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Effects of ω-3 Fatty Acids and Catechins on Fatty Acid Synthase in the Prostate: a Randomized Controlled Trial

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Abstract

Animal and human studies suggest fish oil and green tea may have protective effect on prostate cancer. Fatty acid synthase (FAS) has been hypothesized to be linked to chemoprotective effects of both compounds. This study evaluated the independent and joint effects of fish oil (FO) and green tea supplement (epigallocatechin-3-gallate, EGCG) on FAS and Ki-67 levels in prostate tissue. Through a double-blinded, randomized controlled trial with 2x2 factorial design, 89 men scheduled for repeat prostate biopsy following an initial negative prostate biopsy were randomized into either FO alone (1.9g DHA+EPA/day), EGCG alone (600 mg/day), a combination of FO and EGCG, or placebo. We used linear mixed effects models to test the differences of prostate tissue FAS and Ki-67 by immunohistochemistry between pre- and post-intervention within each group, as well as between treatment groups. Results did not show significant difference among treatment groups in pre-to-post-intervention changes of FAS (p=0.69) or Ki-67 (p=0.26). Comparing placebo group with any of the treatment groups, we did not find significant difference in FAS or Ki-67 changes (all p>0.05). Results indicate FO or EGCG supplementation for a short duration may not be sufficient to produce biologically meaningful changes in FAS or Ki-67 levels in prostate tissue.

Keywords

Catechins; ω-3 fatty acids; fatty acid synthase; Ki-67; prostate

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INTRODUCTION

Prostate cancer is the most common non-skin cancer among men in the U.S. Men at high risk for prostate cancer may also have increased awareness of prostate cancer and a strong desire to identify methods to reduce the likelihood of ultimately developing this disease. Unfortunately, within traditional Western medicine, there is little to offer these men. Thus, many men may seek out complementary and alternative medicines (CAM) or a change in their lifestyles in an attempt to modify their risk of prostate cancer.

A number of nutritional and CAM supplements, including fish oil and green tea, have been hypothesized to reduce prostate cancer risk. The major components in fish oil are docosahexaenoic acid (DHA) and eicosapentanoic acid (EPA) - part of the omega-3 polyunsaturated fatty acids (ω-3 PUFA) family. The most common ω-3 PUFA in the U.S. diet is short-chain α-linolenic acid (ALA) (1) which can be metabolized by Δ-6 desaturase and elongase to long-chain EPA or DHA. ALA is commonly found in plants while EPA and DHA are commonly found in fatty fish and fish oil (2). As compared to ALA, in human prostate cancer cell lines, EPA and DHA have greater anti-cancer effects through inhibition of cell proliferation and induction of apoptosis (3). Tea is the second most commonly consumed beverage in the world (4) and around 20% of tea production is green tea (5). Green tea contains an abundant bioactive component known as catechins which have been demonstrated to induce cell-cycle arrest and apoptosis in multiple prostate cancer cell lines (6–9). Catechins include (−)-epigallocatechin-3-gallate (EGCG), (−)-epicatechin (EC), (−)-epigallocatechin (EGC) and (−)-epicatechin-3-gallate (ECG) with EGCG accounting for about two-thirds of catechins (5).

Human studies on the association between ω-3 PUFA or green tea and prostate cancer are inconsistent. Meta-analyses (10–16) and prospective studies (17–20) investigating the association between ω-3 PUFA and prostate cancer generated mixed results with inverse, null or positive associations reported. For the association between green tea and prostate cancer, a meta-analysis pooling thirteen cohort or case-control studies showed green tea's protective effect on prostate cancer was more apparent among Asian populations (21); yet, a more recent meta-analysis including twenty-one cohort or case-control studies reported no association (22). Despite these mixed results from population-based studies, evidence from animal and in vitro work suggest a plausible common biologic mechanism whereby both ω-3 PUFA and EGCG have been demonstrated to decrease prostate cancer risk through altering regulation and activity of fatty acid synthase (FAS) (3, 23–25).

FAS is a lipogenic multienzyme that catalyzes the final step in de novo fatty acid synthesis (26). In normal cells, FAS is expressed at low levels and its transcription is regulated primarily by the sterol regulatory element binding protein-1 (SREBP-1) in response to variations in nutritional status (27); the primary purpose for the synthesis of fatty acids would be to provide an energy source via β-oxidation. In tumor cells, SREBP-1 gene transcription is reported to be constitutively upregulated by growth factors (GF) and GF receptors (GFR) and/or steroid hormones (SHs) and SH receptors (SHRs) signaling, resulting in the overexpression of FAS and excess production of free fatty acids (26). The

rate of fatty acid formation in tumor cells is clearly greater than the cellular needs for energy, suggesting there may be alternative reasons for fatty acid synthesis. Furuta et al. found that FAS gene was upregulated by hypoxia in tumor cells, and both FAS and SREBP-1 expression were localized in hypoxic regions of tumor tissue (28). Due to the highly anaerobic cancer cell environment there may be excess production of acetyl-CoA from lactate and pyruvate, resulting in lactic acidosis. Overexpression of FAS in tumor cells may be in response to the acidic and hypoxic microenvironment of solid tumors. Alternatively, Hochachka proposes that the significance of enhanced FAS expression and fatty acid synthesis is to provide oxidizing power and thus improve the redox balance found in the malignant prostate cell (29). Thus, the FAS overexpression or dysregulation of the FAS appear to play an important role in allowing for continued prostate tumor cell growth.

It has been demonstrated that ω-3 PUFA downregulates FAS mRNA expression (3) and that EGCG inhibits transcribed FAS activity in prostate cancer cells (24). The primary objective of this double-blind, randomized, placebo-controlled clinical trial, is to elucidate, in men at high risk for prostate cancer, a potential biologic mechanism whereby EGCG and ω-3 PUFA, alone or in combination , may alter the expression and activity of FAS and hence reduce cell proliferation, thereby reducing overall risk of prostate cancer. Since there might be molecular abnormalities in histologically normal appearing prostate tissue due to field effect (30, 31), we chose benign tissue to investigate if early dysregulation of normal cells, with the biomarkers FAS and Ki-67 as the primary endpoints, could be modified by natural compounds. FAS is a molecular marker known to be dysregulated early in carcinogenesis (32–34), our study will be the first study to assess whether nutritional intervention could modulate its expression.

METHODS

Participants

This study's participants were recruited from the urology clinics at the Portland VA Medical Center (PVAMC), Oregon Health and Science University's (OHSU) and Kaiser Permanente Northwest (KPNW) and were scheduled for a repeat biopsy subsequent to an earlier negative, but suspicious, biopsy of the prostate. These men were scheduled for initial biopsy based on elevated PSA (> 4μg/dl), abnormal digital rectal exam (DRE), or suspicious findings by transrectal ultrasound (TRUS). We included men ≥ 21 years of age who signed informed consent. Exclusion criteria included: 1) definitive invasive prostate cancer on initial biopsy; 2) significant active medical illness that in the opinion of the clinician would preclude protocol treatment; 3) history of ventricular tachycardia or ventricular fibrillation; 4) subject reported use of fish oil (greater than 1 gram per day) or green tea supplement within 30 days before day 1 of study treatment or subject reported use ≤ 1 gram per day of fish oil and unwilling to discontinue use for the duration of the study; 5) use of warfarin or need for therapeutic anticoagulation at time of biopsy or at any time during the course of the trial; 6) subject reported allergy or sensitivity to fish oil, olive oil or green tea; 7) subject reported history of hemophilia, van Willebrands disease or other bleeding disorder, except when the subject is evaluated by a hematologist who determines that fish oil supplementation is not contraindicated; 8) total bilirubin greater than institutional upper

limit of normal; and 9) PVAMC subjects participating in another greater than minimal risk study. Eligible men met with study coordinators prior to the initiation of any research task to review the study's purpose and exclusion criteria. The original study protocol was approved by the Institutional Review Boards (IRB) of all participating institutions. At study initiation, the inclusion criteria were as follows: 1) initial negative prostate biopsy result, 2) scheduled repeat biopsy due to a continued elevated PSA, 3) are positive for high grade prostatic intraepithelial neoplasia (PIN) and/or suspicious findings by TRUS or DRE, 4) hemoglobin > 10 g/dL (within 4 weeks), 5) creatinine ≤ 1.5 mg/dL, and 6) Adequate Eastern Cooperative Oncology Group (ECOG) performance status < 2. After 28 subjects were accrued, the Knight Cancer Institute Data & Safety Monitoring Committee conducted an internal audit and found that inclusion criteria were not consistently documented. This was reported to the IRB and the protocol was modified to having only 1 criterion, "clinician recommends repeat biopsy of the prostate". Of the original 28 patients accrued, 25 met the revised criteria and were included in this analysis; 3 patients recruited prior to the change were deemed ineligible and not included in our final analyses.

Study Design

The study sample size flowchart is depicted in Figure 1 following CONsolidated Standards Of Reporting Trials (CONSORT) guidelines (35). The original fish oil and FAS expression study initiated in 2005 was modified in 2006 to add green tea plus fish oil and green tea plus placebo arm. Overall, consented subjects (N =89) were randomized to one of the four groups in a 2 x 2 factorial design: (1) fish oil alone (FO), (2) green tea alone (EGCG), (3) combined fish oil and green tea (EGCG+FO), or (4) placebo group (no fish oil or green tea). Randomization was stratified by age (<65, ≥65 years) and site (VA, OHSU/KPNW). Within each stratum a permuted block randomization with a random block size was used. Study supplements were provided to the study coordinator from the institutional research pharmacies and delivered to the participants. The study coordinator remained blinded to the patient's study status throughout the intervention. Subjects were enrolled and initiated supplementation at approximately 90 days prior to their scheduled follow up biopsy, ensuring the last day of supplementation would correspond to the date of re-biopsy.

The green tea capsules and matching placebos were donated by Sabinsa Corporation® (Piscataway, NJ). Each green tea capsule was 300 mg and the capsules were considered decaffeinated with less than 2% caffeine per 1000 mg capsule. The composition of the green tea capsules include Epigallocatechin (2.62%), Epicatechin (6.31%), Epigallocatechin gallate (EGCG, 52.67%), Epicatechin gallate (9.02%), catechins (1.32%), gallocatechin gallate (4.23%) and multiple other ingredients. Total identified catechins by HPLC is 76.17%. Total polyphenols by UV method is 82.00%. The placebo for green tea treatment consisted of dicalcium phosphate with a food grade coloring substance. The placebo gelatin capsule matches the active product (green tea) in form and color. Green tea was supplemented at 1 capsule two times a day, providing subjects 600 mg EGCG daily, which is the equivalent of approximately 4–6 regular 8-oz cups of green tea's EGCG value (5).

The fish oil capsules and the placebos were provided by Perfect Source® Natural Products (Fullerton, CA) with fish oil active ingredients manufactured by DSM® Nutritional Products

Inc. (Parsippany, NJ). The fish oil supplement is characterized as refined ethyl esters of fish oil; predominantly as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Each fish oil capsule contained a total of around 0.6 grams of ethyl esters of EPA and DHA. The placebo for fish oil treatment consisted of pure extra virgin olive oil, a significant source of oleic acid, for which there had been no described fatty acid synthase effect. The olive oil placebo gelatin capsule matches the active product (fish oil) in form and color. Fish oil was supplemented at 1 pill 3 times per day; this provided subjects 1.9 g EPA+DHA/daily with an EPA/DHA ratio of 1:1.

We stored all the supplements at room temperature (68 – 77° F) in a locked, monitored research pharmacy facility. The quality of the supplements was checked regularly and the study subjects were regularly instructed by the study research coordinator to comply with the intervention protocols.

Research coordinators completed the informed consent, a modified National Cancer Institute (NCI) diet history questionnaire (36), a risk factor questionnaire assessing other potential risk factors for prostate cancer, and an adverse events questionnaire based on known side effects to both supplements. A 10ml blood sample was collected at baseline and prior to the follow-up biopsy visit. Adverse events questionnaires that included common adverse events for EGCG and fish oil were completed at approximately 30 and 60 days during the trial and 30-day follow-up after the trial. For any reported adverse event grade 3 or higher, according to the NCI Common Terminology Criteria for Adverse Events Version 3.0, the responsible clinician was notified; the event was triaged and followed to resolution, depending upon the severity of event. At mid-study, study exit and 30 days post-intervention, subjects were asked about any changes to medications, supplement use or dietary intake over the past one-month. At study exit, blood and flash-frozen prostate biopsy samples were collected. Participant's capsule containers were returned to the Research Pharmacies and remaining capsules were counted and recorded. Subjects who took ≥80% of the prescribed pills were considered treatment-compliant. If repeat biopsy was delayed for reasons unrelated to study treatment, treatments could extend up to a total of 20 weeks. The last dose of treatment would be given no earlier than 12 hours prior to repeat biopsy.

Biopsy Tissue Specimens Collection and Processing

Prostate biopsies were conducted per standard clinical practice. Tissue was collected, paraffin-embedded and stored as part of the standard prostate biopsy procedure. At the examining physician's discretion, 6 to 24 biopsy cores were obtained at the initial examination and at the time of the post-intervention examination. Institutional-standard biopsy templates were followed for the biopsy procedure.

All clinical biopsy specimens were immediately placed into 10% neutral buffered formalin. The anatomic location from which each core biopsy was extracted was also recorded by the urology nurse. The specimens were embedded in paraffin at the institution's histology laboratory. Routine hematoxylin and eosin staining were carried out and the presence of prostate cancer was determined by a pathologist. Reviewed specimens were stored and available for immunohistochemical (IHC) studies. For pre-intervention prostate biopsy tissue, we selected one benign core from each subject for IHC studies; for post-intervention

prostate repeat biopsy tissue, we selected one benign core of each subject and one cancer/PIN core if the subject had cancer/PIN. However, only benign cores were used for pre-to-post-intervention comparison.

Immunohistochemical Staining for FAS and Ki-67

The IHC protocol for FAS was adapted from published studies (37, 38). We used published IHC procedures for Ki-67 in paraffin-embedded tissue. Five micron sections of paraffin-embedded tissue from each research core were prepared on Fisher Plus slides and air dried at room temperature over an air vent. Care was taken to orient pre- and post-intervention slides, such that similar locations in the prostate can be compared. Slides were deparaffinized in xylenes, rehydrated with graded alcohols and washed in Tris-buffered saline (TBS). The slides for Ki-67 antigen expression were boiled in a microwave with 0.01 M citrate buffer. Slides were then treated with 3% aqueous solution of hydrogen peroxide and incubated with 3% goat serum for one hour to block nonspecific binding. Slides were incubated at room temperature for one hour with appropriate antibodies FAS (Transduction Laboratories, Lexington KY, dilution 1/20) and Ki-67 (Zy-Med) followed by mouse Envision (Dako, Glostrup, Denmark). Slides were then counterstained with Gill's hematoxylin and blued in TBS. The slides were finally dehydrated with graded alcohols and xylene, and then coverslipped using Permount.

Stained slides were evaluated by a pathologist on a Leica DMLS microscope. 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, pre-neoplastic (prostatic intraepithelial neoplasia) and cancer tissue were each assessed by our collaborating pathologist and percentage of positive cells was recorded for FAS, average staining intensity [none (0), weak (1), moderate (2), strong (3)] was also recorded for FAS. We used a modified Histo-score (H-score) ranging from 0–300 multiplying percentage of positive cells and intensity for FAS analyses (39); For Ki-67, we used number of positive cells per high-power field (200x, which is 10x objective eyepiece x 20x magnification) for analyses.

Plasma EGCG Measurement

We adapted the method of Masukawa et al (40) using liquid chromatography electrospray tandem mass spectrometry (LC-MS/MS) for plasma EGCG measurement. The internal standard, epicatechin (1 ng), was used. The extracts were analyzed on an ABSciex 4000 QTRAP hybrid/triple quadrupole linear ion trap mass spectrometer (Foster City, CA).) with electrospray ionization (ESI) in negative mode. The mass spectrometer was interfaced to a Shimadzu (Columbia, MD) SIL-20AC XR auto-sampler followed by 2 LC-20AD XR LC pumps. Data were acquired and analyzed using Analyst 1.6.2 software.

Plasma Fatty Acids Measurement

The fatty acids in plasma were analyzed by a modification of the methods described by Langerstedt et al (41). Deuterated fatty acids were added to samples prior to extraction as

internal standards. After hydrolysis and extraction, fatty acids were derivatized to the pentafluorobenzyl (PFB)-esters and analyzed by gas chromatography-mass spectroscopy (GC-MS) on a Trace DSQ (Thermoelectron) operating in the negative ion chemical ionization mode with methane as the reagent gas. Each fatty acid was matched to the deuterated internal standard closest in length and retention time. Peak area ratios of known amounts of standard fatty acids and the internal standards were used to generate calibration curves to quantify unknowns using Xcalibur software.

Statistical Methods

All statistical analyses were performed using SAS 9.4 (SAS Institute Inc., Cary, NC, USA). The intent-to-treat analysis was performed for the primary outcomes and included all randomized participants. Baseline characteristics were expressed as means and standard errors (SEs) for continuous variables, and counts (n) and percentages (%) for categorical variables, stratified by treatment group. The comparability of the four treatment groups for baseline characteristics were tested using one-way analysis of variance (ANOVA) for continuous variables and Chi-square tests for categorical variables.

The key primary outcomes are FAS and Ki-67. Our primary interest was to determine whether changes from pre- to post-intervention were significantly different between the four treatment groups. The analysis was conducted separately for FAS and Ki-67. Shapiro-Wilk Normality tests were conducted for all continuous variables.

Both FAS and Ki-67 biomarkers were log2 transformed in order to obtain approximate normality. To assess treatment group differences in the changes in primary outcome biomarkers, linear mixed effects models were conducted separately for each outcome to calculate least-squares means (LSMEANS) and 95% confidence intervals (95% CI), and to test the statistical significance of the difference between pre- and post-intervention within each group, as well as to compare the magnitude of differences among four groups. These comparisons were performed using appropriate statistical contrasts (e.g., green tea vs. placebo, green tea/fish oil vs. placebo, fish oil vs. placebo). We used the false discovery rate (FDR) method to adjust for multiple comparisons of the primary endpoints (42).

Adverse events and compliance between the treatment groups were analyzed using Fisher-Freeman-Halton tests as appropriate. Subjects who took ≥80% of the prescribed pills were considered treatment-compliant. The proportions of the compliant subjects were compared between FO, EGCG, EGCG+FO groups and placebo groups using a chi-square test. To further determine the compliance using biological samples, we also used mixed-effect models to determine whether changes of plasma EGCG from pre- to post-intervention were significant across the four treatment groups. Comparisons of plasma fatty acid levels in FO and EGCG+FO group before and after treatment were determined using pared t-test. Tests of statistical significance were conducted using two-sided tests, and a p-value ≤ 0.05 was considered statistically significant unless otherwise noted.

The sample size and power analyses were initially performed for five primary endpoints and one secondary endpoint using NCSS PASS (43), assuming two-sided 5% overall significance level. It was expected that 144 men would participate in the study with 36

subjects in each treatment arm. Overall, the initially projected sample size of 144 subjects would provide an adequate power to detect a clinically meaningful difference in laboratory-based efficacy parameters for key comparisons. However, we were unable to meet recruitment goals.

RESULTS

Patient Characteristics and Adverse Events

From January 2005 to September 2011, a total of 89 participants aged 50–78 years (63 ± 6.3 years) were randomized into FO group (n=29), EGCG group (n=15), FO+EGCG group (n=14) or placebo group (n=31 with n=28 included in the final analyses). Table 1 describes the baseline characteristics of the 86 subjects by treatment group. There was no statistically significant difference in baseline characteristics including age, BMI, PSA, race, marital status, income, education, smoking, alcohol, family history of prostate cancer or NSAIDs use. Repeat prostate biopsy, post-intervention diagnoses are as follows; 54 (62.8%) were diagnosed as benign, 13 (15.1%) were diagnosed with PIN and 15 (17.4%) subjects had malignant disease. There was no treatment group difference among post-intervention diagnoses (p=0.41).

There were no treatment group differences noted for each specific type of adverse event and total number of adverse events (Table 2). No subjects experienced grade ≥ 3 adverse events. One subject in the FO group and one subject in the placebo group withdrew from the study (Figure 1). In addition, no statistically significant difference was observed in terms of compliance to the treatment plan between the four treatment groups (p = 0.86).

Immunohistochemistry Biomarkers

Among the 86 men included in the analysis, 84 (97.7%) had prostate biopsy tissue for IHC analysis. Table 3 shows the log2-transformed LSMEANS of FAS and Ki-67 by treatment groups, the p-values comparing pre- and post-intervention biomarker values within each treatment group, and most importantly, the p-values comparing pre-to-post changes of biomarkers between treatment groups. We also show these results in Figure 2 for FAS and Figure 3 for Ki-67. A significant decrease of Ki-67 was present within EGCG group (p=0.02) and a significant increase of FAS was present within placebo group (p=0.03). After multiple comparisons by utilizing the FDR, the significance disappeared (both p-value=0.12). There was no overall statistical significance among the four treatment groups for pre-to-post changes of both FAS (p=0.69) and Ki-67 (p=0.26).

Table 4 demonstrates the pre-to-post treatment LSMEANS difference for each of the intervention treatment compared to placebo groups. For the three groups who received supplementation, there was no significant difference in FAS and Ki-67 level changes (all p>0.05).

Plasma EGCG and Fatty Acids Levels

Among subjects in green tea group, 11 out of 15 (73%) had EGCG level increased; among subjects in FOGT group, 12 out of 15 (80%) had increased EGCG level; among subjects in

fish oil group, 2 out of 29 subjects (6.9%) had increased EGCG level; and in placebo group, none of the subjects (0%) had increased EGCG level. The pre-to-post-intervention changes of plasma EGCG level (least square mean ± standard error) in EGCG (30.71 ± 14.19 ng/mL) and EGCG+FO (48.96 ± 14.19 ng/mL) group were much higher than FO (0.68 ± 11.06 ng/mL) and placebo group (−1.68 ± 11.32 ng/mL) (overall p=0.02). Comparing pre- and post-intervention fatty acids levels within FO and EGCG+FO groups using paired t-test (mean increase ± standard deviation) , the plasma DHA significantly increased in FO (120.9 ± 101.3, p<0.0001) and EGCG+FO group (180.7 ± 113.8, p<0.0001); similarly, EPA also significantly increased in FO (118.0 ± 107.4, p<0.0001) and EGCG+FO group (196.9 ± 102.6, p=0001). All these results indicate the subjects had taken the supplements following the intervention protocols.

DISCUSSION

In this randomized, placebo-controlled trial, we found no significant effect of fish oil or green tea supplement in decreasing prostate tissue FAS or Ki-67 levels among men scheduled for prostate biopsy.

One possible reason that we did not observe an effect may be that the supplementation intervention period was too short or the supplementation doses were too low. In our study, the mean treatment intervention period was 14.4 weeks. Compared to an Italian study which showed a significant chemoprevention effect of green tea catechins (GTC) on prostate cancer (44), our study's intervention doses (EGCG 600mg) were similar to the GTC study (600 mg); but our intervention period (12–20 weeks) was much short than the GTC study (1 year). Compared to a clinical trial showing a 4–6 week low-fat diet with fish oil supplementation (1g EPA+ 1.8g DHA) intervention could significantly decrease prostate tissue Ki-67 level (45), our fish oil doses (1.9g EPA+DHA) were lower. The shorter duration or lower doses may restrict our ability to discern an effect of supplementation on FAS or Ki-67 expression, especially if the variation in FAS or Ki-67 protein expression caused by fish oil or green tea supplementation is a cumulative effect.

Additionally, not all of the participants were prostate cancer cases. For instance, the green tea group had a lower proportion of malignant cases (6.7%) and the fish oil group had a higher proportion of malignant cases (20.7%). FAS expression is consistently greater in cancer tissue vs. normal tissue (46) and overexpression of FAS is one of the earliest and most common events in prostate cancer development (47). In addition, nuclear localization of FAS captured by IHC analysis correlates with prostate cancer Gleason grade (48). Therefore, the average levels of FAS are lower in our study population as compared to what may have been seen should all study subjects have had confirmed prostate cancer. In a double blinded clinical trial with subjects all diagnosed with high-grade PIN (HGPIN), only 1 out of 30 subjects in the GTC (600 mg/day for 12 months) group vs. 9 out of 30 subjects in the placebo group developed prostate cancer (44). Given this trial included only men diagnosed with HGPIN (a similar disease stage), a future trial should consider including only patients with confirmed HGPIN or prostate cancer diagnosis.

The protective effect of inhibiting FAS level in preventing prostate cancer growth might occur via the inhibition of Ki-67, a marker of cell proliferation. Since most of our study participants were benign cases, cell proliferation Ki-67 level measured from our study participants' prostate biopsy tissue was relatively low and did not change significantly from pre-to-post-intervention in the entire intervention group after multiple adjustment comparison. Our result on green tea effect is consistent with a clinical trial of ninety-three men who completed the intervention of 6 cups/day brewed green and black tea prior to prostatectomy, which showed no significant effect of tea consumption on Ki-67 level in prostatectomy tissues (49). However, our non-significant finding of fish oil supplementation is not consistent with a clinical trial of low-fat diet with fish oil supplementation among men undergoing radical prostatectomy, which showed a significant decreased tumor tissue Ki-67 level (45) in the intervention group. This suggests that should the alteration of FAS expression slow prostate cancer development or progression it may not function by altering cell proliferation in non-malignant tissue.

Two recent clinical trials evaluating green tea extract and prostate cancer incidence showed promising effect (44, 50). In the Italian study among men with HGPIN mentioned earlier, a statistically significant protective effect of GTC against prostate cancer development was observed after a 1-year intervention (44); after 2-year follow-up in a subset of the original participants, a lasting protective effect against prostate cancer was also found (51). A U.S.-conducted clinical trial detected a statistically significant difference in the cumulative rate of prostate cancer plus atypical small acinar proliferation (ASAP) between a GTC group (3/26) and placebo group (10/25) among men with HGPIN without ASAP (50). In our study, we observed non-statistically significant lower rate of prostate cancer in EGCG group (1/14) compared to placebo group (6/28), the direction of the observation is consistent with the other two trials.

Our study has several strengths. First, to our knowledge, our study is the first to assess in human tissue the joint effect of EGCG and FO on the FAS pathway. Second, we have acquired almost all of the prostate biopsy tissue samples from our study participants (97%); our withdrawn subjects were the only men from whom we did not acquire prostate tissue. Therefore, our FAS and Ki-67 expression IHC analyses were nearly complete. Third, through the 2×2 randomized blinded trial design, we were able to examine two types of supplements together to best utilize the study resources. Despite these strengths, we have identified a few study limitations. First, the sample size is limited, however, we did not observe a trend of effects that are in line with our hypothesis. If we had achieved our recruitment goal, we still likely may not have observed a significant effect given the current intervention dosage and period. Second, our hospital-based design among men scheduled for repeat prostate biopsy may restrict the generalizability of our results. However, our study results are applicable to men with high risk for prostate cancer identified from clinical examination. As we know, up to 75% of men with elevated PSA who undergo prostate biopsy will have no diagnosed cancer. A portion of these men will have suspicious biopsy findings, abnormal digital rectal exams or continue to present with increasing PSA and hence warrant a repeat biopsy. These men, considered at higher risk of having prostate cancer are often left waiting for 4–6 months before a follow up biopsy with little or no option given about how they may reduce their risk for prostate cancer development or

progression. More research targeted to this population will contribute toward the gap of literature.

In conclusion, short duration of fish oil or green tea supplements could not reduce FAS or Ki-67 substantially. Future trials with longer treatment durations among a more homogenous study population should be considered.

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Abbreviations

ANOVA	analysis of variance
ALA	α-linolenic acid
ASAP	atypical small acinar proliferation
BMI	body mass index
CAM	alternative medicine
CONSORT	CONsolidated Standards Of Reporting Trials
DHA	docosahexaenoic acid
DRE	digital rectal exam
EC	(−)-epicatechin
ECG	(−)-epicatechin-3-gallate
ECOG	Eastern Cooperative Oncology Group

EGC	(−)-epigallocatechin
EGCG	epigallocatechin-3-gallate
EPA	eicosapentanoic acid
FAS	fatty acid synthase
FDR	false discovery rate
FO	fish oil
GF	growth factors
GFR	growth factor receptors
GTC	green tea catechins
HGPIN	high-grade prostatic intraepithelial neoplasia
IHC	immunohistochemistry
KPNW	Kaiser Permanente Northwest
NCI	National Cancer Institute
LSMEANS	least-squares means
NSAIDs	nonsteroidal anti-inflammatory drug
OHSU	Oregon Health & Science University
PSA	prostate specific antigen
SE	standard error
SHs	steroid hormones
SHRs	steroid hormone receptors
PIN	prostatic intraepithelial neoplasia
ω-3 PUFA	omega-3 polyunsaturated acids
PVAMC	Portland VA Medical Center
SREBP-1	sterol regulatory element binding protein-1
TBS	Tris-buffered saline
TRUS	transrectal ultrasound;

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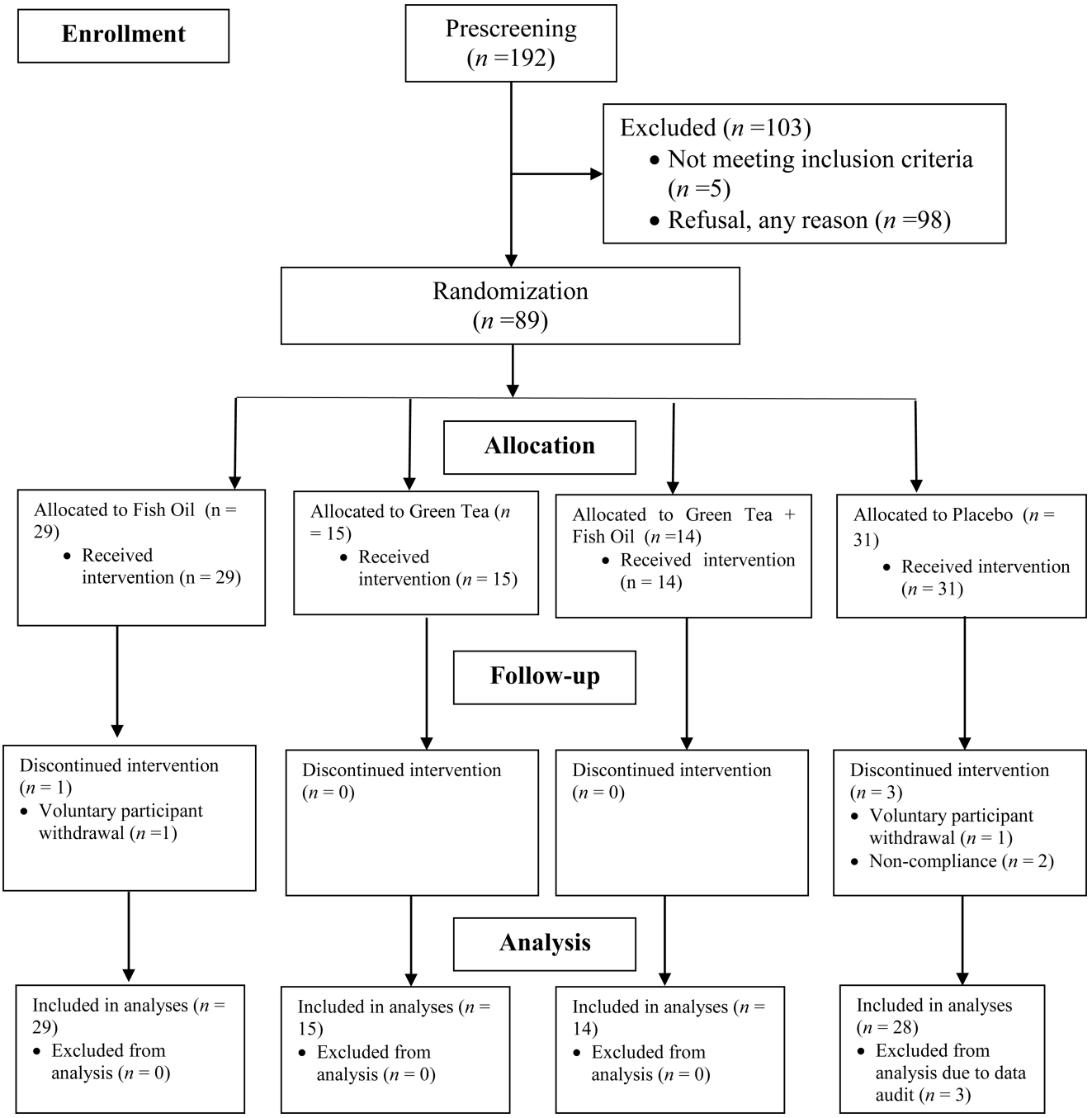

Figure 1.
Randomized controlled trial sample size flow chart

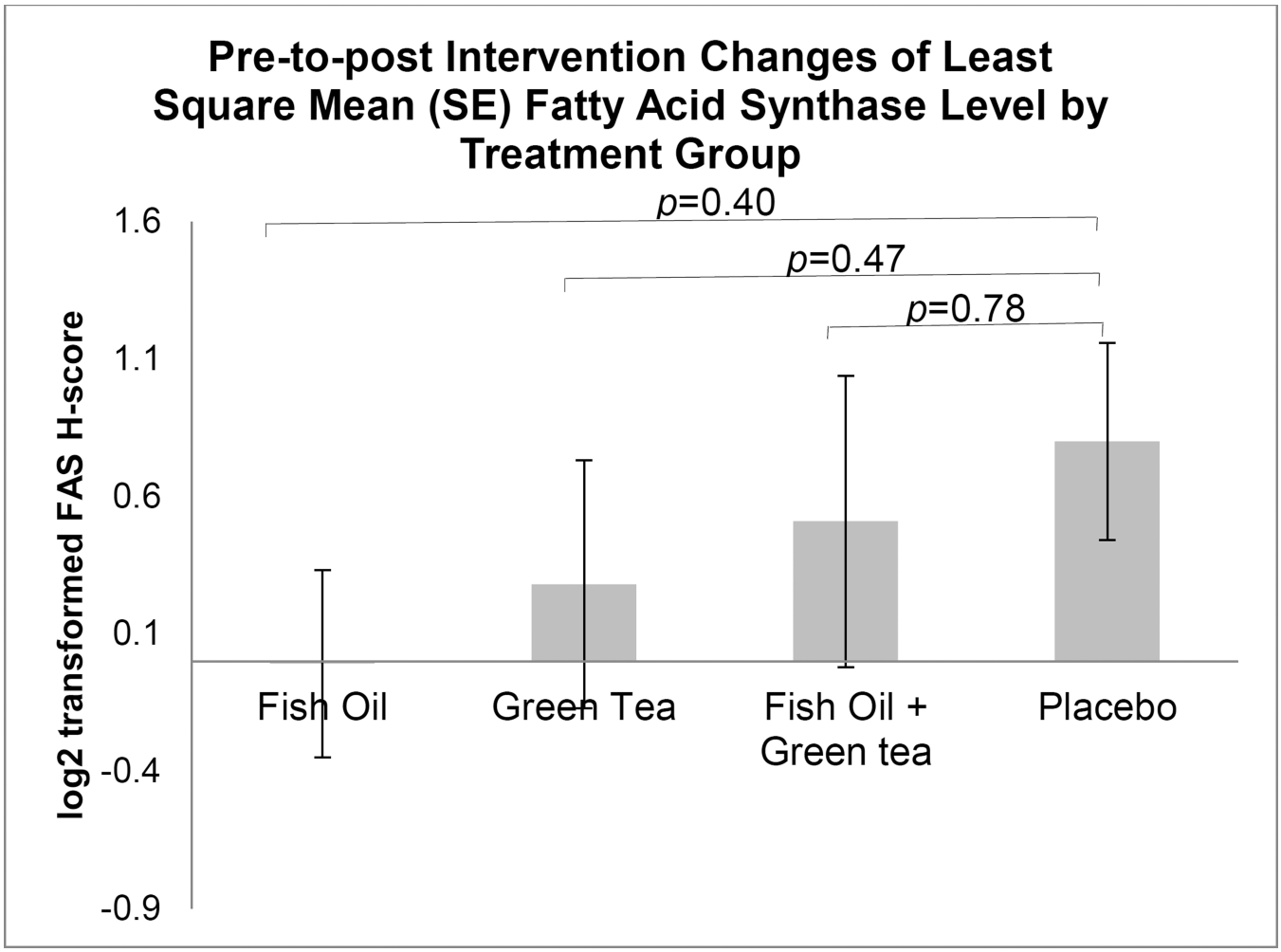

Figure 2.

Comparison of prostate benign tissue fatty acid synthase (FAS) immunohistochemistry H-score level changes between treatment groups. Changes in FAS level from pre- to post-intervention between treatment groups were compared using mixed effect model. Values shown indicate least-squares means of (LSMEANS ± SE) pre-to-post change of FAS level by treatment group.

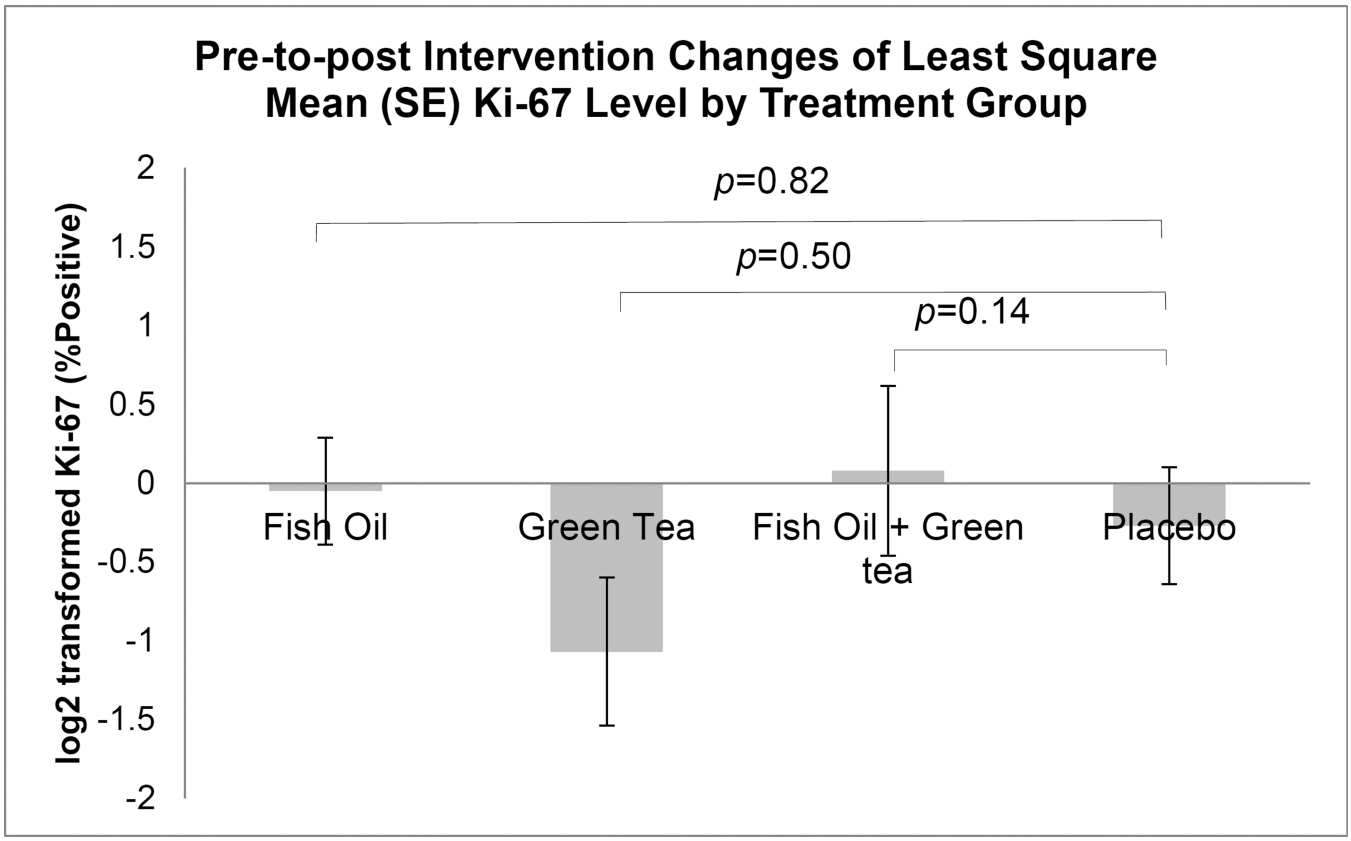

Figure 3.

Comparison of prostate benign tissue Ki-67 immunohistochemistry number of positive cells/high-power field (200x, which is 10x objective eyepiece x 20x magnification) level changes between treatment groups. Changes in Ki-67 level from pre- to post-intervention between treatment groups were compared using mixed effect model. Values shown indicate least-squares means of (LSMEANS ± SE) pre-to-post change of Ki-67 level by treatment group.

Table 1

Basic characteristics of men at baseline by randomization group

Participant Characteristics	**Fish Oil (*n* = 29)**	**Green Tea (*n* = 15)**	**Fish Oil + Green Tea (*n*=14)**	**Placebo (*n* = 28)**	***p*[1, 2, 3]**
	Mean (SD)	**Mean (SD)**	**Mean (SD)**	**Mean (SD)**	
Age,yr	63.3 (5.0)	64.5 (7.2)	62.6 (7.5)	61.8 (6.4)	0.57
BMI (kg/m2) at baseline	28.5 (5.0)	30.1 (3.7)	29.0 (4.0)	28.1 (4.1)	0.54
Prostate Specific Antigen (PSA) Pre-intervention	6.5 (4.2)	6.9 (2.6)	4.8 (2.5)	6.9 (4.2)	0.34
Prostate Specific Antigen (PSA) Post-intervention	6.0 (4.2)	6.4 (3.3)	10.8 (18.3)	7.1 (6.1)	0.38
FAS H-score Pre-intervention	138.3 (63.8)	158.8 (84.6)	142.7 (102.8)	114.2 (85.4)	0.22
FAS H-score Post- intervention	145.2 (72.2)	159.3 (75.5)	140.7 (100.3)	157.3 (81.5)	0.79
Ki-67 number of positive cells per high power field Pre-intervention	13.5 (10.3)	18.3 (15.9)	17.9 (12.1)	15.7 (13.6)	0.68
Ki-67 number of positive cells per high power field Post- intervention	11.8 (7.9)	7.9 (6.6)	21.8 (17.2)	13.1 (9.6)	0.05
	n (%)	*n* (%)	*n* (%)	*n* (%)	
Race					0.45
White	28 (96.6)	13 (86.7)	14 (100.0)	27 (96.4)	
Non-White	1 (3.4)	2 (13.3)	0 (0.00)	1 (3.6)	
Marital status					0.34
Single, Divorced, Widowed	3 (10.3)	3 (20.0)	0 (0.0)	4 (10.7)	
Married/partner	26 (89.7)	11 (73.3)	14 (100.0)	21 (75.0)	
Missing	0 (0.0)	1 (6.7)	0 (0.0)	3 (14.3)	
Income					0.74
≤$50,000	5 (17.3)	3 (20.0)	1 (7.1)	2 (7.1)	
>$50,000	13 (44.8)	5 (33.3)	4 (28.6)	11 (39.3)	
Refuse/Don’t know/missing	11 (37.9)	7 (46.7)	9 (64.3)	15 (53.6)	
Education					0.70
≤ Some college/technical	6 (20.7)	3 (20.0)	3 (21.4)	3 (10.7)	
≥ College graduate	8 (27.6)	6 (40.0)	5 (35.7)	11 (39.3)	
Missing	15 (51.7)	6 40.0)	6 (42.9)	14 (50.0)	

Participant Characteristics	**Fish Oil (*n* = 29)**	**Green Tea (*n* = 15)**	**Fish Oil + Green Tea (*n*=14)**	**Placebo (*n* = 28)**	***p*[1, 2, 3]**
	Mean (SD)	**Mean (SD)**	**Mean (SD)**	**Mean (SD)**	
Smoking					0.10
Current	5 (17.2)	0 (0.0)	2 (14.3)	6 (21.4)	
Former	14 (48.3)	11 (73.3)	7 (50.0)	7 (25.0)	
Never	9 (31.0)	2 (13.3)	3 (21.4)	10 (35.7)	
Missing	1 (3.5)	2 (13.3)	2 (14.3)	5 (17.9)	
Alcohol					0.13
Current	14 (48.3)	5 (33.3)	3 (21.4)	17 (60.7)	
Former	7 (24.1)	6 (40.0)	8 (57.1)	6 (21.4)	
Never	7 (24.1)	3 (20.0)	3 (21.4)	2 (7.1)	
Missing	1 (3.5)	1 (6.7)	0 (7.1)	3 (10.7)	
Family history of Cancer					0.52
Yes	9 (31.0)	2 (13.3)	5 (35.7)	7 (25.0)	
No	20 (69.0)	13 (86.7)	9 (64.3)	21 (75.0)	
Use of NSAIDs					0.76
Yes	17 (58.6)	6 (40.0)	7 (50.0)	14 (50.0)	
No	10 (34.5)	7 (46.7)	6 (42.9)	9 (32.1)	
Missing	2 (6.9)	2 (13.3)	1 (7.1)	5 (17.9)	
Repeat Pathology Biopsy Diagnosis[4]					0.41
Benign	16 (55.2)	11 (73.3)	9 (64.3)	18 (64.3)	
Malignant	6 (20.7)	1 (6.7)	2 (14.3)	6 (21.4)	
Prostatic Intraepithelial Neoplasia (PIN)	6 (20.7)	3 (20.0)	3 (21.4)	1 (3.6)	
Missing	1 (3.4)	0 (0.0)	0 (0.0)	3 (10.7)	

[1] One-way analysis of variance (ANOVA) tests were conducted for continuous variables (age, BMI, PSA); Kruskal–Wallis test was used for comparing variables without normal distribution (FAS H-score and Ki-67 % positivity), chi-square tests were conducted for categorical variables with expected cell frequencies ≥ 5 (Use of NSAIDs); and Fisher-Freeman-Halton tests were conducted for categorical variables with expected cell frequencies < 5.

[2] *p*-value ANOVA or chi-square tests between supplement and placebo groups, $^{*}p < 0.05$. All the p-values were calculated excluding missing category.

[3] Percentages may not add up to 100 due to rounding values.

[4] All the baseline biopsies were diagnosed as benign (n=61, 71%) or PIN (n=25, 29%).

Table 2

Incidence of reported grade 2 adverse events in the fish oil green tea trial (treatment-related). Data represent the number of adverse events (%)

Adverse Events (AE)	Fish Oil (*n* = 29)	Green Tea (*n* = 15)	Fish Oil + Green tea (*n*=14)	Placebo (*n* = 28)
	Number (%)	Number (%)	Number (%)	Number (%)
Bloating	0 (0.0)	0 (0.0)	0 (0.0)	1 (3.6)
Burping	0 (0.0)	1 (6.7)	0 (0.0)	1 (3.6)
Diarrhea	2 (6.9)	0 (0.0)	0 (0.0)	0 (0.0)
Nausea/vomiting	1 (3.4)	1 (6.7)	0 (0.0)	0 (0.0)
Bruising	1 (3.4)	1 (6.7)	0 (0.0)	2 (7.1)
Headache	2 (6.9)	0 (0.0)	0 (0.0)	1 (3.6)
Upset Stomach	3 (10.3)	0 (0.0)	0 (0.0)	3 (10.7)
Heartburn	1 (3.4)	0 (0.0)	0 (0.0)	1 (3.6)
Abdominal Pain	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Muscle Pain	0 (0.0)	1 (6.7)	1 (7.1)	1 (3.6)
Other[1]	0 (0.0)	0 (0.0)	1 (7.1)	1 (3.6)
All[2, 3]	7 (24.1)	2 (13.3)	2 (14.3)	5 (17.9)

[1] Other changes to health included back/neck pain and mental health issue.

[2] Count of subjects who experienced at least one of the grade 2 adverse events.

[3] *p*-value for all adverse events comparing the four groups through Fisher-Freeman-Halton test is 0.53.

Table 3

Log2-transformed least-squares means (LSMEANS) of immunohistochemistry H-score of FAS and Ki-67 positivity in men scheduled for prostate biopsy

	Fish Oil		Green Tea		Fish Oil + Green tea		Placebo		
	Pre-to-Post Change LSMEANS (SE)	***p* comparing pre- and post-treatment values**	**Pre-to-Post Change LSMEANS (SE)**	***p* comparing pre- and post-treatment values**	**Pre-to-Post Change LSMEANS (SE)**	***p* comparing pre- and post-treatment values**	**Pre-to-Post Change LSMEANS (SE)**	***p* comparing pre- and post-treatment values**	***p* comparing treatment groups[1, 2]**
FAS	−0.009 (0.34)	0.98	0.28 (0.45)	0.54	0.51 (0.53)	0.35	0.80 (0.36)	**0.03**	0.69
Ki-67	−0.05 (0.34)	0.89	−1.07 (0.47)	**0.02**	0.08 (0.54)	0.89	−0.27 (0.37)	0.47	0.26

[1]Mixed effect model adjusting treatment and time

[2]Each subject has maximum of 2 observations.

Table 4

Pairwise comparisons of pre-post differences of log2-transformed least-squares means (LSMEANS) of immunohistochemistry H-score of FAS and Ki-67 positivity in men scheduled for prostate biopsy

	Pairwise Comparison	**Pre-to-Post Difference Estimate (SE)**	***p***
FAS	Fish oil vs. Placebo	0.27 (0.32)	0.40
	Green tea vs. Placebo	0.27 (0.37)	0.47
	Fish oil + Green tea vs. Placebo	−0.12 (0.41)	0.78
Ki-67	Fish oil vs. Placebo	−0.06 (0.26)	0.82
	Green tea vs. Placebo	−0.21 (0.31)	0.50
	Fish oil + Green tea vs. Placebo	0.51 (0.34)	0.14