Award Number: W81XWH-04-1-0296

TITLE: Fish Oil Supplementation and Fatty Acid Synthase Expression in the Prostate: A Randomized Controlled Trial

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REPORT DATE: July 2011

TYPE OF REPORT: Final Addendum

PREPARED FOR: U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release; Distribution Unlimited

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Fish Oil Supplementation and Fatty Acid Synthase Expression in the Prostate: A Randomized Controlled Trial

One in seven men over the age of 60 will be diagnosed with prostate cancer. Elucidation of early cellular changes that may predict progression to prostate cancer and the identification of factors that may inhibit or reverse these cellular changes would be of great clinical significance. Alteration of the fatty acid synthase (FAS) pathway is an early cellular change that has recently come under investigation. Overexpression of the lipogenic enzyme FAS has been noted in several tumor and pre-cancerous tissue types, including prostatic intraepithelial neoplasia (PIN) and prostate cancer and has been suggested as an independent predictor of disease stage. Additionally, inhibition of FAS has been demonstrated to induce apoptosis and reduce cell proliferation in cancer cells. Fatty acid synthase expression in cancer and normal cells is regulated by the transcription factor sterol regulatory element binding protein 1c (SREBP-1). The up-regulation of SREBP-1 in tumor cells results in increased FAS expression and fatty acid synthesis. Research in normal cells has demonstrated that dietary supplementation with polyunsaturated fatty acids (PUFA), particularly omega-3 fatty acids, inhibits SREBP-1 activation, resulting in a decreased transcription of FAS.
# Table of Contents

- Introduction .............................................................................................. 4
- Body ........................................................................................................... 5
- Key Research Accomplishments ............................................................... 9
- Reportable Outcomes ............................................................................. 9
- Conclusions .............................................................................................. 10
- References ................................................................................................. 11
- Appendix (Metabolomics Analysis) ......................................................... 12
INTRODUCTION

One in seven men over the age of 60 will be diagnosed with prostate cancer [1]. While improvements in screening and diagnostic procedures (e.g. prostatic specific antigen (PSA) and transrectal ultrasound (TRUS)) have allowed clinicians to better identify men at high risk of having or developing prostate disease, no established means of preventing prostate cancer are currently available. Elucidation of early cellular changes that may predict progression to cancer and the identification of factors that may inhibit or reverse these cellular changes would be of great clinical significance.

Alteration of the fatty acid synthesis pathway is one early cellular change that has only recently come under investigation [2]. Fatty acid synthase (FAS) is a lipogenic multienzyme that catalyzes the final step in de novo fatty acid synthesis. FAS more recently has been characterized as an oncogene and is reported to be overexpressed in several tumor types including prostate. While FAS expression in cancer cells does not appear to be normally regulated by nutritional controls, Menendez et al demonstrated that addition of omega-3 fatty acids (ω-3 FA), docosahexanoic acid (DHA), α-linolenic acid, and ω-6 FA, γ-linolenic acid, resulted in significant inhibition of FAS expression and fatty acid production in FAS expressing SK-Br3 human breast cancer cells [3]. We have conducted a double-blind, placebo-controlled, randomized intervention study to evaluate the effects of Fish Oil (FO) supplementation on markers of lipid metabolism in prostate tissue samples. The protocol for this trial was modified in 2006 to describe addition of a green tea plus fish oil and green tea plus placebo arm. Based on discussion with the Project Officer at the time (Julie Wilberding) it was determined this change to protocol did not affect the aims supported by DOD for the fish oil only trial. Thus no changes were made to the aims, Statement of Work or primary outcomes for the DOD funded trial. The two additional green tea arms are funded through a separate source (NCCAM), thus, in the following final report we will not discuss the green tea portion of this trial.

The primary aims for this trial were:
Among men considered to be at high risk of prostate cancer,

1) to determine if fish oil supplementation as compared to placebo would decrease fatty acid synthase expression, both absolute expression and pre- to post-intervention change in expression, in all tissue types sampled, and
2) to determine if fish oil supplementation as compared to placebo would decrease Ki-67, a marker of cell proliferation, in all tissue types sampled.

The secondary aims include measuring the expression of SREBP-1, a transcription factor for fatty acid synthase, cell death (apoptotic fraction using TUNEL), red blood cell (RBC) fatty acid concentration and change in PSA. Subjects were men from the Portland VA Medical Center (PVAMC), Oregon Health & Science University (OHSU) and Kaiser Permanente Northwest (KPNW) urology clinics who were scheduled for a repeat biopsy. These men had an initial negative biopsy yet are still considered at high risk. All eligible and consenting subjects were randomized to receive three months of either fish oil capsules and placebo capsules (treatment 1), green tea capsules and placebo capsules (treatment 2), fish oil capsules and green tea capsules (treatment 3) or two placebo capsules (treatment 4). As stated above, the DOD funded comparison of fish oil to placebo will be reported herein. Potential confounding variables were assessed through completion of a comprehensive diet history questionnaire and risk factor questionnaire, assessment of pre and post-treatment PSA and surveillance of medication and supplement use. Compliance was assessed using pill count and evaluation of RBC fatty acid concentrations. While this study population is limited to men at high risk of disease, the results may be more broadly generalizable to any man.
considered at risk of prostate cancer due to standard clinical indicators such as a PSA >4μg/ml.

**BODY**

**Specific Aims:** As noted above the primary aims to be addressed in this double-blind randomized controlled trial include evaluating the effect of fish oil supplementation on fatty acid synthase expression and on cell proliferation (Ki67) in post intervention prostate biopsy samples.

**Studies and Results:** During this final budget period, we have completed our recruitment efforts and have conducted immunohistochemical analyses of FAS and Ki67. Recruitment totals are shown below using the CONSORT Statement Randomized Controlled Trial flow diagram, modified to reflect the factorial design of this trial [4].

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**Table 1. Baseline Values by Randomization Group**

<table>
<thead>
<tr>
<th></th>
<th>Fish oil</th>
<th>Placebo</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at Diagnosis (mean SD)</td>
<td>63.3 (4.97)</td>
<td>61.5 (6.32)</td>
</tr>
<tr>
<td>Body Mass Index (mean SD)</td>
<td>28.6 (4.85)</td>
<td>28.5 (4.25)</td>
</tr>
<tr>
<td>Pre-Intervention PSA (mean SD)</td>
<td>6.5 (4.18)</td>
<td>6.79 (4.04)</td>
</tr>
<tr>
<td>Post-Intervention PSA (mean SD)</td>
<td>5.95 (4.16)</td>
<td>6.90 (5.83)</td>
</tr>
<tr>
<td>Post- Pre difference in PSA (Mean SD)</td>
<td>-0.28 (3.91)</td>
<td>0.05 (3.42)</td>
</tr>
</tbody>
</table>
total of 89 men were randomized to receive supplement or placebo and 86 men successfully completed the trial. All analyses were completed following an intention to treat approach. Of these men, compliance was good with 74% of men taking 80% of prescribed study supplements and 85% taking 70% of supplements. Mean number of days on trial was 101 (3 months, 10 days) with a maximum of 172 (5.7 months). All randomized subjects received allocated treatment and none experienced adverse events of grade 3 or higher as determined by CTCAE version 3. Table 2 shows the number and percent of individuals reporting expected adverse events by treatment arm. As shown, the majority of reports were of grade 1 (mild) adverse event. Adverse events noted as “Other” were reported changes to health status; none were determined as related to the treatment.

Table 2. Adverse Event Reports

<table>
<thead>
<tr>
<th>Adverse Event</th>
<th>Placebo (n=31)</th>
<th>Fish Oil (n=29)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. Reporting Grade 2 or higher</td>
<td>Total No. (%) of subjects reporting (Grade 1 &amp; 2)</td>
</tr>
<tr>
<td>Bloating</td>
<td>1</td>
<td>3 (9.7)</td>
</tr>
<tr>
<td>Gas/Flatulence</td>
<td>0</td>
<td>7 (22.6)</td>
</tr>
<tr>
<td>Burping</td>
<td>1</td>
<td>1 (3.2)</td>
</tr>
<tr>
<td>Diarrhea/loose stool</td>
<td>0</td>
<td>5 (16.1)</td>
</tr>
<tr>
<td>Nausea/vomiting</td>
<td>1</td>
<td>3 (9.7)</td>
</tr>
<tr>
<td>Bruising</td>
<td>0</td>
<td>5 (16.1)</td>
</tr>
<tr>
<td>Headache</td>
<td>1</td>
<td>5 (16.1)</td>
</tr>
<tr>
<td>Upset Stomach</td>
<td>1</td>
<td>3 (9.7)</td>
</tr>
<tr>
<td>Heartburn</td>
<td>1</td>
<td>3 (9.7)</td>
</tr>
<tr>
<td>Abdominal Pain</td>
<td>0</td>
<td>1 (3.2)</td>
</tr>
<tr>
<td>Muscle Pain</td>
<td>0</td>
<td>1 (3.2)</td>
</tr>
<tr>
<td>Other*</td>
<td>1</td>
<td>14 (45.2)</td>
</tr>
</tbody>
</table>

*Other changes to health - all designated as unrelated

Pre and post-intervention biopsy specimens (paraffin embedded and frozen research only biopsy cores) have been obtained from the first OHSU, PVAMC and KPNW subjects. Immunohistochemical analyses have been conducted at OHSU for fatty acid

Figure 2. Fatty acids concentrations in frozen prostate biopsy cores (n=2) by GC-MS
synthase, and cell proliferation (Ki-67) on all pre and post-specimens. Numerous efforts were made to complete immunohistochemical analyses for sterol-regulatory element binding protein; however no antibody was adequately specific in paraffin-embedded tissue to allow for quantitation of changes in expression. As reported in 2009-2010, Dr. Donald Jump, our colleague at Oregon State University developed procedures to extract and quantify fatty acids in the frozen biopsy cores (shown in figure 2). However, this is a labor intensive procedure and Dr. Jump is unable to conduct analyses for all tissue samples. Hence we have developed a collaboration with Dr. Arun Sreekumar, at Baylor College of Medicine, who has kindly offered to conduct metabolomic analyses of all frozen tissue samples. These analyses will measure a panel of polar, mid polar and non-polar compounds that will include amino acids, nucleotides, fatty acids, sugars and their derivatives as well as a select group of lipids. The methods for the metabolomic analyses are provided in Appendix A. These analyses will provide one of the first analyses of fatty acid and lipid concentrations in prostate tissue following supplementation and will allow us to indirectly estimate the effect of fish oil supplementation on FAS activity.

**Immunohistochemical (IHC) analyses:** Fatty acid synthase and Ki67 staining was completed in all pre and post-intervention biopsy specimens. All pre-intervention samples contained only benign tissue, as expected based on inclusion criteria. Tumor tissue samples from post-intervention biopsy were available on only 3% of post-intervention subjects (2 of 60); therefore analyses of malignant tissue were not completed as the sample size was inadequate to identify possible treatment differences.

The IHC protocol for FAS was adapted from published studies [5, 6]. We used published IHC procedures for Ki67 in paraffin-embedded tissue. Briefly, five micron sections of paraffin-embedded tissue from each available biopsy core were prepared on Fisher Plus slides, air dried at room temperature over an air vent until all visible water was evaporated and further dried at 60°C for 15 minutes. Care was taken that specimens were mounted in the same order on pre and post-intervention slides, such that we had the capacity to compare similar locations on the prostate. Slides were then deparaffinized in xylene (3 x 3 minutes), then rehydrated with graded alcohols and washed for 10 minutes in Tris-buffered saline (TBS), pH 7.2-7.6.

The slides evaluated for Ki-67 antigen were boiled in a microwave oven for 10 minutes in a 0.01 M. citrate buffer, pH 6.0. Slides were then treated with a 3% aqueous solution of hydrogen peroxide for 5 minutes. Blocking of nonspecific binding was done with a 1-hour incubation in 3% goat serum. Slides were then incubated for one hour at room temperature with the appropriate antibody, FAS (Transduction Laboratories, Lexington KY, dilution 1/20), Ki-67 (Zy-Med) followed by mouse Envision (Dako, Glostrup, Denmark). The slides were counterstained with Gill’s hematoxylin for 1 minute then rinsed in tap water until clear. They were then blued in TBS for 10 seconds and rinsed in tap water again. The slides were dehydrated with graded alcohols and xylene, and then coverslipped using Permount.

Stained slides were evaluated by the collaborating pathologist, Dr. George Thomas, director of the OHSU Knight Cancer Institute’s Histopathology Shared Resource, on a Leica DM LS microscope with 5, 10, 20, and 40x objectives. Dr. Thomas did not have knowledge of any clinical parameters associated with each case. Positive and negative control slides were examined to ensure technical adequacy of staining. Breast cancer
cells with known high FAS expression (SKBR-3) served as positive controls for FAS and similarly prepared slides of each subject’s prostate tissue with substitution of normal mouse serum for the primary antibody served as negative controls. Benign prostate glands stained for FAS were assessed for the following: percent of glands stained (0-25% (1), 26-50% (2), 51-75% (3), >75% (4)), average staining intensity (none (0), weak (1), moderate (2), strong (3)) and range of staining intensity. A summary score was calculated as percent glands stained * average staining intensity. For Ki67, percent staining 0-100% was recorded.

**Immunohistochemistry for FAS and Ki67:** Figure 3a and 3b show the mean (SD) of FAS and Ki67 staining by treatment arm. Using intent to treat analyses, there was a significant increase in the FAS staining summary score in subjects receiving placebo (mean increase 1.88, p=0.03) and virtually no change among subjects receiving fish oil (mean increase of 0.31, p=0.60). There was a non-significant difference in the mean pre to post change in FAS summary score between fish oil and placebo (difference in mean change = 1.57 (p=0.12). Mean percent of cells stained for Ki67 did not change significantly from pre to post intervention in either of the treatment arms and there was not a significant difference in the mean pre-post change between treatment arms (difference in mean change= 3.6% (p=0.45)

**PSA Results:** Prostate specific antigen (PSA) was measured in all men at pre-intervention and post-intervention, and pre to post-change in PSA was calculated. There was no significant difference in PSA at post-intervention or change in PSA in either treatment group. However, there was a small mean decrease in PSA among men receiving fish oil supplementation as compared to a small mean increase in PSA among men receiving placebo (Table 1).

**Human Subjects Review:** Oversight for our protocol was transferred to USAMRMC HRPO on 1 September 2006; all minor modifications were reported to HRPO at the time of Continuing Review for all three sites. We utilize the HRPO Continuing Review Checklist annually and write corresponding explanation memos for these submissions to HRPO. Summary of local human subjects review follows:

For log number A-12538.a (PVAMC), the DOD HRPO received PVAMC's last Continuing Review (PVAMC approval: 14 September 2011) on 2 November 2011. For
log number A-12538.b (OHSU), the DOD HRPO received OHSU’s continuing review submission (OHSU approval: 4 January 2011) on 11 March 2011. For log number A-12538.c (KPNW), the DOD HRPO received KPNW’S continuing review submission (KPNW approval: 21 April 2011) 13 May 2011. The trial underwent a final Data Safety Monitoring Committee Audit by the OHSU Knight Cancer Institute on 7 September 2011. The final report from this audit is to be generated by the OHSU Knight Cancer Institute’s Data Safety Monitoring Committee after their meeting on 18 November 2011.

**KEY RESEARCH ACCOMPLISHMENTS:**

Our key accomplishments over this project include:

1. Complete recruitment of 89 men into a novel trial to evaluate a dietary supplement in men at high risk for prostate cancer. This novel approach of recruiting men at high risk based on previous biopsy into a cancer prevention study allowed us to address the impact of fish oil in a population most likely to benefit from positive results.
2. Obtained dietary and other risk factor data from all participating subjects.
3. Completed immunohistochemical analyses of fatty acid synthase and Ki67 expression in pre and post-intervention prostate biopsy tissue.
4. Obtain blood specimens to allow for analyses of red cell fatty acids at pre and post-intervention; analyses in progress.
5. Obtained flash frozen prostate biopsy core for analyses of fatty acid concentration in the prostate tissue; analyses to be completed and funded by NCCAM grant.

**REPORTABLE OUTCOMES:**

Work from this project was presented at the American Society for Clinical Oncology under ‘Trials in Progress’ from 4-6 June 2010 in Chicago, Illinois. In addition, initial findings were presented at the Innovative Minds in Prostate Cancer Today (IMPaCT) meeting in Orlando, Florida 9-12 March 2011.

We have also developed and obtained Portland VA IRB approval for a biorepository and linked data repository for all specimens collected under this protocol. This repository will support future analyses that may be conducted to further elucidate the role of multiple dietary factors in prostate cancer prevention.

This project spurred the submission and successful funding of a VA Merit Review application to investigate the interaction between dietary fatty acids and variations in genetic profiles in families and individuals with a high familial risk for prostate cancer.

**PERSONNEL SUPPORTED BY THIS AWARD:**

Ms. Lisa Blyele and Ms. Karyn Foster; laboratory technicians with Dr. Dennis Koop, director of the OHSU BioAnalytical Shared Resource and PharmacoKinetics Core Laboratory. Dr. Jutta Deininger and Ms. Cara Poage; laboratory technicians with Drs. Christopher Corless and George Thomas, past and current directors of the OHSU Knight Histopathology Shared Resource. Ms. Paige Farris, Mr. Wesley Stoller, Ms. Amy Palma, Ms. Gretchen Luhr, Ms. Alysia Cox and Ms. Courtney Maxcy; all current and previous research coordinators who worked with this study's subjects under Dr. Shannon. Drs. Mark Garzotto, Motomi Mori and Jackilen Shannon; current key
personnel. Dr. Garzotto served as responsible clinician at the Portland VA Medical Center and also provided the study with eligible men to recruit from his biopsy clinics. Dr. Mori has served as the biostatistician from the time of grant proposal, through additions in funding and changes to statistical method and randomization and now into data analysis. Finally, Dr. Shannon has provided leadership and full study oversight with three different recruitment sites, remained on top of the most current findings with fish oil supplementation and prostate cancer throughout the life of the study in addition to responding to regular requests from the DOD program officer, DOD’s annual report and submission of continuing review to the DOD HRPO annual report.

**CONCLUSIONS:**

Though quickly falling out of favor, PSA screening remains very common as a method to identify early prostate cancer in men over the age of 50 years. It is well known that up to 75% of men with elevated PSA who go under prostate biopsy will be determined to not have cancer. However, a portion of these men will have questionable biopsy findings or continue to present with increasing PSA and hence warrant a second or follow-up biopsy. These men, considered at higher risk of having prostate cancer are often left waiting for 3 to 6 months before this follow up biopsy with little or no options for how they may reduce their risk for prostate cancer development or progression. The goal of the current trial was to identify whether a common dietary supplement, fish oil, altered fatty acid synthase expression in prostate tissue. Fatty acid synthase (FAS), a necessary enzyme in the de novo production of free fatty acids has been shown to be elevated early in the cancer process and to continue to increase with cancer progression. Now considered an oncogene, there is in vitro evidence supporting the potential benefit of inhibiting FAS expression to slow the development or progression of prostate cancer. In our randomized, double-blind clinical trial, we found that among men undergoing repeat biopsy, FAS expression increased significantly in individuals taking placebo, while there was very minimal increase in expression among individuals receiving the fish oil supplement. However, the pre to post-intervention change in FAS expression was not significantly different between men receiving fish oil versus those receiving placebo (difference between fish oil and placebo mean change =1.57, p=0.12). One potential mechanism whereby FAS may inhibit prostate cancer growth is through inhibition of cell proliferation. We evaluated this possible mechanism by determining expression of Ki67, a marker of cell proliferation, in tissue of men receiving placebo or fish oil. As expected in non-malignant tissue, cell proliferation was relatively low and did not change significantly from pre to post-intervention in either study group, suggesting that should the alteration of FAS expression slow prostate cancer development or progression it may not function by altering cell proliferation in the non-malignant tissue.

In the coming months, we will continue to elucidate the impact of fish oil supplementation on prostate cancer tissue as we conduct metabolomics analyses of post-intervention prostate biopsy tissue. This analysis will allow us to determine the extent to which low-dose supplementation with long-chain omega-3 fatty acids, in the form of fish oil, changes the concentration of these fatty acids in the prostate and potentially not just the expression, but the activity of fatty acid synthase. Although this work does not address the stated specific aims of the current study, it is a natural progression of the DOD funded work. Metabolomics analyses will be funded by Dr. Shannon’s institutional funds.
REFERENCES:

APPENDIX A.

Metabolomic Assessment: To determine the metabolic fingerprint associated with overexpression of FAS as well as to determine biochemical changes that may result from treatment with omega-3 fatty acids and EGCG, we will collaborate with Dr. Arun Sreekumar to conduct a metabolic assessment of prostate tissue from men participating in a clinical trial of these compounds. 4

Sample Preparation. Prostate tissues will be frozen until analysis. At the start of the profiling the tissues will be weighed, spiked with equimolar amounts of recovery standards and homogenized. Following this metabolite extraction will be performed using sequential organic and aqueous solvents. The resulting organic and aqueous extracts will be independently filtered, dried, resuspended in identical volumes of the injection solvent and subjected to liquid chromatography (LC) mass spectrometry analysis. Various quality control samples include defined mixture of standards as well as aliquots of characterized liver will be included at multiple times in the profiling process in a randomized scheme to monitor the variation in the profiling process.

Liquid Chromatography/Mass Spectroscopy (LC/MS). The LC/MS portion of the platform is based on a 1200 SL Rapid resolution LC and either a 6520 Q-TOF mass spectrometer (Quadrupole Time Of Flight) or a 6510 QQQ mass spectrometer (Triple Quadrupole). In each of these the samples will be independently analyzed in both positive and negative ionization modes. All the unbiased profiling experiments will be performed using a Q-TOF mass spectrometer equipped with Dual electrospray ionization (ESI) source. For Positive mode ionization \( m/z \) 121.050873, 922.009798 will be used as reference ions were used for online mass correction while the corresponding ions for the negative mode ionization will be \( m/z \) 119.03632, 966.009725. The typical conditions used for operating the Q-TOF will be capillary voltage, 4000 V (negative mode 3500 V) and source temperature 325°C, drying gas 10 l/min, nebulizer 45 psig (reference ion nebulizer 10 psig), fragmentor voltage 140, and skimmer 65 V. Ultra high pure nitrogen will be used as both the nebulizer and the collision gas. For the collision induced dissociation (CID) experiments, the precursor ion will be selected using the quadrupole analyzer and the corresponding product ions will be examined by TOF analyzer. The collision energies for fragmentation will be set at 10-40 eV unless otherwise stated. All the spectra will be recorded under identical experimental conditions for all the prostate samples. The LC Solvents used for the entire study will be purchased from Burdick & Jackson (NJ, USA).

The LC coupled QQQ Mass Spectrometry will be employed for targeted compound assessment using multiple reaction monitor (MRM). The typical operating conditions for this mass spectrometer will be capillary voltage, 3000 V and source temperature 350°C, drying gas 10 l/min, nebulizer 35 psig, fragmentor voltage 70. The collision energies used for fragmenting the parent ions will be set between 10-40 eV. Some compounds are redundantly visualized across more than one of these data-streams, however, not only is the sensitivity and linearity vastly different from interface to interface but these redundancies, in some instances, will actually be used as part of the quality control program. The chromatography in all cases is highly standardized and never allowed to vary. Internal standards as well as well characterized matrix samples (liver extract) will be employed to ensure injection and chromatographic consistency.

Metabolomic Libraries. These will be used to search the mass spectral data. The library was created using approximately 1000 commercially available compounds that
were examined by both reverse phase positive and negative ionization and cataloged into the Metlin library (Agilent). This library will be employed to identify the compounds detected using unbiased mass spectrometry. Compound naming will rely on both mass and retention time information for each compound which would then be matched with the entries listed in the library. Furthermore, compounds that cannot be named by this conventional database search will be subjected to targeted MS/MS analysis to delineate their chemical structure as a prelude to their identification.