluate the FAs pre-surgery and pre-intervention. We anticipate that approximately 10-20 patients will be appropriate for a core biopsy for research purposes only.

Table 2 – Samples to be Collected

PUFA	Plasma /RBC	Breast, tumor[1]	Breast, normal	Metabolites	Breast, tumor[1]	Breast, normal
FA pre-study	60			OH #1	60	60
FA at surgery	60	60	60	OH #2	60	60
				PGE's	60	60

[1]Includes 30 patients with invasive ductal cancer in each dietary treatment group. PUFA are analyzed by GC, metabolites by MRM-LC/MS/MS; OH (i.e. HETE/ HODE/HDHA) metabolites require two separate MRM-LC/MS/MS runs.

9.0 Statistical Considerations

9.1 Study Design/Endpoints

This is a randomized, placebo controlled phase II trial assessing the effect of omega-3 dietary supplementation on PUFA levels (LA, AA, EPA, DHA, total omega-6 PUFA, total omega-3 PUFA and n-6/n-3 PUFA ratios), metabolites of omega-3 and omega-6 PUFA (see Table 1), and proliferation and apoptosis in women with stage I-III breast cancer. Patients who meet the eligibility criteria will be randomized to omega-3 dietary supplementation or a matching placebo with equal probability. Randomization will occur following the diagnosis of breast cancer and at least one week prior to the scheduled surgical resection. Patients will take two capsules per day from the time of randomization through the day before surgery. The primary objective is to assess the effect of omega-3 supplementation on PUFA levels in normal and metastatic breast tissue and in plasma and red blood cells. Secondary objectives include assessing the effect of the omega-3 supplementation on the metabolites of omega-3 and omega-6 PUFA in malignant and normal breast tissue and assessing the effect of the supplementation on proliferation and apoptosis in malignant breast tissue.

9.2 Analyses

Descriptive reports will consist of summary statistics (means, standard deviations, proportions, etc.) for patient characteristics and outcome measures (including toxicities) by treatment arm. Tables, graphs, and charts will be used to illustrate the data when appropriate. Accrual and toxicity are followed by the Safety and Toxicity Review Committee, and adverse events will be reported to the IRB.

Analysis of variance (ANOVA) will be used to assess the effect of omega-3 dietary supplementation on PUFA levels separately in normal and malignant breast tissue. Additionally, we will fit the normal and malignant breast tissue jointly as repeated measures to see if there are PUFA differences by tissue type and to determine if the omega-3 effect differs in the two tissue types (tissue type by treatment interaction). Since we will have pretreatment PUFA levels in the serum, we will use analysis of covariance (ANCOVA) to assess the omega-3 effect in plasma and RBC, where the baseline levels of the PUFAs will be included as covariates. In all analyses, residuals will be assessed to determine if the assumptions of variance homogeneity and normality (and linearity for the ANCOVA models) are met, and transformations will be used if needed. The secondary outcomes will be analyzed using the same methods as described for the primary outcomes.

9.3 Sample Size/Power

Sixty patients will be accrued to this study, approximately 30 in each arm. This sample size will allow us to detect a one standard deviation (SD) difference between treatment groups in PUFA levels with 80% power at the 5% overall level of significance (allowing for multiple comparisons by using a Bonferroni correction – each test done at the 0.05/7 level of significance), assuming a 10% drop-out rate. The anticipated accrual rate for this study is two patients per month.

9.4 Stratification/Randomization

There are no strata in this study. All eligible patients will be randomized to omega-3 supplementation or a matching placebo with equal probability using a variably sized permuted block randomization scheme. Block sizes will be chosen randomly to ensure that future randomizations cannot be inferred from past assignments.

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Appendix A – Eligibility and Source Document Checklists; Registration Form

REGISTRATION GUIDELINES

The following guidelines have been developed in order to ensure timely registration of your patient.

All patients entered on any CCCWFU trial, whether treatment, companion, or cancer control trial, **must** be registered with the CCCWFU Protocol Registrar or entered into ORIS Screening Log within 24 hours of Informed Consent. Patients **must** be registered prior to the initiation of treatment.

In order to ensure prompt registration of your patient, please:

1. Complete the Eligibility Checklist (attached)
2. Complete the Protocol Registration Form (attached)
3. Alert the WFUHS registrar by phone, *and then* send the signed Informed Consent Form, Eligibility Checklist and Protocol Registration Form to the registrar, either by fax or e-mail.

   Contact Information:
   Protocol Registrar PHONE (336) 713-6767
   Protocol Registrar FAX (336) 713-6772
   Protocol Registrar E-MAIL (registra@wakehealth.edu)
   *Protocol Registration is open from 8:30 AM - 4:00 PM, Monday-Friday.
4. Please fax/e-mail ALL eligibility source documents with registration. Patients **will not** be registered without all required supporting documents.

Note: If labs were performed at an outside institution, please provide a printout of the results. Please ensure that the most recent lab values are sent.

CCCWFU # 98113 Eligibility Checklist Page 1

Yes	No	N/A	Inclusion Criteria (All responses must be **YES** in order to enter study)	**Eligibility Confirmation (registrar)**
☐	☐	☐	Does the patient have newly diagnosed stage I to III breast cancer and carcinoma in situ (including lobular carcinoma in situ [LCIS] and ductal carcinoma in situ [DCIS])?	
☐	☐	☐	Is the patient scheduled to undergo breast surgery (lumpectomy or mastectomy) at least 7 days from the day of enrollment?	
☐	☐	☐	Is the patient ≥18 years of age?	
☐	☐	☐	Is the patient able to understand and willing to sign an IRB-approved written informed consent document?	
☐	☐	☐	Does the patient's tumor measure at least 1 centimeter on imaging or physical exam?	
Yes	**No**	**N/A**	Exclusion Criteria (All responses must be **NO** in order to enter study)	
☐	☐	☐	Is the patient scheduled for breast surgery sooner than 7 days after biopsy?	
☐	☐	☐	Will the patient require the use of any NSAID or full-dose ASA-containing NSAID while taking study drug?	
☐	☐	☐	Is the patient scheduled to receive neoadjuvant chemotherapy?	
☐	☐	☐	Is the patient currently taking omega-3 fatty acids?	
☐	☐	☐	Has the patient previously taken omega-3 fatty acid within 1 month prior to study enrollment?	
☐	☐	☐	Does the patient have an allergy or known hypersensitivity to fish?	
☐	☐	☐	Is the patient pregnant or breastfeeding?	

Signature: _____ Date: _____

Please send source documentation with Eligibility Form.

CCCWFU # 98113 Protocol Registration Form **Page 2**

DEMOGRAPHICS

Patient: Last Name: _____ First Name: _____

MRN: ___ ___ ___ ___ ___ ___ ___ ___ DOB (mm/dd/yy): __ __ / __ ___ / __ __

SEX: ☐ Male ☐ Female

Ethnicity (choose one): ☐ Hispanic ☐Non-Hispanic

Race (choose all that apply):
☐ WHITE ☐BLACK ☐ ASIAN ☐ PACIFIC ISLANDER ☐ NATIVE AMERICAN

Height: ___ ___.___ inches Weight: ___ ___ ___.___lbs.(actual)

Surface Area: ___ ___.___m^2

Zip Code: ___ ___ ___ ___ ___

Primary Diagnosis:
_____

Date of Diagnosis: ___ ___ /___ ___/___ ___

Date of scheduled surgery: ___ ___ / ___ ___ / ___ ___

PROTOCOL INFORMATION

Date of Registration:	___ ___ / ___ ___ / ___ ___
MD Name (last) :	_____
Date protocol treatment started:	___ ___ / ___ ___ / ___ ___
Informed written consent: (consent must be signed prior to registration)	☐ YES ☐ NO
Date Consent Signed:	___ ___ / ___ ___ / ___ ___
PID # (to be assigned by ORIS):	___ ___ ___ ___ ___ ___ ___ ___

Protocol Registrar can be contact by calling 336-713-6767 between 8:30 AM and 4:00 PM, Monday – Friday.

Completed Eligibility Checklist and Protocol Registration Form must be hand delivered, faxed or e-mailed to the registrar at 336-7136772 or registra@wakehealth.edu.

Appendix B – Mandatory STRC SAE Notification Procedure

Mandatory Safety and Toxicity Review Committee (STRC) Serious Adverse Event (SAE) Reporting Requirements – Revised 6/05/2012

This document describes STRC reporting and use of the electronic submission form that is submitted **for unexpected grade 4 and any grade 5 (death during protocol intervention) SAEs on CCCWFU Institutional interventional trial patients**. There are multiple entities that require reporting of SAEs. Each entity has different rules for what is reported, and how it is reported.

Rules used by other entities (Institutional Review Board (IRB), AdEERS, MedWatch, etc.) should NOT be used to evaluate whether an event should be reported to STRC. Only the rules for reporting described in this document should be considered.

As defined in the NCI summary IV reporting guidelines, **CCCWFU Institutional studies covered by these reporting requirements are defined as: In-house, internally reviewed trials, including those collaborative studies conducted with industry sponsorship in which the center is a primary contributor to the design, implementation, and monitoring of the trial, or participation in a multi-site trial initiated by an institutional investigator at another center.** Institutional trials are almost always authored by a researcher here at CCCWFU. Institutional protocols are labeled NCI Code="I" for Institutional on the protocol screen in ORIS. Cooperative group protocols are **not** considered Institutional, but Research Base trials **are** classified as Institutional.

The STRC is responsible for reviewing SAEs for CCCWFU Institutional studies, as defined above. STRC currently requires that unexpected grade 4 and all grade 5 SAEs on these trials be reported to them for review. All Clinical Research Management (CRM) staff members assisting a PI in documenting and reporting an SAE that qualifies for STRC reporting are responsible for informing a clinical member of the STRC by phone, followed by informing the entire committee via the required email notification.

THESE REPORTING REQUIREMENTS APPLY TO EVERYONE WORKING WITH CANCER CENTER INSTITUTIONAL PROTOCOLS.

What is considered an SAE under this mandatory procedure?
Any **unexpected grade 4** event not including routinely experienced events per protocol (e.g. myelosuppression) and **all grade 5 events** (death during protocol intervention) should be reported. The patient is considered "on-treatment" as defined in the protocol, which can extend days/weeks/months past the last date of actual protocol intervention.

Table 1: Summary of STRC Reporting Requirements for Institutional Pilot, Phase 1, Phase 2 and Phase 3 Interventional Trials

	ADVERSE EVENT					
	Grade 1, Grade 2, Grade 3		Grade 4		Grade 5	
	Unexpected	Expected	Unexpected	Expected	Unexpected	Expected
Unrelated	Not Required	Not Required	**REPORT TO STRC**	Not Required	**REPORT TO STRC**	**REPORT TO STRC**
Unlikely	Not Required	Not Required	**REPORT TO STRC**	Not Required	**REPORT TO STRC**	**REPORT TO STRC**
Possible	Not Required	Not Required	**REPORT TO STRC**	Not Required	**REPORT TO STRC**	**REPORT TO STRC**
Probable	Not Required	Not Required	**REPORT TO STRC**	Not Required	**REPORT TO STRC**	**REPORT TO STRC**
Definite	Not Required	Not Required	**REPORT TO STRC**	Not Required	**REPORT TO STRC**	**REPORT TO STRC**

STRC reporting may not be appropriate for specific expected adverse events for protocols. In those situations the adverse events that will not require STRC reporting **must be specified in the text of the approved protocol.**

STRC notification responsibilities of the person handling the reporting/documenting of the SAE:

1. Make a phone call to the appropriate clinical member of the STRC as listed below (page if necessary)—see note 2 below
2. Submit the STRC Notification Form WITHIN 24 HOURS of first knowledge of the event. This form is found at either the ORIS main menu page or by going to http://ccc.wfubmc.edu/oris/strc.aspx.
   This will ensure that all persons that the event applies to will be notified; remember to file a copy of your confirmation. (Form instructions will walk you through the required fields, consult the help page for further instructions.)
3. Ensure that you document that the appropriate persons on the STRC has been contacted.
4. Follow up with/update the clinical member of STRC regarding any new developments or information obtained during the course of the SAE investigation and reporting process.

Elements needed to complete the electronic STRC form:

1. ORIS Patient ID (PID)
2. Name of STRC Clinician notified/Date/Time/Comments.
3. Grade of event.
4. Is this related to protocol intervention or treatment?
5. Is suspension of the protocol needed?
6. Is any change to consent or protocol needed?

7. Was the nature or severity of the event unexpected?
8. Date of the event.
9. Brief description of the event using approved CTC version terminology.
10. Date of last study dose before event.
11. Relevant tests/labs.
12. **Most importantly make sure that the Investigator assigns attribution to the reported event (grade) using the appropriate CTCAE version for the protocol.**

The Clinical Members of STRC to Notify by Phone or Page:

Bayard Powell, MD – Director-at-Large, CCCWFU; Chair, PRC; Section Head,
Glenn Lesser, MD – Hematology Oncology
Kathryn Greven, MD – Vice Chair –
Marissa Howard-McNatt, MD – General Surgery

Definition of Unavailable: As a general guideline if the first clinician that is contacted does not respond to the phone call or page within a reasonable amount of time, then initiate contact with their backup. Give the back-up a reasonable amount of time to respond to a phone call or page before contacting another member. This is a general guideline. You must use your best judgment as a clinical research professional given the time of day, severity of the SAE, and other circumstances as to when it is appropriate to contact backup clinicians. If the event occurs near the end of day, then leave messages (voice or email) as appropriate and proceed with submitting your STRC notification form. The important criteria is that have taken reasonable steps to notify and document that you have initiated some type of contact to one or more of the clinical members of STRC.

STRC CLINICAN RESPONSIBILITY:

It is the responsibility of the STRC clinician to review all reported events, evaluate the events as they are reported; and communicate a response to the Investigator, event reporter and the members of STRC. The review will include but not be limited to the information reported; there may be times when additional information is needed in order for an assessment to be made further communication directly with the investigator may be warranted. STRC reserves the right to agree with the investigator's assessment if STRC does not agree with the investigator STRC reserves the right to suspend the trial pending further investigation.

AMENDMENTS TO PREVIOUS REPORTS

If you are not able to supply all pertinent information with the initial submission, once the additional information is available **do not submit a new report**. Go to the original email that was received by STRC and others "reply to all" and entitle your email "**Amendment** for (list date of event and patient ID) this will avoid duplications of the same event. List the additional information which you are reporting.

Appendix C – Telephone Follow-up Data Collection Form

ORIS PID: _____ Initials of person completing form: _____

Date of call: ____ ____ / ____ ____ / ___ ____

Omega-3 PUFA Supplementation:

Date patient began taking capsules: ____ ____ / ____ ____ / ____ ____

Original date of surgery: ____ ____ / ____ ____ / ____ ____

Has the patient's surgery been rescheduled? Yes ☐ No ☐

If Yes, indicate new date: ____ / ____ / ____

If Yes, does patient have ample capsules to last until surgery? Yes ☐ No ☐

If No, indicate the # of additional capsules mailed to patient and the date of shipment:

capsules: _____ Date shipped: ____ ____ / ____ ____ / ____ ____

Compliance:

Does the patient report compliance with taking capsules? Yes ☐ No ☐

If No, how many doses of medication were missed? _____

Adverse Events:

Have any adverse events been identified? Yes ☐ No ☐

If Yes, Have they been recorded on the CCCWFU AE Log (Appendix F)? Yes ☐ No ☐ NA ☐

Have the adverse events been documented in the medical record? Yes ☐ No ☐ NA ☐

Other issues noted during phone call:

_____

_____

_____

_____

Appendix D – Tissue Procurement Form

- A minimum of 5mg and maximum of 100mg of tissue will be obtained from tumor and normal breast tissue.
- Antioxidant solution will be provided by the Edwards or Kucera lab.
- Divide samples into two sections, record weights and place in labeled eppendorf tubes containing the antioxidant solution.

	Tumor		**Normal**	
Section/tube#	**1**	**2**	**3**	4
% of total mass	~40%	~60%	~40%	~60%
Weight in mg	_____	_____	_____	_____
Antioxidant	1ml	1ml	1ml	1ml

Blood

- Collect in 4ml purple vacutainer tube.
- Invert 8-10 times. Store on ice for immediate pick up by Tiffany Walker-West (6-3443)

Patient identification # _____ **Date** _____

Appendix E – CCCWFU Adverse Event Log

Adverse Event Description	Start Date	Stop Date	AE Type	Grade (1-5) per CTC v. 4.0	Attribution	Serious	Action Taken	Treating Physician Initials/ Date
			☐Expected ☐Unexpected	☐Mild/1 ☐Moderate/2 ☐Severe/3 ☐Life-threatening/4 ☐Death/5	☐Related ☐Probably ☐Possible ☐Unlikely ☐Unrelated	☐No ☐Hospitalization ☐Disability ☐Birth Defect ☐Life-threatening ☐Death ☐Other: ________	☐None ☐Therapy Withheld ☐Therapy D/C ☐Therapy Adjusted ☐Other: ________ ☐N/A	
			☐Expected ☐Unexpected	☐Mild/1 ☐Moderate/2 ☐Severe/3 ☐Life-threatening/4 ☐Death/5	☐Related ☐Probably ☐Possible ☐Unlikely ☐Unrelated	☐No ☐Hospitalization ☐Disability ☐Birth Defect ☐Life-threatening ☐Death ☐Other: ________	☐None ☐Therapy Withheld ☐Therapy D/C ☐Therapy Adjusted ☐Other: ________ ☐N/A	
			☐Expected ☐Unexpected	☐Mild/1 ☐Moderate/2 ☐Severe/3 ☐Life-threatening/4 ☐Death/5	☐Related ☐Probably ☐Possible ☐Unlikely ☐Unrelated	☐No ☐Hospitalization ☐Disability ☐Birth Defect ☐Life-threatening ☐Death ☐Other: ________	☐None ☐Therapy Withheld ☐Therapy D/C ☐Therapy Adjusted ☐Other: ________ ☐N/A	