OMEGA-3 FATTY ACIDS, INFLAMMATION, AND OUTCOME IN MEN WITH AND WITHOUT PROSTATE CANCER

By

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CERTIFICATE OF APPROVAL

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ABSTRACT

Background: Research suggests that inflammation may play a role in the development and progression of prostate cancer. Interleukin-6 (IL-6) and C-reactive protein (CRP) have been linked to prostate cancer progression and inflammatory cytokines have been correlated with an increased risk of prostate carcinogenesis. Omega-3 (n-3) fatty acids are known for their anti-inflammatory properties and have been linked to decreases in prostate cancer risk. Research indicates that levels of IL-6 and CRP decrease with higher n-3 fatty acid intakes. This study investigated the relationship between inflammation and outcome in men with and without prostate cancer as well as the modification of that relationship by n-3 fatty acids. *Methods:* This study was a secondary analysis of the data from a case-control study of diet and prostate cancer risk. IL-6 and CRP were measured in plasma of prostate cancer cases (n = 121) and biopsy negative controls (n = 240) collected at recruitment. Erythrocyte n-3 fatty acids (including eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA)) were analyzed using gas chromatography-mass spectrometry. Prostate tissue inflammation was determined by immunohistochemistry (IHC). New incident cancer and cancer outcome was assessed using electronic patient medical records. *Results:* ALA was significantly higher in the subjects with low-grade cancer compared to biopsy negative controls, and subjects with high-grade cancer had significantly higher levels of IL-6 compared to the controls. Circulating levels of plasma IL-6 and CRP were associated with inflammation in the prostate in the biopsy negative controls. Controls were at the highest risk of having prostate tissue inflammation with IL-6 levels in the middle tertile (OR: 2.61, 95% CI: 1.37 – 4.97). IL-6 was significantly correlated with CRP in the biopsy negative controls

and cancer cases (r = 0.471, 0.252, respectively, p <0.001 and 0.005, respectively). CRP was inversely correlated with DHA (r = -0.177, p < 0.001) and IL-6 was inversely correlated with EPA (r = -0.104, p = 0.48) in the cohort. When stratified by cancer status (biopsy negative controls and cancer cases), significant correlations between IL-6, CRP, and n-3 fatty acids were only observed in the biopsy negative controls. There was no significant association between IL-6, CRP, ALA, DHA, and EPA and the risk of prostate cancer. When the inflammatory markers and n-3 fatty acids were examined together, there was a significant increase in risk of prostate cancer in subjects with CRP levels in the middle category and lower ALA or higher DHA levels. Higher levels of EPA were associated with a significantly higher risk of developing prostate cancer, independently and with rising CRP levels. *Conclusions:* These results conflict with previous research showing a protective effect of DHA and EPA on prostate cancer risk, and an increased risk of cancer with higher levels of ALA. However, the subject population for this study had relatively low n-3 fatty acid levels compared to other cohorts. Research that is conducted in populations with adequate intakes of DHA and EPA or in conjunction with supplementation may provide a more accurate picture of their potential protective effects.

INTRODUCTION

Prostate cancer is the most common non-cutaneous form of cancer in men (1). In 2008, there was an estimated 186,320 new cases of prostate cancer in the United States (U.S.) (2). One in six men will be diagnosed with prostate cancer in their lifetime. After lung cancer, prostate cancer is the second leading cause of cancer death in men with over 28,000 deaths each year. The incidence of prostate cancer varies across the globe. For example, the U.S. has a higher rate of prostate cancer compared to Asian countries such as Japan. Furthermore, when immigrants move from one part of the world to another, their risk of developing prostate cancer changes as well. These shifting trends in the rate of prostate cancer suggest that modifiable risk factors may play a role in prostate carcinogenesis (3, 4). Potentially modifiable risk factors that continue to be under investigation include inflammation and lifestyle choices, such as diet (5, 6).

Before 1990, when the prostate-specific antigen (PSA) test was first introduced, most cases of prostate cancer were diagnosed after a patient presented with symptoms. This resulted in a high proportion of locally advanced or metastatic disease at the time of diagnosis. With the development of more effective screening and diagnostic tools, such as the PSA test and transrectal ultrasound (TRUS), a larger proportion of asymptomatic men are being diagnosed with localized tumors (7). According to the National Cancer Institute, 91% of newly diagnosed cancer cases are confined to the localized or regional tissue (e.g. regional lymph nodes). Only 4% of newly diagnosed cases have already metastasized beyond the prostate (distant stage). The five-year disease-specific survival

rate is dramatically different between these two groups. At diagnosis, the five-year survival rate is 100% for those with localized or regional stage and only 31.7% for those men with distant stage (8). Furthermore, men with intermediate- or high-risk tumors (defined as clinical T2b or T2c tumors, PSA > 10 ug/L, or biopsy Gleason score of 7 or greater) will likely experience tumor progression or metastatic disease within five years of diagnosis (7). There is no consensus on the best treatment for men with metastatic disease. Androgen deprivation therapy (ADT) is commonly prescribed to men with recurrent or advanced prostate cancer. While ADT is initially successful, tumors eventually switch from an androgen-dependent to an androgen-independent phenotype, rendering ADT ineffective. Once this transition occurs, there are few treatment options remaining and patient care often becomes palliative (7).

Given the prevalence rate of prostate cancer as well as the poor prognosis and limited treatment options for men with advanced disease, research focused on the prevention of prostate cancer and new therapies is clinically relevant. Prostate cancer prevention may be linked to modifiable risk factors such as inflammation. In men that have already been diagnosed with cancer, inflammation appears to play a role in the progression of prostate cancer. For these reasons, interfering with the inflammatory process may prove beneficial (9, 10).

Inflammation & Cancer

Inflammation is a complex component of the body's immune system. Tissue injury or infection triggers a cascade of chemical signals that initiates and maintains the inflammatory response. Inflammation can also be triggered in autoimmune disease when

the body mistakenly identifies its own cells as invaders. Leukocytes migrate to the injured area from the venous system. Neutrophils are the first to arrive during the acute response and monocytes are also attracted to the site by chemotactic factors. Upon arrival at the tissue, monocytes differentiate into macrophages, which secrete cytokines, such as interleukin-6 (IL-6), and growth factors. These secretions affect surrounding cells of the endothelium and epithelium as well as mesenchymal cells (9, 11, 12). Cytokines are also known to perpetuate the body's systemic response to inflammation through the production of acute-phase proteins. Acute-phase proteins, such as C-reactive protein (CRP), can be used clinically to evaluate the presence and intensity of systemic inflammation. Acute-phase proteins are beneficial at first but become harmful when the production of the proteins becomes chronic (13). Similar to the acute-phase protein response (APPR), the inflammatory response to injury serves a purpose but the development of chronic inflammation can be detrimental. Normal inflammation subsides once the injury or infection is resolved. However, in chronic inflammation, the inflammatory response persists because of the continued presence of an initiating factor and/or the inability of the immune system to end the inflammatory response. Tumor cells are abnormal cells that can act as an initiating factor for the inflammatory response. If the immune system is functioning properly, tumor cells are recognized as an injury and marked for phagocytosis (13). However, in patients with cancer, pro-inflammatory cytokine production may not be from host cells alone. Tumor cells are capable of directly producing cytokines that lead to inflammation (14). This dual stimulus of the inflammatory response leads to the migration of a diverse population of leukocytes including neutrophils, mast cells, lymphocytes and macrophages. The leukocytes

infiltrate the unorganized structure of the neoplasm. Unlike the structured environment of normal tissue, neoplastic tissue is a chaotic tangle of vascular structures due to tumor-induced angiogenesis and lymphangiogenesis. This type of microenvironment allows for intimate interactions between cytokines, acute-phase proteins, leukocytes and malignant cells (9). The consequences of these interactions have been studied in several types of cancer, including prostate cancer.

Inflammation & Prostate Carcinogenesis

Prostate carcinogenesis is influenced by genetic, biological, and environmental risk factors. Well-documented risk factors include race, age, and family history of prostate cancer. Risk factors that continue to be under investigation include inflammation and lifestyle choices, such as diet (5). Chronic inflammation is characterized by leukocyte infiltration of inflamed tissue and has been implicated in the development of common cancers including lung, gastric, and colon cancer. Prostatic inflammation may develop as a result from infection, hormonal changes, urine reflux, and/or physical trauma. The autoimmune recognition of self-prostatic proteins may also result in chronic prostatitis (12, 15). Studies that have examined the relationship between prostate cancer and inflammation have found a correlation between inflammatory cytokines, chemokines, growth factors and prostate carcinogenesis (5, 16, 17). Researchers have also found an association between chronic inflammation in the prostate and future diagnosis of adenocarcinoma. A five-year follow-up study, using needle biopsy specimens, found that 144 out of 177 subjects (81%) had chronic inflammation of the prostate. Of those 144 subjects, 20% were diagnosed with prostate cancer during the five-year follow-up period.

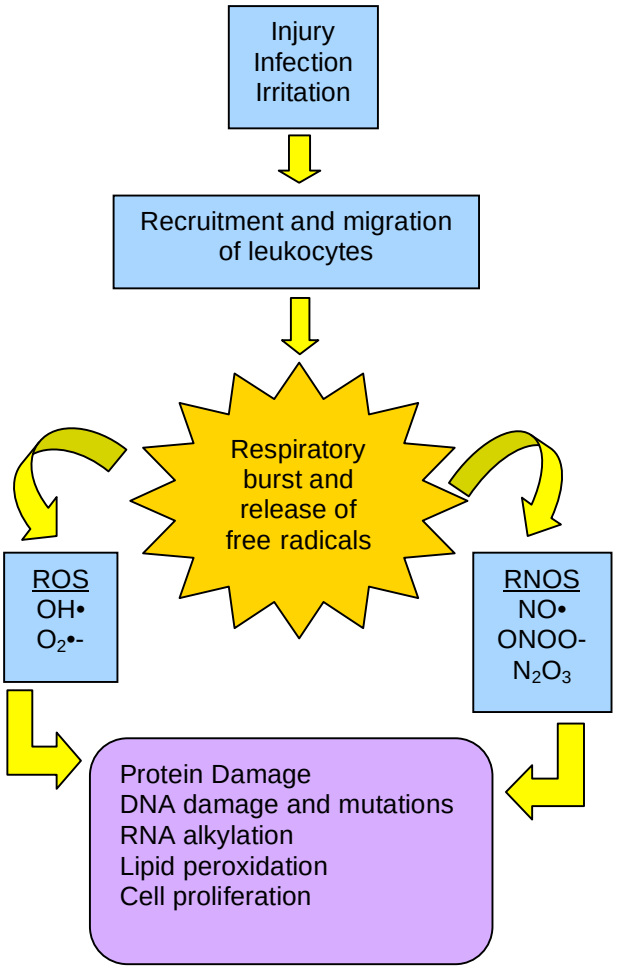

Figure 1. Impact of free radicals released at sites of inflammation on cellular molecules. Reactive oxygen species (ROS), reactive nitrogen oxide species (RNOS), hydroxyl radical (OH•), superoxide (O2–•), nitric oxide (NO•), peroxynitrite (ONOO–), nitrous anhydride (N2O3)

In the 33 subjects without chronic inflammation, only 6% developed prostate cancer (18). However, the mechanism that links chronic inflammation and carcinogenesis has not been elucidated.

Inflammation may lead to cancer development through a variety of pathways including cytokine-mediated cell survival and cell damage caused by oxidative stress. Cytokines are intimately involved with the inflammatory pathway. They are capable of both perpetuating the inflammatory response as well as inhibiting it. Cytokines may also be involved in the activation and deactivation of cancer genes, which may lead to cell differentiation, growth and the prevention of apoptosis. In addition, chemokines may allow for tumor progression and metastasis by causing the release of proteolytic enzymes (16).

Cytokines are a natural component of the inflammatory process and perform a vital function if properly regulated. This is also true for free radicals that are released from leukocytes during inflammation. Reactive oxygen species (ROS) and reactive nitrogen oxide species (RNOS) released during respiratory bursts can cause non-specific DNA damage resulting in mutations and protein modifications (Figure 1) (19). They are also capable of activating signal-transduction pathways. Such alterations in cellular structure and function can lead to abnormalities in apoptosis, cell cycle regulation, and genomic repair. Chronic oxidative insults can increase the likelihood of these abnormalities and tumor development (16, 18, 19). As cancer cells establish themselves in a pro-inflammatory environment, they can manipulate the leukocyte infiltrate to promote tumor growth and metastasis (16).

Inflammation & Prostate Cancer Progression

Inflammation appears to be more prevalent in those with advanced prostate cancer. McArdle et al. investigated the relationship between IL-6 and CRP in patients with benign disease and malignant prostate cancer. They found that circulating concentrations of IL-6 and CRP were not significantly different between those with benign disease (BPH) and those with prostate cancer. However, in the subjects with prostate cancer, there was a significant increase in total PSA ($p < 0.05$), IL-6 ($p < 0.01$) and CRP ($p < 0.01$) with increasing Gleason score. The Gleason score is used to grade prostate cancer and it increases with disease severity. This significant increase translated into a significant correlation between Gleason score and IL-6 ($r = 0.311$, $p = 0.004$) and CRP ($r = 0.304$, $p = 0.004$) (20). CRP has also been found to significantly correlate with PSA

concentrations in men with metastatic disease ($r_s = 0.46$, $p < 0.001$) (11). The association between higher tumor grade and markers of inflammation was echoed in a study that found preoperative IL-6 and its soluble receptor were significantly elevated in patients with a Gleason score of at least seven ($p = 0.042$ and 0.034, respectively) (21). The relationship between inflammation and advanced prostate cancer is important because it appears to affect patient outcome.

Cachexia is a common complication of advanced or recurrent cancer, and it is often accompanied by systemic inflammation, reduced quality of life, and death. In patients with recurrent prostate cancer, cachexia was found to be associated with higher IL-6 levels when compared to untreated patients or patients in remission. Severity of the cachexia also increased as serum IL-6 levels increased (22). Shariat et al. demonstrated another connection between IL-6 and poor outcome in men with prostate cancer. In 120 patients who underwent radical prostatectomy for clinically localized disease, they found that preoperative plasma IL-6 and its soluble receptor (IL-6sR) were strong predictors of biochemical progression after surgery. The median IL-6 and IL-6sR levels were reported to be 1.99 pg/mL and 25.4 ng/mL, respectively, and patients with preoperative levels above the median had a significantly increased probability of PSA progression ($p = 0.0093$, $p = 0.001$). The men in this study with disease progression were classified as having non-aggressive failure or aggressive failure. Non-aggressive failure was characterized by a PSA doubling time longer than 10 months and/or a complete response to local salvage radiotherapy. Men identified as having aggressive failure had a PSA doubling time less than 10 months, positive metastatic workup (positive bone scan), and/or a lack of response to local radiotherapy. Both IL-6 and IL-6sR were significantly

higher in the men with aggressive failure than those with non-aggressive failure (p = 0.042, p = 0.03, respectively) (21).

The interaction between inflammation and outcome in men with prostate cancer can also be examined from a histological perspective. Leukocyte infiltrate is an indicator of inflammation, and the relationship between tumor inflammatory infiltrate and patient outcome has been examined in different types of cancer including renal and colorectal cancer. For prostate cancer, McArdle et al. found that an increase in CD4+ T-lymphocyte infiltrate was associated with poor cancer-specific survival. This association was independent of tumor grade, and CD4+ T-lymphocyte infiltrate was an independent predictor of survival for subjects with both localized and locally advanced disease (HR: 2.88, 95% CI: 1.15 – 7.22, p = 0.024) (23). Similar results, found by Irani et al., indicated that high-grade inflammation in the malignant tissue of men having undergone radical prostatectomy was significantly associated with a higher biochemical recurrence probability at five years compared to patients with low-grade inflammation (RR: 2.35, 95% CI: 1.08 – 5.08, p = 0.03) (24). In addition to the research focused on IL-6 and prostate cancer, these findings indicate that inflammation negatively impacts outcome in prostate cancer patients. This conclusion is contrary to what many would assume. In most cases, the presence of inflammation would indicate that the immune system is aggressively acting on the cancer cells. However, in prostate cancer, this appears to be untrue. For this reason, researchers have started to focus on the connection between inflammation and prostate cancer with the hope that the research will lead to new therapies. IL-6 has become a main focus of this effort.

Since IL-6 increases with prostate tumor volume, it has been proposed that IL-6 is directly produced by prostate cancer cells (21). Hobisch et al. examined this question through IL-6 immunohistochemistry on 17 frozen prostate cancer specimens and IL-6 receptor immunostaining in 21 paraffin-embedded prostate tumor specimens. Both assays were also performed on adjacent areas of high-grade prostatic intraepithelial neoplasia (PIN) and benign tissue. IL-6 levels were also measured in the supernatants of the prostate cell cultures. Compared to benign glandular epithelium, the number of IL-6-positive cells was higher in cancer tissue. Gleason patterns correspond to tumor grade and can range from one to five. The Gleason score is the sum of the first and second most dominant Gleason pattern in a tumor, and it can range from two to ten with ten being the most aggressive. When stratified by Gleason pattern, more than half of the tumors with a Gleason pattern of three or greater had over 50% of the cells stain positive for IL-6. This staining pattern was the same for the PIN lesions. In contrast, the two tumors with a Gleason pattern of two did not show positive staining for more than 10% of the cells. Expression of the IL-6 receptor was found in basal and glandular cells in benign prostatic epithelium. IL-6 receptor was also expressed in every prostate tissue and PIN lesion sample, and over three quarters of the tumor samples with a Gleason pattern of three or more had over 50% of the cells express IL-6 receptors. This level of expression was not found in the tumor samples with a Gleason pattern of two. IL-6 levels measured in the supernatant from stromal and epithelial cell cultures confirmed that both stromal and epithelial prostatic cells secrete IL-6. Through the results of this immunohistochemical study, Hobisch et al. were able to conclude that there is morphological evidence that IL-6 autocrine and paracrine loops may exist in prostatic

tumors as well as PIN lesions. Furthermore, the researchers suggest that stromal IL-6 may play a role in prostatic cell growth and differentiation (25).

The evidence suggests that IL-6 contributes to a reduction of tumor cell apoptosis, promotion of tumor invasion, and tumor resistance to chemotherapy. These negative effects appear to be amplified as the length of time of IL-6 exposure increases (11). LNCaP cell lines are derived from prostate cancer lymph node metastases and are commonly used in prostate cancer cell studies. Long-term exposure to IL-6 gives rise to LNCaP-IL6+ cells. Unlike LNCaP cells, LNCaP-IL6+ cells secrete endogenous IL-6 and they are representative of cells found in advanced prostate cancer (26, 27).

Endogenous and exogenous IL-6 can exert its antiapoptotic effects through more than one cellular pathway including the phosphatidylinositol-3 kinase/Akt (PI3K/Akt), extracellular signal-regulated kinase mitogen-activated protein kinase (MAPK/Erk), and Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathways (Figure 2) (28). The PI3K/Akt and MAPK/Erk pathways regulate cyclin A1 and cells

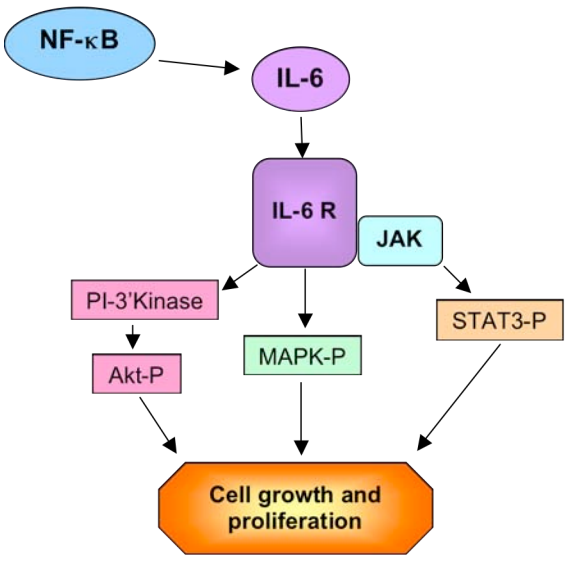

Figure 2. IL-6 signal transduction and influence on cell behavior.

that express cyclin A1 are more resistant to camptothecin-induced apoptosis. Wegiel et al. examined this pathway in LNCaP and LNCaP-IL6+ cell lines and tumor xenografts (27). Treatment with IL-6 induced the PI3K/Akt and MAPK/Erk pathway in both cell lines, which was evidenced by an increase in phosphorylated Akt and Erk. The activation of both pathways correlated with an increase in cyclin A1. The quantity of phosphorylated Erk produced by the LNCaP-IL6+ cells, after treatment with a low dose of IL-6, was similar to the amount of phosphorylated Erk produced by the LNCaP cells, after treatment with a high dose of IL-6. This led the researchers to conclude that activation of the MAPK/Erk pathway in LNCaP-IL6+ cells was caused by both endogenous and exogenous IL-6. In contrast, the level of phosphorylated Akt was higher in LNCaP cells compared to LNCaP-IL6+ after treatment with IL-6. The amount of phosphorylated Akt and Erk were dose-dependent in both cell lines. The production of cyclin A1 by prostate cancer cells after stimulation of IL-6 is important because cyclin A1 promotes prostate cancer cell survival. LNCaP and LNCaP-IL6+ cells that over expressed cyclin A1 were more resistant to camptothecin-induced apoptosis compared to cells without cyclin A1. Cell cycle progression did not differ in either cell line with cyclin A1 or without cyclin A1 indicating the cyclin A1 promotes cell survival. This study also examined LNCaP and LNCaP-IL6+ tumor xenografts in nude mice. Tumors derived from LNCaP-IL6+ cells grew more rapidly and were more malignant compared to LNCaP tumors. The same trend was seen for levels of cyclin A1, which were higher in LNCaP-IL6+ tumor xenografts compared to LNCaP xenografts. Cyclin A1 was significantly correlated with P-Akt expression in the xenografts ($r = 0.809$, $p = 0.05$). From this research, it can be concluded that IL-6 promotes prostate cancer cell survival

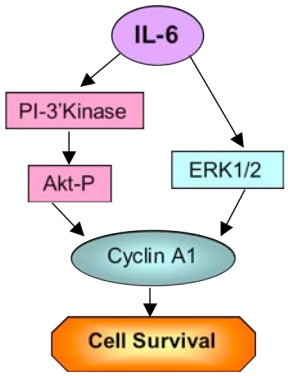

Figure 3. Schematic graph of the role of signaling pathways and cyclin A1 in the survival of prostate cancer cells in response to IL-6.

through the PI3K/Akt and MAPK/Erk pathways via expression of cyclin A1, and the effect of IL-6 is amplified in LNCaP-IL6+ cells (Figure 3) (27).

IL-6 has also been shown to cause antiapoptotic effects through the MAPK/Erk pathway by increasing production of myeloid cell leukemia-1 (Mcl-1) protein. Mcl-1 is another regulator of apoptosis and it is a member of the Bcl-2 family of proteins, which are known to contribute to the antiapoptotic properties of malignant cells. Cavarretta et al. examined both the resistance of LNCaP-IL6+ cells to apoptosis as well as the effects of Mcl-1 on apoptosis (29). LNCaP-IL6- cells, the control for this study, have the same passage as LNCaP-IL6+ cells but in the absence of IL-6. Induction of apoptosis was examined after separate stimulus by IL-6 and ionophore A23187, a substance known to induce calcium-triggered cell death. Compared to the control cells, LNCaP-IL6+ cells were significantly more resistant to apoptosis when exposed to both apoptotic stimuli. When endogenous IL-6 was inhibited in the LNCaP-IL6+ cells, the rate of apoptosis increased, indicating that the IL-6 autocrine loop may be responsible for LNCaP-IL6+ cell's ability to resist apoptosis. To examine the target of endogenous IL-6, Mcl-1 was quantified in both cell lines. It was found that the concentration of Mcl-1 was two times greater in LNCaP-IL6+ cells compared to controls. Similar to the inhibition of IL-6, LNCaP-IL6+ cells became more sensitive to apoptosis when Mcl-1 was inhibited. The same increase in apoptosis was found in Du145 cells after inhibition of Mcl-1. The

Du145 cell line is from brain metastases of prostate cancer. They are similar to LNCaP-IL6+ cells in that they secrete IL-6. Mcl-1 levels decreased in LNCaP-IL6+ cells and Du145 cells after IL-6 was neutralized and in LNCaP-IL6+ cells when the MAPK/Erk pathway was inhibited. This led to the conclusion that endogenous IL-6 activates the MAPK/Erk pathway leading to a production of Mcl-1 protein and resistance to apoptosis.

In addition to the antiapoptotic effects of IL-6, it appears that IL-6 can contribute to tumor invasion through its effect on vascular endothelial growth factor (VEGF) and promatrilysin. Vascular endothelial growth factor is associated with tumor angiogenesis and proliferation. VEGF is only capable of stimulating prostate cancer cell proliferation if the cell expresses VEGF receptor 2 (VEGFR-2). Steiner et al. found that, *in vitro*, LNCaP-IL6+ cells express VEGFR-2 and exhibit a VEGF autocrine loop, unlike LNCaP-IL6- cells. Furthermore, IL-6 stimulates this loop through the PI3K pathway (30). When the PI3K pathway was inhibited, IL-6 no longer induced VEGF production by the LNCaP-IL6+ cells. Exogenous VEGF did not stimulate cell proliferation in either cell line, but the neutralization of VEGFR-2 in the LNCaP-IL6+ cells resulted in a significant inhibition in cell growth ($p < 0.05$). Thus, the observed proliferative advantage of LNCaP-IL6+ cells over LNCaP-IL6- cells was attributed to the presence of the VEGF autocrine loop. IL-6 may promote tumor invasion by stimulating VEGF production as well as the production of promatrilysin. Promatrilysin is a proteolytic enzyme that promotes tumor progression through the breakdown of the extracellular matrix. In *in vitro* co-cultures of prostate cancer cells, IL-6 secreted by Du145 cells was found to induce promatrilysin production in nearby LNCaP cells (31). The *in vitro* co-cultures used in this study were thought to represent real prostate carcinomas because of the

heterogeneous properties of prostate cancer tumors. This may allow IL-6, through paracrine interactions, to promote tumor progression *in vivo*. IL-6 may further contribute to tumor growth by increasing prostate cancer resistance to chemotherapy.

Several forms of chemotherapy exist for patients with prostate cancer. Borsellino et al. examined the relationship between IL-6 and chemotherapy resistance in PCa3 and Du145 cell lines (32). These cells are androgen-independent and can be resistant to adriamycin, etoposide, and cisplatin chemotherapies. PCa3 and Du145 cells are also know to secrete IL-6. When IL-6 was inhibited *in vitro*, both cell lines showed growth inhibition as well as an increased sensitivity to the cytotoxic effects of cisplatin and etoposide. This study is further evidence that IL-6 may play an important role in the progression of prostate cancer.

Research indicates that inflammation is present in men with prostate cancer, and IL-6 may be a major perpetuator of the inflammatory response. *In vitro* evidence suggests that autocrine and paracrine IL-6 loops exist in prostate cancer cells and that IL-6 may contribute to tumor progression and survival. It is also known that the development of advanced prostate cancer is often associated with a poor prognosis. The literature has not addressed whether factors that may decrease the severity of the inflammatory response, *in vivo*, could improve prostate cancer patient outcome. The role of anti-inflammatory agents in the prevention of prostate cancer development has been examined, but further research into modifiable factors that could interfere with the inflammatory response is warranted. Diet is considered a modifiable factor, and the intake of omega-3 fatty acids, which are known for their anti-inflammatory properties, could prove beneficial to prostate cancer patients.

Inflammation & Omega-3 Fatty Acids

Dietary intake of α-linolenic acid (ALA), an omega-3 (n-3) fatty acid, is essential for humans due to the body's inability to synthesize ALA endogenously from other fatty acids. Once ALA is taken in through the diet, the body has a limited capacity to convert ALA to eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), two other members of n-3 fatty acid family. EPA and DHA can also be consumed directly from fish, shellfish, and supplements. Omega-6 (n-6) fatty acids, including linoleic acid (LA) and arachidonic acid (AA), are also essential in the human diet. N-3 and n-6 fatty acids are important to the inflammatory process because both are precursors to eicosanoids, a family of active substances including thromboxanes, prostaglandins, and leukotrienes. Prostaglandins are responsible for cytokine production by macrophages, including the production of IL-6 (33). Eicosanoids are produced from the phospholipids of inflammatory cells when cytokines or hormones bind plasma membrane receptors. Calcium-dependent cytoplasmic phospholipase A_2 (PLA_2) is activated and begins to hydrolyze AA from the phospholipids of the intracellular membrane (Figure 4). AA is then converted to eicosaniods by the cyclooxygenase (COX), lipoxygenase (LOX), and/or cytochrome P450 pathways. Prostaglandins and thromboxanes are produced by the COX pathway; leukotrienes, lipoxins, and hydroxyeicosatetraenoic acid by the LOX pathway; and epoxyeicosatrienoic acid is produced via the cytochrome P450 pathway (34). The phospholipid precursors can be AA or EPA or both, depending on which fatty acid is abundant in the membrane. The proportion of n-3 fatty acid phospholipids reflects dietary intake, and the incorporation of EPA and DHA into the membrane can result in a decrease in the availability of AA. Studies that have examined the incorporation of n-3

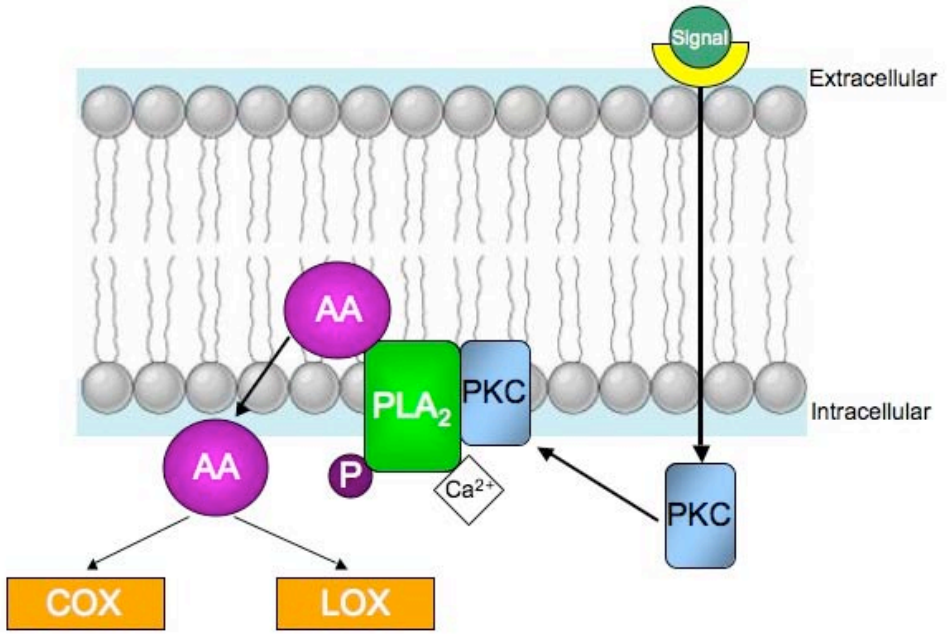

Figure 4. Eicosanoid synthesis begins with the binding of a hormone or other signal to an extracellular receptor. Protein kinase C (PKC) is activated. PKC and intracellular calcium (Ca^{2}+) activate phospholipase A_2 (PLA_2) via phosphorylation (P). PLA_2 hydrolyzes arachidonic acid (AA) from the membrane phospholipids. AA is then converted to eicosanoids via the cyclooxygenase (COX) and/or lipoxygenase (LOX) pathways.

fatty acids into erythrocyte membranes after dietary supplementation have shown that AA is displaced in a dose-dependent manner. Higher intakes of n-3 fatty acids result in a greater decrease in AA in the membrane. These studies also indicate that for every gram increase in dietary EPA and DHA, the percent of EPA and DHA in the erythrocyte membrane increases approximately 1.4% and 1.7%, respectively. This change in membrane composition begins to occur within days of increasing dietary n-3 fatty acids (35, 36). When the level of n-3 fatty acids increases in the membrane, EPA will replace AA as a precursor for eicosanoid production. EPA-derived eicosanoids do not promote the same pro-inflammatory response that is caused by eicosanoids produced from AA. The anti-inflammatory properties of n-3 fatty acids are attributed to this alteration in

eicosanoid synthesis as well as their ability to alter signal transduction pathways and inflammatory gene expression (37). The ability of n-3 fatty acids to reduce inflammation *in vivo* has been examined in small clinical trials as well as large epidemiological studies.

Supplementation with EPA and DHA is often used to study the effects of n-3 fatty acids *in vivo*. One such study examined the effects of EPA+DHA supplementation on unstimulated and LPS-stimulated monocyte expression of IL-6. Treatment subjects received approximately 2 g of EPA and 1.4 g of DHA per day, and erythrocyte membrane analysis was performed to measure compliance. After 18 weeks of supplementation, there was a significant decrease in IL-6 production by both unstimulated and LPS-stimulated monocytes, *in vitro* ($p < 0.05$) (38). A similar trend has been observed in a community-based sample in Italy. Researchers examined the correlation between n-3 fatty acids, n-6 fatty acids, and circulating inflammatory markers in 1,123 subjects (39). Plasma fatty acid analysis was conducted and circulating concentrations of IL-6 and CRP were measured. Supplementation was not used in this study, and the plasma levels of n-3 fatty acids reflected dietary intake. There were significantly lower serum concentrations of IL-6 and CRP in the individuals with the highest levels of plasma n-3 fatty acids. These findings further support the role of n-3 fatty acids as modifiers of the inflammatory response.

In summary, chronic inflammation may play a role in the development of prostate cancer as well as in tumor progression and growth. There is evidence that supports the anti-inflammatory properties of n-3 fatty acids in prostate cancer prevention. However, the ability of n-3 fatty acids to modify the inflammatory response in men with and without prostate cancer has not been thoroughly investigated. Furthermore, if a

relationship does exist between n-3 fatty acids and inflammation, the effect of this relationship on outcome in men with and without prostate cancer is unknown. This study provided further insight into the effects of n-3 fatty acids on inflammation and the possible use of n-3 fatty acids in the prevention and treatment of prostate cancer.

Study Objective & Aims

The purpose of this study was to investigate the relationship between inflammation and outcome in biopsy negative controls and prostate cancer cases as well as the modification of that relationship by n-3 fatty acids.

Primary Aim #1: To determine the association between erythrocyte n-3 fatty acids and circulating concentrations of IL-6 and CRP in biopsy negative controls and prostate cancer cases undergoing primary therapy.

Hypothesis #1: Circulating concentrations of IL-6 and CRP would be lower in subjects with the highest erythrocyte levels of n-3 fatty acids.

Primary Aim #2: To determine the association between patient outcome, measured as recurrence free survival, and circulating concentrations of IL-6 and CRP, with and without modification by erythrocyte n-3 fatty acids in prostate cancer cases.

Hypothesis #2: Better patient outcome would be observed in subjects with lower circulating levels of IL-6 and CRP and this association would be modified by erythrocyte n-3 fatty acid levels.

Primary Aim #3: To determine the relationship between the development of cancer and circulating concentrations of IL-6 and CRP and prostate tissue inflammation, with and without modification by n-3 fatty acids in biopsy negative controls.

Hypothesis #3: Time to development of prostate cancer in the biopsy negative controls would be greater in subjects with lower levels of circulating concentrations of IL-6 and CRP and lower levels of tissue inflammation; this relationship would be modified by erythrocyte n-3 fatty acid levels.

Secondary Aim #1: To determine the correlation between inflammation as measured by circulating concentrations of IL-6 and CRP and prostate tissue, as measured by immunohistochemistry (IHC), in biopsy negative controls.

Secondary Hypothesis #1: Inflammation in the prostate tissue would correlate with markers of inflammation in the blood.

MATERIALS AND METHODS

Subjects

This longitudinal study included subjects from The Diet and Prostate Cancer Study (DPC), a case-control study. The DPC study was conducted at the Portland Veteran Affairs Medical Center (PVAMC) from December 2001 through August 2006. The pilot study was funded by a VA ERIC grant and the project was funded by the National Cancer Institute. Subjects for this secondary analysis were all men referred to the PVAMC urology clinic for a prostate biopsy who provided blood samples upon consenting into the study. The cohorts of interest were men who had a negative biopsy (biopsy negative controls), and men who were diagnosed with prostate cancer (cancer cases) upon entry into the study. The DPC study recruited 728 subjects including 292 biopsy negative controls and 143 cancer cases. From the original cohort, 240 biopsy negative controls and 121 cancer cases provided blood samples at recruitment and were included in this study. The Oregon Health & Science University Institutional Review Board and the PVAMC Institutional Review Board approved the amendment to the original DPC study protocol to perform the secondary analysis. All subjects used for this analysis consented to future studies at the time of entering the DPC study.

Blood Sampling & Storage

Venous blood was collected from eligible study subjects using a 10mL sodium EDTA vacutainer tube. Blood collection tubes were gently inverted after collection and immediately covered with aluminum foil and transported to the laboratory for processing.

Covered vacutainers were stored at +5 to +10 °C until processed, which was no more than five hours after the blood draw. Erythrocytes were separated from the plasma by centrifugation at 1300g for ten minutes. Fifty (50) μL of metaphosphoric acid/dithiothreitol (MPA/DTT) was added to 2-500 μL aliquots of plasma, capped securely, and vortexed for 30 seconds. Remaining plasma was divided into 500 μL aliquots. All plasma samples were stored in cyrovials at -70 °C until analysis.

Plasma IL-6 and CRP Analysis

Plasma IL-6 and CRP analysis was conducted at the core laboratory at the Oregon Clinical and Translational Research Institute (OCTRI) at Oregon Health & Science University (OHSU). Prior to analysis, all plasma samples were randomized to minimize bias from laboratory technique or error. An effort was made to keep the number of freeze-thaw cycles to a minimum.

Plasma IL-6 concentrations were measured using solid-phase enzyme-linked immunosorbent assay (ELISA) (R&D Systems Europe, Ltd.). Standard curves were developed with reconstituted IL-6 standard in Calibrator Diluent with standard stock dilutions of 10 pg/mL, 5 pg/mL, 2.5 pg/mL, 1.25 pg/mL, 0.625 pg/mL, 0.312 pg/mL, and 0.156 pg/mL. All solutions were brought to room temperature. Plasma samples were thawed at room temperature and then vortexed for 30 seconds. Once thoroughly mixed, samples were centrifuged at 1300g for 10 minutes. To each well, standard solutions or plasma samples were added to 100 μL of Assay Diluent. The covered microplate was incubated for two hours at room temperature on a microplate shaker set at 500 ± 50 rpm. The microplate was washed six times with 400 μL of Wash Buffer after incubation. Two

hundred (200) μL of IL-6 Conjugate was added to each well and the covered microplate was incubated at room temperature for two hours on a shaker. After incubation, the microplate was washed again with the procedure previously described. Fifty (50) μL of Substrate Solution was added to each well and the covered microplate was incubated on a bench top at room temperature for 60 minutes. Fifty (50) μL of Amplifier Solution was added to each well and the covered microplate was incubated on a bench top at room temperature. After 30 minutes, 50 μL of Stop Solution was added to each well. Optical density of each well was determined within 30 minutes using a microplate reader set to 490 nm. The wavelength was changed to 650 nm to correct for optical imperfections in the plate. All samples were run in duplicate and analysis was repeated if there was a greater than 10% difference between the duplicates. Samples that had IL-6 values above the upper limit of the standard curve (10 pg/mL) were diluted with Calibrator Diluent and analyzed again. Plasma IL-6 levels were expressed in pg/mL. The average intra-assay coefficient of variation (CV) between duplicates was 4.75%, with a minimum intra-assay CV of 1.82% and a maximum intra-assay of 13.72%. The high CV was due to the accidental addition of 50 μL of extra Amplifier solution to five wells on the 96-well plate. This resulted in a large difference between duplicates for the five samples and a high intra-assay CV.

Plasma CRP concentrations were measured using solid-phase, chemiluminescent immunometric assay by the IMMULITE /IMMULITE 1000 Analyzer. Plasma samples were thawed at room temperature and then vortexed for 30 seconds. Once thoroughly mixed, samples were centrifuged at 1300g for 10 minutes. Plasma samples were pre-diluted in CRP Sample Diluent at a ratio of 1:101 with 10 μL of sample in 1000 μL of

Diluent. Samples were analyzed according to the procedures outlined in the IMMULITE/IMMULITE 1000 Operator's Manual. All samples were run in singlet and CRP values were expressed as mg/L.

Erythrocyte Fatty Acid Analysis

Erythrocyte fatty acid analysis was provided from analysis previously conducted during the DPC study. Erythrocyte fatty acids were analyzed using gas chromatography/mass spectrometry and fatty acids were expressed as a percent of total fatty acids. The n-3 fatty acids of interest for this analysis included α-linolenic acid (ALA), docosahexaenoic acid (DHA), and eicosapentaenoic acid (EPA).

Prostate Tissue Analysis

Inflammation in prostate tissue collected at the time of biopsy was documented in the biopsy negative controls. Prostate tissue inflammation was determined using immunohistochemistry (IHC).

Patient Outcome

Patient outcome was assessed using the IRB-approved DPC study longitudinal database and electronic medical records (CPRS) at the PVAMC. The DPC longitudinal database was approved to conduct passive follow-up for prostate related morbidity and mortality among men that were originally consented and interviewed as part of the DPC study.

Cancer outcome for the prostate cancer cases was described as recurrence free survival, and was assessed only for subjects who had a radical retropubic prostatectomy

(RRP) (n = 52) or localized radiotherapy, including beam radiation and brachytherapy (n = 38). Subjects who chose other treatment options (watchful waiting, proton beam therapy, and hormone therapy only) were not assessed for cancer outcome. For subjects who underwent a RRP, recurrence free survival was defined as the time from the achievement of nadir (PSA $\leq$ 0.1 ng/mL) after surgery to the time of biochemical failure, death, or the end of the study follow-up period. Biochemical failure after a RRP was defined as a PSA of 0.2 ng/mL or greater, with a second confirmatory PSA of > 0.2 ng/mL (40, 41). The defined date of failure was the date of the original PSA elevation, and PSA measurements taken within six weeks of surgery were excluded. For subjects who underwent localized radiotherapy, recurrence free survival was defined as the time from the achievement of nadir (defined as the lowest PSA achieved after treatment) to the time of biochemical failure, death, or the end of the study follow-up period. Biochemical failure after localized radiotherapy was defined as a rise of 2 ng/mL or greater above the nadir PSA (42, 43). The date of failure was the date of the first elevated PSA value to meet the definition of biochemical failure.

New incident prostate cancer was the outcome variable of interest for the biopsy negative controls. Disease free survival was defined as the time from entry into the study to the time of biopsy-confirmed diagnosis of prostate cancer, death, or the end of the study follow-up period. Subjects who were diagnosed with prostate cancer within six months of their initial biopsy were excluded from the outcome analysis. The end of the study follow-up period was June 1, 2008.

Statistical Analysis

All statistical analyses were performed using STATA 10.0 (StataCorp LP, College Station, Texas). Analyses were conducted between the biopsy negative controls and the cancer cases. Analyses were also conducted between the biopsy negative controls and the cancer cases stratified by the severity of their disease using two different definitions of high-grade and low-grade cancer. Gleason score was used to determine disease severity and cancer grade. As mentioned previously, the Gleason score is the sum of the grade given to the first and second most dominant histological tumor patterns in a specimen. The grade for each pattern can range from one to five and the Gleason score can range from two to 10. The first definition used to stratify the cancer cases defined low-grade cancer as a Gleason score ≤ 6 or 7 with a histological tumor pattern grade of 3+4 (LG3+4) (n = 89) and high-grade cancer as a Gleason score of 7 with a histological tumor pattern grade of 4+3 or ≥ 8 (HG4+3) (n = 32). The second definition used to stratify the cancer cases defined low-grade cancer as a Gleason score ≤ 6 (LG6) (n = 59) and high-grade cancer as a Gleason score ≥ 7 (HG7+) (n = 62). The two different methods of defining low-grade and high-grade cancer were used in this analysis in an effort to reflect the past literature as well as more recent research that has concluded a Gleason score of 7 can reflect two levels of disease severity when the tumor pattern grade (3+4 or 4+3) was taken into account. Tumors with a Gleason score of 7 with a tumor pattern grade of 4+3 are known to be more aggressive compared to tumors with a Gleason score of 7 with a tumor pattern grade of 3+4 (44-46).

Differences in demographics, medication use and prostate cancer risk factors were examined between the biopsy negative controls and cancer cases before stratification by

Gleason score. Differences in categorical covariates were determined using a chi-square test, and the Student's *t* test was used to compare continuous covariates. The primary exposure variables (IL-6, CRP, ALA, DHA, and EPA) were presented as median values, and differences in the primary exposure variables between the biopsy negative controls and cancers case, as a complete cohort and stratified by Gleason score, were determined using the Wilcoxon rank-sum test. Calculated plasma markers of inflammation (IL-6 and CRP) and erythrocyte n-3 fatty acids were compared using Pearson's correlation coefficient and Goodman and Kruskal's gamma coefficient to determine the relationship between systemic inflammation and dietary intake of n-3 fatty acids. Partial correlations were used to assess the relationship between plasma markers of inflammation and n-3 fatty acids while adjusting for the other primary covariates. Correlations and gamma coefficients were calculated in the biopsy negative controls and cancer cases as a complete cohort and independently. Correlations and gamma coefficients were also calculated in the cancer cases stratified by Gleason score. IL-6, CRP, ALA, DHA, and EPA were logarithmically transformed due to their skewed distribution. Continuous variables were used for the Pearson's and partial correlations, and the gamma coefficients were calculated using categorical variables. A statistical p-value of < 0.05 was considered significant for all performed tests. Missing values were imputed using the median or mean value for that covariate depending on the distribution of the variable. Due to an additive in the plasma of eight subjects, CRP was unable to be determined. For these subjects, CRP was imputed using calculations based in their IL-6 values, which were significantly correlated. Additive-free plasma for two of the eight subjects was

located after statistical analyses were completed. The true CRP values for these two subjects were used for the survival analysis component of this study.

The association between systemic inflammation, measured by plasma IL-6 and CRP, and erythrocyte omega-3 fatty acids and prostate cancer was summarized in terms of odds ratios (ORs) and corresponding 95% confidence intervals (CIs) determined by age-adjusted unconditional logistic regression. Primary exposure variables were categorized into tertiles based on the biopsy negative controls for IL-6, ALA, DHA, and EPA. In healthy individuals, CRP is typically < 3 mg/L. CRP can be elevated up to 10 mg/L for minor or insignificant reasons. CRP above 10 mg/L is considered abnormal (47-49). CRP was categorized based on these definitions. The outcome variable (diagnosis of prostate cancer) included the biopsy negative controls and the cancer cases as an entire cohort and stratified by disease severity. Models adjusted for potential confounding factors were also determined. Potential confounding factors, including race, body mass index (BMI), education, smoking status, alcohol status, co-morbidities based on the age-adjusted Charlson co-morbidity index, family history of cancer, family history of prostate cancer, history of non-prostate cancer, PSA and prostate volume and density at time of initial biopsy, statin use, and other cholesterol drug use were examined. Medications that may affect inflammation and/or the development of prostate cancer were also considered possible confounding variables. The use of aspirin and non-steroidal anti-inflammatory drugs (NSAIDs) may interact with plasma concentrations of IL-6 and CRP. Patients were instructed to discontinue the use of aspirin and NSAIDs for two-weeks prior to their biopsy appointments and aspirin and NSAID use was documented. The influence of aspirin and NSAID use was evaluated and variables were

included as appropriate. Dietary factors that may have an association with prostate cancer and inflammation as well as known anti-oxidants were examined. This included alcohol, caffeine, lycopene, trans-fats, grains and whole grains, dark-green and deep-yellow vegetables, dry beans and peas, white potato, tomato, fruit including citrus, melon, and berries, dairy, meat, poultry, fish, eggs, soy products, nuts and seeds, and dietary and supplemental vitamin E, C, B12, iron, zinc, copper, folate, and beta-carotene. Each potential confounding variable was independently examined in a univariate model with each primary outcome variable. Variables with a statistical *p*-value of < 0.25 were included in the preliminary multivariable analysis. Primary exposure variables were independently entered in a model already containing age and a crude odds ratio for the primary exposure variable. During the model building process, primary exposure variables, stratified into categories with ordinal coding beginning at zero with increments of one, were entered as continuous variables. Potential confounding variables were entered, one at a time, into a model with age and each primary exposure variable. Variables were considered confounders if the odds ratio of the primary exposure variable changed by ± 10% from the crude odds ratio after addition of the confounding variable into the model. Final models included the primary exposure variables as categorical variables. Primary exposure variables were entered into the final models as continuous variable to determine trend. Likelihood ratio tests were calculated to determine the overall effect of the primary exposure variables on the outcome variables. Age-adjusted and multivariable-adjusted odds ratios were reported. The relationship between IL-6 and CRP and the omega-3 fatty acids was investigated using interaction terms. ALA, DHA, and EPA were re-categorized based on the median levels of the biopsy negative controls.

Interaction terms were entered into the model and odds ratios were calculated using linear combinations of estimated parameters. The significance of the interaction was tested using the likelihood ratio test. Due to the number of confounding variables in some models and the small sample size after stratification, some odds ratios could not be estimated. Goodness-of-fit was assessed using the Hosmer-Lemeshow goodness-of-fit test; all final models were determined to have adequate fit by this criterion (See Appendix for goodness-of-fit assessments).

Survival analysis was performed to evaluate the relationship between systemic inflammation and erythrocyte n-3 fatty acids on the development of cancer in the biopsy negative controls. Due to the small sample size and inaccuracy of defining recurrence free survival among the prostate cancer cases, survival analysis was not performed on this subset of the cohort. Kaplan-Meier (KM) survival curves were constructed for the primary exposure variables including the presence of inflammation in the prostate. KM survival curves were also calculated with IL-6 stratified by tertiles and CRP stratified by categories, and then by median ALA, DHA, and EPA levels. This was also done for prostate tissue inflammation and the n-3 fatty acids. The differences between the curves were examined by the log-rank and Peto-Peto test for significance. Hazard ratios were calculated using the Cox proportional hazards model. Final models were determined following the same model building strategy that was used for the logistic regression models. Age-adjusted and multivariable-adjusted hazards ratios were reported. Interactions between IL-6 and CRP and the n-3 fatty acids were also performed using the strategy described previously. Models and interactions were also examined with inflammation in the prostate as a primary exposure variable. Goodness-of-fit was

assessed using Cox-Snell generalized residuals (See Appendix for goodness-of-fit assessments).

Calculated plasma IL-6 and CRP and observed tissue inflammation in the biopsy negative controls were compared using the Wilcoxon rank-sum test. Logistic regression models were constructed with inflammation in the prostate as the primary outcome variable and IL-6 and CRP as the primary exposure variables.

With a sample size of 361 (121 prostate cancer cases and 240 biopsy negative controls) and significance level of < 0.05, correlation coefficients between systemic inflammation and n-3 fatty acids of at least 0.13, 0.15, and 0.17 would be detectable with 70%, 80% and 90% power, respectively. The minimum detectable hazard ratio (HR) was calculated using sample size, a power of 80%, a significance level of 0.05, the expected proportion of subjects who would fail, and previously reported standard deviations for IL-6, CRP, ALA, EPA, and DHA (50-52). The minimum detectable HR was adjusted using an R-squared of 10%, which was the estimated proportion of variance of the primary exposure variable explained by additional covariates in a multiple regression. The minimum detectable HR for the interaction of IL-6 and CRP with ALA, DHA, and EPA was calculated by adding an interaction of their respective standard deviations into the equation. The expected biochemical recurrence rate after localized treatment for men with prostate cancer, when biochemical recurrence was defined as a PSA ≥ 0.2 and rising, has been estimated at 26% (40). The sample size of 121 cancer cases allowed for a minimum detectable HR of 1.19, 1.46, 3.73, 1.52, and 4.31 when the primary exposure variable was IL-6, CRP, ALA, DHA, or EPA, respectively. When the interaction between the plasma markers of inflammation and n-3 fatty acids was taken into

consideration, the minimum detectable HR was 2.14, 1.27, and 2.33 with the interaction between CRP and ALA, DHA, and EPA, respectively. The minimum detectable HR for IL-6 and ALA, DHA, and EPA was 3.04, 1.42, and 3.44, respectively. The expected rate of prostate cancer development among men was estimated to be 16% (1, 2). It was assumed that this rate would be stable over the accrual period of four years and follow-up period of four years. The sample size of 240 biopsy negative controls allowed for a minimum detectable HR of 1.17, 1.40, 3.21, 1.45, and 3.65 when the primary exposure variable was IL-6, CRP, ALA, DHA, or EPA, respectively. When the interaction between the plasma markers of inflammation and n-3 fatty acids was taken into consideration, the minimum detectable HR was 1.47, 1.13, and 1.54 with the interaction between CRP and ALA, DHA, and EPA, respectively. The minimum detectable HR for IL-6 and ALA, DHA, and EPA was 2.30, 1.30, and 2.52, respectively.

The outcomes from this study were used to estimate power and sample size in a future study based on the exemplary chi-square method (53) (See Appendix for power calculations).

RESULTS

Demographics

Two hundred forty (240) biopsy negative controls and 121 prostate cancer cases were included in this analysis. The median age for the biopsy negative controls was 63 years, and was 65 years in the cancer cases. There was no significant difference in age, race (categorized as white and non-white) and BMI between the biopsy negative controls and cancer cases (Table 1). The level of education attained was significantly different between the biopsy negative controls and cancer cases ($p = 0.004$) with over 74% of the biopsy negative controls having greater than 12 years of education compared to only 57% of the cancer cases.

For this study, co-morbidities were described with the age-adjusted Charlson Co-morbidity Index (ACCI) (54, 55). The ACCI is a useful tool to assess the impact of age and numerous co-morbidities on the outcome of interest. Subjects were categorized into three groups based on previous use of the ACCI in a population of bladder cancer patients (56). The first group included those with an ACCI ≤ 2 and described subjects who were younger than 60 (yrs) with few to no co-morbidities. The second group included subjects with an ACCI of 3-5 and described subjects both older subjects with few co-morbidities and younger subjects with many co-morbidities. The third group included subjects with an ACCI of greater than 5 and this included older patients with more co-morbidities. The ACCI of the biopsy negative controls was significantly different compared to the cancer cases ($p = 0.002$) with a higher proportion of biopsy negative controls (35.8%) in the lowest ACCI category and a lower proportion (15.4%) in

the highest ACCI category. Among the cancer cases, 38 subjects (31.4%) were in the highest ACCI category.

Table 1. Selected demographic characteristics of the biopsy negative controls and prostate cancer cases

Characteristics		Controls (n = 240)	Cancer Cases (n = 121)	p^a
Age (yrs)		n (%)		
	≤ 59	77 (32.1)	32 (26.5)	0.38
	60 - 69	113 (47.1)	57 (47.1)	
	≥ 70	50 (20.8)	32 (26.5)	
BMI (kg/m^2)				
	≤ 24.9	40 (16.7)	19 (15.7)	0.78
	25 - 29.9	88 (36.7)	49 (40.5)	
	≥30	112 (46.7)	53 (43.8)	
Race				
	White	222 (92.5)	111 (91.7)	0.80
	Non-White[b]	18 (7.5)	10 (8.3)	
Education				
	12 or less years[b]	62 (25.8)	52 (43.0)	0.004
	Some college or tech college	105 (43.8)	42 (34.7)	
	College graduate	73 (30.4)	27 (22.3)	
Age-Adjusted Charlson Score				
	≤ 2	86 (35.8)	34 (28.1)	0.002
	3 - 5	117 (48.8)	49 (40.5)	
	> 5	37 (15.4)	38 (31.4)	

[a] Pearson's chi-square test

[b] Following categories contain subjects with unknown values: Race (n=1); Education (n=9)

Medication Use

Commonly used medications that may have a relationship with prostate cancer risk or inflammation were assessed for the biopsy negative controls and cancer cases (Table 2). Subjects were categorized as either using or not using statins and other cholesterol lowering medications. If subjects used NSAIDs or aspirin, the frequency was also documented. There was no significant difference in medication use between the biopsy negative controls and cancer cases for statins, other cholesterol lowering drugs, NSAIDs or aspirin.

Table 2. Selected medication use prior to biopsy of the biopsy negative controls and prostate cancer cases

Medication		Controls (n = 240)	Cancer Cases (n = 121)	p^a
		n (%)		
Statins				
	No[b]	143 (59.6)	76 (62.8)	0.55
	Yes	97 (40.4)	45 (37.2)	
Other Cholesterol Medications				
	No[b]	225 (93.8)	113 (93.4)	0.89
	Yes	15 (6.3)	8 (6.6)	
Non-steriodal Anti-inflammatories (NSAIDs)				
	No[b]	157 (65.4)	81 (66.9)	0.59
	Less than weekly	27 (11.3)	18 (14.9)	
	Weekly	24 (10.0)	10 (8.3)	
	Greater than or equal to daily	32 (13.3)	12 (9.9)	
Aspirin				
	None[b]	112 (46.7)	55 (45.5)	0.84
	Less than daily	15 (6.3)	6 (5.0)	
	Daily or more than daily	113 (47.1)	60 (49.6)	

[a] Pearson's chi-square test

[b] Following categories contain subjects with unknown values: Statins (n=6); Other Cholesterol Medications (n=8); NSAIDs (n=14); Aspirin (n=11)

Prostate Cancer Risk Factors

In addition to age and race, risk factors for prostate cancer include family history of prostate cancer, prostate volume, and PSA levels (57, 58). Drinking alcohol, smoking and a family history of cancer are also associated with an increase risk of cancer. PSA at the time of biopsy, family history of prostate cancer, family history of cancer, and alcohol status were not significantly different between the biopsy negative controls and cancer cases (Table 3). Smoking status was significantly different ($p = 0.04$) with a higher proportion of current smokers (26.5%) among the cancer cases and a higher proportion of subjects who never smoked (29.2% including 10 subjects with unknown status or 25% excluding the unknowns) in the biopsy negative controls. The median prostate volume at the time of biopsy was significantly higher at 47.5 mL in the biopsy negative controls compared to the median volume of 37.9 mL in the cancer cases ($p < 0.001$).

Table 3. Selected prostate cancer risk factors in the biopsy negative controls and prostate cancer cases

		Controls (n = 240)	Cancer Cases (n = 121)	*p*
		(median)		
Initial prostate volume at time of biopsy (cc/mL)		47.5	37.9	<0.001[a]
PSA at time of biopsy (ng/mL)		n (%)		
	< 4	45 (18.8)	18 (14.9)	0.09[b]
	4 - 10	166 (69.2)	78 (64.5)	
	> 10	29 (12.1)	25 (20.7)	
Smoking Status				
	Never[c]	70 (29.2)	21 (17.4)	0.04[b]
	Past	123 (51.3)	68 (56.2)	
	Current	47 (19.6)	32 (26.5)	
Alcohol Status				
	Never[c]	63 (26.3)	21 (17.4)	0.07[b]
	Past	99 (41.3)	64 (52.9)	
	Current	78 (32.5)	36 (29.8)	
Family History of Cancer				
	Yes	162 (67.5)	78 (64.5)	0.56[b]
	No[c]	78 (32.5)	43 (35.5)	
Family History of Prostate Cancer				
	Yes	32 (13.3)	16 (13.2)	0.73[b]
	No	171 (71.3)	90 (74.4)	
	Unknown	37 (15.4)	15 (12.4)	

[a] Wilcoxon rank-sum test

[b] Pearson's chi-square test

[c] Following categories contain subjects with unknown values: Smoke Status (n=10); Alcohol Status (n=12); Family History of Cancer (n=16)

Plasma Profiles of Erythrocyte Fatty Acids, IL-6 & CRP

The median erythrocyte fatty acids levels for ALA, DHA, and EPA were reported in Table 4 as a percent of total membrane fatty acids. Comparisons of the n-3 fatty acids of interest were made between the biopsy negative controls and the cancer cases as an entire cohort and stratified by low-grade and high-grade cancer. The only significant difference found was between ALA in the biopsy negative controls and the LG3+4 cancer cases (p = 0.012). The median levels for ALA were similar at 0.12% of total fatty acids in the

biopsy negative controls and 0.11% in the LG3+4 cancer cases. However, the LG3+4 cancer cases had a smaller range in values with the highest ALA level reaching 0.27% compared to 0.38% in the biopsy negative controls.

Plasma IL-6 and CRP showed a similar pattern with minimal difference across the groups (Table 5). CRP did not differ significantly between the biopsy negative controls and cancer cases by any stratification. The highest median CRP level was observed in the HG4+3 cancer cases at 1.75 mg/L. The HG4+3 cancer cases also exhibited the only significant difference in plasma IL-6 compared to the biopsy negative controls with a median value of 2.52 pg/mL ($p = 0.03$), which is significantly higher than the biopsy negative control median value of 1.87 pg/mL. As a complete cohort, the cancer cases did show higher levels of plasma IL-6 and CRP over the biopsy negative controls but the difference was not significant.

Relationship between Plasma Markers of Inflammation & Omega-3 Erythrocyte Fatty Acids

The relationship between plasma markers of inflammation (IL-6 and CRP) and n-3 fatty acids (ALA, DHA, and EPA) were examined in the biopsy negative controls and cancer cases. IL-6 and CRP exhibited a significant positive correlation in the biopsy negative controls and cancer cases (Table 6). After adjusting for the n-3 fatty acids, IL-6 and CRP remained significantly correlated (Table 13). There was a significant inverse relationship between IL-6 and EPA ($r = -0.104$, $p = 0.048$) and CRP and DHA ($r = -0.177$, $p < 0.001$) (Table 6). The inverse relationship between CRP and DHA continued to be significant after adjusting for IL-6, EPA, and ALA (Table 13). This was not true for IL-6 and EPA.

Table 4. Median (range) percentage of fatty acids in erythrocyte cell membranes of biopsy negative controls and prostate cancer cases, both as a complete cohort and stratified by Gleason score

			Cancer Cases Stratified by Gleason Score			
	Controls (n = 240)	Cancer Cases (n = 121)	Low Grade 3+4 (LG3+4) (n = 89)	High Grade 4+3 (HG4+3) (n = 32)	Low Grade 6 (LG6) (n = 59)	High Grade 7+ (HG7+) (n = 62)
ALA[b]	0.12 (0.05 - 0.38)	0.12 (0.06 - 0.36)	0.11 (0.06 - 0.27)[a]	0.13 (0.07 - 0.36)	0.12 (0.07 - 0.27)	0.12 (0.06 - 0.36)
DHA[b]	3.37 (1.55 - 7.48)	3.46 (1.72 - 8.04)	3.55 (2.04 - 8.03)	3.36 (1.72 - 5.75)	3.43 (2.07 - 8.04)	3.48 (1.72 - 5.97)
EPA[b]	0.41 (0.14 - 2.50)	0.39 (0.18 - 1.83)	0.41 (0.18 - 1.83)	0.38 (0.22 - 1.46)	0.44 (0.23 - 1.83)	0.39 (0.18 - 1.46)

[a] Significantly different compared to biopsy negative controls (Wilcoxon rank-sum test, $p = 0.012$)

[b] EPA (Eicosapentaenoic acid); DHA (Docosahexaenoic acid (DHA); ALA (alpha-Linolenic acid)

Table 5. Median (range) of IL-6 and CRP plasma levels in biopsy negative controls and prostate cancer cases, both as a complete cohort and stratified by Gleason score

			Cancer Cases Stratified by Gleason Score			
	Controls (n = 240)	Cancer Cases (n = 121)	Low Grade 3+4 (LG3+4) (n = 89)	High Grade 4+3 (HG4+3) (n = 32)	Low Grade 6 (LG6) (n = 59)	High Grade 7+ (HG7+) (n = 62)
IL-6 (pg/mL)[b]	1.87 (0.54 - 26.3)	1.99 (0.45 - 155.5)	1.87 (0.59-155.5)	2.52 (0.45 - 93.8)[a]	2.01 (0.64 - 155.5)	1.93 (0.45 - 93.8)
CRP (mg/L)[b]	1.40 (<0.3 - 42.6)	1.50 (<0.3 - 23.6)	1.30 (<0.3 - 23.6)	1.75 (0.31 - 12.3)	1.60 (<0.3 - 23.6)	1.35 (<0.3 - 12.3)

[a] Significantly different compared to biopsy negative controls (Wilcoxon rank-sum test, $p = 0.03$)

[b] Interleukin-6 (IL-6); C-reactive protein (CRP)

When the relationship between IL-6, CRP, and the n-3 fatty acids was examined within each study population (biopsy negative controls, all cancer cases, LG3+4, HG4+3, LG6, and HG7+), significant inverse correlations were only observed in the biopsy negative controls (Table 7, 8, 9, 10, 11, 12). IL-6 showed a significant inverse relationship with DHA and EPA (r = -0.149 and -0.137, p = 0.021 and 0.034, respectively) and CRP was significantly inversely correlated with DHA (r = -0.246, p < 0.001) (Table 7). Similar to what was observed in the entire cohort, CRP remained significantly inversely correlated with DHA in the biopsy negative controls after adjust for IL-6, ALA, and EPA (Table 13). CRP and IL-6 did exhibit a positive correlation in the biopsy negative controls and the cancer cases, as complete cohort, and stratified by LG3+4 and HG7+ cancer (r = 0.471, 0.252, 0.220, and 0.303, p < 0.001, 0.005, = 0.038 and 0.017, respectively) (Table 7, 8, 9, 12). This was also true after adjusting for the n -3 fatty acids (Table 13, 14).

Table 6. Pearson's correlation coefficients of plasma markers of inflammation and erythrocyte fatty acids in biopsy negative controls and cancer cases*

	IL-6	CRP
IL-6		
CRP	0.385[a]	
ALA	-0.009	0.043
DHA	-0.082	-0.177[c]
EPA	-0.104[b]	-0.083

[a]p <0.001, [b]p =0.048, [c]p <0.001

*IL-6, CRP, ALA, DHA, EPA were logarithmically transformed

Table 7. Pearson's correlation coefficients of plasma markers of inflammation and erythrocyte fatty acids in biopsy negative controls*

	IL-6	CRP
IL-6		
CRP	0.471[a]	
ALA	0.015	0.080
DHA	-0.149[b]	-0.246[d]
EPA	-0.137[c]	-0.112

[a]p <0.001, [b]p = 0.021, [c]p = 0.034, [d]p <0.001

*IL-6, CRP, ALA, DHA, EPA were logarithmically transformed

When the plasma markers of inflammation and the n-3 fatty acids were categorized, there was a significant positive association between IL-6 and CRP in the entire cohort and in each group independently (Table 15,

16). In the entire cohort and biopsy negative controls, IL-6, categorized in tertiles, had a significant inverse association with DHA, categorized dichotomously (Table 15). CRP, categorized into three ordinal groups, had a significant inverse association with DHA in the entire cohort and in the biopsy negative controls (Table 15). CRP was also inversely associated with EPA, categorized dichotomously, in the biopsy negative controls (Table 15).

Table 8. Pearson's correlation coefficients of plasma markers of inflammation and erythrocyte fatty acids in cancer cases*

	IL-6	CRP
IL-6		
CRP	0.252[a]	
ALA	-0.031	-0.031
DHA	0.016	-0.025
EPA	-0.061	-0.026

[a] p <0.005

*IL-6, CRP, ALA, DHA, EPA were logarithmically transformed

Table 9. Pearson's correlation coefficients of plasma markers of inflammation and erythrocyte fatty acids in low grade 3+4 (LG3+4) cancer cases*

	IL-6	CRP
IL-6		
CRP	0.220[a]	
ALA	-0.001	-0.059
DHA	-0.026	0.029
EPA	-0.054	-0.039

[a] p = 0.038

*IL-6, CRP, ALA, DHA, EPA were logarithmically transformed

Table 10. Pearson's correlation coefficients of plasma markers of inflammation and erythrocyte fatty acids in high grade 4+3 (HG4+3) cancer cases*

	IL-6	CRP
IL-6		
CRP	0.267	
ALA	-0.223	-0.169
DHA	-0.183	-0.079
EPA	-0.046	0.073

*IL-6, CRP, ALA, DHA, EPA were logarithmically transformed

Table 11. Pearson's correlation coefficients of plasma markers of inflammation and erythrocyte fatty acids in low grade 6 (LG6) cancer cases*

	IL-6	CRP
IL-6		
CRP	0.230	
ALA	-0.028	-0.052
DHA	0.038	0.030
EPA	-0.050	0.022

*IL-6, CRP, ALA, DHA, EPA were logarithmically transformed

Table 12. Pearson's correlation coefficients of plasma markers of inflammation and erythrocyte fatty acids in high grade 7+ (HG7+) cancer cases*

	IL-6	CRP
IL-6		
CRP	0.303[a]	
ALA	-0.030	-0.015
DHA	-0.005	-0.077
EPA	-0.082	-0.077

[a] $p = 0.017$

*IL-6, CRP, ALA, DHA, EPA were logarithmically transformed

Table 13. Partial correlation coefficients of association between plasma markers of inflammation and erythrocyte fatty acids in biopsy negative controls and cancer cases, as a complete cohort and stratified by cancer status*

	Entire Cohort		Biopsy Negative Controls		Prostate Cancer Cases	
	IL-6	CRP	IL-6	CRP	IL-6	CRP
IL-6						
CRP	0.382[a]		0.458[a]		0.252[c]	
ALA	0.001	0.009	0.002	0.013	-0.004	-0.026
DHA	0.044	-0.157[b]	0.030	-0.199[b]	0.073	-0.033
EPA	-0.085	0.065	-0.088	0.090	-0.086	0.016

[a] $p < 0.001$, [b] $p = 0.002$, [c] $p = 0.006$

*IL-6, CRP, ALA, DHA, EPA were logarithmically transformed

Table 14. Partial correlation coefficients of association between plasma markers of inflammation and erythrocyte fatty acids in prostate cancer cases stratified by Gleason score*

	Low Grade 3+4 (LG3+4)		High Grade 4+3 (HG4+3)		Low Grade 6 (LG6)		High Grade 7+ (HG7+)	
	IL-6	CRP	IL-6	CRP	IL-6	CRP	IL-6	CRP
IL-6								
CRP	0.219[a]		0.276		0.210		0.302[b]	
ALA	0.025	-0.044	-0.167	-0.145	0.009	-0.058	-0.003	-0.005
DHA	0.002	0.069	0.261	-0.192	0.099	-0.008	0.065	0.061
EPA	-0.039	-0.057	-0.128	0.194	-0.107	0.038	-0.085	-0.006

[a] $p = 0.04$, [b] $p = 0.02$

*IL-6, CRP, ALA, DHA, EPA were logarithmically transformed

Table 15. Goodman and Kruskal's gamma coefficients of association between ordinal categories of plasma markers of inflammation and ordered dichotomous categories of erythrocyte fatty acids in biopsy negative controls and cancer cases, as a complete cohort and stratified by cancer status*

	Entire Cohort		Biopsy Negative Controls		Prostate Cancer Cases	
	IL-6	CRP	IL-6	CRP	IL-6	CRP
IL-6						
CRP	0.671[a]		0.734[a]		0.515[a]	
ALA	-0.016	0.099	-0.042	0.277	-0.117	-0.213
DHA	-0.290[a]	-0.390[a]	-0.394[a]	-0.616[a]	-0.099	0.046
EPA	-0.161	-0.192	-0.174	-0.317[b]	-0.138	0.042

[a]p <0.001, [b]p =0.021

*IL-6 categorized into ordinal tertiles based on biopsy negative controls, CRP categorized into three ordinal categories based severity of systemic inflammation; ALA, DHA, and EPA dichotomous categories defined as having fatty acids levels below the median or above or equal to the median with median values based on biopsy negative controls

Table 16. Goodman and Kruskal's gamma coefficients of association between ordinal categories of plasma markers of inflammation and ordered dichotomous categories of erythrocyte fatty acids in prostate cancer cases stratified by Gleason score*

	Low Grade 3+4 (LG3+4)		High Grade 4+3 (HG4+3)		Low Grade 6 (LG6)		High Grade 7+ (HG7+)	
	IL-6	CRP	IL-6	CRP	IL-6	CRP	IL-6	CRP
IL-6								
CRP	0.478[a]		0.533[b]		0.423[b]		0.605[c]	
ALA	-0.134	-0.397	-0.370	-0.063	-0.244	-0.409	0.013	-0.006
DHA	-0.172	-0.084	0.136	0.313	-0.105	-0.141	-0.087	0.302
EPA	-0.085	0.202	-0.232	-0.244	-0.118	0.143	-0.175	-0.085

[a]p = 0.002, [b]p = 0.02, [c]p <0.001

*IL-6 categorized into ordinal tertiles based on biopsy negative controls, CRP categorized into three ordinal categories based severity of systemic inflammation; ALA, DHA, and EPA dichotomous categories defined as having fatty acids levels below the median or above or equal to the median with median values based on biopsy negative controls

Patient Outcome

The median follow-up time for the biopsy negative controls from the time of initial biopsy to a diagnosis of prostate cancer, death, or end of the study period, was 51.5 months with a range of 3.0 to 78.0 months (Table 17). Ten (4.0%) subjects died during the follow-up period; however, none of the deceased were diagnosed with prostate cancer prior to their death. During the follow-up period, 99 (42.3%) of the biopsy negative controls underwent repeat biopsies. Of that group, 20 (8.3%) were diagnosed with

Table 17. New incident prostate cancer and mortality in biopsy negative controls as of June 1, 2008

Outcome[a]	Controls (n = 240) n (%)
Had Repeat Biopsies	99 (41.3)
Developed Prostate Cancer[b]	17 (7.1)
Deceased	10 (4.0)
Prostate Cancer Related Death	0 (0.0)
Other Cancer Related Death	2 (0.8)
Multi-Organ Failure	3 (1.2)
Parkinson's	1 (0.4)
Unknown	4 (2.0)

[a] Median follow-up time in months (range): 51.5 (3.0 - 78.0)

[b] Includes subject who developed prostate cancer before June 1, 2008 and no less than 6 months after their initial biopsy

prostate cancer but three of the subjects were diagnosed within six months of their initial biopsy. Thus, they were not considered as a new incidence prostate cancer for this study and were deducted from the total number of new prostate cancer cases. Seventeen (7.1%) subjects met the study criteria for new incident prostate cancer.

Gleason score for the prostate cancer cases was previously collected during the DPC study. For subjects with a Gleason score of 7, their tumor pattern was determined (3+4 vs. 4+3) for the purposes of this study. Fifty-nine (48.8%) subjects had a Gleason score of 6, 30 (24.8%) subjects had a score of 7 with a tumor pattern of 3+4, 4 (3.3%) subjects had a score of 7 with a tumor pattern of 4+3, 19 (15.7%) subjects had a Gleason score of 8, and 9 (7.4%) subjects had a score of 9 (Table 18). The mortality rate was higher in the cancer cases compared to the biopsy negative controls at 11.6% (n = 14). No deaths were determined to be prostate cancer related. Every cancer case was classified as pursuing a type of primary treatment, including watchful waiting. A complete summary of primary treatments is listed in Table 18. The largest proportion (38.8%) of cancer cases underwent a RRP with the second largest proportion (19.8%) choosing watchful waiting.

Table 18. Gleason score and outcome in the prostate cancer cases as of June 1, 2008.

Outcome	Cases (n = 121) n (%)
Gleason Score[a]	
6	59 (48.8)
7 (3+4)	30 (24.8)
7 (4+3)	4 (3.3)
8	19 (15.7)
9	9 (7.4)
Primary Treatment	
Androgen Deprivation Therapy (ADT)	6 (5.0)
ADT + Orchiectomy	1 (0.8)
ADT + Radiotherapy	11 (9.1)
Brachytherapy	7 (5.8)
Radical Prostatectomy (RRP)	47 (38.8)
RRP + Chemotherapy	4 (3.3)
Radiotherapy	19 (15.7)
Watchful Waiting	24 (19.8)
Other[b]	2 (1.7)
Biochemical Recurrence[c]	
After radiotherapy	2 (1.7)
After RRP	8 (6.6)
After RRP + Chemotherapy	2 (1.7)
Deceased	14 (11.6)
Prostate Cancer Related Death	0 (0)
Other Cancer Related Death	6 (5.0)
Abdominal aortic aneurysm	1 (0.8)
Sepsis	1 (0.8)
Stroke	2 (1.7)
Other[d]	1 (0.8)
Unknown	3 (2.5)

[a] Reported as highest Gleason score found during initial biopsy

[b] One subject had proton beam therapy and one subject died immediately after his biopsy with no treatment

[c] Median time to biochemical recurrence in months (range): 25.5 (6.0 - 43.0)

[d] One subject died of sepsis, one of an abdominal aortic aneurysm, and one of refractory cardiogenic shock

Biochemical recurrence was only documented in subjects who had a RRP, with or without chemotherapy, or radiotherapy, including brachytherapy and radiotherapy combined with ADT. Thus, follow-up data was only collected on 88 cancer cases. Of these 88 cases, biochemical recurrence occurred in 2 (1.7%) subjects after radiotherapy, 8 (6.6%) subjects after RRP without chemotherapy, and 2 (1.7%) subjects after RRP with pre-operative chemotherapy. The median time to biochemical recurrence was 25.5 months with a range of 6 to 43 months.

Circulating Markers of Inflammation and Inflammation in the Prostate

The presence of inflammation in the prostate at the time of biopsy was documented in the biopsy negative controls during the DPC study. One hundred fourteen (47.5%) of the biopsy negative controls had inflammation present in the specimens from their initial biopsy (Table 19). Circulating levels of IL-6 were significantly higher in the subjects with prostate tissue inflammation (median 1.99 pg/mL) compared to the biopsy negative controls without inflammation (median 1.69 pg/mL) (p = 0.038). CRP exhibited the same pattern with a significantly higher median level of 1.7 mg/L in the subjects with prostate tissue inflammation compared to a median value of 1.2 mg/L in those without tissue inflammation (p = 0.047).

Table 19. IL-6 and CRP plasma levels in biopsy negative controls with and without prostate tissue inflammation at the time of initial biopsy

	Prostate Tissue Inflammation		
	Present (n = 114)	Not Present (n = 126)	p[a]
	median (range)		
IL-6 (pg/mL)	1.99 (0.54 - 26.3)	1.69 (0.64 - 22.7)	0.038
CRP (mg/L)	1.70 (<0.3 - 29.6)	1.20 (<0.3 - 42.6)	0.047

[a] Wilcoxon rank-sum test

Table 20. Odds of prostate tissue inflammation in biopsy negative controls

	Age-adjusted		
	No. with inflammation/ no. without inflammation (114/126)	Odds Ratio	95% Confidence Interval
IL-6 (pg/mL)			
< 1.50	26/54	1.00	Referent
1.50 < 2.33	44/35	2.61	1.37, 4.97
≥ 2.33	44/35	2.46	1.29, 4.70
p*Trend*		*0.007*	
p*Effect*		*0.004*	
CRP (mg/L)			
< 3	86/100	1.00	Referent
3 < 10	19/20	1.1	0.55, 2.20
≥ 10	9/6	1.74	0.59, 5.13
p*Trend*		*0.35*	
p*Effect*		*0.59*	

After univariate and multivariable examination of possible confounding variables, the final model for the prediction of inflammation in the prostate included age and the primary exposure variables (Table 20). There was a significant increase in risk of prostate tissue inflammation

with higher levels of IL-6. After adjusting for age, the biopsy negative controls with IL-6 levels in the highest tertile had 2.46 times the odds of having inflammation in the prostate compared to those with IL-6 values in the lowest tertile. The odds increased to 2.61 for those with IL-6 levels in the middle tertile compared to those in the lowest tertile. While not significant, there was increased risk of prostate tissue inflammation with increasing levels of CRP after adjusting for age.

Model Description for the Risk of Prostate Cancer

Age-adjusted risk of prostate cancer, including stratification by disease severity, was determined for each primary exposure variable. After univariate and multivariable analysis, the final model for the risk of prostate cancer by IL-6 exposure included age and NSAID use. The final model for the risk of low-grade cancer, defined as LG3+4 and LG6, with CRP exposure included age and the ACCI. For the risk of high-grade (HG4+3) cancer by IL-6 exposure, the final model included age and smoking status. The final model with CRP included age, smoking status and the ACCI. When high-grade cancer was defined as HG7+, the final model with CRP included age, the ACCI, and prostate volume at the time of initial biopsy.

PSA at time of initial biopsy and age were included in the final model for HG4+3 and HG7+ cancer risk with ALA exposure. Smoking status and age were included in the final model for HG4+3 cancer risk with DHA exposure. The final model with DHA included age and prostate volume at the time of initial biopsy when high-grade cancer was defined as HG7+.

All other models not specified above included only age after univariate and multivariable analyses of potential confounding variables.

Inflammation and the Risk of Prostate Cancer

There was no significant relationship between systemic markers of inflammation (IL-6 and CRP) and age-adjusted risk of prostate cancer (Table 21). While there was no significant association between prostate cancer risk and the three categories of IL-6, each group exhibited the same risk pattern across the tertiles. Prostate cancer risk appeared to decrease in the subjects with IL-6 in the middle tertile compared to subjects in the lowest IL-6 tertile. Furthermore, the risk for prostate cancer then increased in subjects with IL-6 values in the highest tertile compared to the reference group. This U-shaped change in prostate cancer risk appeared across the five stratified cohorts and may indicate a protective effect of IL-6 to a certain threshold. After that threshold, increasing levels of IL-6 lead to an increased risk in prostate cancer. There was a strong trend of increasing risk of high-grade cancer (HG4+3) with increasing levels of IL-6.

While not as strong, a similar trend was seen between CRP and the risk of more aggressive prostate cancer (HG4+3). However, after adjusting for age, smoking status and the ACCI, this trend was eliminated (Table 22). There were no other discernable patterns in the age-adjusted risk of prostate cancer for varying levels of CRP across the degrees of cancer risk (Table 21). After adjusting for age and the ACCI, the risk of low-grade (LG3+4 and LG6+) prostate cancer did not significantly change with CRP. After adjusting for age, the ACCI, and prostate volume at the time of initial biopsy, risk of more aggressive disease (HG7+) did not differ across the CRP categories (Table 22).

Table 21. Age-adjusted odds of prostate cancer in biospy negative controls vs. prostate cancer cases as a complete cohort and stratified by Gleason Score.

				Biopsy Negative Controls vs. Cancer Cases Stratified by Gleason Score											
	Biopsy Negative Controls vs. Cancer Cases			Low Grade 3+4 (LG3+4)			High Grade 4+3 (HG4+3)			Low Grade 6 (LG6)			High Grade 7+ (HG7+)		
	No. of cases/ no. of controls (121/240)	Odds Ratio	95% Confidence Interval	No. of cases/ no. of controls (89/240)	Odds Ratio	95% Confidence Interval	No. of cases/ no. of controls (32/240)	Odds Ratio	95% Confidence Interval	No. of cases/ no. of controls (59/240)	Odds Ratio	95% Confidence Interval	No. of cases/ no. of controls (62/240)	Odds Ratio	95% Confidence Interval
IL-6 (pg/mL)															
< 1.50	41/80	1.00	Referent	34/80	1.00	Referent	7/80	1.00	Referent	21/80	1.00	Referent	20/80	1.00	Referent
1.50 < 2.33	28/79	0.67	0.38, 1.19	21/79	0.61	0.33, 1.15	7/79	0.97	0.32, 2.91	10/79	0.49	0.21, 1.10	18/79	0.87	0.42, 1.77
≥ 2.33	52/81	1.18	0.70, 2.00	34/81	0.96	0.54, 1.70	18/81	2.33	0.91, 5.95	28/81	1.33	0.69, 2.56	24/81	1.08	0.55, 2.14
p*Trend*		*0.48*			*0.87*			*0.051*			*0.35*			*0.81*	
p*Effect*		*0.13*			*0.25*			*0.08*			*0.03*			*0.81*	
CRP (mg/L)															
< 3	89/186	1.00	Referent	68/186	1.00	Referent	21/186	1.00	Referent	41/186	1.00	Referent	48/186	1.00	Referent
3 < 10	25/39	1.34	0.76, 2.35	18/39	0.40	0.67, 2.34	7/39	1.58	0.62, 4.00	15/39	1.75	0.88, 3.48	10/39	0.99	0.46, 2.13
≥ 10	7/15	0.96	0.38, 2.46	7/15	0.54	0,15, 1.93	4/15	2.43	0.72, 8.18	3/15	0.93	0.26, 3.36	4/15	0.99	0.31, 3.15
p*Trend*		*0.52*			*0.78*			*0.11*			*0.40*			*0.97*	
p*Effect*		*0.59*			*0.43*			*0.81*			*0.29*			*1.00*	

Table 22. Multivariable-adjusted odds of prostate cancer by levels of CRP, in biospy negative controls vs. prostate cancer cases stratified by Gleason Score.

	Biopsy Negative Controls vs. Cancer Cases Stratified by Gleason Score											
	Low Grade 3+4 (LG3+4)[a]			High Grade 4+3 (HG4+3)[b]			Low Grade 6 (LG6)[a]			High Grade 7+ (HG7+)[c]		
	No. of cases/ no. of controls (89/240)	Odds Ratio	95% Confidence Interval	No. of cases/ no. of controls (32/240)	Odds Ratio	95% Confidence Interval	No. of cases/ no. of controls (59/240)	Odds Ratio	95% Confidence Interval	No. of cases/ no. of controls (62/240)	Odds Ratio	95% Confidence Interval
CRP (mg/L)												
< 3	68/186	1.00	Referent	21/186	1.00	Referent	41/186	1.00	Referent	48/186	1.00	Referent
3 < 10	18/39	1.05	0.54, 2.01	7/39	1.37	0.51, 3.7	15/39	1.46	0.71, 2.99	10/39	0.67	0.29, 1.53
≥ 10	7/15	0.46	0.13, 1.70	4/15	1.25	0.33, 4.74	3/15	0.79	0.21, 2.95	4/15	0.76	0.22, 2.58
p*Trend*		*0.43*			*0.59*			*0.75*			*0.40*	
p*Effect*		*0.44*			*0.81*			*0.52*			*0.59*	

[a] Adjusted for age and age-adjusted Charlson Comorbidity Index (ACCI)

[b] Adjusted for age, ACCI, and smoking status

[c] Adjusted for age, ACCI, and prostate volume at time of biopsy

The strong trend of increasing risk of high-grade (HG4+3) prostate cancer risk with increasing levels of IL-6 was diminished after adjusting for age and smoking status (Table 23). The relationship between IL-6 and prostate cancer risk was relatively unchanged in the biopsy negative controls and prostate cancer cases after adjusting for age and NSAID use.

Table 23. Multivariate-adjusted odds of prostate cancer by levels of IL-6, in biospy negative controls vs. prostate cancer cases as a complete cohort and stratified by HG4+3.

	Biopsy Negative Controls vs. Cancer Cases[a]			Biopsy Negative Controls vs. Cancer Cases Stratified by Gleason Score: High Grade 4+3 (HG4+3)[b]		
	No. of cases/ no. of controls (121/240)	Odds Ratio	95% Confidence Interval	No. of cases/ no. of controls (32/240)	Odds Ratio	95% Confidence Interval
IL-6 (pg/mL)						
< 1.50	41/80	1.00	Referent	7/80	1.00	Referent
1.50 < 2.33	28/79	0.68	0.38, 1.21	7/79	0.86	0.28, 2.64
≥ 2.33	52/81	1.21	0.72, 2.06	18/81	1.61	0.60, 4.32
p*Trend*		*0.43*			*0.27*	
p*Effect*		*0.18*			*0.38*	

[a] Adjusted for age and NSAID use

[b] Adjusted for age and smoking status

Omega-3 Fatty Acids and the Risk of Prostate Cancer

Erythrocyte levels of ALA, DHA, and EPA were not related to prostate cancer risk, after adjusting for age (Table 24). When the data was stratified by low-grade and high-grade cancer, there was a strong trend between increasing levels of ALA and a decreasing risk of low-grade (LG3+4) prostate cancer. While above the level of significance, the subjects in the highest tertile of ALA had a 48% reduction in prostate cancer risk compared to subjects with the lowest ALA levels.

Table 24. Age-adjusted odds of prostate cancer in biospy negative controls vs. prostate cancer cases as a complete cohort and stratified by Gleason Score.

		Biopsy Negative Controls vs. Cancer Cases			Biopsy Negative Controls vs. Cancer Cases Stratified by Gleason Score											
					Low Grade 3+4 (LG3+4)			High Grade 4+3 (HG4+3)			Low Grade 6 (LG6)			High Grade 7+ (HG7+)		
		No. of cases/ no. of controls (121/240)	Odds Ratio	95% Confidence Interval	No. of cases/ no. of controls (89/240)	Odds Ratio	95% Confidence Interval	No. of cases/ no. of controls (32/240)	Odds Ratio	95% Confidence Interval	No. of cases/ no. of controls (59/240)	Odds Ratio	95% Confidence Interval	No. of cases/ no. of controls (62/240)	Odds Ratio	95% Confidence Interval
ALA*																
	< 0.103	42/80	1.00	Referent	35/80	1.00	Referent	7/80	1.00	Referent	22/80	1.00	Referent	20/80	1.00	Referent
0.103	< 0.137	45/79	1.07	0.63, 1.81	35/79	1.00	0.57, 1.76	10/79	1.45	0.52, 4.02	23/79	1.06	0.55, 2.06	22/79	1.11	0.56, 2.21
	≥ 0.137	34/81	0.75	0.43, 1.30	19/81	0.52	0.27, 0.98	15/81	1.91	0.73, 5.00	14/81	0.62	0.29, 1.30	20/81	0.89	0.44, 1.81
	p*Trend*		*0.32*			*0.055*			*0.19*			*0.23*			*0.75*	
	p*Effect*		*0.40*			*0.07*			*0.38*			*0.30*			*0.82*	
DHA*																
	< 2.962	36/80	1.00	Referent	26/80	1.00	Referent	10/80	1.00	Referent	19/80	1.00	Referent	17/80	1.00	Referent
2.96	< 3.70	38/79	1.03	0.59, 1.81	25/79	0.95	0.50, 1.80	13/79	1.26	0.52, 3.08	18/79	0.98	0.47, 2.02	20/79	1.10	0.53, 2.28
	≥ 3.70	47/81	1.20	0.69, 2.07	38/81	1.40	0.77, 2.55	9/81	0.76	0.29, 2.01	22/81	1.16	0.57, 2.33	25/81	1.28	0.63, 2.59
	p*Trend*		*0.51*			*0.25*			*0.58*			*0.68*			*0.49*	
	p*Effect*		*0.78*			*0.38*			*0.55*			*0.88*			*0.79*	
EPA*																
	< 0.350	43/80	1.00	Referent	30/80	1.00	Referent	13/80	1.00	Referent	20/80	1.00	Referent	23/80	1.00	Referent
0.350	< 0.476	29/79	0.67	0.38, 1.19	22/79	0.74	0.39, 1.39	7/79	0.51	0.19, 1.36	15/79	0.76	0.36, 1.59	14/79	0.59	0.28, 1.23
	≥ 0.476	49/81	1.09	0.65, 1.84	37/81	1.20	0.67, 2.15	12/81	0.85	0.36, 2.01	24/81	1.18	0.60, 2.31	25/81	1.03	0.53, 2.00
	p*Trend*		*0.73*			*0.51*			*0.70*			*0.62*			*0.92*	
	p*Effect*		*0.20*			*0.28*			*0.37*			*0.48*			*0.24*	

*Expressed as a percent of total erythrocyte membrane fatty acids

Table 25. Multivariable-adjusted odds of prostate cancer by levels of ALA and DHA, in biospy negative controls vs. prostate cancer cases stratified by Gleason Score.

	Biopsy Negative Controls vs. Cancer Cases Stratified by Gleason Score					
	High Grade 4+3 (HG4+3)[a]			High Grade 7+ (HG7+)[b]		
	No. of cases/ no. of controls (32/240)	Odds Ratio	95% Confidence Interval	No. of cases/ no. of controls (62/240)	Odds Ratio	95% Confidence Interval
ALA*						
< 0.103	7/80	1.00	Referent	20/80	1.00	Referent
0.103 < 0.137	10/79	1.52	0.51, 4.53	22/79	1.14	0.56, 2.33
≥ 0.137	15/81	1.58	0.55, 4.54	20/81	0.73	0.34, 1.57
p*Trend*		*0.41*			*0.44*	
p*Effect*		*0.75*			*0.35*	
DHA*						
< 2.962	10/80	1.00	Referent	17/80	1.00	Referent
2.96 < 3.70	13/79	1.55	0.61, 3.93	20/79	1.52	0.71, 3.26
≥ 3.70	9/81	1.10	0.39, 3.14	25/81	1.68	0.80, 3.53
p*Trend*		*0.82*			*0.18*	
p*Effect*		*0.62*			*0.35*	

*Expressed as a percent of total erythrocyte membrane fatty acids

[a] ALA odds ratios adjusted for age and PSA at time of initial biopsy; DHA odds ratios adjusted for age and smoking status

[b] ALA odds ratios adjusted for age and PSA at time of initial biopsy; DHA odds ratios adjusted for age and prostate volume at time of initial biopsy

Similar to IL-6, it appears that there may be a U-shaped relationship between n-3 fatty acids and the risk of prostate cancer. This was particularly true for DHA and EPA. Compared to the reference group, subjects with EPA and DHA in the middle tertile had the lowest prostate cancer risk. This was seen with and without stratification by Gleason score. The exception to this observation was observed in the risk of more aggressive disease (HG4+3 and HG7+). Subjects with DHA levels in the middle tertile had the highest risk of high-grade (HG4+3) prostate cancer compared to the reference group. A similar relationship persisted after adjusting for age and smoking status (Table 25). When high-grade cancer was categorized as HG7+, the risk of prostate cancer increased as DHA levels increased. This trend was strengthened after adjusting for age and prostate volume at the time of initial biopsy.

Inflammation, Omega-3 Fatty Acids, and the Risk of Prostate Cancer

When subjects were stratified by median ALA, DHA, and EPA levels, the risk of prostate cancer was significantly associated with systemic inflammation. Median n-3 fatty acid levels were based on the biopsy negative controls and were 0.12, 3.37, and 0.41 for ALA, DHA, and EPA, respectively. Subjects with n-3 fatty acids below the median and IL-6 in the lowest tertile or CRP levels in the lowest category were used as the reference group. Before stratification by Gleason score, IL-6 did not have any significant relationship with prostate cancer risk at any n-3 fatty acid level (Table 26). This continued to be true after stratifying by severity of disease (Table 27, 28, 29, 30). It was noted that the risk of prostate cancer exhibited a U-shaped pattern in a majority of the IL-6 tertiles when stratified by median n-3 fatty acid levels. There did not appear to be any pattern between IL-6 and cancer risk when comparisons were made between subjects with levels above the median and subjects with levels below the median for ALA, DHA, or EPA. After adjusting for age, subjects with ALA levels below the median and CRP levels in the middle category had a significant increased risk of prostate cancer compared to the reference group with 2.24 times of the odds of prostate cancer (Table 26). Subjects with CRP levels in the same category but DHA levels above or equal to the median also had a significant increased risk of prostate cancer with 3.56 times the odds of cancer compared to those with the lowest CRP levels and DHA below the median. While not significant, the same association was observed between CRP, DHA, and less aggressive disease (LG3+4) after adjusting for age and the ACCI (Table 27). Subjects with CRP levels in the middle category and DHA levels above or equal to the median also had a significant increased risk of aggressive disease (HG4+3) with 5.63 times the odds of the reference

Table 26. Odds of prostate cancer by levels of IL-6 and CRP in biospy negative controls vs. prostate cancer cases stratified by median erythrocyte omega-3 fatty acids.

	ALA				DHA				EPA			
	< Median		≥ Median		< Median		≥ Median		< Median		≥ Median	
	Odds Ratio	95% Confidence Interval	Odds Ratio	95% Confidence Interval	Odds Ratio	95% Confidence Interval	Odds Ratio	95% Confidence Interval	Odds Ratio	95% Confidence Interval	Odds Ratio	95% Confidence Interval
IL-6 (pg/mL)[a]												
< 1.50	1.00	Referent	0.85	0.39, 1.82	1.00	Referent	0.73	0.33, 1.58	1.00	Referent	0.79	0.37, 1.69
1.50 < 2.33	0.62	0.29, 1.36	0.61	0.25, 1.47	0.49	0.20, 1.22	0.61	0.27, 1.40	0.55	0.24, 1.29	0.63	0.28, 1.44
≥ 2.33	1.54	0.74, 3.21	0.77	0.35, 1.66	0.77	0.36, 1.66	1.49	0.65, 3.45	1.11	0.54, 1.28	1.01	0.46, 2.23
p*INT**	*0.44*				*0.20*				*0.81*			
CRP (mg/L)[b]												
< 3	1.00	Referent	0.94	0.57, 1.57	1.00	Referent	0.80	0.48, 1.34	1.00	Referent	0.76	0.46, 1.26
3 < 10	2.24[c]	1.04, 4.84	0.68	0.28, 1.64	0.64	0.29, 1.41	3.56[d]	1.32, 9.58	0.93	0.43, 2.04	1.52	0.66, 3.48
≥ 10	1.64	0.42, 6.44	0.60	0.16, 2.29	0.55	0.14, 2.12	1.42	0.36, 5.61	0.73	0.22, 2.47	1.05	0.24, 4.63
p*INT*	*0.12*				*0.005*				*0.37*			

[a] Adjusted for age and NSAID use

[b] Adjusted for age

[c] Significantly different from reference group (Wald Test*p*-value = 0.04)

[d] Significantly different from reference group (Wald Test*p*-value = 0.012)

*Likelihood Ratio Test for Interaction

group (Table 28). Interestingly, when high-grade cancer was defined as HG7+, there was an 85% reduction in risk of prostate cancer in subjects with CRP levels in the middle category and DHA levels below the median (Table 30). The increased risk of aggressive disease (HG7+) was no longer significant for subjects with the same CRP levels but DHA levels above or equal to the median even though the risk of cancer remained elevated. There was an overall significant interaction between CRP and DHA and the risk of high-grade (HG 4+3 and HG7+) cancer with DHA levels below the median showing a protective effect against HG7+ cancer for those with CRP levels in the middle and highest categories. The risk of low-grade cancer, defined as LG3+4 and LG6, was associated with a significant interaction between CRP and ALA (Table 27 and 29). While there was no significant difference for any sub-group, subjects with ALA levels below the median appeared at greater risk of less aggressive cancer when they had CRP levels in the middle or highest categories. The significant interaction between CRP and ALA was also observed with the risk of HG4+3 cancer, but not distinct pattern of risk could be determined (Table 28).

Table 27. Odds of prostate cancer by levels of IL-6 and CRP in biospy negative controls vs. Low Grade 3+4 prostate cancer cases stratified by median erythrocyte omega-3 fatty acids.

	ALA				DHA				EPA			
	< Median		≥ Median		< Median		≥ Median		< Median		≥ Median	
	Odds Ratio	95% Confidence Interval	Odds Ratio	95% Confidence Interval	Odds Ratio	95% Confidence Interval	Odds Ratio	95% Confidence Interval	Odds Ratio	95% Confidence Interval	Odds Ratio	95% Confidence Interval
IL-6 (pg/mL)[a]												
< 1.50	1.00	Referent	0.68	0.30, 1.52	1.00	Referent	0.93	0.40, 2.16	1.00	Referent	0.89	0.39, 1.97
1.50 < 2.33	0.59	0.26, 1.32	0.37	0.13, 1.04	0.57	0.21, 1.54	0.60	0.24, 1.52	0.55	0.22, 1.38	0.59	0.24, 1.45
≥ 2.33	1.20	0.55, 2.59	0.47	0.20, 1.11	0.69	0.29, 1.63	1.42	0.56, 3.63	0.85	0.38, 1.90	0.95	0.40, 2.23
p*INT**	*0.62*				*0.37*				*0.91*			
CRP (mg/L)[b]												
< 3	1.00	Referent	0.76	0.43, 1.35	1.00	Referent	1.01	0.57, 1.81	1.00	Referent	0.85	0.48, 1.49
3 < 10	1.55	0.66, 3.68	0.45	0.16, 1.31	0.64	0.27, 1.54	3.00	0.98, 9.16	0.77	0.32, 1.87	1.29	0.51, 3.31
≥ 10	1.47	0.33, 6.58	*nd*		0.23	0.03, 1.95	0.92	0.16, 5.16	*nd*		*nd*	
p*INT*	*0.03*				*0.07*				*0.05*			

[a] Adjusted for age

[b] Adjusted for age and age-adjusted Charlson Comorbidity Index (ACCI)

*Likelihood Ratio Test for Interaction

nd - Inadequate date to provide an estimate of odds ratios

Table 28. Odds of prostate cancer by levels of IL-6 and CRP in biospy negative controls vs. High Grade 4+3 prostate cancer cases stratified by median erythrocyte omega-3 fatty acids.

	ALA				DHA				EPA			
	< Median		≥ Median		< Median		≥ Median		< Median		≥ Median	
	Odds Ratio	95% Confidence Interval	Odds Ratio	95% Confidence Interval	Odds Ratio	95% Confidence Interval	Odds Ratio	95% Confidence Interval	Odds Ratio	95% Confidence Interval	Odds Ratio	95% Confidence Interval
IL-6 (pg/mL)[a]												
< 1.50	1.00	Referent	2.15	0.38, 12.1	1.00	Referent	0.25	0.04, 1.47	1.00	Referent	0.53	0.11, 2.61
1.50 < 2.33	0.71	0.09, 5.47	2.27	0.39, 13.1	0.25	0.04, 1.47	0.67	0.17, 2.72	0.51	0.10, 2.52	0.75	0.17, 3.35
≥ 2.33	3.23	0.62, 16.7	2.16	0.41, 11.3	0.63	0.18, 2.22	1.34	0.37, 4.89	1.42	0.40, 5.10	0.83	0.20, 3.48
p*INT**	*0.24*				*0.07*				*0.59*			
CRP (mg/L)[b]												
< 3	1.00	Referent	1.39	0.52, 3.75	1.00	Referent	0.70	0.25, 1.93	1.00	Referent	0.63	0.24, 1.67
3 < 10	2.19	0.53, 9.10	1.28	0.30, 5.50	0.34	0.07, 1.75	5.63[c]	1.33, 23.8	0.69	0.16, 2.92	1.74	0.46, 6.55
≥ 10	1.26	0.12, 13.1	1.69	0.31, 9.12	0.48	0.08, 2.98	2.49	0.40, 15.7	1.64	0.38, 7.18	*nd*	
p*INT*	*0.69*				*0.01*				*0.08*			

[a] Adjusted for age and smoking status

[b] Adjusted for age, age-adjusted Charlson Comorbidity Index (ACCI), and smoking status

[c] Significantly different from reference group (Wald Test*p*-value = 0.019)

*Likelihood Ratio Test for Interaction

nd - Inadequate date to provide an estimate of odds ratios

Table 29. Odds of prostate cancer by levels of IL-6 and CRP in biospy negative controls vs. Low Grade 6 prostate cancer cases stratified by median erythrocyte omega-3 fatty acids.

	ALA				DHA				EPA			
	< Median		≥ Median		< Median		≥ Median		< Median		≥ Median	
	Odds Ratio	95% Confidence Interval	Odds Ratio	95% Confidence Interval	Odds Ratio	95% Confidence Interval	Odds Ratio	95% Confidence Interval	Odds Ratio	95% Confidence Interval	Odds Ratio	95% Confidence Interval
IL-6 (pg/mL)[a]												
< 1.50	1.00	Referent	0.95	0.36, 2.49	1.00	Referent	0.68	0.26, 1.83	1.00	Referent	0.85	0.32, 2.23
1.50 < 2.33	0.54	0.19, 1.56	0.36	0.09, 1.42	0.35	0.10, 1.25	0.42	0.14, 1.30	0.27	0.07, 1.07	0.61	0.21, 1.78
≥ 2.33	1.90	0.77, 4.69	0.79	0.29, 2.15	0.84	0.33, 2.13	1.59	0.57, 4.46	1.14	0.45, 2.85	1.33	0.51, 3.48
p*INT**	*0.47*				*0.32*				*0.52*			
CRP (mg/L)[b]												
< 3	1.00	Referent	0.90	0.45, 1.80	1.00	Referent	0.89	0.44, 1.80	1.00	Referent	0.90	0.46, 1.79
3 < 10	2.08	0.77, 5.49	0.82	0.27, 2.47	1.00	0.40, 2.51	3.16	0.91, 11.0	1.19	0.45, 3.13	1.72	0.60, 4.92
≥ 10	2.56	0.56, 11.7	*nd*		0.37	0.04, 3.13	1.47	0.25, 8.54	*nd*		*nd*	
p*INT*	*0.04*				*0.18*				*0.07*			

[a] Adjusted for age

[b] Adjusted for age and age-adjusted Charlson Comorbidity Index (ACCI)

*Likelihood Ratio Test for Interaction

nd - Inadequate date to provide an estimate of odds ratios

Table 30. Odds of prostate cancer by levels of IL-6 and CRP in biospy negative controls vs. High Grade 7+ prostate cancer cases stratified by median erythrocyte omega-3 fatty acids.

	ALA				DHA				EPA			
	< Median		≥ Median		< Median		≥ Median		< Median		≥ Median	
	Odds Ratio	95% Confidence Interval	Odds Ratio	95% Confidence Interval	Odds Ratio	95% Confidence Interval	Odds Ratio	95% Confidence Interval	Odds Ratio	95% Confidence Interval	Odds Ratio	95% Confidence Interval
IL-6 (pg/mL)[a]												
< 1.50	1.00	Referent	0.65	0.24, 1.76	1.00	Referent	0.75	0.27, 2.10	1.00	Referent	0.77	0.28, 2.06
1.50 < 2.33	0.64	0.24, 1.68	0.78	0.28, 2.23	0.67	0.21, 2.09	0.77	0.27, 2.20	0.85	0.31, 2.31	0.65	0.23, 1.86
≥ 2.33	1.17	0.46, 3.00	0.65	0.25, 1.72	0.63	0.22, 1.78	1.47	0.51, 4.26	1.07	0.43, 2.71	0.76	0.27, 2.15
p*INT**	*0.51*				*0.27*				*0.99*			
CRP (mg/L)[b]												
< 3	1.00	Referent	0.85	0.43, 1.68	1.00	Referent	1.02	0.50, 2.07	1.00	Referent	0.75	0.37, 1.48
3 < 10	1.23	0.42, 3.66	0.28	0.07, 1.09	0.15[c]	0.03, 0.72	3.49	0.99, 12.4	0.38	0.11, 1.32	0.88	0.29, 2.73
≥ 10	0.62	0.06, 6.15	0.77	0.18, 3.27	0.41	0.08, 2.17	2.18	0.33, 14.2	0.96	0.26, 3.61	*nd*	
p*INT*	*0.29*				*0.002*				*0.14*			

[a] Adjusted for age

[b] Adjusted for age, age-adjusted Charlson Comorbidity Index (ACCI), and prostate volume at time of initial biopsy

[c] Significantly different from reference group (Wald Test *p*-value = 0.018)

*Likelihood Ratio Test for Interaction

nd - Inadequate date to provide an estimate of odds ratios

Model Description for the Development of Prostate Cancer

Age-adjusted hazard ratios for the risk of developing prostate cancer were determined for each primary exposure variable. After univariate and multivariable analysis, the final model for the risk of developing prostate cancer included age and the ACCI with IL-6 as the primary exposure variable. When CRP was the primary exposure variable, the final model included age and NSAID use. For the risk of developing prostate cancer with inflammation of the prostate at the time of initial biopsy being the primary exposure variable, the final model included age, and PSA and prostate volume at the time of biopsy. The final model with ALA as the primary exposure variable included age and the average daily consumption of meat products from red meat, poultry and fish (oz). The final model for DHA included age and the average daily consumption of caffeine (mg). After univariate and multivariable analyses of potential confounding variables, the final model with EPA as the primary exposure variable only included age.

Inflammation and the Development of Prostate Cancer

As stated previously, 240 subjects were followed for a median of 51.5 months. During the follow-up period, 20 subjects developed cancer. Three of the subjects were diagnosed with cancer within six months of their initial biopsy and they were excluded from analysis. One subject died within three months of his initial biopsy and his contribution to the follow-up data was also excluded. Kaplan-Meier (KM) survival curves were calculated to assess the relationship between inflammation and patient survival, defined as being cancer free at the end of the follow-up period.

The KM survival curves were not significantly different between tertiles of IL-6 and CRP (Figure 5 and 6). This was also true for the KM curves of subjects with and without inflammation of the prostate

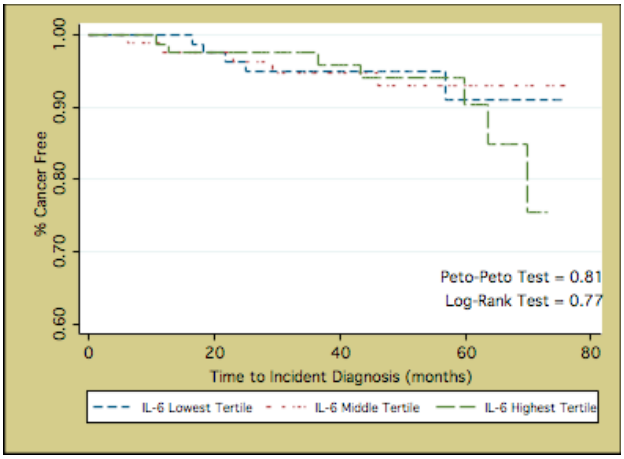

Figure 5. Kaplan-Meier estimates of prostate cancer-free survival functions by tertiles of IL-6.

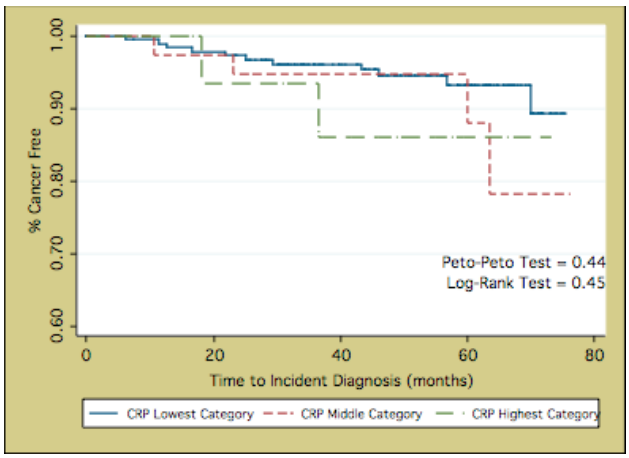

Figure 6. Kaplan-Meier estimates of prostate cancer-free survival functions by categories of CRP.

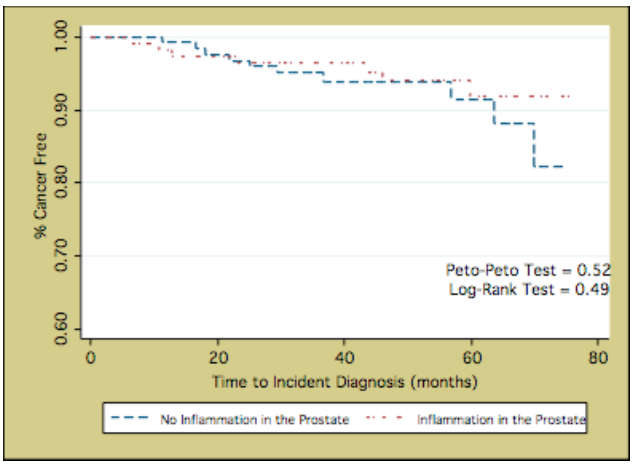

Figure 7. Kaplan-Meier estimates of prostate cancer-free survival functions by the presence of inflammation in the prostate.

at the time of initial biopsy (Figure 7). It did appear that subjects who had IL-6 levels in the lowest and middle tertile were at lower risk of developing prostate cancer compared to subjects with IL-6 levels in the highest tertile. Those subjects with inflammation in the prostate at the time of initial biopsy also appeared to be at lower risk for developing prostate cancer compared to those who did not have inflammation in the prostate.

After adjusting for age, there was no significant difference in risk of developing prostate cancer and levels of IL-6 (Table 31). Compared to the reference group, subjects with IL-6 levels in the middle were at the lowest risk of developing prostate cancer with a hazard ratio of 0.89. The hazard ratio increased to 1.36 for those with levels of IL-6 in the highest tertile. The same pattern was seen after adjusting for age and the ACCI.

As with IL-6, the risk of developing prostate cancer did not significantly differ

Table 31. Hazard ratios of developing prostate cancer by markers of systemic inflammation and prostate tissue inflammation

			Age-Adjusted		Multivariable-Adjusted[a]	
		No. of failures/ No. of censored (17/219)	Hazard Ratio	95% Confidence Interval	Hazard Ratio	95% Confidence Interval
IL-6 (pg/mL)						
	< 1.50	5/73	1.00	Referent	1.00	Referent
	1.50 < 2.33	5/74	0.89	0.26, 3.08	0.68	0.19, 2.41
	≥ 2.33	7/72	1.36	0.42, 4.38	1.04	0.32, 3.44
	p*Trend*		*0.59*		*0.89*	
	p*Effect*		*0.75*		*0.74*	
CRP (mg/L)						
	< 3	11/172	1.00	Referent	1.00	Referent
	3 < 10	4/34	1.71	0.54, 5.37	1.82	0.57, 5.82
	≥ 10	2/13	2.18	0.47, 9.63	2.83	0.62, 12.9
	p*Trend*		*0.23*		*0.12*	
	p*Effect*		*0.50*		*0.35*	
Prostate Tissue Inflammation						
	Not Present	10/114	1.00	Referent	1.00	Referent
	Present	7/105	0.71	0.27, 1.87	0.58	0.21, 1.59
	p*Trend*		*0.49*		*0.29*	
	p*Effect*		*0.48*		*0.29*	

[a] IL-6 hazard ratios adjusted for age and age-adjusted Charlson Comorbidity Index (ACCI); CRP hazard ratios adjusted for age and NSAID use; Prostate tissue inflammation hazard ratios adjusted for age, and PSA and prostate volume at the time of initial biopsy

with levels of CRP (Table 31). Unlike the U-shaped pattern seen with IL-6, the age-adjusted hazard ratio and multivariable-adjusted hazard ratio increased with increasing levels of CRP. Subjects with CRP levels in the highest category had the greatest risk for developing prostate cancer compared to those with the lowest CRP levels.

Interestingly, after adjusting for age, subjects were at lower risk of developing prostate cancer if they had inflammation in the prostate present at the time of initial biopsy compared to those who did not (Table 31). This continued to be true after adjusting for age, the ACCI, and PSA and prostate volume at the time of initial biopsy. Again, the difference in risk for those with inflammation in the prostate and those without inflammation in the prostate was not significantly different.

Omega-3 Fatty Acids and the Development of Prostate Cancer

The relationship between n-3 fatty acids and the development of prostate cancer was first examined with KM curves. There was no significant difference in KM curves between tertiles of ALA or DHA (Figure 8 and 9). There was a significant difference in the KM curves when the risk of developing prostate cancer was examined by tertiles of EPA (Figure 10). Subjects in with EPA levels in the middle tertile appeared to be at higher risk for developing prostate cancer compared to subjects in the lowest and highest tertiles. When the KM curve for subjects in the middle tertile for EPA was compared to the curve for those with EPA values in the lowest tertile, there was a significant difference (log-rank test p-value = 0.02). However, when the KM curve for those in the middle tertile was compared to those in the highest tertile, there was no significant difference between the estimated survival functions (log-rank test p-value = 0.13). There was also no

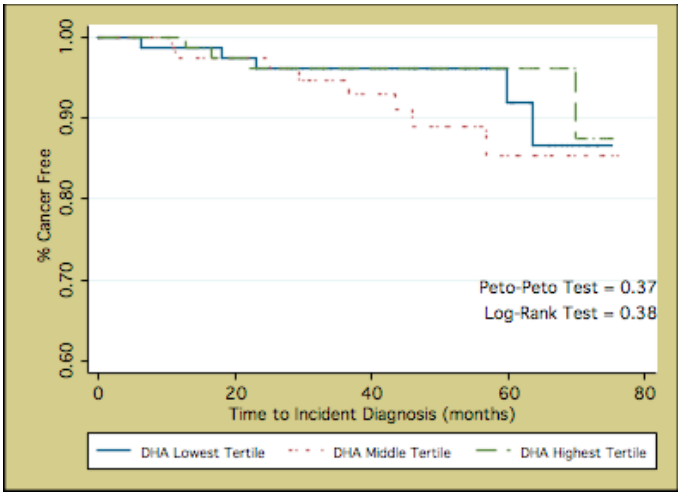

Figure 8. Kaplan-Meier estimates of prostate cancer-free survival functions by levels of DHA.

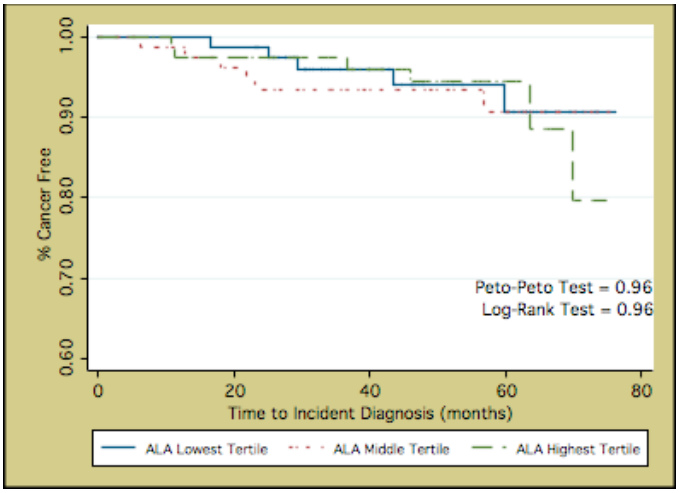

Figure 9. Kaplan-Meier estimates of prostate cancer-free survival functions by levels of ALA.

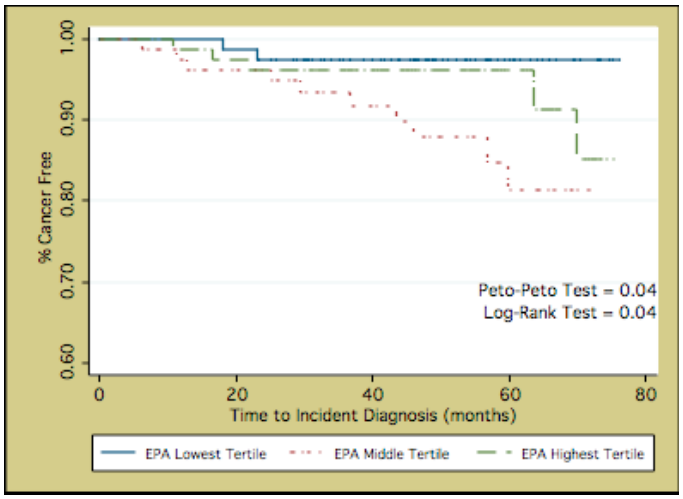

Figure 10. Kaplan-Meier estimates of prostate cancer-free survival functions by levels of EPA.

significant difference between those in the highest tertile and those in the lowest tertile (log-rank test p-value = 0.29).

After adjusting for age, subjects with EPA levels in the middle tertile had a significantly higher risk of developing prostate cancer compared to those in the lowest tertile (Table 32). The estimated hazard was markedly higher at 4.96 compared to the reference group. The risk of developing decreased for subjects with EPA levels in the highest tertile. While not significant, a similar pattern of risk was seen across the tertiles for DHA and ALA after adjusting for age and in the multivariable analysis for DHA. After adjusting for age and the average daily

consumption of meat products from red meat, poultry and fish (in ounces), the risk for developing prostate cancer increased with increasing levels of ALA. Again, the difference in hazard ratios was not significantly different.

Table 32. Hazard ratios ofdeveloping prostate cancer by levels of erythrocyte n-3 fatty acids expressed as a percent of total membrane fatty acids.

	No. of failures/ No. of censored (17/219)	Age-Adjusted Hazard Ratio	Age-Adjusted 95% Confidence Interval	Multivariable-Adjusted[a] Hazard Ratio	Multivariable-Adjusted[a] 95% Confidence Interval
ALA					
< 0.103	5/75	1.00	Referent	1.00	Referent
0.103 < 0.137	6/70	1.15	0.35, 3.76	1.15	0.35, 3.78
≥ 0.137	6/74	1.13	0.34, 3.81	1.37	0.40, 4.68
p*Trend*		*0.84*		*0.61*	
p*Effect*		*0.97*		*0.89*	
DHA					
< 2.962	5/73	1.00	Referent	1.00	Referent
2.96 < 3.70	8/70	1.46	0.47, 4.54	1.36	0.44, 4.26
≥ 3.70	4/76	0.64	0.17, 2.43	0.51	0.13, 1.97
p*Trend*		*0.51*		*0.31*	
p*Effect*		*0.39*		*0.26*	
EPA					
< 0.350	2/75	1.00	Referent	n/a	
0.350 < 0.476	10/68	4.96[b]	1.08, 22.7	n/a	
≥ 0.476	5/74	2.31	*0.45, 12.0*	n/a	
p*Trend*		*0.45*			
p*Effect*		*0.05*			

[a] ALA hazard ratios adjusted for age and the average daily consumption of meat products from red meat, poultry and fish (oz); DHA hazard ratios adjusted for age and average daily intake of caffeine (mg); EPA hazard ratios adjusted for age only

[b] Significantly different from the reference group (Wald test *p-value* = 0.039)

Inflammation, Omega-3 Fatty Acids and the Relative Risk of Prostate Cancer

KM curves were used to investigate the relationship between inflammation, n-3 fatty acids, and the risk of developing prostate cancer. Subjects were stratified into six categories for IL-6 and CRP defined by their inflammatory marker category and whether they were above or below the median for ALA, DHA, or EPA. For prostate tissue

inflammation, subjects were grouped by being with or without tissue inflammation and being above or below the median for the fatty acids.

Risk for developing prostate cancer appeared to be higher for subjects with IL-6 levels in the highest tertile and fatty acid levels above or equal to the median. This difference in risk was not significant between the KM curves for ALA and DHA (Figure 11 and 12). However, there was a significant difference when subjects were stratified by IL-6 and EPA (Figure 13). Those with IL-6 levels in the highest tertile and EPA levels above or equal to the median appeared to be at greater risk for developing prostate cancer. When the KM curve for this category was compared to the other curves, there was only a significant difference between those with IL-6 levels in the highest tertile and EPA levels above or equal to the median and the subjects with the same IL-6 levels but EPA levels below the median (log-rank test p-value = 0.002).

The KM curves for CRP did not show any significant differences when stratified by ALA, DHA, or EPA (Figure 14, 15, 16). This finding was also true for the KM curves for prostate tissue inflammation and the n-3 fatty acids (Figure 17, 18, 19). The most noticeable differences between the KM curves were found when CRP and prostate tissue inflammation were stratified by EPA (Figure 16 and 19). Subjects with EPA levels above or equal to the median exhibited the poorest outcome across all markers of inflammation.

After adjusting for age and the ACCI, there was only a significant interaction between IL-6 and EPA and the risk of developing prostate cancer (Table 33). The risk of developing prostate cancer was the lowest in subjects with IL-6 levels in the middle tertile and EPA levels below the median. Compared to the reference group, subjects with

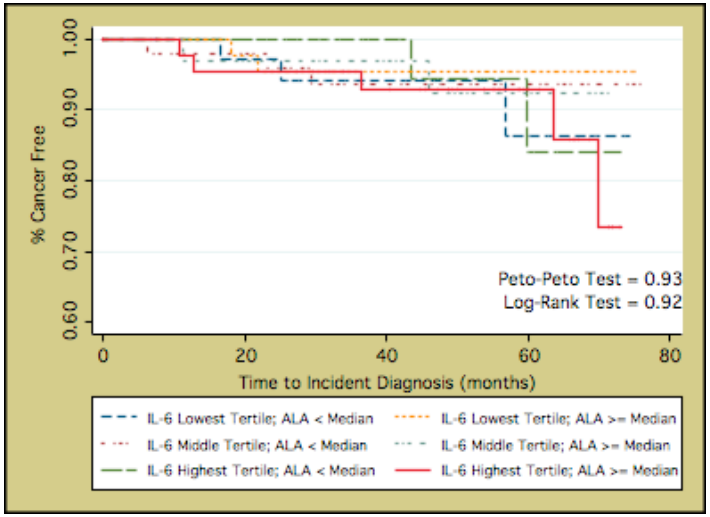

Figure 11. Kaplan-Meier estimates of prostate cancer-free survival functions by levels of IL-6 and ALA.

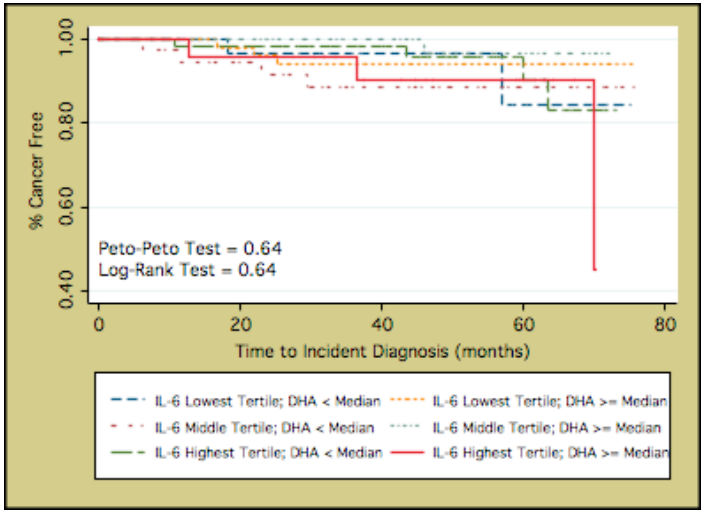

Figure 12. Kaplan-Meier estimates of prostate cancer-free survival functions by levels of IL-6 and DHA.

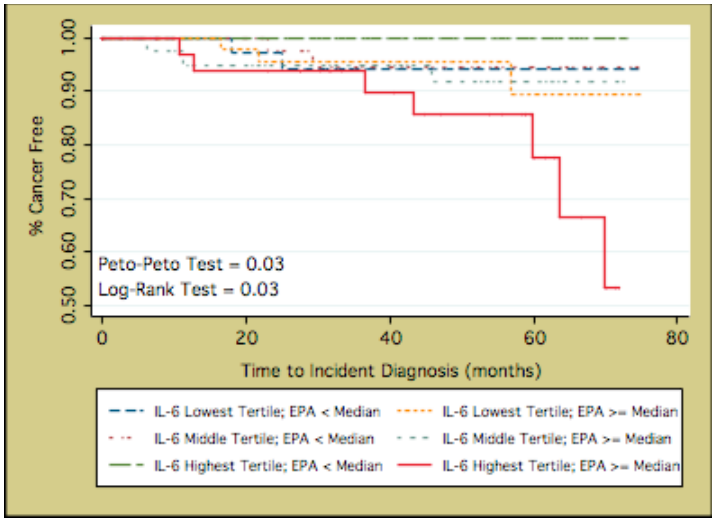

Figure 13. Kaplan-Meier estimates of prostate cancer-free survival functions by levels of IL-6 and EPA.

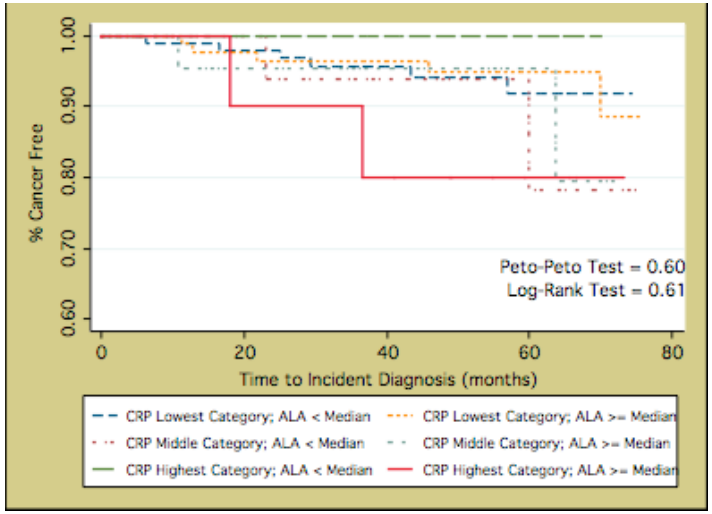

Figure 14. Kaplan-Meier estimates of prostate cancer-free survival functions by levels of CRP and ALA.

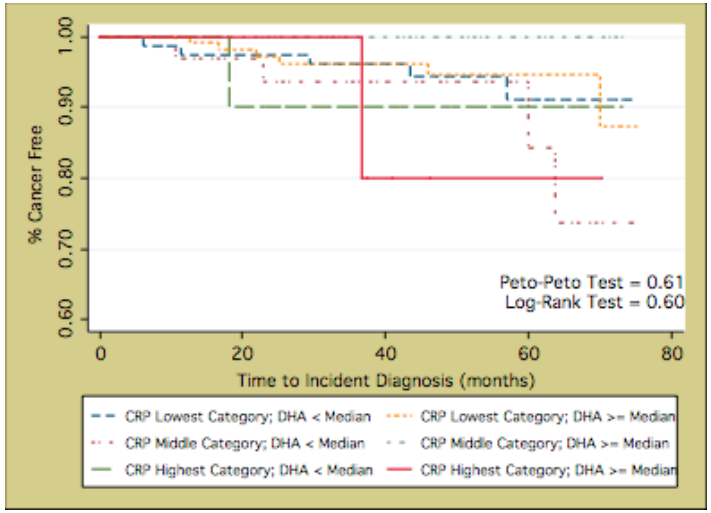

Figure 15. Kaplan-Meier estimates of prostate cancer-free survival functions by levels of CRP and DHA.

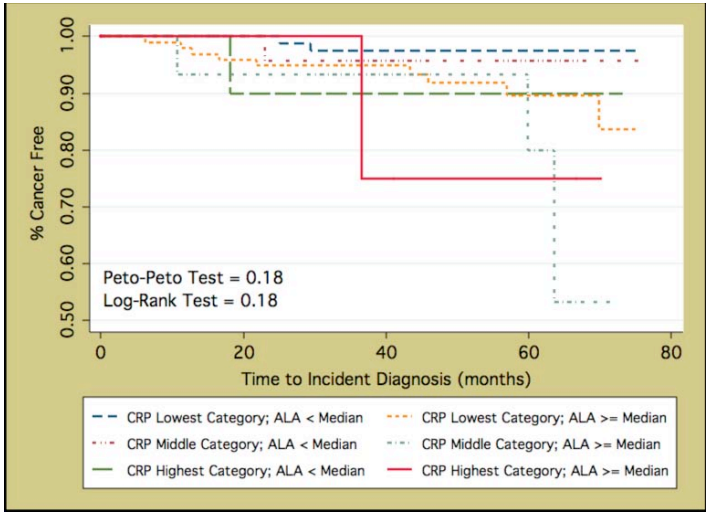

Figure 16. Kaplan-Meier estimates of prostate cancer-free survival functions by levels of CRP and EPA.

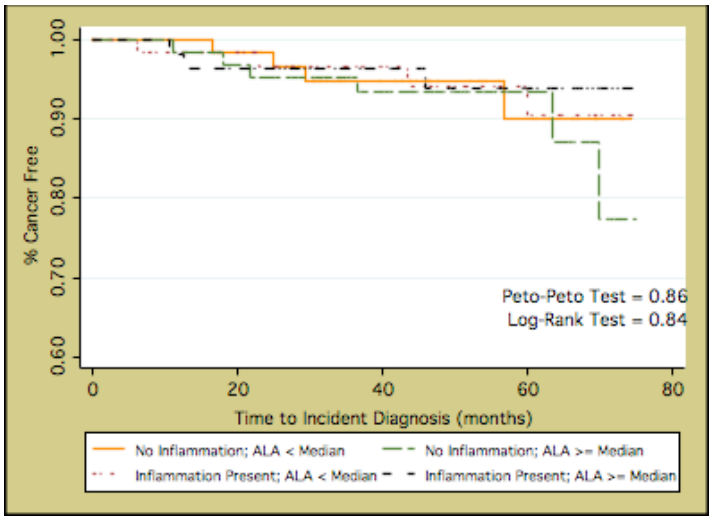

Figure 17. Kaplan-Meier estimates of prostate cancer-free survival functions by prostate tissue inflammation and ALA.

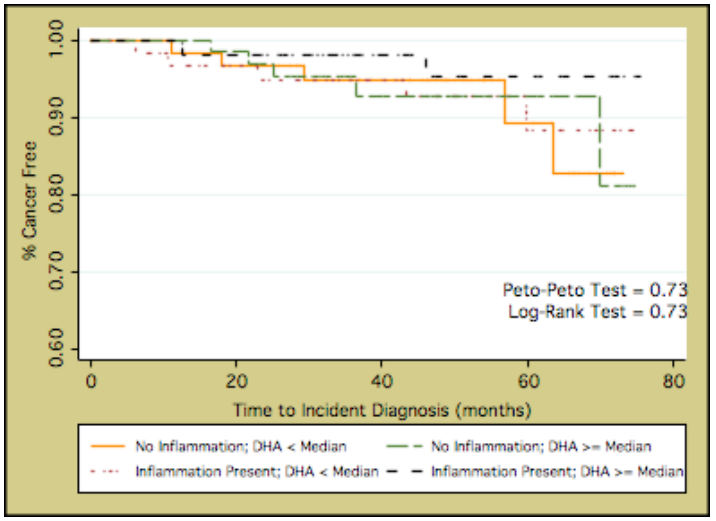

Figure 18. Kaplan-Meier estimates of prostate cancer-free survival functions by prostate tissue inflammation and DHA.

EPA levels above or equal to the median were at higher risk for developing prostate cancer. As stated above, the KM curve for IL-6 and EPA showed that those subjects with IL-6 in the highest tertile and EPA levels above or equal to the median had to poorest outcome. Unfortunately, there was insufficient data to develop a hazard ratio for this category as well as for those with the same IL-6 levels but EPA levels below the median.

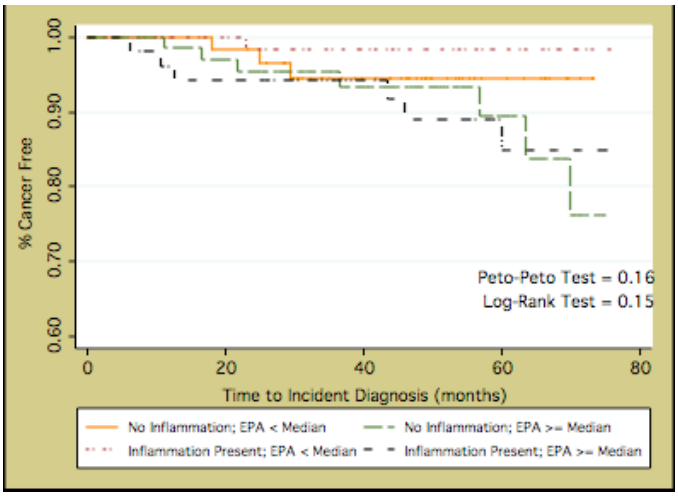

Figure 19. Kaplan-Meier estimates of prostate cancer-free survival functions by prostate tissue inflammation and EPA.

EPA levels above or equal to the median significantly increased the risk of developing prostate cancer when evaluated with CRP. After adjusting for age and NSAID use, there was a significant increased risk of developing prostate cancer for subjects with EPA level above or equal to the median and CRP levels in the middle and highest categories. Compared to the reference group, the risk for developing prostate cancer was the greatest for those with CRP levels in the highest category, with a hazard ratio of 26.6. The hazard ratio decreased to 9.05 for those with CRP levels in the middle category. It must be noted that the 95% confidence intervals for these estimates was extremely wide and the estimated hazard ratios should be reported with caution. There was no significant interaction between CRP, IL-6, ALA and DHA. The presence or lack of inflammation in the prostate was not significantly associated with any omega-3 fatty acids. However, it can be noted that the risk for developing prostate cancer was the

greatest in subjects without inflammation in the prostate and omega-3 fatty acid levels above the median. As with the other markers of inflammation, the highest risk of developing cancer was associated with EPA.

Table 33.Hazard ratios of developing prostate cancer stratified by systemic and prostate tissue inflammation and median erythrocyte omega-3 fatty acids.

	ALA				DHA				EPA			
	< Median		≥ Median		< Median		≥ Median		< Median		≥ Median	
	Hazard Ratio	95% Confidence Interval	Hazard Ratio	95% Confidence Interval	Hazard Ratio	95% Confidence Interval	Hazard Ratio	95% Confidence Interval	Hazard Ratio	95% Confidence Interval	Hazard Ratio	95% Confidence Interval
IL-6 (pg/mL)[a]												
< 1.50	1.00	Referent	0.45	0.07, 2.75	1.00	Referent	0.79	0.12, 5.16	1.00	Referent	0.94	0.15, 5.71
1.50 < 2.33	0.43	0.09, 2.21	0.51	0.08, 3.16	1.06	0.19, 5.99	0.21	0.02, 2.46	0.50	0.07, 3.66	0.85	0.14, 5.24
≥ 2.33	0.54	0.09, 3.29	0.82	0.18, 3.78	0.75	0.13, 4.25	1.26	0.19, 8.15	*nd*		*nd*	
p*INT**	*0.59*				*0.26*				*0.04*			
CRP (mg/L)[b]												
< 3	1.00	Referent	0.86	0.26, 2.87	1.00	Referent	0.84	0.25, 2.77	1.00	Referent	3.77	0.81, 17.5
3 < 10	2.13	0.40, 11.4	1.41	0.28, 7.16	2.19	0.58, 8.35	*nd*		1.77	0.16, 19.7	9.05[d]	1.47, 55.6
≥ 10	*nd*		*nd*		1.56	0.18, 13.4	7.64	0.84, 69.9	4.06	0.37, 45.0	26.6[e]	2.28, 309.3
p*INT*	*0.59*				*0.20*				*0.94*			
Prostate Tissue Inflammation[c]												
Not Present	1.00	Referent	0.91	0.24, 3.50	1.00	Referent	1.06	0.28, 3.92	1.00	Referent	1.82	0.44, 7.48
Present	0.65	0.15, 2.79	0.48	0.09, 2.24	0.73	0.20, 2.71	0.42	0.08, 2.28	0.22	0.02, 2.27	1.57	0.37, 6.75
p*INT*	*0.78*				*0.56*				*0.27*			

[a] Adjusted for age and age-adjusted Charlson Comorbidity Index (ACCI)
[b] Adjusted for age, ACCI, NSAID use, and average daily consumption of meat from meat, poultry and fish (oz)
[c] Adjusted for age, ACCI, and PSA and prostate volume at time of initial biopsy
[d] Significantly different from reference group (Wald Test*p*-value = 0.017)
[e] Significantly different from reference group (Wald Test*p*-value = 0.009)
*Likelihood Ratio Test for interaction
nd - Inadequate date to provide an estimate of hazard ratio

DISCUSSION

Summary of Significant Findings

The subject population for this study was relatively homogenous. There was no significant difference in age, race, BMI, medication use, or a majority of the prostate cancer risk factors between the biopsy negative controls and cancer cases. There was a significant difference for level of education, the ACCI, smoking status, and prostate volume. Overall, the biopsy negative controls attained a higher level of education compared to the cancer cases. The cancer cases suffered from more co-morbidities compared to the biopsy negative controls with a higher proportion of cancer cases in the highest ACCI category. There were also a greater number of current smokers among the cancer cases and more non-smokers among the biopsy negative controls. Similar to other studies, prostate volume at the time of initial biopsy was higher in the biopsy negative controls compared to the cancer cases (58, 59).

There was minimal difference in the primary exposure variables between the biopsy negative controls and cancer cases. The only significant differences occurred when the cancer cases were stratified by disease severity. ALA was significantly higher in the subjects with LG3+4 cancer compared to controls. Subjects with high-grade cancer, defined as HG4+3, had significantly higher levels of IL-6 compared to the controls with a median value of 2.52 pg/mL. When the biopsy negative controls were stratified by the presence of inflammation in the prostate at the time of initial biopsy, CRP and IL-6 were significantly higher in the controls with inflammation in the prostate. Circulating levels of IL-6 were also related to the risk of prostate inflammation. After

adjusting for age, controls with elevated IL-6 levels were at a significantly higher risk of having prostate tissue inflammation with the highest risk occurring in controls with IL-6 levels in the middle tertile (OR: 2.61, 95% CI: 1.37 - 4.97).

When the relationship between IL-6, CRP, ALA, DHA, and EPA was examined, there was a significant inverse correlation between IL-6 and EPA, and CRP and DHA. Interestingly, when the relationship between the primary exposure variables was examined in the controls and cancer cases independently, significant correlations were only found in the biopsy negative controls. In addition to the significant inverse correlation between IL-6 and EPA, the controls exhibited a significant inverse relationship between IL-6 and DHA. CRP continued to have a significant inverse correlation with DHA. In the entire cohort and biopsy negative controls, CRP had a significant inverse association with DHA after adjusting for IL-6, ALA, and EPA, but there was no significant inverse relationship between IL-6 and EPA and DHA after adjusting for CRP and the other n-3 fatty acids. In the entire cohort and biopsy negative controls, IL-6, categorized in tertiles, had a significant inverse association with DHA, categorized dichotomously. CRP, categorized into three ordinal groups, had a significant inverse association with DHA in the entire cohort and in the biopsy negative controls. CRP was also inversely associated with EPA, categorized dichotomously, in the biopsy negative controls. As expected, CRP and IL-6 exhibited a positive correlation in the biopsy negative controls and cancer cases. The positive correlation was also found in the biopsy negative controls, cancer cases, LG3+4 cancer cases, and HG7+ cancer cases when each group was examined alone. The positive association between IL-6 and CRP was also found in the same groups after adjusting for the n-3 fatty acids. When the

variables were categorized, there was a significant positive association between IL-6 and CRP in the entire cohort and in each group examined independently. There were no significant relationships between the primary exposure variables in the HG4+3 and LG6 cancer cases.

After adjusting for age and other covariates, there was no significant association between the primary exposure variables and the risk of prostate cancer. When the interaction between the plasma inflammatory markers and the n-3 fatty acids was added to the model, there were significant findings for the risk of prostate cancer. After adjusting for age, there was a significant increase in risk of prostate cancer in subjects with ALA levels below the median and CRP levels in the middle category. The risk of prostate cancer and HG4+3 cancer was also found to be higher in subjects with CRP levels in the same category and DHA levels above or equal to the median. However, there was a significant decrease in risk of HG7+ cancer in subjects with DHA levels below the median but the same levels of CRP. There was a significant interaction between CRP and ALA and the risk of LG3+4 and LG6 cancer, but there was no significant difference in risk within each group.

EPA was the only primary exposure variable that exhibited a significant association with the risk of developing prostate cancer. Levels of EPA between 0.350% and 0.476% were associated with a significantly higher risk of developing prostate cancer compared to those with levels below 0.350%. When the interaction between CRP and EPA was taken into consideration, there was a significant increased risk of developing prostate cancer in subjects with EPA levels above or equal to the median and increasing levels of CRP. However, there was question regarding the validity of the estimate of risk

due to the wide confidence interval. There was also a significant interaction between EPA and IL-6 but there was no significant difference in the risk of developing prostate cancer within the group. The KM estimate of cancer-free survival did indicate that subjects had the poorest outcome when they had levels of IL-6 in the highest tertile and EPA levels above or equal to the median.

Correlations between Plasma Markers of Inflammation and Omega-3 Fatty acids

As hypothesized, there was an inverse relationship between levels of IL-6 and CRP and the n-3 fatty acids, DHA and EPA, in the study population. Similar to other studies, IL-6 and CRP were positively correlated in the controls and cancer cases (60). Surprisingly, the inverse correlations between plasma markers of inflammation and n-3 fatty acids were not found when the cancer cases were examined independently. The loss of the anti-inflammatory effects of DHA and EPA in the cancer cases could be a result of changes in the composition of their membrane fatty acids. Stepovaya et al. have analyzed erythrocyte membrane fatty acids in patients with various malignancies (61). They reported that arachidonic acid (AA) was significantly decreased in the erythrocyte membranes of cancer patients. Novitskii et al. also found a decrease in the phosphatidylcholine and phosphatidylethanolamine fractions of erythrocyte membranes in patients with stomach and head and neck cancer (62). There may also be fluctuations in the fatty acid composition of prostate tissue. It has been shown that the concentration of AA was lower in malignant prostate tissue compared to controls (63-65). It was thought that this decline in AA may be due to an up-regulation of the COX and LOX pathways. In contrast, Hagstrup et al. reported that the concentration of ALA was

elevated in the prostate of men with cancer compared to the concentration found in their leukocyte membranes (66). The same study also found that EPA and DHA were strongly correlated in the leukocytes and prostate tissue of men with and without cancer. The present study found no significant difference in erythrocyte n-3 fatty acids in the biopsy negative controls and cancer cases. The only exception was the elevated ALA levels in the LG3+4 cancer cases. The lack of difference in n-3 fatty acids among prostate cancer cases and controls has been observed in other studies (51, 63). It has also be found that dietary n-3 fatty acids reported on a diet recall correlated with erythrocyte n-3 fatty acid levels in men with and without prostate cancer (67). This study did not examine the levels of AA, but a disruption in the levels of AA in the erythrocyte membranes of the prostate cancer cases, as found in other studies, may explain the absence of correlations between circulating markers of inflammation and n-3 fatty acids. It is possible that the anti-inflammatory effects of n-3 fatty acids may be lost in patients with cancer secondary to changes in the composition of their membrane fatty acids. There may also be changes in the key enzymes of the eicosanoid pathway that occur with malignancy. The research into the effect of cancer on membrane fatty acids and related metabolic pathways may provide further insight into the findings from this study.

Inflammation, Omega-3 Fatty Acids and Prostate Cancer Risk

Research focused on the relationship between n-3 fatty acids and the risk of prostate cancer has not been consistent. Some studies have found an increased risk of prostate cancer with higher levels of ALA and no association between EPA, DHA, and the risk of prostate cancer (51, 52, 68). Others have found that the association between an increased

risk in prostate cancer and ALA was related to advance prostate cancer only (69). There has also been research that indicated that the risk of prostate cancer decreased with increasing levels of DHA and EPA (69-71). The results of the present study conflicted with previously conducted studies and contradicted the hypotheses that subjects with the highest n-3 fatty acid levels would be at the lowest risk for cancer. As stated previously, ALA has been implicated in the increased risk of prostate cancer by other research. The opposite was true for this population where ALA levels below the median were associated with an increased risk in prostate cancer when subjects had CRP levels in the middle category. Furthermore, higher levels of DHA have shown protective effects against the risk of prostate cancer. Recent research by Edward et al. has documented the role of DHA in the regulation of syndecan-1 (SDC-1), a proteoglycan that has been found to prevent tumor progression (72). DHA, via peroxisome proliferator activated receptor γ (PPARγ), up-regulated SDC-I in the prostate of mice *in vitro* and in human prostate cancer cells *in vivo*. However, in the present study, there was an increased risk in high-grade (HG4+3) cancer in men with DHA levels above or equal to the median and CRP levels in the middle category. There was also a decreased risk in high-grade (HG7+) cancer in men with DHA levels below the median and similar CRP levels. As with DHA, EPA has been associated with a decreased risk in prostate cancer but this was not supported by the present findings. When examined independently, men with EPA levels in the middle tertile were at a higher risk for developing prostate cancer. There was also an increased risk in developing prostate cancer in men with EPA levels above or equal to the median and CRP levels in the middle and highest category or IL-6 in the highest tertile.

This study also hypothesized that the risk of prostate cancer would increase with increasing levels of IL-6 and CRP. The increased risk of prostate cancer described above was found in subjects with IL-6 and CRP levels in the middle and highest categories when considered with EPA and DHA, but CRP and IL-6 were not independently related to the risk of prostate cancer. The relationship between IL-6 and CRP and prostate cancer risk has been investigated with conflicting results. IL-6 has been linked to the development and progression of prostate cancer but other studies have found no association between IL-6 and the risk of prostate cancer (50, 60). The same has been true for circulating levels of CRP.

This was the first study to investigate the risk of prostate cancer as it is relates to circulating plasma inflammatory markers and erythrocyte n-3 fatty acids. Thus, it is difficult to compare the results discussed here with previously published research. Three plausible reasons for the discrepancy between this study and others are discussed here. First, there may be genetic variations in the IL-6 and CRP genes as well as in the genes that are responsible for the COX-2 enzyme. This genetic variation has been attributed to the presence of single nucleotide polymorphisms (SNPs) located in or near the promoter region of the genes. SNPs can alter gene expression and protein synthesis, and they have been associated with a both an increase and decrease in prostate cancer risk (50, 70, 71). Polymorphisms have also been associated with changes in circulating IL-6 and CRP concentrations (73), while other researchers have found genetic variations in the IL-6 gene that are not associated with increases in circulating IL-6 (50, 60). Genetic variations that alter IL-6, CRP and eicosanoid production may confound the association between inflammation, n-3 fatty acids and prostate cancer.

Secondly, the n-3 fatty acid, IL-6 and CRP levels in this study population were lower compared to similar research. The median ALA, DHA, and EPA levels in the biopsy negative controls were 0.12, 3.37 and 0.41, respectively. In the prostate cancer cases, the median values were 0.12, 3.46, and 0.39, respectively. Studies that have found significant associations between the risk of prostate cancer and n-3 and n-6 fatty acids have had median ALA, DHA, and EPA levels around 0.18-0.20, 4.07-6.84, and 0.54-0.67, respectively (51, 52, 67). Research focused on the use of EPA to treat rheumatoid arthritis has suggested that the maximum benefit from EPA occurs with daily intakes of approximately 1.6 g/day and mononuclear cell phospholipid EPA concentrations above 1% (74, 75). EPA concentrations in erythrocyte membranes increase approximately 1.4% per gram of intake (35, 36). Daily intakes of 1.6 g/day may result in erythrocyte membrane EPA levels of 2.2% of total membrane fatty acids. The median levels in this study were 0.41 in the controls and 0.39 in the cancer cases, which is markedly lower than levels recommended for the treatment of rheumatoid arthritis. This may indicate that there is a threshold that must be reached before the protective effects of n-3 fatty acids can be observed (67). The present study and previous research may not have reached that threshold resulting in a lack of significant results. This may be especially true for population-based studies in countries where the intake of n-3 acids is below recommended levels (76). It is interesting to note that evidence that supports the protective role of n-3 fatty acids in the prevention of prostate cancer and inflammation has been reported by studies conducted in countries that have diets rich in n-3 fatty acids when compared to Western countries (39, 70).

Lastly, it has been proposed that an increase in the ratio of n-3 to n-6 fatty acids is more important to a reduction in prostate cancer risk versus the intake of n-3 fatty acids alone (37, 63, 70). Some researchers go further suggesting that it is an increase in the ratio of EPA+DHA to n-6 fatty acids that may be protective against prostate cancer. This is supported by research that implicates higher ALA intake with an increasing risk of prostate cancer and the poor conversion of ALA to EPA in humans. The ratio of n-6 to n-3 fatty acids has risen dramatically in the past century (76). In the 1990s, it was estimated that the ratio of n-6 to n-3 fatty acids was 10 to 6 and some suggest that the ratio should be 2.3 to 1. The intimate connection between n-3 and n-6 fatty acids, particularly in the eicosanoid pathway, may play a role in the risk of prostate cancer. It is plausible that an increase in n-3 fatty acid needs to be accompanied by a decrease in n-6 fatty acid intake for there to be any benefit. This study did not examine the relationship between n-6 and n-3 fatty acids or the association between n-6 fatty acids and prostate cancer risk. Such analyses might have enhanced the findings in this population.

Circulating Plasma Markers of Inflammation and Inflammation in the Prostate

This was the first study to examine the relationship between circulating levels of IL-6 and CRP and inflammation in the prostate in men without prostate cancer. Men with IL-6 values in the highest tertile had 2.5 times the odds of having inflammation in the prostate compared to men with IL-6 levels in the lowest tertile. The risk was even higher for men with IL-6 levels in the middle tertile at 2.6 the odds. While more research is needed to validate the results of this study, the use of circulating IL-6 to detect inflammation in the prostate may be a useful, non-invasive, diagnostic tool for researchers and practitioners.

This study indicates that men may be at greater risk for inflammation in the prostate with IL-6 levels between 1.50 and 26.3 pg/mL.

Strengths

This study had several strengths. The study design combined both prospective and case-control analyses. Case-control studies are highly informative because they allow for the evaluation of several causal hypotheses as well as the evaluation of interactions and confounding factors. Prospective studies are considered to be a stronger study design. Subjects are disease-free at the time of entry into the study and are then followed for a specified period of time. Thus, the covariates of interest are not confounded by the presence of disease like they can be in case-control studies. This study was able to benefit from the strengths of both study designs.

The use of erythrocyte membrane n-3 fatty acids levels as a biomarker for n-3 fatty acid intake can be considered a more accurate measurement compared to dietary intake assessed from food frequency questionnaires which often suffer from recall bias. Since erythrocytes have an average life span of 120 days, the fatty acids values reflect long-term exposure and dietary patterns.

The data collected during the DPC study included extensive information on dietary intake, medication use, and common risk factors for disease. This made it possible to adjust for several possible confounding variables including aspirin and NSAID use, and a variety of nutrients that may interact with inflammation or prostate cancer.

Limitations

This study was not without limitations. Case-control studies are often associated with recall and selection bias. The recall bias related to the intake of n-3 fatty acids was eliminated by the use of erythrocyte measurements. However, recall bias would still impact other covariates such as medication use and other nutrient data. This study was also a secondary analysis of the DPC study. This prevented changes to the study design, inclusion/exclusion criteria, methods, or sample size. For example, the small sample size made examination within sub-groups problematical. For the prospective analyses, the follow-up period was relatively short and there was insufficient outcome data to exam the relationship between inflammation, n-3 fatty acids, and biochemical recurrence.

This study was only able to analyze measurements of IL-6, CRP and erythrocyte n-3 fatty acids at a single time point. This prevents the assessment for trends that might be related to the disease process as well as physiological fluctuations. The values for ALA, DHA, and EPA fell within a narrow range and this lack of variance may have prevented the detection of change within the data set.

Research focused on the connection between prostate cancer and n-3 fatty acids has used a variety of methods to assess n-3 fatty acids including dietary recalls, plasma phospholipids, adipose tissue, and the membranes of leukocytes and erythrocytes. It was difficult to accurately compare the results from this study to previously published research because of the variation in the measurement of n-3 fatty acids.

SUMMARY & CONCLUSION

This study found an inverse relationship between plasma markers of inflammation and the n-3 fatty acids, DHA and EPA. This supports the hypothesis that higher intakes of n-3 fatty acids interfere with the perpetuation of the inflammatory response. N-3 fatty acids may exert this effect through several pathways including the displacement of AA as a precursor for eicosanoid production, competition for key enzymes of the LOX and COX pathways, and suppression of pro-inflammatory transcription factors (77). Interestingly, this inverse relationship was only found in men without prostate cancer for reasons that cannot be elucidated without further research. The associations between inflammation, n-3 fatty acids, and the risk of prostate cancer described by this study did not correlate with the findings of others. However, the availability of related research was limited and the population examined in this study may have had levels of n-3 fatty acids and inflammatory markers that were inadequate to observe any valid associations.

In summary, this study supports the anti-inflammatory effects of n-3 fatty acids in men without prostate cancer. It was also concluded the circulating levels of IL-6 can be indicative of inflammation in the prostate. Further research is needed to examine the relationship between n-3 fatty acids, inflammation, and outcome in men with and without prostate cancer. In particular, research that is conducted in populations with adequate intakes of DHA and EPA or in conjunction with supplementation may provide a more accurate picture of the potential protective effects of these fatty acids.

REFERENCES

1. Mayo Clinic. Prostate Cancer Information Page. Available at: http://www.mayoclinic.com/health/prostate-cancer/DS00043. Accessed 5/1, 2008.

2. National Cancer Institute. Cancer Topics: Prostate Cancer. Available at: http://www.cancer.gov/cancertopics/types/prostatecancer.gov. Accessed 5/1, 2008.

3. Li H, Stampfer MJ, Giovannucci EL, Morris JS, Willett WC, Gaziano JM, Ma J. A prospective study of plasma selenium levels and prostate cancer risk. *J Natl Cancer Ins*t. 2004;96:696-703.

4. Berquin IM, Min Y, Wu R, Wu J, Perry D, Cline JM, Thomas MJ, Thornburg T, Kulik G, Smith A, Edwards IJ, D'Agostino R, Zhang H, Wu H, Kang JX, Chen YQ. Modulation of prostate cancer genetic risk by omega-3 and omega-6 fatty acids. *J Clin Inves*t. 2007;117:1866-1875.

5. De Marzo AM, Platz EA, Sutcliffe S, Xu J, Gronberg H, Drake CG, Nakai Y, Isaacs WB, Nelson WG. Inflammation in prostate carcinogenesis. *Nat Rev Cance*r. 2007;7:256-269.

6. Center for Disease Control and Prevention. Prostate Cancer Initiatives. Available at: http://www.cdc.gov/cancer/prostate/prospdf/0607_prostate_fs.pdf. Accessed 5/2, 2007.

7. David AK, Khwaja R, Hudes GR. Treatments for improving survival of patients with prostate cancer. *Drugs Agin*g. 2003;20:683-699.

8. National Cancer Institute. SEER Stat Fact Sheet-Cancer of the Prostate. Available at: http://seer.cancer.gov/statfacts/html/prost.html?statfacts_page=prost.html&x=8&y=9. Accessed 5/6, 2008.

9. Coussens LM, Werb Z. Inflammation and cancer. *Natur*e. 2002;420:860-867.

10. Culig Z, Steiner H, Bartsch G, Hobisch A. Interleukin-6 regulation of prostate cancer cell growth. *J Cell Biochem*. 2005;95:497-505.

11. Gabay C. Interleukin-6 and chronic inflammation. *Arthritis Res Ther*. 2006;8 Suppl 2:S3.

12. Pontari MA, Ruggieri MR. Mechanisms in prostatitis/chronic pelvic pain syndrome. *J Urol*. 2004;172:839-845.

13. Gabay C, Kushner I. Acute-phase proteins and other systemic responses to inflammation. *N Engl J Med*. 1999;340:448-454.

14. Deans C, Wigmore SJ. Systemic inflammation, cachexia and prognosis in patients with cancer. *Curr Opin Clin Nutr Metab Care*. 2005;8:265-269.

15. Alexander RB, Brady F, Ponniah S. Autoimmune prostatitis: Evidence of T cell reactivity with normal prostatic proteins. *Urology*. 1997;50:893-899.

16. Hold GL, El-Omar ME. Genetic aspects of inflammation and cancer. *Biochem* J. 2008;410:225-235.

17. Haverkamp J, Charbonneau B, Ratliff TL. Prostate inflammation and its potential impact on prostate cancer: A current review. *J Cell Biochem*. 2008;103:1344-1353.

18. MacLennan GT, Eisenberg R, Fleshman RL, Taylor JM, Fu P, Resnick MI, Gupta S. The influence of chronic inflammation in prostatic carcinogenesis: A 5-year followup study. *J Urol*. 2006;176:1012-1016.

19. Hussain SP, Hofseth LJ, Harris CC. Radical causes of cancer. *Nat Rev Cancer*. 2003;3:276-285.

20. McArdle PA, McMillan DC, Sattar N, Wallace AM, Underwood MA. The relationship between interleukin-6 and C-reactive protein in patients with benign and malignant prostate disease. *Br J Cance*r. 2004;91:1755-1757.

21. Shariat SF, Andrews B, Kattan MW, Kim J, Wheeler TM, Slawin KM. Plasma levels of interleukin-6 and its soluble receptor are associated with prostate cancer progression and metastasis. *Urology*. 2001;58:1008-1015.

22. Kuroda K, Horiguchi Y, Nakashima J, Kikuchi E, Kanao K, Miyajima A, Ohigashi T, Umezawa K, Murai M. Prevention of cancer cachexia by a novel nuclear factor {kappa}B inhibitor in prostate cancer. *Clin Cancer Re*s. 2005;11:5590-5594.

23. McArdle PA, Canna K, McMillan DC, McNicol AM, Campbell R, Underwood MA. The relationship between T-lymphocyte subset infiltration and survival in patients with prostate cancer. *Br J Cance*r. 2004;91:541-543.

24. Irani J, Goujon JM, Ragni E, Peyrat L, Hubert J, Saint F, Mottet N. High-grade inflammation in prostate cancer as a prognostic factor for biochemical recurrence after radical prostatectomy. pathologist multi center study group. *Urology*. 1999;54:467-472.

25. Hobisch A, Rogatsch H, Hittmair A, Fuchs D, Bartsch G,Jr, Klocker H, Bartsch G, Culig Z. Immunohistochemical localization of interleukin-6 and its receptor in benign, premalignant and malignant prostate tissue. *J Patho*l. 2000;191:239-244.

26. Hobisch A, Ramoner R, Fuchs D, Godoy-Tundidor S, Bartsch G, Klocker H, Culig Z. Prostate cancer cells (LNCaP) generated after long-term interleukin 6 (IL-6) treatment express IL-6 and acquire an IL-6 partially resistant phenotype. *Clin Cancer Re*s. 2001;7:2941-2948.

27. Wegiel B, Bjartell A, Culig Z, Persson JL. Interleukin-6 activates PI3K/Akt pathway and regulates cyclin A1 to promote prostate cancer cell survival. *Int J Cance*r. 2008;122:1521-1529.

28. Paule B, Terry S, Kheuang L, Soyeux P, Vacherot F, de la Taille A. The NF-kappaB/IL-6 pathway in metastatic androgen-independent prostate cancer: New therapeutic approaches? *World J Uro*l. 2007;25:477-489.

29. Cavarretta IT, Neuwirt H, Untergasser G, Moser PL, Zaki MH, Steiner H, Rumpold H, Fuchs D, Hobisch A, Nemeth JA, Culig Z. The antiapoptotic effect of IL-6 autocrine loop in a cellular model of advanced prostate cancer is mediated by mcl-1. *Oncogen*e. 2007;26:2822-2832.

30. Steiner H, Berger AP, Godoy-Tundidor S, Bjartell A, Lilja H, Bartsch G, Hobisch A, Culig Z. An autocrine loop for vascular endothelial growth factor is established in prostate cancer cells generated after prolonged treatment with interleukin 6. *Eur J Cance*r. 2004;40:1066-1072.

31. Stratton MS, Sirvent H, Udayakumar TS, Nagle RB, Bowden GT. Expression of the matrix metalloproteinase promatrilysin in coculture of prostate carcinoma cell lines. *Prostat*e. 2001;48:206-209.

32. Borsellino N, Belldegrun A, Bonavida B. Endogenous interleukin 6 is a resistance factor for cis-diamminedichloroplatinum and etoposide-mediated cytotoxicity of human prostate carcinoma cell lines. *Cancer Re*s. 1995;55:4633-4639.

33. Gropper SS, Smith JL, Groff JL. Lipids. In: *Advanced Nutrition and Human Metabolism.* 4th ed. Belmont, CA: Thomson Wadsworth; 2005:128.

34. Stipanuk M. Essential fatty acids. In: *Biochemical, Physiological, Molecular Aspects of Human Nutrition.* 2nd ed. Philadelphia, PA: Saunders Elsevier; 2006:518.

35. Cao J, Schwichtenberg KA, Hanson NQ, Tsai MY. Incorporation and clearance of omega-3 fatty acids in erythrocyte membranes and plasma phospholipids. *Clin Che*m. 2006;52:2265-2272.

36. Katan MB, Deslypere JP, van Birgelen AP, Penders M, Zegwaard M. Kinetics of the incorporation of dietary fatty acids into serum cholesteryl esters, erythrocyte membranes, and adipose tissue: An 18-month controlled study. *J Lipid Re*s. 1997;38:2012-2022.

37. Calder PC. N-3 polyunsaturated fatty acids, inflammation, and inflammatory diseases. *Am J Clin Nut*r. 2006;83:1505S-1519S.

38. Abbate R, Gori AM, Martini F, Brunelli T, Filippini M, Francalanci I, Paniccia R, Prisco D, Gensini GF, Neri Serneri GG. n-3 PUFA supplementation, monocyte PCA expression and interleukin-6 production. *Prostaglandins Leukot Essent Fatty Acid*s. 1996;54:439-444.

39. Ferrucci L, Cherubini A, Bandinelli S, Bartali B, Corsi A, Lauretani F, Martin A, Andres-Lacueva C, Senin U, Guralnik JM. Relationship of plasma polyunsaturated fatty acids to circulating inflammatory markers. *J Clin Endocrinol Meta*b. 2006;91:439-446.

40. Cookson MS, Aus G, Burnett AL, Canby-Hagino ED, D'Amico AV, Dmochowski RR, Eton DT, Forman JD, Goldenberg SL, Hernandez J, Higano CS, Kraus SR, Moul JW, Tangen C, Thrasher JB, Thompson I. Variation in the definition of biochemical recurrence in patients treated for localized prostate cancer: The american urological association prostate guidelines for localized prostate cancer update panel report and

recommendations for a standard in the reporting of surgical outcomes. *J Uro*l. 2007;177:540-545.

41. Stephenson AJ, Kattan MW, Eastham JA, Dotan ZA, Bianco FJ,Jr, Lilja H, Scardino PT. Defining biochemical recurrence of prostate cancer after radical prostatectomy: A proposal for a standardized definition. *J Clin Onco*l. 2006;24:3973-3978.

42. Roach M,3rd, Hanks G, Thames H,Jr, Schellhammer P, Shipley WU, Sokol GH, Sandler H. Defining biochemical failure following radiotherapy with or without hormonal therapy in men with clinically localized prostate cancer: Recommendations of the RTOG-ASTRO phoenix consensus conference. *Int J Radiat Oncol Biol Phy*s. 2006;65:965-974.

43. Kuban DA, Levy LB, Potters L, Beyer DC, Blasko JC, Moran BJ, Ciezki JP, Zietman AL, Zelefsky MJ, Pisansky TM, Elshaikh M, Horwitz EM. Comparison of biochemical failure definitions for permanent prostate brachytherapy. *Int J Radiat Oncol Biol Phy*s. 2006;65:1487-1493.

44. Kang DE, Fitzsimons NJ, Presti JC,Jr, Kane CJ, Terris MK, Aronson WJ, Amling CL, Freedland SJ, SEARCH Database Study Group. Risk stratification of men with gleason score 7 to 10 tumors by primary and secondary gleason score: Results from the SEARCH database. *Urology*. 2007;70:277-282.

45. Chan TY, Partin AW, Walsh PC, Epstein JI. Prognostic significance of gleason score 3+4 versus gleason score 4+3 tumor at radical prostatectomy. *Urology*. 2000;56:823-827.

46. Sakr WA, Tefilli MV, Grignon DJ, Banerjee M, Dey J, Gheiler EL, Tiguert R, Powell IJ, Wood DP. Gleason score 7 prostate cancer: A heterogeneous entity? correlation with pathologic parameters and disease-free survival. *Urology*. 2000;56:730-734.

47. Shimada H, Nabeya Y, Okazumi S, Matsubara H, Miyazawa Y, Shihratori T, Hayashi H, Aoki T, Sugaya M, Gunji Y, Kobayashi S, Ochiai T. Prognostic value of preoperative serum immunosuppressive acidic protein in patients with esophageal squamous cell carcinoma. *Dis Esophagu*s. 2003;16:102-106.

48. Ballou SP, Kushner I. C-reactive protein and the acute phase response. *Adv Intern Me*d. 1992;37:313-336.

49. Miki C, Konishi N, Ojima E, Hatada T, Inoue Y, Kusunoki M. C-reactive protein as a prognostic variable that reflects uncontrolled up-regulation of the IL-1-IL-6 network system in colorectal carcinoma. *Dig Dis Sc*i. 2004;49:970-976.

50. Pierce BL, Biggs ML, Decambre M, Reiner AP, Li C, Fitzpatrick A, Carlson CS, Stanford JL, Austin MA. C-reactive protein, interleukin-6, and prostate cancer risk in men aged 65 years and older. *Cancer Causes Control*. 2009.

51. Park SY, Wilkens LR, Henning SM, Le Marchand L, Gao K, Goodman MT, Murphy SP, Henderson BE, Kolonel LN. Circulating fatty acids and prostate cancer risk in a nested case-control study: The multiethnic cohort. *Cancer Causes Control*. 2009;20:211-223.

52. Newcomer LM, King IB, Wicklund KG, Stanford JL. The association of fatty acids with prostate cancer risk. *Prostate*. 2001;47:262-268.

53. Shieh G, O'Brein RG. A simpler method to compute power for likelihood ratio tests in generalized linear models.

54. Charlson M, Szatrowski TP, Peterson J, Gold J. Validation of a combined comorbidity index. *J Clin Epidemiol.* 1994;47:1245-1251.

55. Charlson ME, Pompei P, Ales KL, MacKenzie CR. A new method of classifying prognostic comorbidity in longitudinal studies: Development and validation. *J Chronic Dis.* 1987;40:373-383.

56. Koppie TM, Serio AM, Vickers AJ, Vora K, Dalbagni G, Donat SM, Herr HW, Bochner BH. Age-adjusted charlson comorbidity score is associated with treatment decisions and clinical outcomes for patients undergoing radical cystectomy for bladder cancer. *Cancer.* 2008;112:2384-2392.

57. Bostwick DG, Burke HB, Djakiew D, Euling S, Ho SM, Landolph J, Morrison H, Sonawane B, Shifflett T, Waters DJ, Timms B. Human prostate cancer risk factors. *Cancer*. 2004;101:2371-2490.

58. Schroder FH, Roobol MJ, Andriole GL, Fleshner N. Defining increased future risk for prostate cancer: Evidence from a population based screening cohort. *J Urol*. 2009;181:69-74; discussion 74.

59. Briganti A, Chun FK, Suardi N, Gallina A, Walz J, Graefen M, Shariat S, Ebersdobler A, Rigatti P, Perrotte P, Saad F, Montorsi F, Huland H, Karakiewicz PI. Prostate volume and adverse prostate cancer features: Fact not artifact. *Eur J Cancer*. 2007;43:2669-2677.

60. Stark JR, Li H, Kraft P, Kurth T, Giovannucci EL, Stampfer MJ, Ma J, Mucci LA. Circulating prediagnostic interleukin-6 and C-reactive protein and prostate cancer incidence and mortality. *Int J Cancer*. 2009;124:2683-2689.

61. Stepovaya EA, Novitskii VV, Ryazantseva NV, Goldberg VE, Tkachenko SB, Kolosova MV. Structure and properties of lipid bilayer of erythrocyte membranes in patients with malignant tumors. *Bull Exp Biol Med.* 2003;136:490-493.

62. Novitskii VV, Stepovaia EA, Bazhenova NG, Gol'dberg VE, Kolosova MV, Saprykina EV, Gubert EA, Sokolova IB, Koreshkova KG, Bulavina I. Lipid composition of erythrocyte membranes in patients with cancer. *Biull Eksp Biol Med*. 1998;126:204-206.

63. Ritch CR, Wan RL, Stephens LB, Taxy JB, Huo D, Gong EM, Zagaja GP, Brendler CB. Dietary fatty acids correlate with prostate cancer biopsy grade and volume in jamaican men. *J Urol*. 2007;177:97-101; discussion 101.

64. Freeman VL, Meydani M, Yong S, Pyle J, Flanigan RC, Waters WB, Wojcik EM. Prostatic levels of fatty acids and the histopathology of localized prostate cancer. *J Urol*. 2000;164:2168-2172.

65. Faas FH, Dang AQ, White J, Schaefer RF, Johnson DE. Decreased prostatic arachidonic acid in human prostatic carcinoma. *BJU Int*. 2003;92:551-554.

66. Christensen JH, Fabrin K, Borup K, Barber N, Poulsen J. Prostate tissue and leukocyte levels of n-3 polyunsaturated fatty acids in men with benign prostate hyperplasia or prostate cancer. *BJU Int*. 2006;97:270-273.

67. Godley PA, Campbell MK, Gallagher P, Martinson FE, Mohler JL, Sandler RS. Biomarkers of essential fatty acid consumption and risk of prostatic carcinoma. *Cancer Epidemiol Biomarkers Prev*. 1996;5:889-895.

68. Brouwer IA. Omega-3 PUFA: Good or bad for prostate cancer? *Prostaglandins Leukot Essent Fatty Acids*. 2008;79:97-99.

69. Leitzmann MF, Stampfer MJ, Michaud DS, Augustsson K, Colditz GC, Willett WC, Giovannucci EL. Dietary intake of n-3 and n-6 fatty acids and the risk of prostate cancer. *Am J Clin Nutr*. 2004;80:204-216.

70. Hedelin M, Chang ET, Wiklund F, Bellocco R, Klint A, Adolfsson J, Shahedi K, Xu J, Adami HO, Gronberg H, Balter KA. Association of frequent consumption of fatty fish with prostate cancer risk is modified by COX-2 polymorphism. *Int J Cancer*. 2007;120:398-405.

71. Fradet V, Cheng I, Casey G, Witte JS. Dietary omega-3 fatty acids, cyclooxygenase-2 genetic variation, and aggressive prostate cancer risk. *Clin Cancer Res*. 2009;15:2559-2566.

72. Edwards IJ, Sun H, Hu Y, Berquin IM, O'Flaherty JT, Cline JM, Rudel LL, Chen YQ. In vivo and in vitro regulation of syndecan 1 in prostate cells by n-3 polyunsaturated fatty acids. *J Biol Chem*. 2008;283:18441-18449.

73. Walston JD, Fallin MD, Cushman M, Lange L, Psaty B, Jenny N, Browner W, Tracy R, Durda P, Reiner A. IL-6 gene variation is associated with IL-6 and C-reactive protein levels but not cardiovascular outcomes in the cardiovascular health study. *Hum Genet*. 2007;122:485-494.

74. Cleland LG, Proudman SM, Hall C, Stamp LK, McWilliams L, Wylie N, Neumann M, Gibson RA, James MJ. A biomarker of n-3 compliance in patients taking fish oil for rheumatoid arthritis. *Lipids*. 2003;38:419-424.

75. Caughey GE, Mantzioris E, Gibson RA, Cleland LG, James MJ. The effect on human tumor necrosis factor alpha and interleukin 1 beta production of diets enriched in n-3 fatty acids from vegetable oil or fish oil. *Am J Clin Nutr*. 1996;63:116-122.

76. Kris-Etherton PM, Taylor DS, Yu-Poth S, Huth P, Moriarty K, Fishell V, Hargrove RL, Zhao G, Etherton TD. Polyunsaturated fatty acids in the food chain in the united states. *Am J Clin Nutr*. 2000;71:179S-88S.

77. Larsson SC, Kumlin M, Ingelman-Sundberg M, Wolk A. Dietary long-chain n-3 fatty acids for the prevention of cancer: A review of potential mechanisms. *Am J Clin Nutr*. 2004;79:935-945.

Power Calculations

Minimum detectable Pearon's correlation coefficients for plasma markers of inflammation and omega-3 fatty acids.

Correlation Coefficient	Sample Size	Power	p-value (two-sided)
0.13	361	0.70	0.05
0.15	361	0.80	0.05
0.17	361	0.90	0.05

Minimum detectable hazard ratio for the risk of developing prostate cancer by primary exposure variable.

Primary Exposure Variable	Sample Size	Expected Event Rate	Power	p-value (two sided)	Minimum Detectable Hazard Ratio
IL-6	240	0.17	0.80	0.05	1.17
CRP	240	0.17	0.80	0.05	1.40
ALA	240	0.17	0.80	0.05	3.21
DHA	240	0.17	0.80	0.05	1.45
EPA	240	0.17	0.80	0.05	3.65

Minimum detectable hazard ratio for the risk of developing prostate cancer with an interaction between plasma markers of inflammation and omega-3 fatty acids.

Primary Exposure Variable	Sample Size	Expected Event Rate	Power	p-value (two sided)	Minimum Detectable Hazard Ratio
CRP x ALA	240	0.17	0.80	0.05	1.47
CRP x DHA	240	0.17	0.80	0.05	1.13
CRP x EPA	240	0.17	0.80	0.05	1.54
IL-6 x ALA	240	0.17	0.80	0.05	2.30
IL-6 x DHA	240	0.17	0.80	0.05	1.30
IL-6 x EPA	240	0.17	0.80	0.05	2.52

Minimum detectable hazard ratio for the risk of biochemical recurrrence by primary exposure variable.

Primary Exposure Variable	Sample Size	Expected Event Rate	Power	p-value (two sided)	Minimum Detectable Hazard Ratio
IL-6	121	0.26	0.80	0.05	1.19
CRP	121	0.26	0.80	0.05	1.46
ALA	121	0.26	0.80	0.05	3.73
DHA	121	0.26	0.80	0.05	1.52
EPA	121	0.26	0.80	0.05	4.31

Minimum detectable hazard ratio for the risk of biochemical recurrence with an interaction between plasma markers of inflammation and omega-3 fatty acids.

Primary Exposure Variable	Sample Size	Expected Event Rate	Power	p-value (two sided)	Minimum Detectable Hazard Ratio
CRP x ALA	121	0.26	0.80	0.05	2.14
CRP x DHA	121	0.26	0.80	0.05	1.27
CRP x EPA	121	0.26	0.80	0.05	2.33
IL-6 x ALA	121	0.26	0.80	0.05	3.04
IL-6 x DHA	121	0.26	0.80	0.05	1.42
IL-6 x EPA	121	0.26	0.80	0.05	3.44

Number of subjects needed to detect a significant interaction between plasma markers of inflammation and omega-3 fatty acids based the non-centrality parameter calculated from this study.

Interaction	Sample Size	Likelihood Ratio Test Statistic*	Non-Centrality Parameter	Chi-Square Statistic (2df)	Power	p-value (two sided)	Number of Subjects Needed
IL-6 x ALA	240	1.04	9.63	5.99	0.80	0.05	2,222
IL-6 x DHA	240	2.72	9.63	5.99	0.80	0.05	850
IL-6 x EPA	240	6.68	9.63	5.99	0.80	0.05	346
CRP x ALA	240	1.05	9.63	5.99	0.80	0.05	2,201
CRP x DHA	240	3.22	9.63	5.99	0.80	0.05	718
CRP x EPA	240	0.13	9.63	5.99	0.80	0.05	17,778

* Test of significance of interaction from this study

Goodness-of-fit Assessment

Hosmer-Lemeshow goodness-of-fit test for final regression models.

Final Model	Primary Exposure Variable Continuous: Hosmer-Lemeshow Chi-Squared	Primary Exposure Variable Continuous: p-value	Primary Exposure Variable Categorical: Hosmer-Lemeshow Chi-Squared	Primary Exposure Variable Categorical: p-value
Biopsy Negative Controls & Cancer Cases				
IL-6 + Age + NSAID use	5.27	0.73	5.14	0.74
CRP + Age	0.88	0.83	0.92	0.82
ALA + Age	3.91	0.69	4.22	0.65
DHA + Age	4.77	0.57	4.80	0.57
EPA + Age	4.82	0.44	2.82	0.73
Biopsy Negative Controls & LG3+4 Cancer Cases				
IL-6 + Age	6.76	0.34	1.71	0.89
CRP + Age + ACCI	8.20	0.32	12.20	0.06
ALA + Age	2.57	0.77	2.50	0.87
DHA + Age	3.76	0.81	2.90	0.72
EPA + Age	9.24	0.24	3.47	0.63
Biopsy Negative Controls & HG4+3 Cancer Cases				
IL-6 + Age + Smoking status	5.34	0.72	1.40	0.99
CRP + Age + ACCI + Smoking status	3.55	0.89	7.35	0.50
ALA + Age + PSA	5.76	0.67	7.54	0.48
DHA + Age + Smoking status	8.09	0.42	8.32	0.40
EPA + Age	0.99	0.91	0.99	0.91
Biopsy Negative Controls & LG6 Cancer Cases				
IL-6 + Age	9.13	0.24	3.48	0.75
CRP + Age + ACCi	5.93	0.55	4.10	0.66
ALA + Age	5.88	0.55	4.82	0.68
DHA + Age	1.33	0.97	1.72	0.94
EPA + Age	5.14	0.40	6.82	0.45
Biopsy Negative Controls & HG7+ Cancer Cases				
IL-6 + Age	3.54	0.62	1.11	0.89
CRP + Age + ACCI + Prostate volume	4.11	0.85	3.50	0.90
ALA + Age + PSA	2.67	0.95	4.29	0.83
DHA + Age + Prostate volume	3.59	0.89	4.38	0.82
EPA + Age	3.94	0.56	1.49	0.91

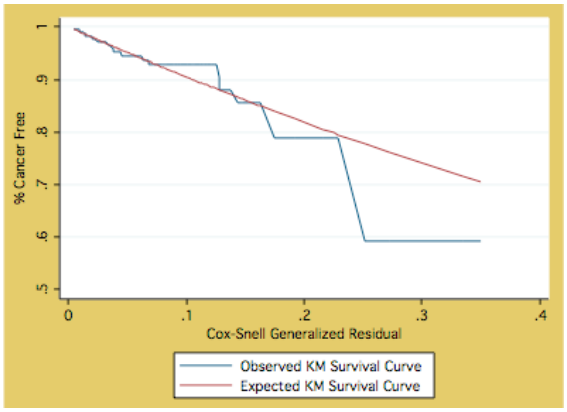

Figure 20. Cox-Snell generalized residual plot for the risk of developing cancer by IL-6 levels.

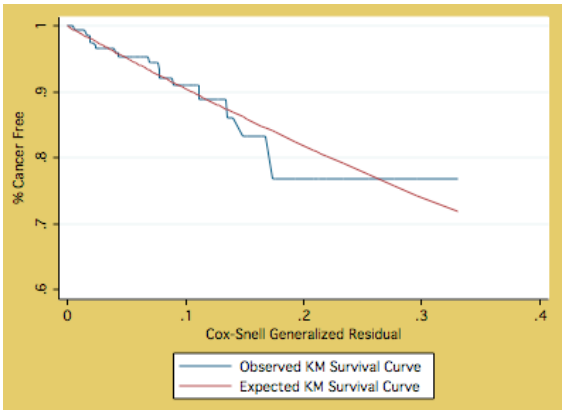

Figure 21. Cox-Snell generalized residual plot for the risk of developing cancer by CRP levels.

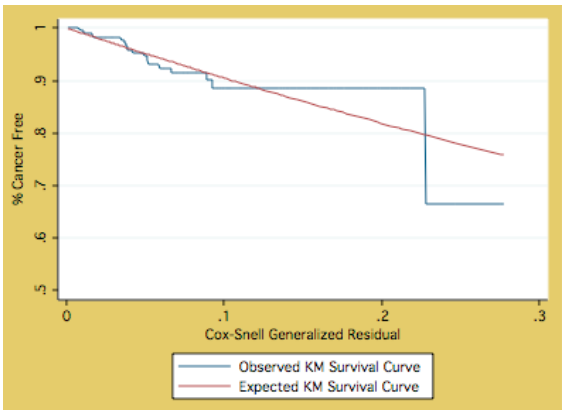

Figure 22. Cox-Snell generalized residual plot for the risk of developing cancer by ALA levels.

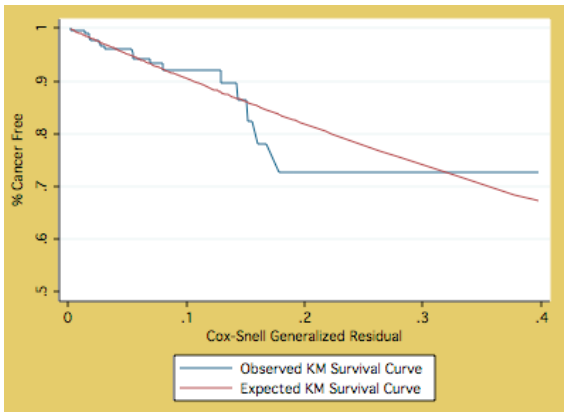

Figure 23. Cox-Snell generalized residual plot for the risk of developing cancer by DHA levels.

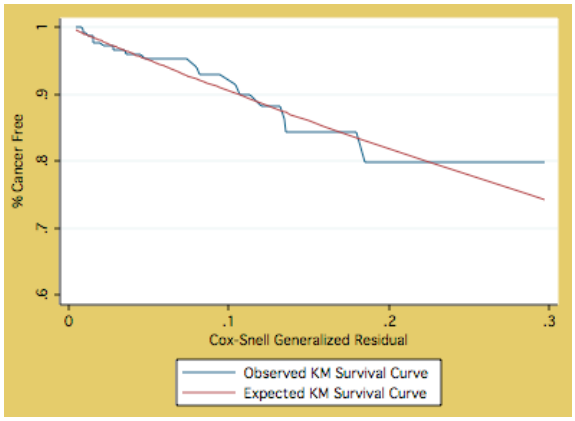

Figure 24. Cox-Snell generalized residual plot for the risk of developing cancer by EPA levels.

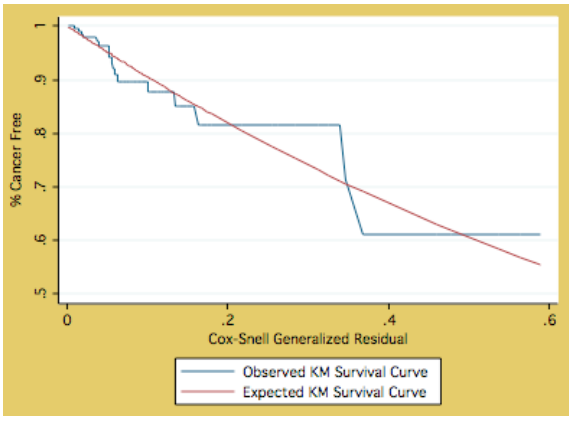

Figure 25. Cox-Snell generalized residual plot for the risk of developing cancer by inflammation in the prostate.