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Dietary Interventions Against Mammary Glands and Prostate Cancers

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**DIETARY INTERVENTIONS AGAINST MAMMARY GLAND AND
PROSTATE CANCERS**

Dissertation

Submitted to the
Graduate College of
Marshall University

In Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy
In Biomedical Sciences

By
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ABSTRACT

Dietary Interventions against Mammary Gland and Prostate Cancers

By Juliana Adedayo Akinsete

Dietary components modulate normal cellular functions and, in cancer cells, alter processes that lead to cancer and/or its progression. The change in the patterns of human food production and consumption over time has contributed to increasing risk for diseases including cancer. Omega-3 and omega-6 fatty acids are classes of essential polyunsaturated fatty acids in the human diet and are required for normal growth and development. Omega-3 and omega-6 fats are thought to be required in the ratio of 1:1 to 1:4 on which humans are thought to have evolved however, the Western diet has greatly shifted from this ratio. The Western diet consists of high omega-6 and low omega-3 fat ratio as high as 50:1. Consumption of a diet high in omega-3 fat is associated with reduced risk for some cancers, whereas consumption of a diet high in omega-6 fat is associated with increased risk. We hypothesized that canola oil in the maternal diet or, regular walnut consumption, or fish oil in adult diet as sources of omega-3 fat to increase omega-3 fat and reduce omega-6 fat in the diet, might reduce the risk for breast and prostate cancers in the C3(1)TA_g mouse. Consumption of high omega-3 diet from canola oil by mothers suppressed mammary gland tumorigenesis in the female offspring. Walnut consumption suppressed mammary gland tumorigenesis more than high omega-3 diet from canola oil, and high omega-3 diet from fish oil suppressed prostate tumorigenesis.

Dedication

First and foremost I thank God for His grace and strength I daily experienced throughout my study. I appreciate Him for giving me a wonderful and supportive family. To my husband, Alfred, I say thank you for being ever loving and caring. To my children, Ayokunle and Moyosore, thank you for your understanding and forbearance. I love you all. This work is dedicated to the glory of God and to my family.

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LIST OF ABBREVIATIONS

AA	Arachidonic acid
ADC	Adenocarcinoma
ALA	Alpha linolenic acid
AP	Anterior prostate
AR	Androgen receptor
BHT	Butylated hydroxyl toluene
C3(1)	Prostate steroid binding protein T-antigen
CA	Canola oil containing diet
CEBP β	CCAAT/ enhancer binding protein β
CO	Corn oil containing diet
CO/CO	Mother fed with corn oil containing diet, baby fed with corn oil containing diet after weaning
CO/FS	Mother fed with corn oil containing diet, baby fed with fish oil containing diet at puberty
CO/walnut	Mother fed with corn oil containing diet, baby fed with walnut containing diet after weaning
DHA	Docosahexaenoic acid
DL	Dorsolateral prostate
DPA	Docosapentaenoic acid
EPA	Eicosapentaenoic acid
ER α	Estrogen receptor alpha
Fas	Fatty acid synthase

FS	Fish oil containing diet
GAPDH	Glyceraldehyde 3 phosphate dehydrogenase
GU	Genitourinary bloc
H & E	Hematoxylin and eosin
HGPIN	High grade prostatic intraepithelial neoplasia
Ikkkb (IKK β)	Inhibitor of κ B kinase beta
IPA	Ingenuity pathway analysis software
LA	Linoleic acid
LGPIN	Low grade prostatic intraepithelial neoplasia
Nfkbia (IK α)	NF κ B inhibitor alpha
NOS2	Nitric oxide synthase Type II
PCNA	Proliferating cell nuclear antigen
PIN	Prostatic intraepithelial neoplasia
PUFA	Polyunsaturated fatty acid
SV40 Tag	SV40 Large T-antigen
VP	Ventral prostate
Walnut/CO	Mother fed with walnut containing diet, baby fed with corn oil containing diet after weaning
ω -3(n-3)	Omega 3 fatty acid
ω -6(n-6)	Omega 6 fatty acid

CHAPTER ONE

INTRODUCTION

Diet and the risk of cancer

There have been remarkable changes in the production and consumption of food due to industrialization and/or urbanization. Such changes have resulted in reduced risk of some dietary deficiencies, increased food production and improved overall nutrition. However, in many societies these changes have also resulted in adverse shifts in diet composition so as to include high fat content and higher consumption of omega-6 fatty acids (Simopoulos, 2008). For example, the Western world consumes more total fat, with high amounts of omega-6 and low amounts of omega-3 fatty acids in their diet relative to the rest of the world (Cordain *et al.*, 2002). These changes in diet composition are thought to influence the risk of obesity, cardiovascular disease, cancer, inflammatory and autoimmune diseases (Simopoulos, 2002).

Cancers in the human can be caused by genetic factors and the incidence may also be determined by external factors such as diet (Doll and Peto, 1981). Dietary components modify several processes in normal as well as in cancer cells (Milner, 2004). In normal cells, food and their nutrients are needed for normal functioning of all biological processes. In cancer cells, over 500 dietary components have already been identified as possible modifiers of cancer processes (Milner, 2002). As the adage goes, we are what we eat. Dietary components can either inhibit processes that lead to cancer or contribute to its development. For example, feeding a diet that is high in fat, but low in calcium and vitamin D, increased colon cancer in rodents (Wargovich, *et al.*, 1990 ; Newmark *et al.*, 1990), and feeding with black raspberries inhibited esophageal tumorigenesis in mice (Kresty *et al.*, 2001).

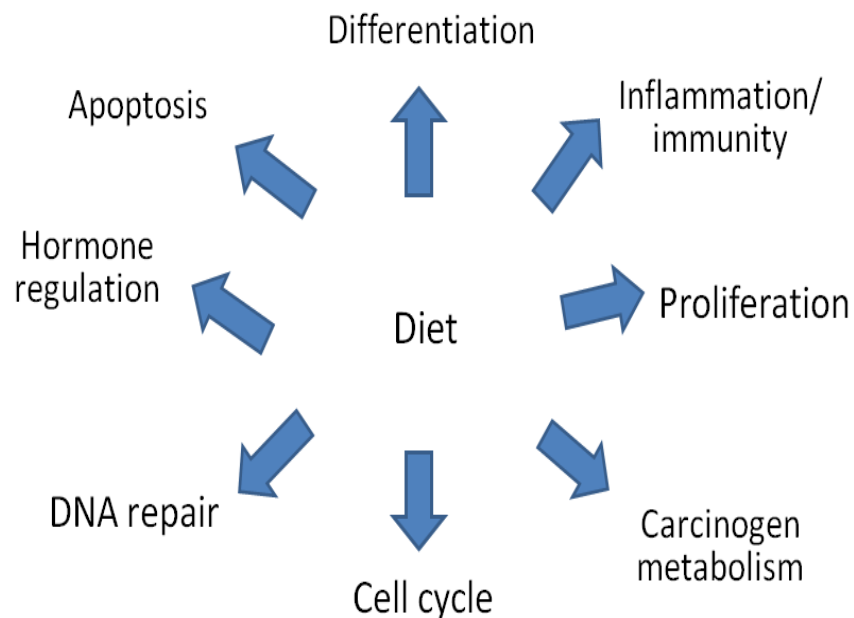


Fig. 1.1: Diet and cellular processes linked to cancer

Diet can influence several fundamental processes (Fig. 1.1) involved in the promotion or inhibition of the development and progression of cancer. For example, retinoids can inhibit proliferation of cancer cells by inducing apoptosis or convert abnormal cells to normal cells by inducing differentiation (Sporn *et al.*, 1984). Phytoestrogens, which are abundant in soya beans, can inhibit the proliferation of cancer cells (Adlercreutz, 2002). Vitamin A induces cell cycle arrest (Bohnsack and Hirschi, 2004) and high insulin, which may increase body fat, can promote tumor growth (Yakar *et al.*, 2005). Some studies also showed that feeding mice with a diet high in omega-3 fatty acid from canola oil or walnuts, compared to feeding mice with a diet containing corn oil diet, resulted in reduced growth rate of implanted human breast cancer

(Hardman, 2007; Hardman and Ion, 2008). Furthermore, fish oil supplements have been shown to decrease the number of tumors in an animal model of colorectal cancer (Rao *et al.*, 2001). Long-chain omega-3 (eicosapentaenoic acid), abundant in fish oil (Petrik *et al.*, 2008), can suppress tumor proliferation (Nkondjock *et al.*, 2003) by inhibiting the signaling of activated oncogenes (Collett *et al.*, 2001).

The incidences and rates of cancer vary from one region of the world to another, with overall incidence rates being higher in the developed world than in the developing world, but the overall mortality rates are generally similar (Jemal *et al.*, 2011). There is a rapid change observed in cancer rate after migration from one region to another. In general, migration results in dietary changes which are followed by changes in disease pattern within one or two generations. Studies have shown that people migrating between areas with different cancer incidence rates tend to acquire the cancer rates characteristic of their new location (McMichael *et al.*, 1980), implying that lifestyle factors, such as diet, profoundly affect the risk for cancer and therefore cancer can be prevented.

Omega-3 and omega-6 fatty acids

Alpha linolenic acid (ALA) and linoleic acid (LA) were discovered as essential fatty acids some 80 years ago (Burr and Burr, 1973). ALA and LA belong to the families of omega-3 (ω -3) and omega-6 (ω -6) fatty acids respectively. Fatty acids consist of a chain of hydrogen and carbon atoms with a methyl group at the tail (ω) end and a carboxyl group at the head (δ) end (Fig. 1. 2). The carbon atoms may be connected by single or double bonds. Saturated fatty acids contain the maximum level of hydrogen atoms possible and are therefore single bonds. Polyunsaturated fatty acids have bonds not saturated with hydrogen and therefore have two or more double bonds

connecting carbon atoms. In omega-3 fatty acids, the first double bond occurs on the third carbon atom from the ω end, whereas in omega-6 fatty acids, the first double bond is on the sixth carbon atom from the ω end.

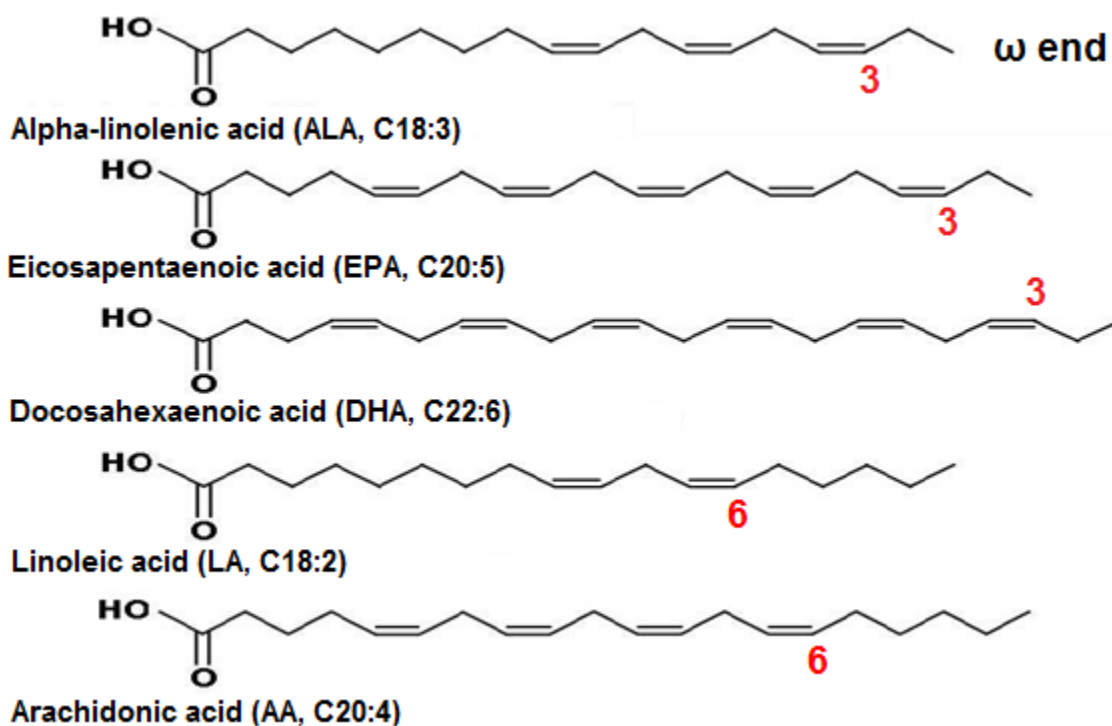


Fig. 1.2. Structures of omega-3 and omega-6 fatty acids

Omega-3 and omega-6 fatty acids are the two major classes of polyunsaturated fatty acids found in the human diet. Both are essential dietary components for normal growth and development of human beings as they cannot be synthesized by humans. Omega-3 and omega-6 fatty acids are known to have opposing physiological functions (Simopoulos, 2008). They are not interconvertible because mammalian cells lack the converting enzyme, omega-3 (δ -15) desaturase (Kang, 2004). ALA is a precursor for the synthesis of other members of the ω -3

family, namely eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). ALA is found in high amounts in flaxseed, walnuts, perilla oil, canola oil and green leaves, particularly from wild plants (Simopoulos and Childs, 1990; Simopoulos, 2008). EPA and DHA are abundant in fish and fish oils (Hardman, 2004). Arachidonic acid (AA) is the other member of the omega-6 family and can be synthesized from LA. Vegetable oils such as corn oil, sunflower, safflower and linseed oil are rich in LA (Simopoulos, 2008), whereas AA is abundant in red meat and red meat fat (Hardman, 2004). Western diets are low in omega-3 fatty acids and have excessive amounts of high omega-6 fatty acids, which may promote pathogenesis of many diseases including cancer (Simopoulos, 2008). For example, mammary carcinogenesis was suppressed when equal parts of ω -6 and ω -3 fatty acids were fed to rats (Cohen *et al.*, 1993; Sasaki *et al.*, 1998). An ω -6 / ω -3 ratio of about 1:1, as found in wild animals, is believed to be ideal for normal biological processes and is closer to the ratio on which humans evolved (Simopoulos *et al.*, 1999; Simopoulos, 2008).

Metabolism of omega-3 and omega-6 fatty acids and their effects on cancer

Polyunsaturated fatty acids (PUFA) are essential components of structural membrane lipids. Both ω -3 and ω -6 PUFA are absorbed and integrated into cell membrane phospholipids either as consumed in the diet (Li *et al.*, 1998), or they can be elongated and desaturated by delta 5- and delta 6-desaturases to form longer-chain PUFA, namely AA, EPA and DHA (Hagve and Christophersen, 1984 ; Denys *et al.*, 2005) (Fig. 1.3). Humans and rodents can convert LA to AA and ALA to EPA and DHA but at a slow rate, and delta 6-desaturase is the rate-limiting enzyme in the synthesis of longer chain PUFA (de Gomez Dumm and Brenner, 1975). For example, the production of AA from LA is suppressed by ALA, EPA and DHA which outcompete LA for the

activity of the desaturases (Hagve and Christophersen, 1984). A study of human subjects by Cleland *et al.*, (1992) showed LA to inhibit the incorporation of EPA from dietary fish oil supplements in neutrophil membrane phospholipids. However, decreasing the levels of LA with ALA in dietary fats was found to increase EPA level in plasma phospholipids in human (Liou *et al.*, 2007).

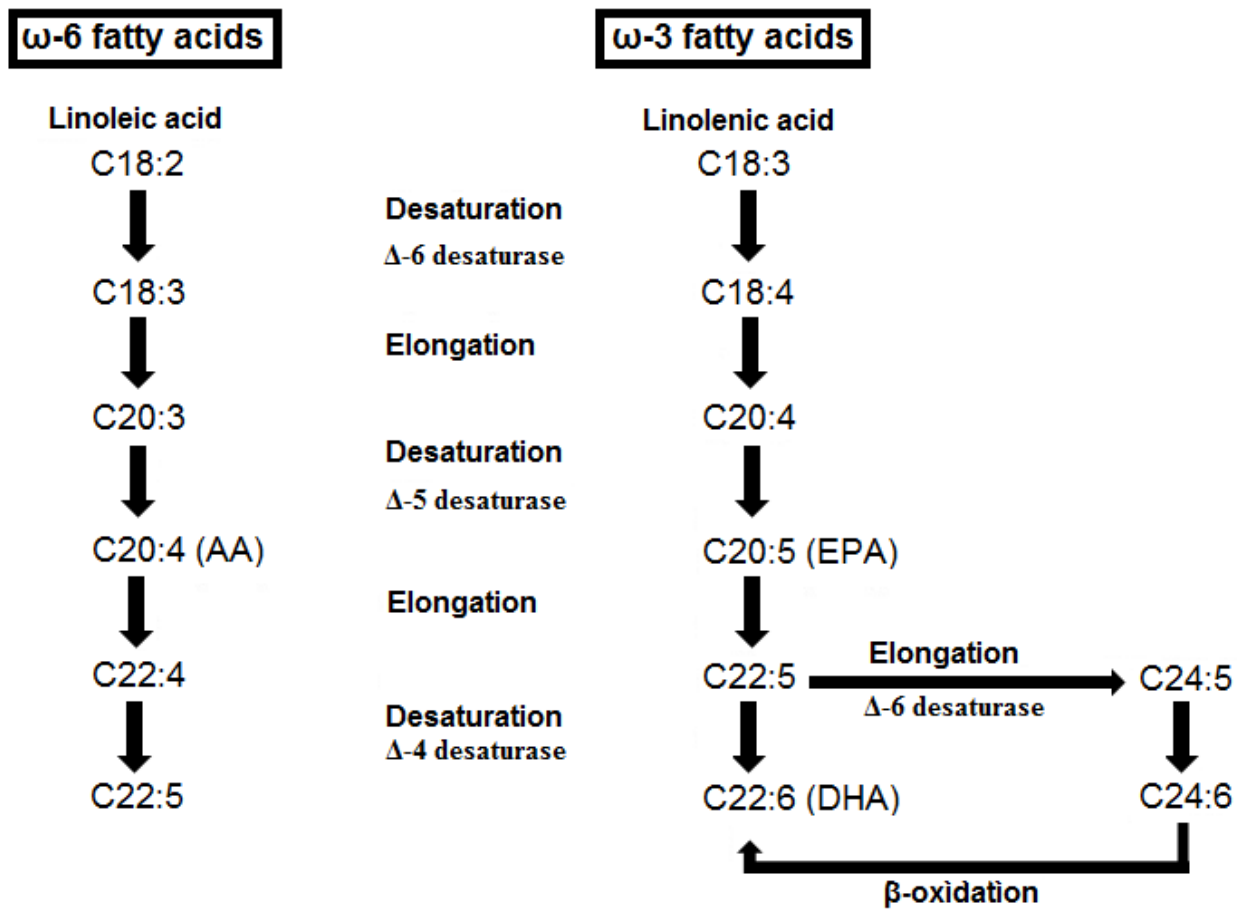


Fig 1.3. Synthesis of longer-chain omega-6 and omega-3 fatty acids.

Fatty acids released from membrane phospholipids are important cell signaling molecules (Simopoulos, 2008). Omega-3 and omega-6 fatty acids can be cleaved from cell membrane by phospholipase A₂ (PLA₂) for metabolism into eicosanoids by cyclooxygenase (COX) and lipoxygenase (LOX) enzymes (Denys *et al.*, 2005). These eicosanoids induce signaling at the autocrine or paracrine level (Simopoulos., 2001). In most tissues cyclooxygenases (COX-1 and COX-2) can metabolize AA into the 2-series prostaglandins (including PGE₂) and EPA into 3-series prostaglandins (including PGE₃). PGE₂ favors cell proliferation while PGE₃ is anti-inflammatory and anti-proliferative (Al Olama *et al.*, 2009; Yang *et al.*, 2004). EPA is a better substrate for COX and can compete more successfully for COX activity than AA for the synthesis of prostaglandin (Yang *et al.*, 2002; Needleman *et al.*, 1979). COX metabolites of ω -6 fatty acids can stimulate angiogenesis (Rose and Connolly, 2000), whereas exposure to ω -3 fatty acids is able to suppress vessel formation via the inhibition of VEGF and VEGFR (Tsuzuki *et al.*, 2007; Tsuzuki and Kawakami, 2008). Omega-3 fatty acid can inhibit NF κ B activation and restore apoptosis in cancer cells (Hering *et al.*, 2007).

Several investigations have reported the decreased growth of various cancers due to consumption of an ω -3 diet. Kim *et al.*, (1998) showed that colon cell proliferation was lower in rats fed ω -3 PUFA from fish oil compared to rats fed ω -6 PUFA from corn oil, and diets containing ω -3 PUFA from fish oil also decreased primary breast tumor growth and its metastasis in mice (Hubbard *et al.*, 1998). Paulsen *et al.*, (1998) reported that EPA and DHA inhibited the formation and growth of aberrant crypt foci during tumorigenesis in rat colon, and Chang *et al.*, (1998) found fish oil enriched diet to lower the incidence of adenocarcinoma compared to a corn oil diet via increased apoptosis and differentiation. Furthermore, Rose & Connolly (1991) showed that the PC-3 human prostate cancer cell line growth is stimulated when

treated with LA, whereas EPA and DHA inhibited its growth. Berquin *et al.*, (2007) reported that omega-3 fatty acids reduced prostate tumor growth, slowed histopathological progression, and increased survival, whereas omega-6 fatty acids had opposite effects in a transgenic mouse model. PUFA can also interact with pro-oxidants to influence tumorigenesis as reported by Hardman *et al.*, (1997), in which iron supplementation combined with ET-18-OCH₃, a pro-oxidative drug resulted in the slowest growth rate, lowest mitotic index, highest level of lipid peroxidation products and increased the cytotoxic index in implanted human breast tumors in mice consuming a fish oil diet. Furthermore, omega-3 and omega-6 fatty acids can rapidly and directly alter the transcription of some genes (Simopoulos, 1996). Simopoulos, (1996) showed that EPA and DHA suppressed the transcription of IL-1 β , an inflammatory gene, while AA did not.

Few studies have investigated the levels of plasma omega-3 and omega-6 fatty acids in humans. Bailey and Southon (1998) reported the following normal average plasma levels of omega-3 and omega-6 fatty acids in adult humans: ALA – 27.5 μ g/mL (98.8 μ mol/L), EPA – 23.0 μ g/mL (76.0453 μ mol/L), DHA – 54.9 μ g/mL (166 μ mol/L); LA – 1221.0 μ g/mL (4353.7 μ mol/L), AA – 228.8 μ g/mL (751.47 μ mol/L). A report by Harper *et al.*, (2005) showed average plasma levels of ALA – 19.19 μ mol/L, EPA – 24.09 μ mol/L, DHA – 80.46 μ mol/L; LA – 1487.31 μ mol/L, AA – 650.5 μ mol/L in individuals with multiple illnesses that included hypertension and diabetes. Unpublished data from our laboratory showed that patients with early stage chronic lymphocytic leukemia (CLL) had average ALA – 74.97 μ mol/L, EPA – 65.9 μ mol/L, DHA – 64.9 μ mol/L; LA – 2435.0 μ mol/L, AA – 666.4 μ mol/L in the plasma. After patients received omega-3 as fish oil supplements for 3 months, the plasma levels of the longer-chain omega-3 fatty acids increased and omega-6 fatty acids decreased (ALA – 56.2 μ mol/L,

EPA – 296.4 μ mol/L, DHA – 188.5; LA – 1979.5 μ mol/L, AA – 444.9 μ mol/L). A study by Sekikawa *et al.*, (2008) compared the normal serum levels of omega-3 and omega-6 fatty acids in Japanese in Japan and White Americans. These results showed the average proportions of individual fatty acids to total fatty acids in Japanese as ALA – 0.2%, EPA – 2.5%, DHA – 5.9%; LA – 26.8%, AA – 6.6%, and in American men as ALA – 0.3%, EPA – 0.8%, DHA – 2.4%; LA – 29.9%, AA – 9.0%. From these reports, the ratio of omega-6/omega-3 was higher in individuals with illnesses that included hypertension, diabetes and early stage CLL compared to normal individuals, and lower in Japanese who are known for lower incidence for cardiovascular diseases and cancer compared to Americans with higher incidence for cardiovascular disease and cancer.

Breast cancer

Breast cancer is the cancer that originates from breast tissue. Breast cancer can occur in both women and men but it is far more common in women than in men (NCI, 2011). It is the most frequently diagnosed noncutaneous cancer and the leading cause of cancer death in females worldwide, accounting for 23% (1.38 million) of the total new cancer cases and 14% (458,400) of the total cancer deaths in 2008 (Jemal *et al.*, 2011). The incidence rates are high in the Western world and lower in Asia (Jemal *et al.*, 2011). Breast cancer is the most frequently diagnosed noncutaneous cancer and the second leading cause of cancer related death in women in US, accounting for 28% and 15% of total expected cancer incidence and death respectively in women in 2010 (Jemal *et al.*, 2010).

Breast cancer is a complex disease that involves the endocrinology of the female interacting with exogenous factors such as environmental chemicals, and lifestyle such as diet and obesity (Dewaard, 1992; Davies *et al.*, 1997).). Estrogen stimulates cell proliferation, which may lead to accumulation of random genetic errors that result in neoplasia (Henderson, 1993). The incidence of breast cancer increases with age with the majority of women diagnosed after the age of 40 years (Breast Cancer Facts & Figures, 2010). Early menarche and late menopause lead to an increased total lifetime number of menstrual cycles and increased estrogen exposure, thereby increasing the risk for breast cancer. In contrast, late menarche and early menopause lead to a reduction in breast cancer risk due to less exposure to estrogen (Vogel, 2000). The use of postmenopausal estrogen and progestin is associated with increased risk for breast cancer (Schairer *et al.*, 2000), and in utero exposure to high estrogen level has also been reported to increase the risk (Potischman and Troisi, 1999). Nulliparity and late age at first birth increase the risk for breast cancer but pregnancy at a young age, especially before the age of 20, and early age at birth of a second child are associated with reduced risk (McPherson *et al.*, 2000; Vogel, 2000). Breast cancer can be inherited as family history of the disease increases a woman's own risk for breast cancer (Hankinson, 2004). About 10% of breast cancer in Western countries is attributed to genetic predisposition (McPherson *et al.*, 2000). BREast CAncer1 and 2 (BRCA1 and BRCA2) genes account for most of the cases of familial breast cancer (McPherson *et al.*, 2000). Individuals who harbor mutations in these genes have a 60% to 80% risk of developing breast cancer in their lifetimes (Malone *et al.*, 1998). Treatments for breast cancer include surgery, hormonal therapy, chemotherapy, radiation and/or immunotherapy (Florescu *et al.*, 2011)

Morphology of the mammary gland

The female breast consists of the lobules which are involved in milk production, the ducts which convey milk from the lobules to the nipple, and the stroma which consists of fatty tissue, connective tissue, blood vessels, and lymphatic vessels (Fig. 1.4). Most breast cancers begin in the epithelial cells that line the ducts (ductal cancers). Some begin in the epithelial cells that line the lobules (lobular cancers), while a small number start in other tissues. Mammary gland development occurs throughout life beginning from childhood at a slow pace until the onset of puberty during which the process increases rapidly (Russo and Russo, 2004).

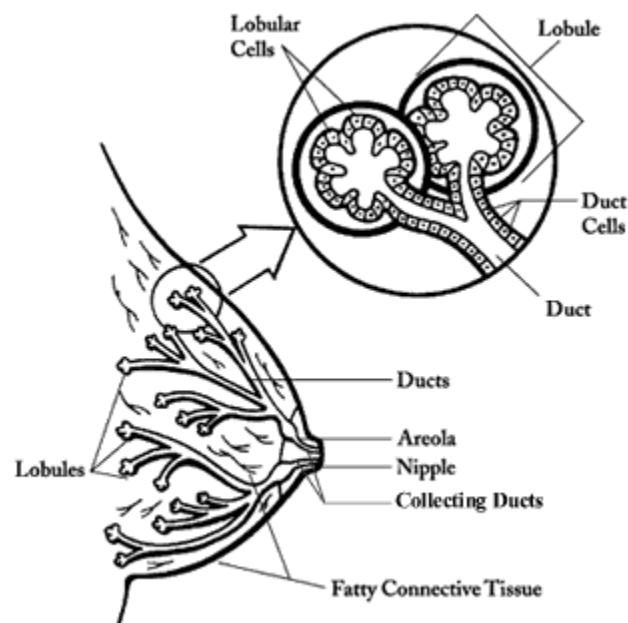


Fig.1.4. Schematic view of mammary gland morphology (Reprinted by the permission of the American Cancer Society, Inc. from www.cancer.org. All rights reserved)

Each ductal branch ends with zones of highly proliferative cells referred to as the terminal ductal lobular units (called terminal end buds in mice) which differentiate into

lobuloalveolar units (called alveolar buds in mice) during pregnancy and later stages of development (Russo and Russo, 2004). These highly proliferative structures are the initiation sites in tumorigenesis (Russo and Russo, 2004). Nulliparous women have predominantly undifferentiated structures in their breasts, whereas parous women have predominantly most differentiated structures in their breasts (Russo *et al.*, 1992).

Studies of cancer incidence in Asian-American women have strongly suggested that lifestyle and environmental factors such as diet are important factors that contribute to breast cancer risks (Davies *et al.*, 1997). Asian women, with typically a lower rate of breast cancer and higher consumption of fish oil compared with Western women, experience an increase in incidence of breast cancer after emigration to the West, increasing to equal to the indigenous population level within a generation (Ziegler *et al.*, 1993). Several studies have therefore suggested that timing of exposure to dietary intervention is important in modulating cancer risk at multiple stages such as in utero, prepuberty, puberty, pregnancy and lactation (Hilakivi-Clarke, 2001; De and Hilakivi-Clarke, 2006; Lo *et al.*, 2009). As treatment for breast cancer is sometimes unsuccessful or may be too late, and, where possible, the side effects are significant and distressing. Thus, preventing cancer is a desirable strategy.

Breast cancer classification

Breast cancer is classified into several categories according to multiple different schemes that describe each individual breast cancer in a way that helps select which treatment approach will yield the best outcome. A full classification includes histopathological type, grade of tumor, stage (TNM) of tumor, receptor status, and the presence or absence of genes as determined by DNA testing.

The commonly used grading system is the Bloom-Richardson-Elston grading system (Genestie *et al.*, 1998), which examines the cells and tissue structure of the cancer to determine how aggressive and invasive the cancer is. Grade 1 tumor is well differentiated tumor with the best prognosis, grade 2 is moderately differentiated tumor with a medium prognosis and grade 3 is poorly differentiated tumor with the worst prognosis. Lower grade tumors can be treated with less aggressive surgery and medication and have a better survival rate, whereas higher grade tumors require more aggressive surgery, radiation, and drugs, and have a worse survival rate.

The TNM (tumor, node, metastasis) staging system describes the extent of cancer in a patient's body (Denoix, 1946). It uses the size and extension of the primary tumor, its lymphatic involvement, and the presence of metastases to classify the progression of cancer. 'T' describes the size of the tumor and whether it has invaded nearby tissue, 'N' describes regional lymph nodes involved, and 'M' describes distant metastasis. The TNM descriptors are often combined into a single overall stage grouping number called Prognostic Group, labelled as Stage 0 to IV. Most Stage I tumors are curable whereas most Stage IV tumors are incurable.

Immunohistochemistry is used to identify the presence of estrogen receptors (ER), progesterin receptors (PR) and Human Epidermal growth factor Receptor 2 (HER2) all of which are routinely assayed in breast cancers to determine the suitability of using targeted treatments such as tamoxifen, an antagonist of the estrogen receptor.

DNA classification uses DNA microarrays for comparing normal cells to breast cancer cells to identify differences in gene expression levels for disease prognosis and to help determine therapeutic strategies (Sparano and Solin, 2010).

Prostate cancer

Prostate cancer is a cancer that develops in the prostate of the male reproductive system. It is the second most common noncutaneous cancer in men worldwide and the sixth leading cause of cancer death in males (Jemal *et al.*, 2011). It accounted for 14% (903,500) of the total new cancer cases and 6% (258,400) of the total cancer deaths in males in 2008 (Jemal *et al.*, 2011). The incidence of prostate cancer is highest in the developed nations of North America, Europe, and Oceania, and lowest in Asia (Jemal *et al.*, 2011). In the US, prostate cancer remains the most frequently diagnosed noncutaneous cancer and the second leading cause of cancer related death among men, accounting for 28% of total expected cancer incidence in men in 2010 (Jemal *et al.*, 2010). The risk for prostate cancer increases with age and is common among men aged 65 and above (Gronberg, 2003). Prostate cancer, in most cases, is slow-growing but can also be fast developing and aggressive and may metastasize from the prostate to other parts of the body, particularly bones and lymph nodes. Conventional treatment regimens include active surveillance (monitoring for tumor progress or symptoms), radical prostatectomy, radiation therapy, chemotherapy, hormonal therapy, or some combination of these modalities.

Prostate cancer can be caused by genetic factors which may be inherited (Al Olama *et al.*, 2009). Men with a family history of prostate cancer have a higher risk of developing the cancer. There is also the shared familial risk for prostate and breast cancers. For example, mutations in the *BRCA-2* gene (Breast Cancer 2 susceptibility gene) increase the risk for prostate cancer that is more aggressive and develops at a younger age (Cancer Facts and Figures, 2010). Some epidemiologic and more experimental studies have reported that nutrition also plays a role in the development of prostate cancer. Consumption of high fat diets is associated with high risk for prostate cancer (Kolonel *et al.*, 1981; Giovannucci *et al.*, 1993). A study by Ritch *et al.*, (2007)

found that omega-6 fatty acids stimulated prostate cancer cell growth, whereas omega-3 fatty acids inhibited cancer cell growth in Jamaican men known for high consumption of omega-6 fatty acids and with the highest incidence of prostate cancer. A possible protective role in reducing prostate cancer has also been reported for foods containing Vitamin B6 (Kasperzyk *et al.*, 2009), selenium, vitamin E, lycopene, and soy (Lee *et al.*, 2003).

Although treatment for prostate cancer has advanced greatly, the cost is enormous and treatment is not very effective. Identifying and establishing dietary factors that may reduce the risk for prostate cancer will no doubt reduce the human and economic burden for its treatment.

Anatomy and histology of the human and mouse prostate

The human prostate lacks discernible lobular structure, but it is described as a zonal architecture consisting of the central, transition, and peripheral zones, together with an anterior fibromuscular stroma (McNeal, 1988, Timms, 2008) (Fig. 1.5A). The peripheral zone occupies the most volume and harbors the majority of prostate carcinomas (Shappell *et al.*, 2004). In contrast to the human prostate, the mouse prostate consists of multiple lobes corresponding to the anterior, ventral, lateral and dorsal lobes (Fig. 1.5B). The dorsal and lateral lobes are often combined and referred to as the dorsolateral lobe, which is most homologous to the human peripheral zone (Shappell *et al.*, 2004).

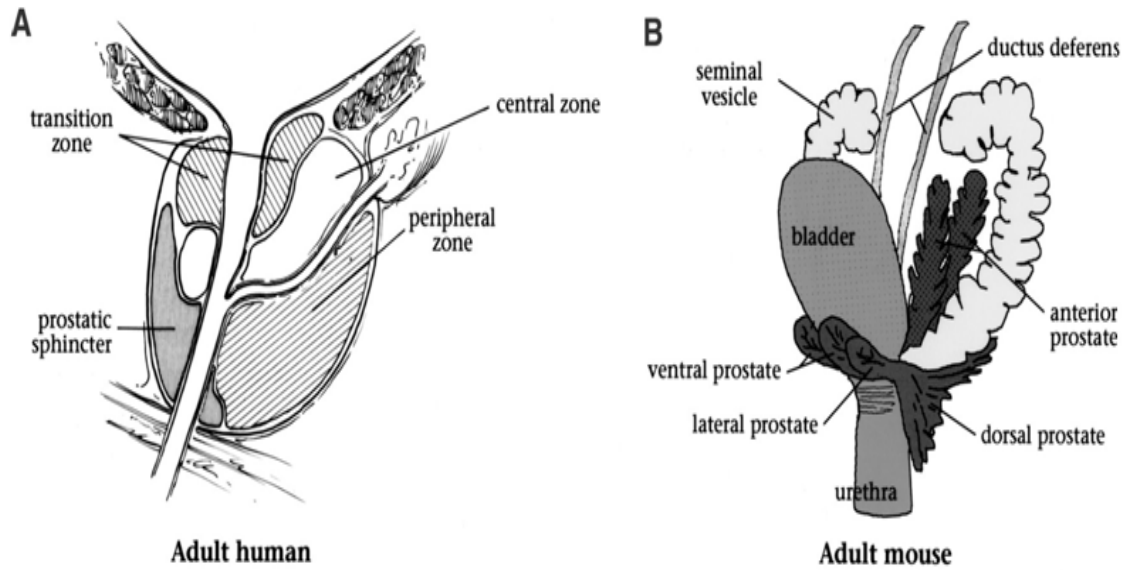


Fig. 1.5. Schematic view of the anatomy of the human and mouse prostate (Cory Abate-Shen and Michael M. Shen, *Genes & Dev.*, 2000. With kind permission of Cold Spring Harbor Laboratory Press)

Histologically, both the human and mouse prostate contain a pseudostratified epithelium with three differentiated epithelial cell types: luminal, basal, and neuroendocrine (Shappell *et al.*, 2004) (Fig. 1.6).

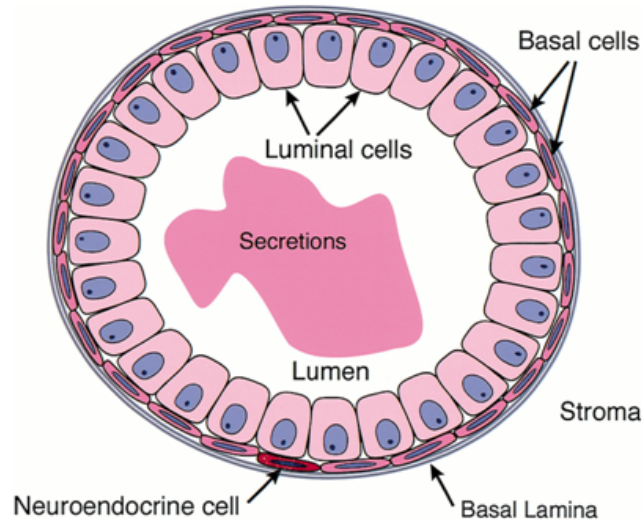


Fig. 1.6. Schematic view of the cell types in the human and mouse prostatic duct (Cory Abate-Shen and Michael M. Shen, *Genes & Dev.*, 2000. With kind permission of Cold Spring Harbor Laboratory Press)

The luminal epithelial cells form a continuous layer of columnar cells that produce protein secretions. Basal cells and the neuroendocrine cells are located beneath the luminal epithelium. The neuroendocrine cells are rare cells in the normal prostate.

Hormone responsiveness in prostate cancer

Androgen and estrogen are involved in the normal development of the prostate as well as in prostate cancer pathogenesis (King *et al.*, 2006). The most abundant androgen is testosterone, which is produced mostly by the testis. Additional testosterone can be synthesized in the adrenal gland. Testosterone is converted into the more potent metabolite dihydrotestosterone (DHT) in prostate tissue through the activity of 5 α -reductase. The direct effect of androgen is mediated by the androgen receptor (AR). The dependency of prostate tissue on androgen is manifested by

rapid cell apoptosis and regression following androgen withdrawal (Watson *et al.*, 2005; Gao *et al.*, 2004). Circulatory estrogens are produced in a very low amount in the testis. Estrogen may also be produced locally within the prostate via conversion of testosterone to estrogen by the aromatase enzyme (Risbridger *et al.*, 2003). The direct effects of estrogen on prostate are mediated through estrogen receptors (ER) (Griffiths *et al.*, 1997; Bohnsack and Hirsch, 2004; Omoto, 2008) and indirectly by increasing the sensitivity of the prostate tissue to low levels of testosterone via up-regulation of the androgen receptor (Moore *et al.*, 1979; Richer *et al.*, 2007). Estradiol is reported to induce high-grade PIN and prostate cancer in the aging dog (Ho, 2004) whereas toremifene, an antiestrogen, prevented prostate cancer in mouse (Raghow *et al.*, 2002).

Pathological classification of prostate cancer

Most prostate cancers are adenocarcinoma (Shen and Shen, 2010). Prostatic intraepithelial neoplasia (PIN) is the precursor for prostate cancer (Shappell *et al.*, 2004) (Fig. 1.7). PIN can be classified as low-grade PIN (LGPIN) or high-grade PIN (HGPN). The histology of PIN is characterized by epithelial hyperplasia, enlargement of nuclei and nucleoli, cytoplasmic hyperchromasia, and nuclear atypia (Shappell *et al.*, 2004).

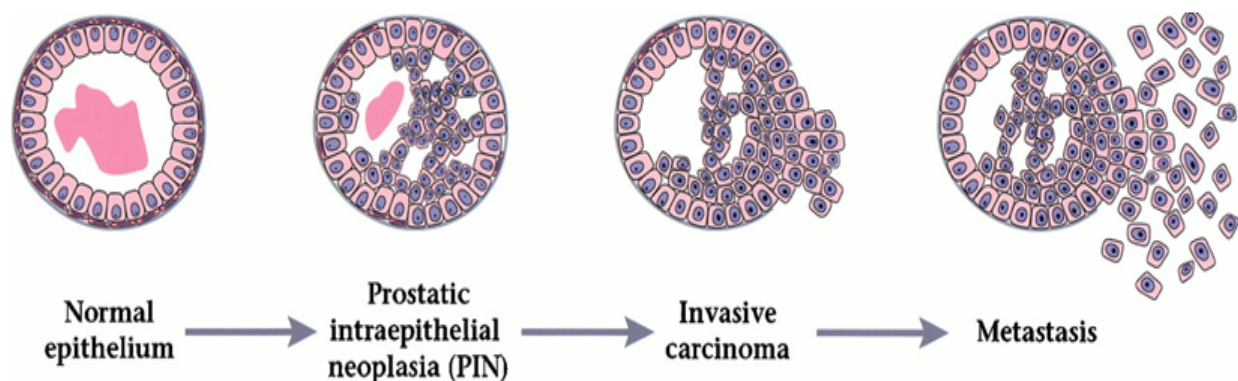


Fig. 1.7. Schematic illustration of prostate cancer progression (Cory Abate-Shen and Michael M. Shen, *Genes & Dev.*, 2000. With kind permission of Cold Spring Harbor Laboratory Press)

Histopathological grading of prostate tissue is done by Gleason grading system (Gleason and Mellinger, 1974), which evaluates the tumor's degree of differentiation and anaplasia. The shape, size and staining properties, as well as whether they are well, moderately or poorly differentiated cells, are taken into account. The Gleason score ranges between 1 and 10. Well differentiated cells are given Gleason score 1, score 2 – 4 is low grade, score 5 – 7 is moderate, and score 8 – 10 is a high grade tumor.

Mouse model

The C3(1) TAg transgenic mouse model, developed in Dr Jeffrey Green's laboratory of the National Cancer Institute, was chosen in our intervention studies because mammary and prostate cancers from these mice are histologically similar to human breast and prostate cancers

respectively (Green *et al.*, 2000). This model has been used to study omega-3 intervention against tumorigenesis in the mammary gland and well as in the prostate.

The mice carry the C3(1)SV40 TAg construct in which the C3(1) rat prostate steroid binding protein promoter drives the expression of the SV40 large T (TAg) in the epithelium of the mammary and prostate glands (Maroulakou *et al.*, 1994). SV40 TAg induces transformation and tumorigenesis by binding to and functionally inactivating p53 and Rb (Mietz *et al.*, 1992; Dyson *et al.*, 1989). Male and female hemizygotes were generated by crossing the C3(1) TAg mice (having FVB/N background) and SV129 wild type female mice to obtain C3 (1)TAg/129 hemizygote offspring . Female hemizygotes developed hyperplasia in the mammary duct at 3 months of age, progressing to adenocarcinoma at 4 months. Metastasis to the lung occurred by 6 months. This model has been successfully used in studies of chemoprevention of mammary tumorigenesis (Green *et al.*, 2000).

The homozygous male transgenic mice developed prostatic hyperplasia by 3 months which progressed to adenocarcinoma by 7 months (Maroulakou *et al.*, 1994). Prostate cancer metastases to lung occurred after 8 months (Maroulakou *et al.*, 1994). Hemizygous FVB/C3(1)TAg male mice developed prostatic intraepithelial lesion (PIN) that histologically resembled those found in human PIN (Shibata *et al.*, 1996). Low-grade PIN appeared at 2 months and progressed to high grade at 5 months, and prostate carcinomas developed after 7 months (Shibata *et al.*, 1996). The C3(1)/TAg transgenic model develops prostate cancer slowly and has a well characterized disease progression (Shibata *et al.*, 1996), which makes it well suited for prevention studies.

STUDY OBJECTIVES

The main objectives of our studies were to determine whether increasing omega-3 and decreasing omega-6 fatty acids via consumption of diets that contain canola oil, walnut or fish oil supplements instead of corn oil will slow the development for breast cancer and prostate cancer in our transgenic mouse model. In chapter two, we investigated whether increasing omega-3 and reducing omega-6 fatty acids using canola oil instead of corn oil in the maternal diet might reduce the risk for breast cancer in female offspring. We found this change to the maternal diet suppressed tumorigenesis in the mammary glands of the offspring. In chapter three, we investigated whether regular walnut consumption might reduce the risk for developing breast cancer. We found that consumption of an equivalent of 2 servings of walnut per day slowed mammary gland tumorigenesis. In chapter four I investigated whether changing from corn oil diet to a fish oil-supplemented diet at adulthood might reduce the risk for prostate cancer. We found that consumption of fish oil-supplemented diet suppressed prostate tumorigenesis.

In all of these studies, we showed that a shift from a diet with a high omega-6/omega-3 ratio, as prevalent in Western diets, to lower omega-6/omega-3 diets was beneficial for suppressing breast and prostate cancers in these mice. The diets were chosen to be an easy change for humans to make. Such changes may be beneficial for reducing the risk for breast and prostate cancer in humans.

CHAPTER TWO

Maternal consumption of canola oil suppressed mammary gland tumorigenesis in C3(1) TAg mice offspring

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Juliana A. Akinsete assisted with all facets of the study and performed the protein analyses.

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Abstract

Background

Maternal consumption of a diet high in omega-6 polyunsaturated fats (n-6 PUFA) has been shown to increase risk whereas a diet high in omega-3 polyunsaturated fats (n-3 PUFA) from fish oil has been shown to decrease risk for mammary gland cancer in female offspring of rats. The aim of this study was to determine whether increasing n-3 PUFA and reducing n-6 PUFA by using canola oil instead of corn oil in the maternal diet might reduce the risk for breast cancer in female offspring.

Methods

Female SV 129 mice were divided into two groups and placed on diets containing either 10% w/w corn oil (which is 50% n-6 PUFA, control diet) or 10% w/w canola oil (which is 20% n-6 PUFA, 10% n-3 PUFA, test diet). After two weeks on the diets the females were bred with homozygous C3(1) TAG transgenic mice. Mother mice consumed the assigned diet throughout gestation and nursing of the offspring. After weaning, all female offspring were maintained on the control diet.

Results

Compared to offspring of mothers fed the corn oil diet (CO/CO group), offspring of mothers fed the canola oil diet (CA/CO group) had significantly fewer mammary glands with tumors throughout the experiment. At 130 days of age, the CA/CO group had significantly fewer tumors per mouse (multiplicity); the tumor incidence (fraction of mice with any tumor) and the total tumor weight (per mouse that developed tumor) was less than one half that of the CO/CO group. At 170 days of age, the total tumor weight per mouse was significantly less in the CA/CO group and if a tumor developed the rate of tumor growth rate was half that of CO/CO group. These

results indicate that maternal consumption of canola oil was associated with delayed appearance of mammary gland tumors and slowed growth of the tumors that developed.

Conclusions

Substituting canola oil for corn oil is an easy dietary change for people to make; such a change to the maternal diet may decrease risk for breast cancer in the daughter.

Background

It has been shown that diets that contain high amounts of omega-6 polyunsaturated fatty acids (PUFA) increase the growth rates of cancers (Rose and Hatala, 1994) whereas omega-3 PUFA have been shown to reduce cancer growth rates (Rose and Connolly, 1993) and have been suggested as cancer preventive agents (Rose, 1997). The type of fat consumed by the mother during pregnancy and nursing of the offspring has also been shown to influence mammary gland cancer risk in the offspring. A maternal diet that contained a high (versus low) amount of omega-6 fatty acids increased the risk for mammary gland cancer in the carcinogen-treated offspring (Hilakivi-Clarke *et al.*, 1997). Compared to a corn oil diet, maternal consumption of either an olive oil containing diet (high in omega 9 fatty acid) (Stark *et al.*, 2003) or of a diet containing long chain omega 3 PUFA from fish oil (Hilakivi-Clarke *et al.*, 2002) has been shown to decrease carcinogen-induced mammary gland cancer in rat offspring. We have reported that the 18 carbon omega-3 PUFA found in canola oil also effectively slowed the growth of implanted mammary gland cancers (Hardman, 2007). We hypothesized that a maternal diet that contained

canola oil instead of corn oil would increase maternal dietary omega 3 PUFA and thus reduce the risk for mammary gland cancer in the offspring.

The C3(1) SV40 T-antigen transgenic mouse was selected as the model for this study. This mouse is a well characterized model developed in the laboratory of Dr. Jeffrey E. Green (jeffrey.green1@nih.gov). The transgene uses the 5' flanking region of the rat C3(1) prostate steroid binding protein to target expression of the SV 40 large T-antigen in the mammary gland and prostate (Green *et al.*, 2000). The female mice develop invasive mammary gland tumors by 16 weeks of age (Green *et al.*, 2000). The tumors are similar to human infiltrating ductal carcinomas, are hormone responsive at early stages but become hormone independent in later stages (Green *et al.*, 2000) similar to advanced human breast cancers. The lesions develop in a predictable time, thus, this mouse is a good model to study alterations in tumorigenesis and progression (Green *et al.*, 2000). The T-antigen is thought to induce cancer by inactivation of p53 (Mietz *et al.*, 1992) and Rb (Dyson *et al.*, 1989), two proteins that are involved in cell cycle regulation. Both p53 and Rb are tumor suppressors and are frequently mutated in human breast cancer. Because the mouse has a strong transgenic tumor promoter, complete prevention of tumors would not be expected but a delay in tumor development or slower tumor growth compared to a control group would indicate cancer preventive benefit.

Most studies that have tested effects of omega 3 fatty acids on cancer have used the long chain omega 3 fatty acids, eicosapentaenoic (EPA, 20 carbons, 5 unsaturations) or docosahexaenoic (DHA, 22 carbons, 6 unsaturations) as the test fatty acids (Cave, 1991; Clarke *et al.*, 1999; Germain *et al.*, 1999; Chen and Istfan, 2000; Hardman *et al.*, 2002). These fatty acids are most commonly found in fish, thus fish oil or fish oil concentrates are frequently incorporated into diets. However, the double bond in the omega-3 position is produced by plants,

not by animals, and can be found in plant products as the 18 carbon fat, α -linolenic acid (ALA). Good dietary sources of ALA include canola oil, walnuts and “omega-3 eggs” (Bourre, 2007). Flaxseed or flaxseed oil is also a good source of ALA (Demark-Wahnefried *et al.*, 2001) but is often consumed as a dietary supplement rather than a part of the regular diet. Canola oil contains about 10% ALA and about 20% linoleic acid (LA), an 18 carbon omega 6 fatty acid (USDA, 2009), whereas corn oil contains about 50% LA and about 1% ALA (USDA, 2009). A ratio of omega-3 to omega-6 fatty acids of somewhere between 1:1 and 1:4 has been suggested to be much healthier than the 1:10 to 1:25 ratio of omega-3 to omega-6 fatty acids contained in the usual Western diet (Simopoulos, 1991).

The ALA that is consumed by animals (whether fish, mouse or human) may be metabolized without change or it may be elongated and desaturated to longer chain lipids of the omega-3 series (Simopoulos, 1991). Humans elongate and desaturate ALA. However, there is controversy about the efficiency of this conversion (Simopoulos, 1991). Recent reports indicate that humans do convert ALA to measurable amounts of EPA and docosapentaenoic acid (22 carbons, 5 unsaturations, omega-3, DPA) and that the conversion is adequate to reduce measures of inflammation, indicating biologic activity of the omega 3 fatty acids in humans (Zhao *et al.*, 200; Zhao *et al.*, 2007). Long chain omega-3 PUFA incorporated in tissues have been shown to slow cancer growth in animal models by multiple mechanisms, including slowing proliferation, increasing apoptosis (Rose *et al.*, 1995), increasing lipid peroxidation (Hardman *et al.*, 2002) and increasing oxidative damage in cancer cells but not normal cells (Hardman *et al.*, 2002). Induction of these mechanisms is correlated with an increased amount of the omega-3 PUFA in the tissues.

Johnson *et al.*, (2007) used data from the National Health and Nutrition Survey, 1999-2002, to estimate the actual consumption of ALA and LA in the average American diet. They report that the usual diet of adult Americans contains about 14.7 g of LA and about 1.5 g of ALA per day (Johnson *et al.*, 2007), almost 10 times as much LA as ALA. They estimated that, if Americans replaced vegetable oils, butter, and margarine with canola oil or canola oil based margarine, the intakes of ALA would increase to 2.6 g per day and of LA would decrease to 8.1 g per day (Johnson *et al.*, 2007). This would change the ratio of these omega-3 and omega-6 fatty acids in the diet to about 1:3, a much healthier ratio that would better meet current dietary fat recommendations (Johnson *et al.*, 2007).

We have shown that the use of canola oil as a source of omega-3 PUFA significantly slowed the growth rate of MDA-MB 231 human breast cancers implanted in nude mice and that the decreased tumor growth rate was associated with increased long chain omega-3 PUFA in mouse tissues (Hardman, 2007). There is a suggestion that canola oil use may be beneficial against cancer in humans in that women who used canola oil for cooking had a lower risk for breast cancer than those who used hydrogenated fats or corn oil (Wang *et al.*, 2008). The use of canola oil instead of corn oil is a diet change that would be easy to make because canola oil could be substituted for corn oil in baking, frying and salad dressings. Our results indicate that such a dietary change could result in important health benefits, perhaps decreasing risk for cancer in the next generation.

Methods

Animals

Twenty female SV129 mice, 6 weeks old were obtained from Charles River Laboratories (Wilmington, MA). Breeding pairs of mice bearing a transgene for the SV40 large T antigen with a C3(1) rat prostate steroid binding protein promoter were obtained from the Dr. Jeffrey Green. The female transgenic mice are expected to develop mammary gland cancer due to expression of the large T antigen in the mammary gland (Maroulakou *et al.*, 2001). The transgenic line is maintained in the laboratory and all mice are genotyped to ensure presence of the transgene. All animal work was approved by the Marshall University School of Medicine Institutional Animal Care and Use Committee.

Study design

Mice were quarantined for 2 weeks and then moved to a study room. SV129 females were split into 2 groups and numbered for identification. Ten female mice were placed on a diet containing 10% w/w corn oil (control diet, see below) and ten female mice were placed on a diet containing 10% w/w canola oil (test diet). After 2 weeks these females were bred with homozygous C3(1)/TAg male mice. The hemizygous female pups from these breedings were the experimental mice NOT the wild type mother mice. Pups were weaned at 21 days old and placed on the corn oil containing diet, generating two experimental groups: corn/corn (CO/CO) and canola/corn (CA/CO) (the first diet is the maternal diet, the second diet is the pup's diet). Only the maternal diet of the CA/CO group contained canola oil, not the diet of the experimental pups. The offspring were housed 3 to 4 in a cage, individually numbered for identification, and weighed weekly.

Diet

Diets were prepared in the Marshall University School of Medicine animal diet prep room. Diet composition is shown in Table 2.1 and was formulated to be isocaloric, isonutrient and more relevant to human consumption than the high fat diets used in many studies. (If a Western diet contains about 14.7g of LA/day (Johnson *et al.*, 2007) the calories from LA are 6.6% of a 2000 calorie diet. The 10% corn oil mouse diet contained 10.9% of calories from LA.) The AIN-76A diet is adequate for the nutritional support of the mice (American Institute of Nutrition, 1977). The dry ingredients of the diet, except sugar, were obtained in bulk from MP Biomedicals (Solon, Ohio), and sugar and oil were purchased locally (100% canola oil or 100% corn oil, no additives or preservatives). Batches of diet were prepared as needed, about every two weeks.

Table 2.1 Composition of the diets

Ingredient	% of wt	Amount /100g	Calories/100g
Casein (protein)	20%	20 g	80
Sucrose	45%	45 g	180
Corn starch (carbohydrate)	15%	15 g	60
Alphacel (fiber)	5%	5 g	0
Choline bitartrate	0.2%	0.2 g	0
DL-methionine	0.3%	0.3 g	0
Mineral mix	3.5%	3.5 g	0
Vitamin mix	1.0%	1 g	0
Oil (fat) either corn oil or canola oil	10%	10 g	90
Total	100%	100 g	410
Total fat		10g	90
Total protein		20g	80
Total carbohydrate		60g	240

The AIN-76A was slightly modified to contain 10% w/ w corn oil (control diet) or 10% w/w canola oil (test diet). Control and canola diets were balanced for calories, nutrients, protein, fat and carbohydrate. The AIN-76A diet is adequate for growth and nutrition of mice.

The diet mixture was pressed into trays and cut into small squares. Individual cage sized portions (25-30 g) were stored in sealed containers at -20⁰C to prevent oxidation of the fat and bacterial growth in the food. Mice had free access to food and water and were fed fresh food 5 days per week. Food removed from cages was discarded.

Transgene copy number

Real time PCR was used to verify the presence of the transgene in all experimental pups. Ear punches (two 2 mm punches, stored at -20⁰C until processing) were digested in digestion buffer [50mM KCl, 1.5mM MgCl₂, 10mm Tris pH 8.5, 0.01% Gelatin, 0.45% NP-40, 0.45% Tween 20 containing 140mg/ml proteinase K (Shelton Scientific, Shelton, CN)], followed by dilution of the samples 1:40 in milliQ water (Milli-Q Advantage, Millipore, Massachusettes). Primers for the transgene (**SV40 forward**: ATA TGC CTT CAT CAG AGG AAT ATT C; **SV40 reverse**: TAA AGT TTT AAA CAG AGA GGA ATC TTT GC) and the VIC labeled **SV40PROBE** (VICCCC AGG CAC TCC TTT CAA GAC CTA GAA GGMGBNFQ) were purchased from Applied Biosystems (Foster City, CA). Beta-actin primers (for an internal control) and PCR Master Mix were also purchased from Applied Biosystems. The rtPCR assay was performed according to the Applied Biosystems instructions on an ABI Prism 7000 (Applied Biosystems, Foster City, CA) instrument.

Tumor growth rates, incidence, multiplicity and weight

Mice were palpated for tumors 3 times weekly from 90 days of age. Lengths and widths of palpable tumors were measured from the time of detection until euthanasia to estimate tumor volumes. Tumor volume was estimated using the formula: (Length X Width X Width) /2. Prism[©] software (Graphpad, Inc., La Jolla, CA) was used to plot tumor growth curves and for regression

analyses to determine the growth rate of each palpable tumor. A T-test was then used to compare the mean tumor growth rates between groups of mice. Total tumor incidence, multiplicity and weights were determined at necropsy. The differences between groups and across time were statistically analyzed by two way analyses of variance, T-test, Fisher exact test or Mann-Whitney test as appropriate using Prism[®] software.

Necropsy

Mice were euthanized at 21, 110, 130, 150 and 170 days of age. Twenty one days of age was the time of weaning. The earliest time for tumors was expected to be 110 days of age, mice were euthanized each 20 days thereafter to follow the increase in tumor incidence and multiplicity. The left 4th mammary gland was quickly removed and frozen in liquid nitrogen. All ten mammary glands were examined for the presence of a tumor 1 mm or larger. All tumors detected were measured, removed and weighed, thus total tumor weight and numbers includes many tumors that were too small to be detected by palpation. If tumor was large enough for further assay, it was flash frozen in liquid nitrogen. The number of tumors in each gland and the number of glands with tumor were recorded for every mouse. Samples of inguinal fat and liver were removed and frozen in liquid nitrogen until further analyses.

Body weights

Body weights were measured each week and terminally. Statistical differences in mean body weight change between groups were determined using a T-test and Prism[®] (Graphpad, Inc) software.

Gas chromatography

The fatty acid compositions of mammary glands and liver at 3 weeks of age and 130 days of age were analyzed by gas chromatography. Frozen tissues were thawed and homogenized in distilled water containing 0.1% BHT to prevent oxidation of the fatty acids. Lipids were extracted with chloroform/methanol, the fatty acids were methylated followed by separation and identification using gas chromatography, as previously described (Hardman, 2007). Gas chromatography was done using a PerkinElmer Clarus 500 Gas Chromatograph (Shelton, CT) with a Elite-5 (5% Diphenyl) Dimethyl-polisiloxane Series Capillary Column (Length: 30m, Inner Diameter: 0.25mm), under the following conditions: initial temperature 150⁰C, ramp 1 at 175⁰C for 15min, ramp 2 at 225⁰C for 50min, ramp 3 at 250⁰C for 10min, helium carrier gas flow rate of 1.60ml/min. Fatty acid methyl ester standards (Nu-Chek-Prep, Elysian, MN) were used for peak identification. For a better identification of the peaks two standards were used: GLC #464 which contains 52 fatty acids and a custom preparation, GLC #704, which contains 10 fatty acids, methyl esters of stearate, oleate, linoleate, alpha linolenate, gamma linolenate, homogamma linolenate, arachidonate, eicosapentaenoate, docosapentaenoate, and docosahexaenoate. The fatty acid methyl esters were reported as the percent of the total methylated fatty acids (area under the curve). Using this protocol and column, we could not clearly separate the oleic acid (18:1n-9) and alpha linolenic acid (ALA 18:3n-3) peaks thus we report these results as 18:1 + 18:3 ALA. However, the differences in the peaks due to diet should be mostly ALA since it is the dietary ALA that was altered. A T-test was used to determine statistical differences of individual fatty acids between dietary groups.

Gene expression assay

The Mouse Signal Transduction Pathway Finder™ RT² Profiler™ PCR Array, PAMM-014 (SuperArray Bioscience Corporation, Frederick, MD) was used to analyze the expression of genes in 3-4 mammary glands per group at 130 days of age mice. (The complete list of genes on the plate can be found at http://www.sabiosciences.com/rt_pcr_product/HTML/PAMM-014A.html.) Frozen tissue was homogenized in Tri Reagent (Sigma-Aldrich, St. Louis, Mo) following the protocol of the manufacturer to isolate the RNA. RNA quality control was performed for all samples to insure the purity and integrity of the RNA on an Agilent 2100 Bioanalyzer (Santa Clara, CA). The RT2 First Strand Kit was used to make cDNA; the cDNA was then quantitatively amplified by real time PCR using an ABI Prism 7000 (Applied Biosystems, Foster City, CA) and RT2 qPCR Master Mix (Superarray) according to the manufacturer's protocol. The protocol and software provided by SuperArray were followed to determine relative fold difference in gene expression using the $\Delta\Delta\text{ct}$ method and for statistical analyses of the data by T-test.

Immunoblot analysis

Frozen mammary gland tissues were homogenized in tissue extraction buffer (50mM Tris, pH 7.4, 250mM NaCl, 5mM EDTA, 2mM Na₃VO₄, 1mM NaF, 20mM Na₄P₂O₇, 0.02% NaN₃ and proprietary detergent) from BioSource International, Inc. at 4°C to prepare cell lysates. Protein concentration was determined by BCA protein Assay Kit (EMD Biosciences, Inc. Darmstadt, Germany) following the manufacturer's protocol. Ten micrograms of protein were applied to each lane of a 4-15% Tris-HCl polyacrylamide gradient gel (Bio-Rad, Hercules, CA), separated by electrophoresis and then transferred onto a nitrocellulose membrane. The blots were blocked with 5% BSA in TBST overnight at 4°C and probed with primary antibodies against [CCAAT-

enhancer binding proteins β (C/EBP β) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), fatty acid synthase (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), glyceraldehyde 3 phosphate dehydrogenase (GAPDH) (CHEMICON International, Billerica, MA) or cytokeratin 8 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA,)] in blocking buffer for 1 hour at room temperature. The membrane was thereafter incubated with antimouse (Santa Cruz Biotechnology, Inc.) secondary antibody horseradish peroxidase (HRP) conjugate followed by signal detection with chemiluminescence ECL Kit (PIERCE, Inc.). Densitometry was used to quantify bands. A ChemDoc XRS system (Bio-Rad Laboratories Inc., Hercules, CA) was used to acquire the image then image analysis was done using 'Quantity One' software, V. 4.5.2 (Bio-Rad Laboratories Inc., Hercules, CA). Data was normalized by cytokeratin (for size of the epithelial compartment) and by GAPDH (for protein loading).

Results

Group nomenclature

Groups will be referred to as: 1) CO/CO – mothers fed the 10% corn oil diet, pup weaned to the 10% corn oil diet or 2) CA/CO – mothers fed the 10% canola oil diet, pups weaned to the 10% corn oil diet. Pups were NOT exposed to the 10% canola oil diet after weaning.

Body weight gain

Mice were allowed free access to food. There was no difference due to maternal diet in the amount of weight gained between weaning and 170 days of age by the groups of experimental

mice ($p=0.95$ by T-test). Figure 2.1 shows the mean amount of weight gained per mouse per day from weaning until 170 days.

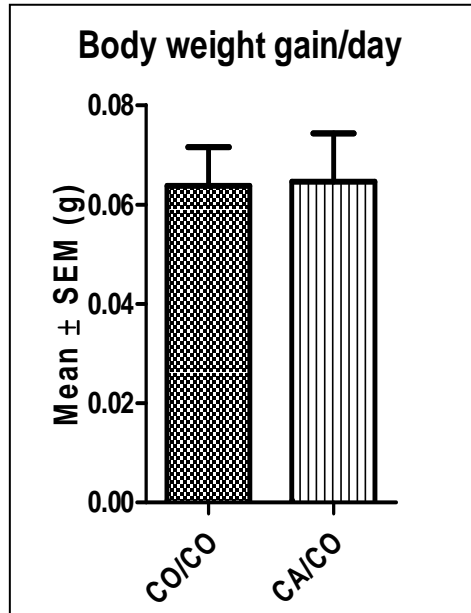


Fig. 2.1 Mean body weight gain between 21 and 170 days of age. Body weight gain between 21 and 170 days of age was determined for each mouse. There was no significant difference between the two groups in body weight gain between weaning (21 days) and 170 days of age. CO/CO $n=14$; CA/CO $n=17$ mice, $p=0.95$ by T-test.

Diet influence on tissue lipid composition

The lipid compositions of the livers and mammary glands at 21 and 130 days of age are shown in Table 2.2.

At 21 days of age (the time of weaning), both the livers and the mammary glands of pups from the CA mothers contained significantly less ($p<0.05$ by T-test) of the n-6 PUFA (LA and AA) than the livers and mammary glands of pups from the CO mothers.

At 21 days of age, the livers of pups from the CA mothers contained significantly more ($p<0.05$ by T-test) of the omega-3 PUFA, ALA, and significantly more ($p<0.05$ by T-test) of the

products of ALA metabolism, EPA and DHA than the livers of pups from the CO mothers. The only source of the omega-3 bond was the ALA in the canola oil. Thus, the ALA in the canola oil

Table 2.2 Major omega 6 and omega 3 fatty acids in tissues of mice.

Liver 21 days			
Fatty acid	Corn oil diet	Canola diet	p value
18:2 LA	17.35 ± 1.22	12.10 ± 0.90	<0.0001*
18:1+18:3 ALA	9.59 ± 2.16	20.29 ± 2.04	<0.0001*
20:4 AA	18.40 ± 1.60	10.08 ± 0.57	<0.0001*
20:5 EPA	0.09 ± 0.02	1.39 ± 0.20	<0.0001*
22:6 DHA	5.04 ± 0.81	11.22 ± 0.46	<0.0001*
22:5 DPA	0.64 ± 0.08	0.53 ± 0.06	0.023*
Liver 130 days			
18:2 LA	23.96 ± 2.74	22.91 ± 0.93	0.5565
18:1+18:3 ALA	18.19 ± 2.47	24.14 ± 1.01	0.0289*
20:4 AA	13.82 ± 0.46	10.33 ± 0.49	0.0041*
20:5 EPA	0.045 ± 0.04	0.10 ± 0.04	0.2382
22:6 DHA	5.37 ± 0.49	3.83 ± 0.17	0.0127*
22:5 DPA	0.315 ± 0.04	0.24 ± 0.03	0.0817
Mammary gland 21 days			
18:2 LA	26.25 ± 1.32	6.74 ± 1.99	0.0000*
18:1+18:3 ALA	29.26 ± 0.70	50.99 ± 1.98	0.0000*
20:4 AA	0.67 ± 0.12	0.31 ± 0.11	0.0043*
20:5 EPA	0.06 ± 0.01	0.08 ± 0.05	0.4050
22:6 DHA	0.08 ± 0.02	0.02 ± 0.01	0.0008*
22:5 DPA	0.01 ± 0.01	0.06 ± 0.03	0.0124*
Mammary gland 130 days			
18:2 LA	37.15 ± 0.78	35.77 ± 2.30	0.2879
18:1+18:3 ALA	0.40 ± 0.01	0.38 ± 0.03	0.1996
20:4 AA	0.13 ± 0.01	0.11 ± 0.03	0.3218
20:5 EPA	0.02 ± 0.01	0.00 ± 0.00	0.0000*
22:6 DHA	0.01 ± 0.00	0.01 ± 0.01	0.2415
22:5 DPA	0.02 ± 0.00	0.00 ± 0.00	0.0000*

*values in row are significantly different by T-test

was effectively elongated and desaturated to the longer chain omega 3 PUFAs, EPA and DHA, for incorporation into lipids in the liver.

At 21 days of age, the mammary glands of pups from the CA mothers contained significantly more ($p < 0.05$ by T-test) ALA and DPA than the pups from the CO mothers. The content of the longer chain fatty acids; AA, EPA, DHA and DPA, in the mammary glands of both groups was less than 1% each indicating less incorporation of long chain fatty acids or less activity of elongation and desaturation enzymes in the mammary glands than in the liver.

All mice were fed the corn oil diet from 21 to 130 days of age. At 130 days of age the livers of the CA/CO group still contained significantly more ($p < 0.05$ by T-test) ALA (n-3 PUFA) and significantly less ($p < 0.05$ by T-test) AA (n-6 PUFA) than the CO/CO group. The LA, ALA, and AA in the mammary glands of the two groups were not different. EPA, DHA and DPA were almost undetectable in the mammary glands at 130 days of age, though the small differences between groups for EPA and DPA were statistically significant. Both EPA and DHA were significantly less ($p < 0.05$ by T-test) in the mammary glands of the CA/CO than in the CO/CO group.

Effect of maternal consumption of canola oil instead of corn oil on tumor multiplicity, incidence and growth

The total number of tumors and the tumor weight per mouse were assessed in C3(1) $T_{AG}/129$ female offspring at 110, 130, 150, 170 days of age. The presence of the transgene was confirmed in all pups used in the experiment (data not shown).

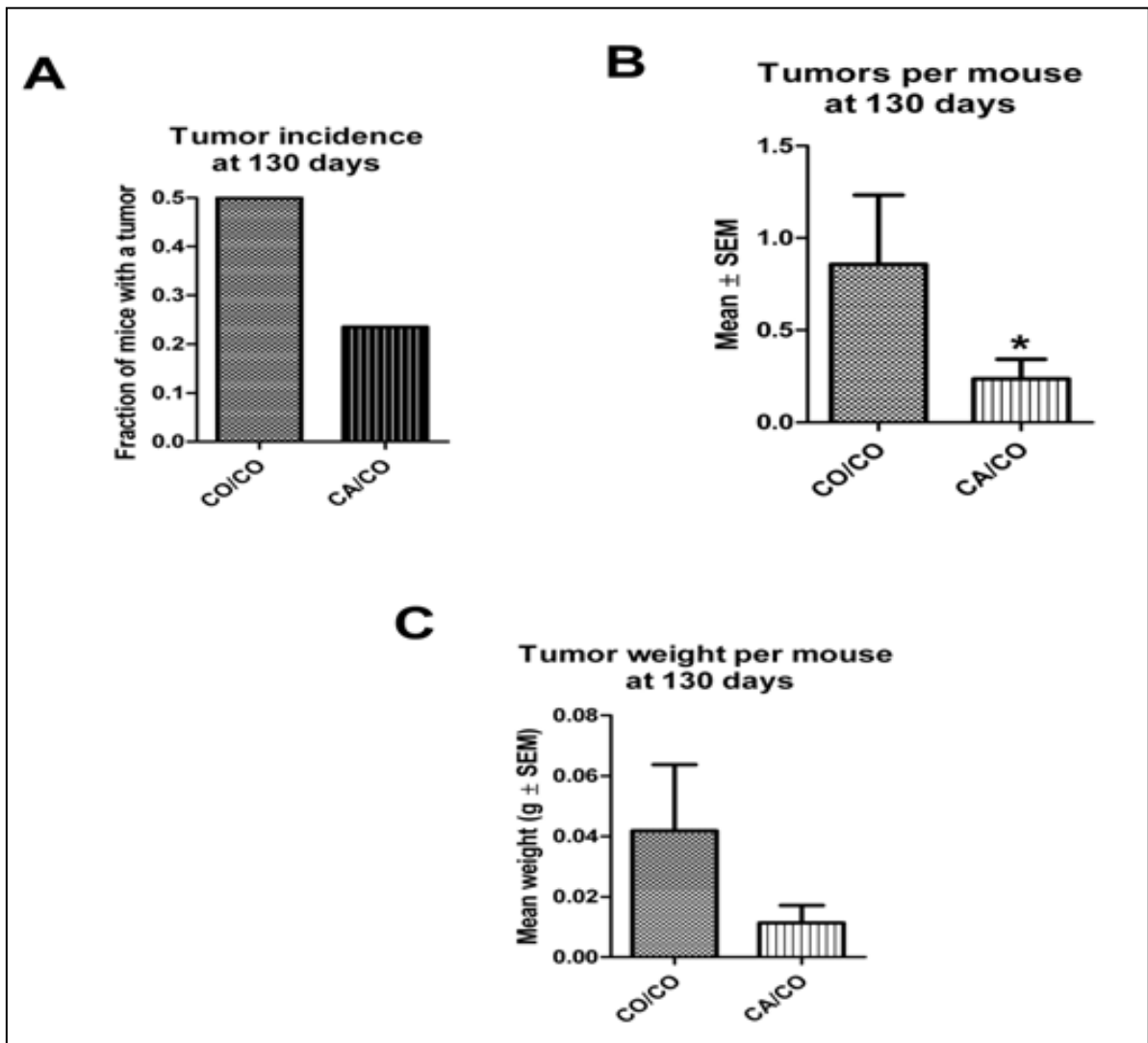


Fig. 2.2. - Tumor incidence and multiplicity at 130 days of age. A) Tumor incidence (fraction of mice with any tumor) at 130 days of age was not quite significantly different due to the diet of the mother. CO/CO – 7 of 14 mice had tumors, CA/CO – 4 of 17 mice had tumors. Fisher’s exact test, $p=0.1$. B) Tumor multiplicity, the mean number of tumors per mouse at 130 days of age. There were significantly fewer tumors per mouse by Mann-Whitney, $p<0.001$. CO/CO $n=14$ mice; CA/CO $n=17$ mice. C) The total tumor weight per mouse was not quite significantly less in the CA/CO group than in the CO/CO group, $p=0.15$ by Mann-Whitney test. CO/CO $n=14$ mice; CA/CO $n=17$ mice.

Incidence

At 110 days of age, no mice had tumors. The tumor incidence (whether or not a mouse has a tumor) at 130 days of age is shown in Figure 2.2A. The tumor incidence of the CA/CO group, (4/17 or 23%) was not quite significantly less ($p = 0.1$) by Fisher's exact test than the tumor incidence of the CO/CO group, (7/14 or 50%). At 150 and 170 days of age, all mice had at least one tumor. Thus, the tumor incidence was 100%.

Multiplicity

Since these mice all bear a tumor promoting transgene, all mice are expected to develop tumors at some point. As shown in Figure 2.2B, the tumor multiplicity (number of tumors per mouse) at 130 days of age was significantly less ($p < 0.001$ by Mann-Whitney) in the CA/CO group than in the CO/CO group. Even at 170 days of age, Figure 2.3A, the multiplicity of tumors in the CA/CO group was slightly less than in the CO/CO group.

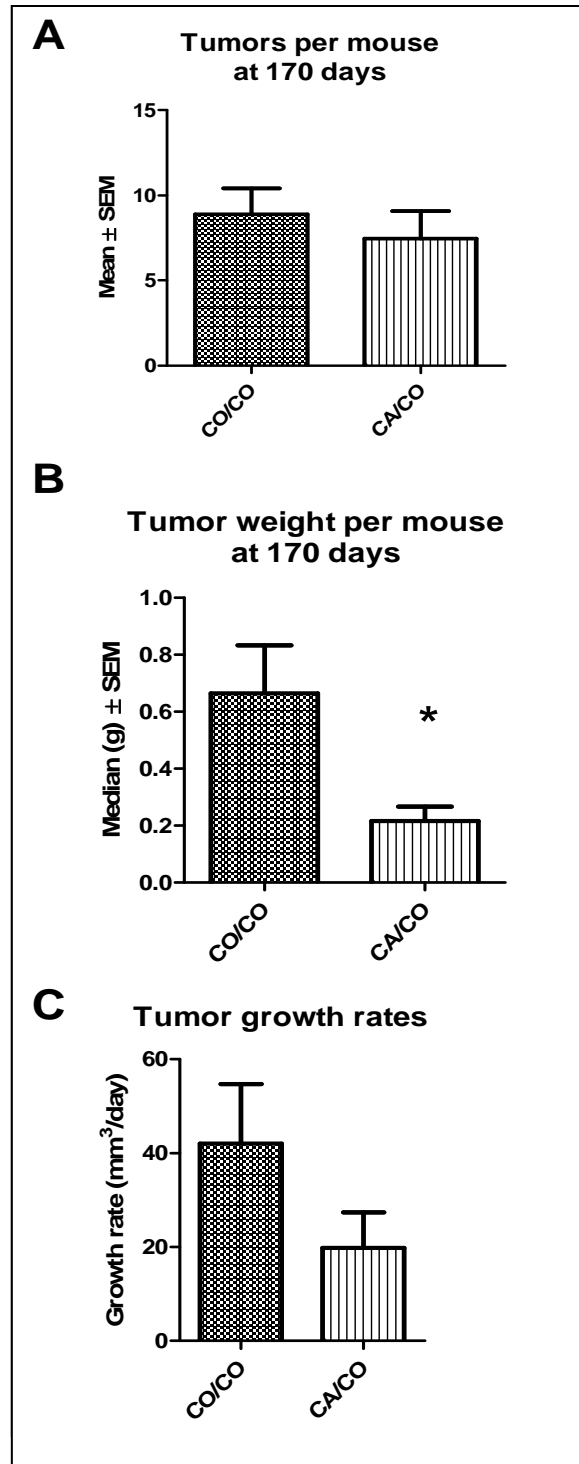


Fig. 2.3 Tumor multiplicity at 170 days and growth rate. A) The mean number of tumors per mouse at 170 days was less, but not significantly different, in the CA/CO group (n=11) than in the CO/CO group (n=9). B) The tumor weight per mouse at 170 days was significantly less in the CA/CO group (n=11) than in the CO/CO group (n=9), $p < 0.02$ by Mann-Whitney. C) The mean tumor growth rate in the CA/CO group from detection until 170 days of age was $\frac{1}{2}$ the growth rate of the CO/CO group.

Total tumor weight

The total tumor weight per mouse, calculated from autopsy data, indicates a difference in tumor burden due to the diet of the mother during gestation and lactation of the offspring. At 130 days of age, Figure 2.2C, the tumor weight in the CA/CO group was not quite significantly less ($p = 0.15$ by Mann-Whitney) than that of the CO/CO group. By 170 days of age, Figure 2.3B, the tumor weight per mouse (due to both fewer tumors/mouse and slower growth of tumors that developed) in the CA/CO group was significantly less ($p = 0.02$ by Mann-Whitney) than that of the CO/CO group.

Tumor growth rate

The mean tumor growth rates, calculated from measured tumor growth of 11 tumors for the CO/CO group and 8 tumors for the CA/CO group, are shown in Figure 2.3C. Linear regression analyses were used to determine the growth rate of each tumor (data not shown). A T-test of the tumor growth rates showed that the mean tumor growth rates were slower in the CA/CO group but not quite significantly different, $p=0.11$.

Number of glands with tumor

The number of glands with tumor at each time point is illustrated in Figure 2.4. Two-way analysis of variance revealed that there were significant effects due to both diet and time. The number of glands with tumor was significantly less in the CA/CO group than in the CO/CO group, $p=0.02$, and as expected, the number of glands with tumor was significantly increased with time, $p<0.0001$.

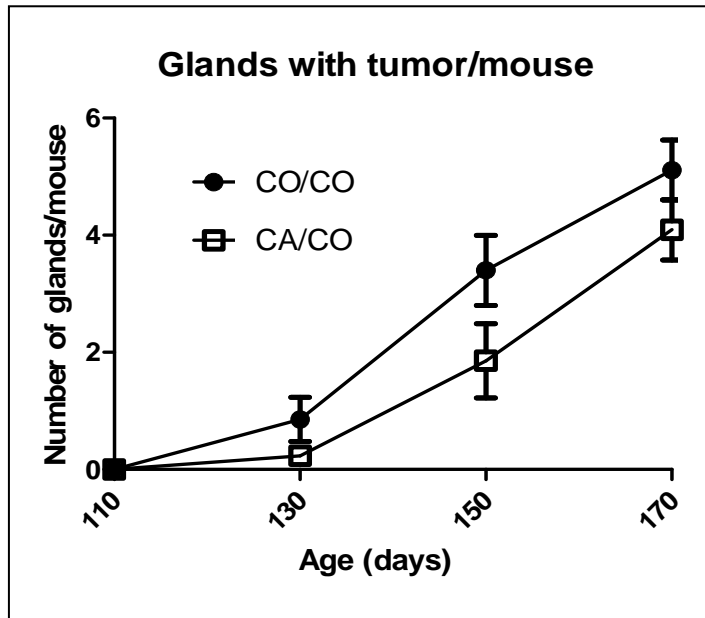


Fig. 2.4 Glands with tumor per mouse with time. Two way analyses of variance showed that the number of glands with tumor per mouse with time was significantly different due to treatment ($p=0.02$) and to time ($p=0.0001$). Consumption of the canola oil containing diet by the mothers of these mice significantly decreased the number of glands with tumor. As expected, with time the number of glands with tumor increased in both groups. Number of mice per group at 110, 130, 150 and 170 days of age are: CO/CO - 14, 14, 5, 9, and CA/CO - 6, 17, 7, 11 respectively.

Taken together, these data indicate that maternal consumption of canola oil delayed appearance of tumors in these transgenic mice and slowed the growth rate of the tumors that arose, resulting in significantly reduced tumor burden at 170 days of age.

Table 2.3 Gene expression in mammary glands at 130 days of age.

Gene name	Symbol	Fold change CA/CO vs CO/CO
Bcl2-associated X protein	Bax	22.71
B-cell leukemia/lymphoma 2	Bcl2	4.01
Bcl2-like 1	Bcl2l1	-9.92
Baculoviral IAP repeat-containing 3	Birc3	-2.35
Breast cancer 1	Brcal	-2.35
Chemokine (C-C motif) ligand 2	Ccl2	-2.25
Cyclin-dependent kinase inhibitor 2A	Cdkn2a	6.36
CCAAT/enhancer binding protein (C/EBP), beta	Cebpb	550.65
Chemokine (C-X-C motif) ligand 9	Cxcl9	2.02
Early growth response 1	Egr1	13.13
Etoposide induced 2.4 mRNA	Ei24	-2.15
Engrailed 1	En1	3.48
Fatty acid synthase	Fasn	-7.09
Fibronectin 1	<td>-4.42</td>	-4.42
Hedgehog-interacting protein	Hhip	-2.58
Hexokinase 2	Hk2	4.63
Homeo box A1	Hoxa1	-2.07
Intercellular adhesion molecule	Icam1	-2.05
Insulin-like growth factor binding protein 3	Igfbp3	-2.51
Inhibitor of kappaB kinase beta	Ikkkb	-3.13
Interleukin 1 alpha	Il1a	-2.06
Interleukin 2 receptor, alpha chain	Il2ra	-3.33
Interleukin 4 receptor, alpha	Il4ra	-2.19
Lymphoid enhancer binding factor 1	Lef1	-3.02
Lymphotoxin A	Lta	-3.19
Matrix metalloproteinase 10	Mmp10	2.87
Matrix metalloproteinase 7	Mmp7	2.97
Ngfi-A binding protein 2	Nab2	-4.71
Nitric oxide synthase 2, inducible, macrophage	Nos2	-3.31
Patched homolog 1	Ptch1	-3.32
Transcription factor 7, T-cell specific	Tcf7	2.53
Telomerase reverse transcriptase	Tert	-2.55
Transferrin receptor	Tfrc	-2.04
Transmembrane, prostate androgen induced RNA	Tmepai	-2.52
Tumor necrosis factor	Tnf	-5.27
Transformation related protein 53	Trp53	-2.74
Vascular cell adhesion molecule 1	Vcam1	-3.16
Wingless-related MMTV integration site 2	Wnt2	-2.24

Mice were exposed to maternal consumption of either corn or canola oil diets. Real time, reverse transcriptase PCR was performed to determine mRNA abundance in mammary glands of experimental mice at 130 days of age. Genes with greater than 2 fold differences in mRNA abundance, of the genes analyzed (see methods), are shown.

Effect of maternal diet on gene and protein expression at 130 days of age.

Analysis of expression of genes involved in multiple cell signaling pathways in mammary glands of mice at 130 days of age was used to identify potential mechanisms for how maternal consumption of canola oil might slow mammary gland tumorigenesis in C3(1)TA_g/129 mice offspring. We chose this time point because, at 130 days of age, there were mammary glands without tumor in each group so that changes in gene expression due to the maternal diet but not those due to the presence of a tumor could be assessed. *A priori*, we did not know which signal transduction pathway(s) might be important for any alterations in tumor development. The Mouse Signal Transduction Pathway Finder™ reverse transcriptase, real time PCR (rtPCR) panel profiles the expression of 84 key genes representative of 18 different signal transduction pathways. The CO/CO group was the control group, the CA/CO group was the experimental group for analysis. The presence of the large T antigen protein in each mammary gland was confirmed by Western Blot (data not shown).

Table 2.3 presents genes that were analyzed and found to be at least 2 fold different between the two groups at 130 days of age. Differences in gene expression between groups at 130 days of age must be due to sustained gene expression changes induced by the maternal diet since: 1) both groups were weaned to the same diet 109 days previously and 2) there are only very small differences in fat composition in the mammary gland at 130 days of age and these differences do not support the notion that increased omega-3 PUFA in the CA/CO group is influencing gene expression. The possible significances of some of the changes in gene expression are presented in the discussion.

The differences in mRNA should be reflected in changes in protein abundance. Figure 2.5 illustrates the results of Western blot for two genes of interest, fatty acid synthase (Fas) and

CCAAT/ enhancer binding protein β (C/EBP β). The mouse mammary gland contains a high percentage of adipocyte as well as the epithelial cells that form cancer, thus, the protein change was normalized by the size of the epithelial cell compartment in the specimen, using cytokeratin, and for protein loading using GAPDH. Compared to the CO/CO group, at 130 days of age, Fas protein was significantly less in the CA/CO group whereas C/EBP β protein was increased in the CA/CO group.

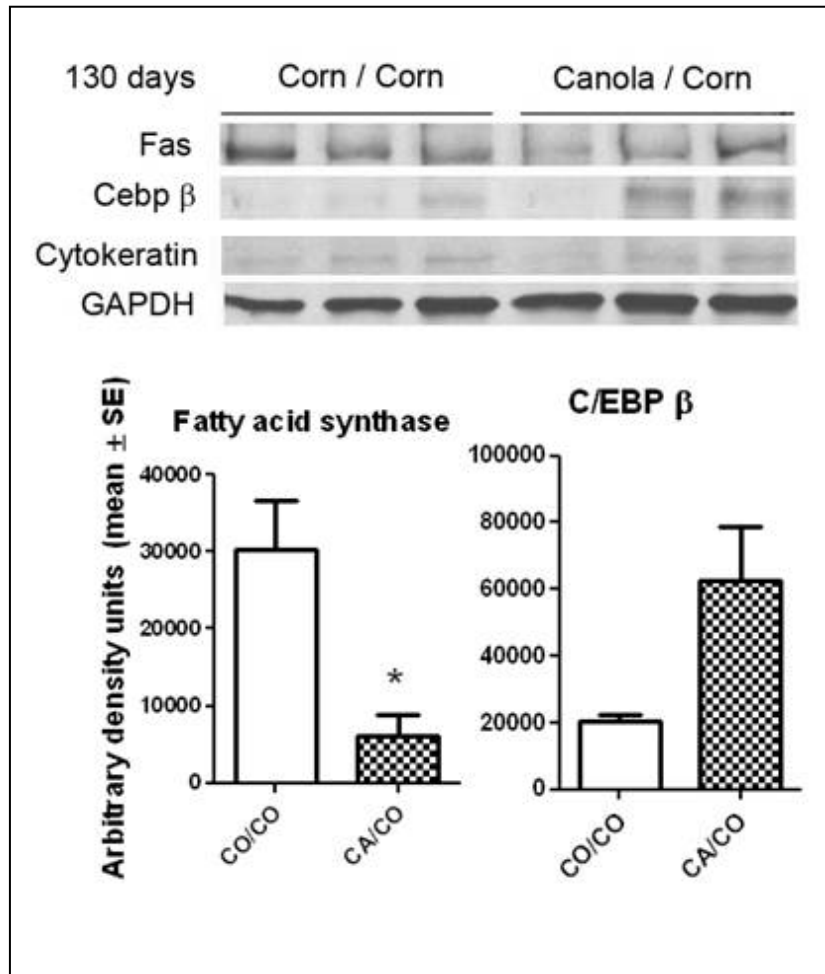


Fig. 2.5 Results of protein analyses for fatty acid synthase (Fas) and CCAAT/enhancer binding protein (C/EBP), beta at 130 days of age. Western blot for fatty acid synthase showed that Fas protein was significantly less ($p = 0.02$ by T-test, $n = 3/\text{group}$) and that C/EBP β was not quite significantly higher ($p = 0.06$ by T-Test, $n=3/\text{group}$) in mammary glands of CA/CO mice than in CO/CO mice at 130 days. Values shown are relative density and have been corrected for protein loading (GAPDH) and for cytokeratin (to correct for the epithelial compartment of the assayed sample).

Discussion

The results of this study indicate that incorporation of canola oil in the maternal diet delayed the development of mammary gland cancer in this transgenic mouse model. The mice exposed to canola oil during gestation and lactation had significantly fewer tumors per mouse and the tumor incidence was $\frac{1}{2}$ that of the control mice at 130 days of age. By 170 days of age the canola exposed mice had almost as many tumors as the control mice. However, because the growth rate of the tumors that developed in the canola oil exposed mice was about $\frac{1}{2}$ that of the control mice and the appearance of tumors was delayed, the tumor burden (tumor weight) in the canola exposed mice at 170 days of age was significantly less than that of the control mice. If these results can be extrapolated to humans they are important for 2 reasons: 1) incorporation of canola oil in the diet is an easy dietary change for humans to make and 2) the maternal diet can have a life-long influence on development of breast cancer in the daughter.

Epidemiologic studies can be interpreted to support the notion that the maternal diet can influence breast cancer risk in the daughter. When Chinese, Japanese or Filipino women migrate to the United States, breast cancer rates rise over two generations to approach that of US women (Ziegler *et al.*, 1993). Breast cancer incidence in first generation migrants (who consumed a Western diet but whose mothers consumed the traditional diet) was increased almost 3 fold over that of the Asian-born mother but was still lower than that of the general Western population, indicating that there remained some protection from breast cancer due to the mother's traditional diet. In second generation migrants, whose mothers consumed a Western diet during gestation and lactation of the daughter, breast cancer risk was 5-fold higher than the breast cancer risk for the Asian born grandmother (Kolonel *et al.*, 1980) and was the same as the general Western population.

What could explain the benefit of exposure to maternal consumption of the omega 3 fatty acids in canola oil? The fatty acid composition of the mammary glands was different between the two groups at 21 days of age, but by 130 days of age there were no real differences in fatty acid composition. However, at 130 days of age, there were significant differences in gene expression in the mammary glands of these mice. The PCR array that we chose assays mRNA abundance in 84 key genes representative of 18 different signal transduction pathways that are important to the development of cancer. We found that there were multiple differences in gene expression between the 2 groups of mice when the mice were 130 days of age, 109 days after the last exposure to the diet that contained canola oil.

Among the differential changes that were of special interest was the large increase in CEBP β (CCAAT/ enhancer binding protein β) mRNA. Western blot confirmed that the mRNA was being translated to protein and that the quantity of protein was higher in the CA/CO group. CEBP β is a leucine zipper transcription factor. The expression of CEBP β in the liver has been shown to respond to dietary changes (Chapin *et al.*, 1994). Homodimers and heterodimers of CEBP β initiate transcription of multiple factors involved in proliferation, differentiation and apoptosis in the mammary gland (Grimm and Rosen, 2003). Multiple isoforms of the protein, (including some that are dominant negatives) may be generated by truncation or proteolysis of the CEBP β transcript (Zahnow, 2002). Slowed proliferation and increased differentiation could result in reduced tumor incidence while promotion of apoptosis could slow tumor growth. These mechanisms are supported by the phenotypic data however the exact meaning of increased CEBP β expression in the mammary gland in this model will require additional study.

The 7 to 8 fold decrease of fatty acid synthase mRNA and significant decrease in fatty acid synthase protein by maternal consumption of the canola oil diet is particularly interesting.

Increased expression of fatty acid synthase has been associated with the early steps of human mammary carcinogenesis (Esslimani-Sahla *et al.*, 2007). Conversely, inhibition of fatty acid synthase has been associated with apoptosis of human breast cancer cells (Bandyopadhyay *et al.*, 2006) and has been suggested as a target for chemoprevention of breast cancer (Lu and Archer, 2005). In cell culture studies, α -linolenic acid (increased in the canola oil diet) has been shown to be tumoricidal to breast cancer cells and to inhibit the overexpression of fatty acid synthase (Menendez *et al.*, 2004). The long term suppression of fatty acid synthase would contribute to reduced tumor burden seen in this model.

Another gene expression change that could reduce tumor burden was the 13-fold increased expression of Egr1 mRNA in the CA/CO group. Egr1 is a tumor suppressor gene that has been associated with suppression of proliferation (Singletary and Ellington, 2006). The expression of this gene has been shown to be increased by genistein and by retinoids (Singletary and Ellington, 2006; Liu *et al.*, 2005) providing precedent for the regulation of this gene by dietary components.

Even though we did not directly assess NF κ B activation, the mRNA results suggest that activation of the transcription factor NF κ B was reduced in mammary glands of pups from mothers that consumed the canola oil diet. The mRNA for inhibitor of κ B kinase β (IKK β) was reduced 3-fold in CA/CO pups at 130 days of age. Activation of IKK β results in the phosphorylation of I κ B (inhibitor of κ B) and allows formation of NF κ B dimers that can translocate to the nucleus and activate transcription of downstream genes (Hayden and Ghosh, 2004). Activation of IKK β is an important regulatory step in NF κ B activity (Hayden and Ghosh, 2004). Thus, reduced IKK β would be expected to result in decreased activation of NF κ B and reduction in mRNA of genes that are transcribed following NF κ B binding. Genes downstream

from NFκB include inducible nitric oxide synthase (iNOS), tumor necrosis factor (TNF) and vascular cell adhesion molecule 1 (Vcam1). The mRNA from each of these genes was decreased 2-to 5-fold, supporting the idea that NFκB activation was reduced in the CA/CO pups. Clearly there is much work to be done to verify this notion and to identify mechanisms.

The mRNA changes in the Bcl-2 apoptotic pathway were also intriguing. Progression to apoptosis is a balance between pro-apoptotic genes such as Bcl-2 associated X (BAX) and anti-apoptotic genes such as Bcl-2 and Bcl-2-like-1 (Letai, 2008). At 130 days of age, the mammary glands of CA/CO mice had BAX mRNA at 22-fold and BCL-2-like 1 at 10-fold that of the CO/CO mice. Bcl2 was increased 4 fold but clearly the overall balance is to promotion of apoptosis of defective epithelial cells in the CA/CO mammary glands.

Conclusions

Clearly, consumption of canola oil by mothers of the experimental mice delayed mammary gland tumor development in this model. Our gene expression data have provided clues to mechanisms employed but identifying and verifying the mechanism(s) remains to be done. Because long term changes in the expression of multiple proteins, such as seen in this study, are often related to epigenetic modification of the promoter region of genes, we have initiated epigenetic studies to identify these changes. Future work includes verification of protein changes and developing reasonable pathways for the delay in cancer development seen in this model.

It has been suggested that 30% or more of cancers could be prevented by dietary changes (World Cancer Research, 2007). Substituting canola oil for corn oil would increase the ratio of omega 3

fatty acids in the diet and is an easy, cost effective dietary change for people to make. Many animal studies have shown that increasing omega 3 fatty acids in the adult diet provides multiple benefits against cancer. This work suggests that substituting canola oil for the corn oil in the maternal diet may decrease risk for breast cancer in the daughter in addition to providing benefit for the mother.

Competing interests

Authors have no competing interests.

Acknowledgements

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Diets are consumed as whole foods, and the multiple components in foods could work synergistically and independently to reduce the risk for cancer. Walnuts contain ingredients that may slow cancer growth including omega 3 fatty acids in the form of alpha linolenic acids, phytosterols and antioxidants. In the previous study, we investigated the maternal consumption of a diet that differs only in omega-3 to omega-6 ratio compared to the control diet, on the risk for breast cancer in female offspring. We found that substituting canola oil for corn oil to increase omega-3 and reduce omega-6 fatty acids in the maternal diet suppressed mammary gland tumorigenesis in the offspring. In the next chapter, we investigated the regular consumption of walnut and its influence on breast cancer risk. The regular consumption of walnut in a population means that offspring in such a population are exposed to the nutrients from walnut during gestation, lactation and at adulthood. These offspring as well as individuals who consume walnut after weaning as would occur when children move to a different environment or choose to make walnut part of the diet, and individuals exposed to walnut via gestation and lactation alone were compared with individuals not exposed to walnut to assess for the risk of breast cancer.

CHAPTER THREE

Dietary walnut suppressed mammary gland tumorigenesis in the C(3)1 TAg mouse

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Juliana A. Akinsete assisted with all facets of the study and performed the protein analyses.

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Abstract

Walnuts contain multiple ingredients that, individually, have been shown to slow cancer growth including omega-3 fatty acids, antioxidants and phytosterols. In previous research, consumption of walnuts has slowed the growth of implanted breast cancers. We wanted to determine whether regular walnut consumption might reduce the risk for developing cancer. Homozygous, male C(3)1 TAg mice were bred with female SV129 mice consuming either the control AIN-76 diet or the walnut containing diet. At weaning, the female hemizygous pups were randomized to control or walnut containing diets and followed for tumor development.

Compared to a diet without walnut, consumption of walnut significantly reduced tumor: incidence (fraction of mice with at least one tumor), multiplicity (number of glands with tumor/mouse) and size. Gene expression analyses indicated that consumption of the walnut diet altered expression of multiple genes associated with proliferation and differentiation of mammary epithelial cells. A comparison with another dietary intervention indicated that the omega-3 content alone did not account for the extent of tumor suppression due to the walnut. The results of this study indicate that walnut consumption could contribute to a healthy diet to reduce risk for breast cancer.

Introduction

Many scientists now think that diet can alter carcinogenesis (World Cancer Research fund, 2007). Epidemiology studies have tried to identify individual components of whole foods that might reduce risk for cancer. However, these studies often indicate little or no benefit of consuming a specific dietary component. Clinical trials that have used specific supplements [i.e., calcium (Gregoire *et al.*, 1989), calcium and wheat bran (Alberts *et al.*, 1997), selenium and Vitamin E (Lippman *et al.*, 2009)] often demonstrate little or no effect of the supplement. However, people consume whole foods; the multiple ingredients in individual foods as well as accumulated amounts of these components within a whole diet, could act additively or synergistically to contribute to reduction of risk for disease, including cancer. In support of this idea, one preclinical study reported that very low doses (more similar to dietary doses) of selenium and docosahexaenoic acid in combination were more effective against cancer than either of these components individually at high doses (Narayanan *et al.*, 2004).

Walnuts contain multiple ingredients (Maguire *et al.*, 2004) that individually have been reported to reduce cancer risk or growth rate. These ingredients include: omega 3 fatty acids (Petrik *et al.*, 2000; Berquin *et al.*, 2008), phytosterols, especially β -sitosterol (Ju *et al.*, 2004; Choi *et al.*, 2003) and antioxidants (Han *et al.*, 2006; Liu *et al.*, 2009). We have previously reported that walnut in the diet of mice (human equivalent of two servings a day) would reduce the growth rate of implanted human breast cancers (Hardman and Ion, 2008). We have also reported that canola oil which contains α -linolenic acid, the 18C omega-3 fatty acid, incorporated in the maternal diet during gestation and lactation of offspring, significantly reduced mammary gland cancer risk in the offspring (Ion *et al.*, 2010). Walnuts contain the highest fraction of α -linolenic acid of any tree nut (USDA, 2009). The current study was

designed to determine whether the risk for developing breast cancer might be reduced by regular walnut consumption, perhaps due to the high α -linolenic acid content.

In designing mouse diets that were applicable to human consumption we used a 10% corn oil diet as the “control diet.” This diet has an omega-6 fatty acid (linoleic acid) content that is approximately equivalent to the linoleic acid content American diet. It turned out that the α -linolenic acid content of the walnut diet was equivalent to the α -linolenic acid content of the canola oil diet, giving us the added value of comparing two interventions containing the same amount of α -linolenic acid to determine if the α -linolenic acid content of the walnut diet might be the primary cancer suppressive component of walnut.

The C(3)1 TAg transgenic mouse is a well characterized breast cancer model and the females of these mice develop mammary gland cancer at a predictable rate (Green *et al.*, 2000; Maroulakou *et al.*, 2001). As in human tumors, the early stages are estrogen receptor α (ER α) positive, but the tumors become ER α -negative in later stages (Maroulakou *et al.*, 2001). The cancer development is slow enough that a dietary modification can make a difference in tumor development. Use of this model allowed us to control the maternal diet as well as the diet of the experimental offspring. However, as with any dietary modification study, we cannot determine if subtraction of a detrimental component or addition of a beneficial component determined the difference in tumorigenesis.

When a dietary component is routinely consumed within a population, the offspring are exposed to the component during gestation and lactation as well as by consumption after weaning. This notion is duplicated by the experiment reported here. Exposure to the walnut included in the maternal diet during gestation and lactation as well as by individual consumption after weaning provided significant protection from mammary gland cancer in the offspring. The

reduction in cancer risk cannot be explained solely by the omega-3 content of the diet. Increased consumption of walnut could be part of a healthy diet and reduce risk for cancer in future generations.

Methods and Materials

Animals

Breeding pairs of mice bearing a transgene for the SV40 large T antigen with a C3(1) rat prostate steroid binding protein promoter were obtained from the Dr. Jeffrey Green. The female transgenic mice are expected to develop mammary gland cancer due to expression of the large T antigen in the mammary gland (Maroulakou et al., 2001). The transgenic line is maintained in the laboratory and all mice were genotyped to ensure presence of the transgene. Twenty, female SV129 mice, 6 weeks old were obtained from Charles River Laboratories (Wilmington, MA), quarantined for 2 weeks, and then moved to a study room. All animal work was approved by the Marshall University School of Medicine Institutional Animal Care and Use Committee.

Study design

SV129 females (breeder females) were split into 2 groups and numbered for identification. Ten female mice were placed on a diet containing 10% w/w corn oil (control diet, see below) and ten female mice were placed on a diet containing walnuts (test diet). The compositions of the diet and of the dietary fat are listed in Table 3.1. After 2 weeks these females were bred with homozygous C3(1)/TAg male mice. The hemizygous female pups from these breedings were the experimental mice NOT the wild type mother mice. Pups were weaned at 21 days old and were

randomized to the two diets, generating four experimental groups: corn oil/corn oil (CO/CO), corn oil/walnut (CO/walnut), walnut/walnut, or walnut/corn oil (walnut/CO) (the first diet was the maternal diet, the second diet was the pup's diet). The offspring were housed 3 to 4 in a cage, individually numbered for identification, and weighed weekly.

Diet

Diets were prepared in the Marshall University School of Medicine animal diet prep room. Diet composition is shown in Table 3.1 and was formulated to be isocaloric, isonutrient and more relevant to human consumption than the very high fat diets used in many studies. (If a Western diet contains about 14.7g of linoleic acid/day (Johnson et al., 2007) the calories from linoleic acid are 6.6% of a 2000 calorie diet. The 10% corn oil mouse diet contained 10.9% of calories from linoleic acid. The walnut-containing diet was formulated to contain 18% of calories from walnut. This approximates a human diet that includes 2 servings (2 ounces) of walnut per day. (Two ounces of walnut, 28 halves, equals 370 calories; 370 calories = 18.5% of a 2000 calorie diet). The AIN-76A diet is adequate for the nutritional support of the mice (American Institute of Nutrition, 1977). The dry ingredients of the diet, except sugar, were obtained in bulk from MP Biomedicals (Solon, Ohio), sugar and corn oil were purchased locally (100% corn oil, no additives or preservatives). Walnuts were provided by the California Walnut Commission and kept frozen at -20°C until used. Walnuts (with brown pellicle but without shells) were ground fine in a blender for mixing into the diet. Batches of diet were prepared as needed, about each two weeks. The diet mixture was pressed into trays and cut into small squares. Individual cage sized portions (25-30 g) were stored in sealed containers at -20°C to prevent oxidation of the fat

and bacterial growth in the food. Mice had free access to food and water and were fed fresh food 5 days per week. Food removed from cages was discarded.

Transgene copy number

Real time PCR was used to verify the presence of the transgene in all experimental pups, as previously described (Ion *et al.*, 2010). Primers for the transgene (**SV40 forward**: ATA TGC CTT CAT CAG AGG AAT ATT C; **SV40 reverse**: TAA AGT TTT AAA CAG AGA GGA ATC TTT GC) and the VIC labeled **SV40PROBE** (VICCCC AGG CAC TCC TTT CAA GAC CTA GAA GGMGBNFQ), β -actin primers and PCR Master Mix were purchased from Applied Biosystems (Foster City, CA). The rtPCR assay was performed according to the Applied Biosystems instructions on an ABI Prism 7000 (Applied Biosystems, Foster City, CA) instrument.

Body weights

Body weights were measured each week and terminally. Statistical differences in mean body weight change between groups were determined using ANOVA and Prism[®] (Graphpad, Inc) software.

Tumor incidence, multiplicity and weight

Mice were palpated for tumors 3 times weekly from 90 days of age. Total tumor incidence, multiplicity and weights were determined at necropsy. The differences between groups and across time were statistically analyzed by Kruskal-Wallis, two way analyses of variance, Chi-square, Fisher exact test or Mann-Whitney test as appropriate using Prism[®] software (Graphpad, Inc., La Jolla, CA).

Necropsy

Mice were euthanized at 110, 130, 145 days of age. The earliest time for tumors was expected to be 110 days of age. Mice were euthanized thereafter to follow the increase in tumor incidence and multiplicity. The left 4th mammary gland was quickly removed and frozen in liquid nitrogen. All ten mammary glands were examined for the presence of a tumor 1mm or larger. All tumors detected were measured, removed and weighed, thus total tumor weight and numbers includes tumors that were too small to be detected by palpation. If a tumor was large enough for further assay, it was flash frozen in liquid nitrogen. The number of tumors in each gland and the number of glands with tumor were recorded for every mouse. Some mice in the CO/CO group had to be euthanized (due to large tumor size) before a scheduled 145 days thus slightly skewing (to a smaller size) the results for tumor mass in this group.

Gas chromatography

The fatty acid compositions of mammary glands at 130 days of age were analyzed by gas chromatography. Frozen tissues were thawed and homogenized in distilled water containing 0.1% BHT to prevent oxidation of the fatty acids. Lipids were extracted with chloroform/methanol, the fatty acids were methylated followed by separation and identification using gas chromatography, as previously described (Witte et al., 2010). ANOVA followed by a Bonferroni posttest was used to determine statistical differences of individual fatty acids between dietary groups.

Gene expression assay

The Mouse Signal Transduction Pathway Finder™ RT² Profiler™ PCR Array, PAMM-014 (SuperArray Bioscience Corporation, Frederick, MD) was used to analyze the expression of 84 genes in 3-4 mammary glands per group at 130 days of age mice. (The complete list of genes on the plate can be found at: www.sabiosciences.com/rt_pcr_product/HTML/PAMM-014A.html.)

The Signal Transduction pathway finder array was used because, *a priori*, we did not know what genes or pathways might be influenced by consumption of the walnut diet. Mammary gland without macroscopic tumor was analyzed to obtain changes due to the diet not to the presence of a tumor. This array profiles genes in 18 different pathways that could be applicable to cancer development. Frozen tissue was homogenized in Tri Reagent (Sigma-Aldrich, St. Louis, Mo) following the protocol of the manufacturer to isolate the RNA. RNA quality control was performed for all samples to insure the purity and integrity of the RNA on an Agilent 2100 Bioanalyzer (Santa Clara, CA). The RT2 First Strand Kit was used to make cDNA; the cDNA was then quantitatively amplified by real time PCR using an ABI Prism 7000 (Applied Biosystems, Foster City, CA) and RT2 qPCR Master Mix (Superarray) according to the manufacturer's protocol. The protocol and software provided by SuperArray were followed to determine relative fold difference in gene expression using the $\Delta\Delta\text{Ct}$ method and for statistical analyses of the data by T-test.

Immunoblot analysis

Tri Reagent (SIGMA, Missouri, USA) was used to homogenize and isolate protein from frozen mammary gland tissues following the manufacturer's protocol. Protein concentration was determined by BCA protein Assay Kit (EMD Biosciences, Inc. Darmstadt, Germany) following

the manufacturer's protocol. Ten micrograms of protein were applied to each lane of a 4-15% Tris-HCl polyacrylamide gradient gel (Bio-Rad, Hercules, CA), separated by electrophoresis and then transferred onto a nitrocellulose membrane. The blots were blocked with 5% BSA in TBST overnight at 4°C and probed with primary antibodies against: Nitric oxide synthase Type II (NOS 2), Simian virus 40 Large T antigen (SV40 T Ag) (BD Biosciences); NF kappa B inhibitor alpha (IκB alpha) (Abcam Inc.); or inhibitor of NF kappa-B kinase beta (IKK beta) (Cell Signaling Technology, Inc.), in blocking buffer for 1hr at room temperature. Cytokeratin 8 (Santa Cruz Biotechnology, Inc.) was used to normalize for the epithelial compartment of the mammary gland specimen and GAPDH (CHEMICON International) was used to normalize protein loading. The membrane was thereafter incubated with horseradish peroxidase (HRP) conjugated antimouse (Santa Cruz Biotechnology, Inc.) or antirabbit (PIERCE, Inc.) secondary antibody. Band detection was done with chemiluminescence ECL Kit (PIERCE, Inc.). Densitometry was used to quantify the bands. A ChemDoc XRS system (Bio-Rad Laboratories Inc., Hercules, CA) was used to acquire the image then image analysis was done using 'Quantity One' software, V. 4.5.2 (Bio-Rad Laboratories Inc., Hercules, CA). Tissue availability (many of walnut consuming mice either had no or very small tumors) limited the number of repeats that could be performed.

Results

Body weight and weight gain

There were no differences between groups in terminal body weight at any of the necropsy time points. Body weight gain between 91 and 135 days were (mean g \pm SD): CO/CO = 3.85 ± 1.05 ; CO/walnut = 2.87 ± 1.5 ; walnut/walnut = 2.72 ± 1.1 ; walnut/CO = 2.12 ± 1.1 . ANOVA indicated no significant differences between these weight gains. The groups with the greatest difference in tumor incidence and size were the CO/CO and Wal/Wal groups. However, there was no significant difference in body weight (mean \pm SD of 12 mice/group: CO/CO= 24.85 ± 1.6 g vs Wal/Wal= 24.25 ± 1.4 g) at 135 days (before any tumor cachexia). The mean tumor mass of the CO/CO group was 1/3 of the variance in body weight thus it does not seem that the tumor mass was a significant portion of the mouse weight gain though certainly some of the extra weight gain in the CO/CO group could be due to tumor growth. An important point here is that free access to walnut, a high calorie food, in the diet did not increase weight gain as would be a concern of many readers. Clinical trials have also indicated that addition of walnut to the diet does not increase body weight or cause weight gain in people (Sabate, 2003; Sabate et al., 2005; Gillen et al., 2005).

Table 3.1 Compositions of the diets

Diet compositions	Corn oil (Control) diet	Both diets		Walnut diet (18% of calories from walnut)		
	g/kg diet	calories/g diet	% of total energy	g/kg diet	Additional nutrient contained in 111 g walnut/kg diet	
Casein	200	0.80	19.2	183g	Protein -17.2g	
Sucrose	450	1.800	43.4	450g		
Corn starch	150	0.60	14.46	135g	Carbo – 15.5g	
Alphacel	50	0	0	48g	Fiber – 2.0g	
Choline bitartrate	2.0	0	0	2g		
DL-methionine	3.0	0	0	3g		
AIN-76 Mineral mix	35.0	0.02	0.4	35g		
AIN-76A Vitamin mix	10.0	0.030	0.7	10g		
Ground walnut	0			111g		
Fat (10% total fat)	100g corn oil	0.90	21.7	26.3g corn oil	Fat – 73.7g	
Totals	1000g	4.15cal/g	99.9	1003g	(4.6g water in walnut)	
Fat in diet	From Corn oil g/kg diet			From Corn oil g/kg diet	From Walnut g/kg diet	Total g/kg diet
Oleic acid	27.33			7.2	9.7	16.9
Linoleic acid	53.2			14.0	41.9	55.9
α -Lenolenic acid	1.2			0.3	10.0	10.3
Total saturated fat	12.9			3.3	6.7	10.0

Fat composition (g/100g) of corn oil and walnut from (USDA, 2009). Small amounts of various lipids make up the remainder of the fat and are not listed.

Fatty acid composition of the diet and of the mammary gland:

Table 3.1 shows that the corn oil diet contained more oleic acid (monounsaturated fat) and saturated fat but that the walnut diet contained more linoleic acid and α -linolenic acid. The fat composition of the mammary gland (Figure 3.1) reflects the fat composition of the diet, that is, the mammary glands of mice that consumed the walnut diet after weaning contained

significantly less oleic acid and significantly more linoleic acid, α -linolenic acid and total omega 3 fats than did the mammary glands of the mice that consumed the corn oil diet after

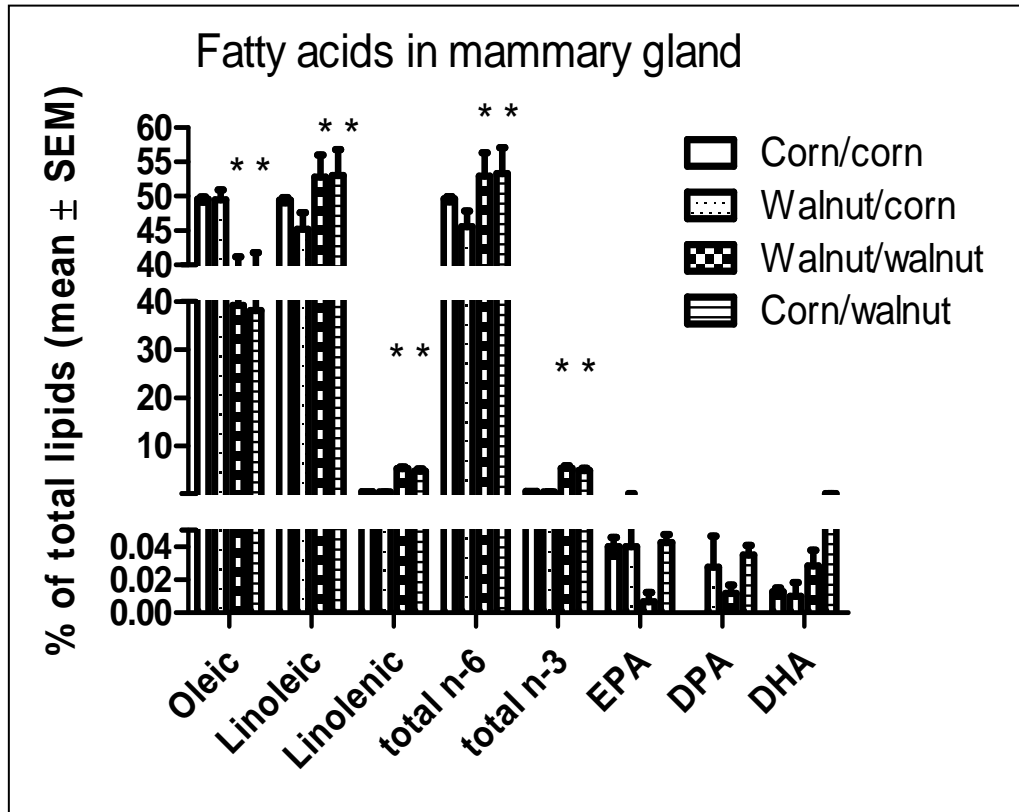


Fig. 3.1 Gas chromatography for fatty acids in mammary gland. Gas chromatography was used to analyze fatty acids in the mammary gland. Shown are the monounsaturated (oleic) and polyunsaturated (omega-6 (n-6), linoleic and omega-3 (n-3), linolenic) fatty acids that were significantly different due to the diet. Total omega-6 includes linoleic acid (18C) and arachidonic acid (20C) and total omega-3 fatty acid includes α -linolenic acid (18C), eicosapentaenoic (20C), docosapentaenoic (22C) and docosahexaenoic (22C) acids. Individually, the fractions of the 20C and 22C fatty acids were not different between diets (by T-test). Fatty acid compositions reflect the current diet and are as expected based on the fat composition of the diet. (n=4-5 per group, p<0.05 by ANOVA)

weaning [two-way ANOVA (fatty acid and diet group) followed by Bonferroni posttest, p<0.05].

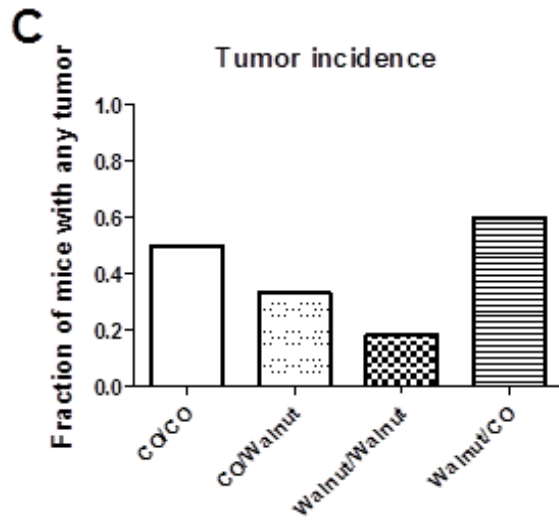
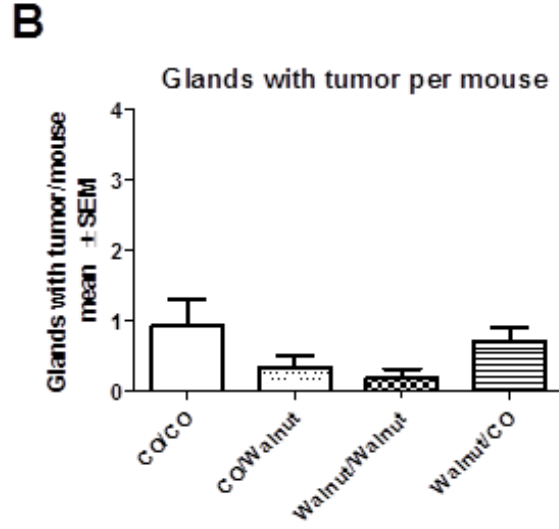
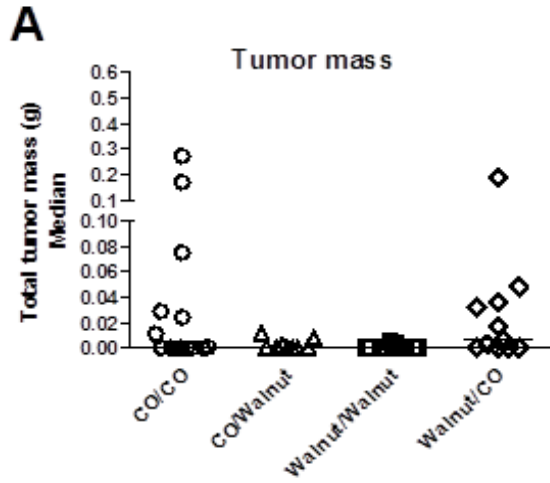
There were no other fatty acids that were significantly different due to diet.

Tumor incidence and multiplicity

At 110 days of age, the first euthanasia point, no mice had tumors. Figure 3.2 shows the tumor size, incidence and multiplicity at 130 and 145 days of age. At 130 days it appears that consumption of walnut is increasing the latency of tumors. However, largely due to the early stage of tumorigenesis, neither the tumor mass (Figure 3.2A, Kruskal Wallis, p-value for Walnut/Walnut vs Co/CO = 0.06) multiplicity (Figure 3.2B, number of glands with tumor, by ANOVA) nor the tumor incidence (Figure 3.2C, fraction of mice with any tumor, by Fisher's exact test) were significantly different.

By 145 days, suppression of tumorigenesis by the walnut containing diet was clearly evident. The median tumor size of the walnut/walnut group was significantly less than of the CO/CO group (Figure 3.2D, $p < 0.05$, Kruskal-Wallis followed by Dunn's multiple comparison test). (Some mice in the CO/CO group had to be euthanized due to large tumor size before the scheduled 145 days thus slightly skewing the results for tumor mass in this group to a smaller mass than if the mice had lived to 145 days old.). The multiplicity of tumor was decreased in both groups that consumed walnut after weaning with the multiplicity of tumor in the walnut/walnut group being significantly less (Figure 3.2E, $p < 0.05$, Kruskal-Wallis followed by Dunn's multiple comparison test) than in the group not exposed to walnut (CO/CO).

130 Days



145 Days

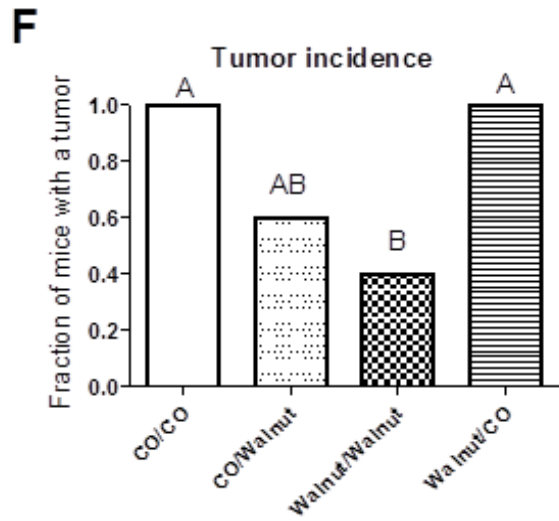
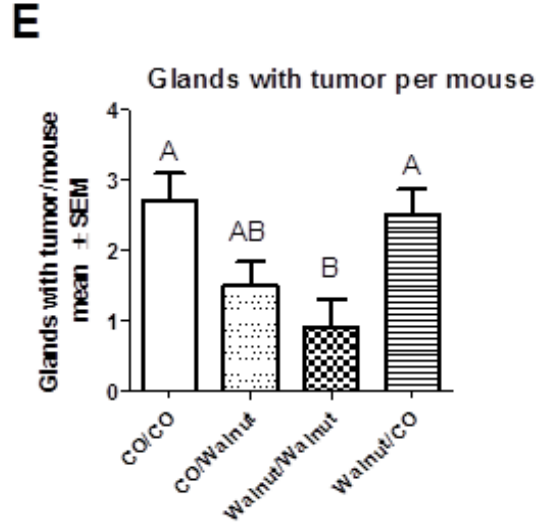
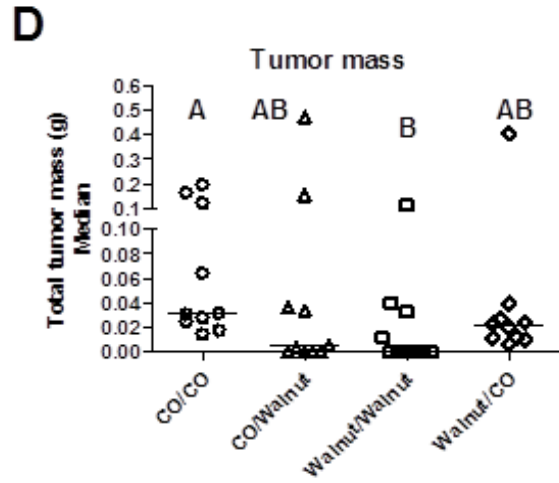


Fig. 3.2 Tumor mass, multiplicity and incidence at 130 and 145 days of age. Mammary gland tumors were quantified at euthanasia at 130 and 145 days of age, n = 10-13 mice per time point and diet group. By 145 days, the total tumor mass, number of glands with tumor (multiplicity) and tumor incidence of the walnut/walnut group was significantly less than of the corn oil/corn oil group. Consumption of walnut after weaning (corn oil/walnut group) also decreased the multiplicity, the mass and the incidence of tumors (groups which share a letter are not significantly different $p < 0.05$, statistical test stated in Results).

The tumor incidence was less in both groups that consumed walnut after weaning than in the group not exposed to walnut (Figure 3.2F, CO/CO) with the tumor incidence in the walnut/walnut group being significantly less than the tumor incidence of the CO/CO group ($P < 0.05$ by Fisher's exact test). Tumor incidence was decreased 40% compared to the CO/CO group when walnut was consumed after weaning, however, with 10 mice in a group this did not reach significance.

PCR array analyses for mRNA expression followed by western blot

The mRNA from a total of 84 genes was quantitatively assayed by the Mouse Signal Transduction Pathway Finder™ RT² Profiler™ PCR Array. Table 3.2 lists the 41 genes of these genes in which expression in the mammary gland was changed either more than 2 fold or with a P-value < 0.05 by T-test (using the manufacturer's statistical analyses software). Genes in the arrays are listed in the applicable pathways at the bottom of Table 3.2, with genes that were significantly altered by walnut consumption shown in italics and green color. The expression of many genes in pathways associated with mitogenesis, survival or NFκB and other signaling has been altered by exposure to dietary walnut.

We noted that a number of the genes with altered expression are in the NFκB signaling pathway so three of these genes [NFκB inhibitor alpha (IκB, Nfκbia), IκB kinase (IKK, Iκbkb) and nitric oxide synthase 2 (iNOS or Nos2)] were chosen for Western blot analyses. Western blot results (Figure 3.3) were in general agreement with the PCR array analyses for these genes and with the actual tumor incidence results. That is, compared to protein abundance in the mammary glands of the CO/CO group, protein abundance of Nfκbia was increased and of Iκbkb was decreased in the walnut/walnut group. Because Nfκbia binds and prevents activation of NFκB and Iκbkb cleaves Nfκbia to allow activation of NFκB, these coordinated changes would be expected to decrease NFκB activation and to be associated with suppression of cancer risk (Aggarwal et al, 2004). Nfκbia was also increased in the CO/walnut and in walnut/CO groups but Iκbkb was not changed. These results would be expected to be associated with less tumor suppression in the CO/walnut and walnut/CO groups than in the walnut/walnut group. Inducible nitric oxide synthase (Nos2) mRNA was slightly decreased but protein was barely detectable in any group and was not changed by a walnut containing diet.

Table 3.2 Gene expression altered by walnut diet

Symbol	*T-TEST	Fold difference		*T-TEST	Fold difference		*T-TEST	Fold difference
	p value	CO/walnut vs CO/CO		p value	walnut/walnut vs CO/CO		p value	walnut/CO vs CO/CO
Activating transcription factor 2 - ATF2	0.004	2.597		0.034	1.737		0.021	1.838
Bcl2-associated X protein - BAX	0.079	107.535		0.074	119.263		0.079	105.420
B-cell leukemia/lymphoma 2 - Bcl2	0.163	27.338		0.292	11.867		0.178	24.098
Baculoviral IAP repeat-containing 2 - Birc2	0.002	4.328		0.076	1.896		0.007	3.375
Bone morphogenetic protein 2 - BMP2	0.109	17.182		0.095	19.865		0.139	12.914
Bone morphogenetic protein 4 - BMP4	0.486	3.254		0.450	3.592		0.399	4.228
Cyclin-dependent kinase inhibitor 2A - Cdkn2a (p16)	0.011	950.691		0.026	216.092		0.024	308.865
CCAAT/enhancer binding protein (C/EBP), beta	0.014	2876.968		0.027	790.342		0.016	2152.305
Chemokine (C-X-C motif) ligand 1 - Cxcl1	0.001	9.309		0.001	4.728		0.000	3.732
Early growth response 1 - EGR1	0.033	472.608		0.037	363.002		0.057	168.410
Engrailed 1 - En1	0.000	21.449		0.162	6.895		0.000	43.236
Fas (TNF receptor superfamily member)	0.000	5.794		0.123	1.782		0.000	5.084
Fatty acid synthase - Fasn	0.365	7.350		0.447	5.280		0.332	8.649
FBJ osteosarcoma oncogene- FOS	0.068	67.275		0.077	59.356		0.124	29.446
Growth arrest and DNA-damage-inducible 45 alpha - Gadd45a	0.067	3.519		0.083	3.156		0.166	2.362
Gene regulated by estrogen in breast cancer protein - Greb1	0.280	-2.328		0.096	-4.923		0.150	-4.134
Glycogen synthase 1, muscle - Gys1	0.002	-2.717		0.009	-2.213		0.000	-2.086
Hedgehog-interacting protein - Hhip	0.035	2.111		0.164	-3.308		0.003	4.285
Hexokinase 2 - Hk2	0.192	35.886		0.237	24.499		0.213	29.719
Heat shock factor 1 - Hsf1	0.200	15.378		0.987	-1.034		0.221	13.462
Insulin-like growth factor binding protein 4 - Igfbp4	0.241	18.214		0.205	24.584		0.229	19.904

Inhibitor of kappaB kinase beta - Ikbkb	0.305	-2.727	0.168	-6.874	0.236	-3.456
Interleukin 2 - Il2	0.182	2.749	0.127	4.986	0.804	1.260
Interleukin 2 receptor, alpha chain - Il2ra	0.091	-4.650	0.042	-12.622	0.103	-5.448
Interleukin 4 receptor, alpha - Il4ra	0.025	-6.221	0.045	-8.601	0.029	-6.317
Jun oncogene - Jun	0.000	506.529	0.336	2.230	0.000	465.456
Transformed mouse 3T3 cell double minute 2 - Mdm2	0.015	71.937	0.036	29.135	0.020	53.199
Matrix metalloproteinase 10 - Mmp10	0.077	2.350	0.167	3.487	0.541	1.275
Matrix metalloproteinase 7 - Mmp7	0.206	2.285	0.004	10.664	0.044	3.647
Myelocytomatosis oncogene - Myc	0.040	1.739	0.089	-4.569	0.223	1.360
Ngfi-A binding protein 2 - Nab2	0.000	-7.938	0.215	-2.961	0.000	-8.042
Nuclear factor of kappa light chain gene enhancer in B-cells inhibitor, alpha - Nfkbia	0.196	20.971	0.267	12.874	0.213	18.358
Nitric oxide synthase 2, inducible, macrophage - Nos2	0.021	-3.080	0.015	-2.922	0.130	-1.813
Patched homolog 1 - Ptch1	0.062	-4.102	0.004	-21.412	0.075	-3.651
TRAF family member-associated Nf-kappa B activator - Tank	0.018	4.188	0.351	-2.890	0.029	3.389
Transcription factor 7, T-cell specific - Tcf7	0.126	70.661	0.337	12.807	0.239	28.003
Telomerase reverse transcriptase - Tert	0.120	-1.423	0.929	-1.042	0.127	-1.422
Tmepai	0.076	-5.109	0.029	-147.067	0.029	-8.545
Tumor necrosis factor - Tnf	0.847	-1.106	0.075	-3.123	0.454	-1.762
Vascular cell adhesion molecule 1 - Vcam1	0.046	1.750	0.040	-2.019	0.510	1.193
Vascular endothelial growth factor A - Vegfa	0.008	-2.094	0.086	-1.621	0.003	-2.612
WNT1 inducible signaling pathway protein 1 - Wispl	0.076	12.556	0.250	4.055	0.097	8.825

*Available statistical software only allowed T-tests of test group versus a control group.

Genes with significantly different expressions as determined by PCR array. All comparisons are to the corn oil/corn oil group. Genes with expression that differed by more than 2 fold or with a $p < 0.05$ are listed.

Pathways associated with genes above. Italics and green color denote genes with altered message in above table.

Mitogenic Pathway: *Egr1 (egr-1), Fos (c-fos), Jun (c-jun), Nab2.*

Wnt Pathway: *Birc5*, Ccnd1 (cyclin D1), *Cdh1*, Fgf4, *Jun (c-jun)*, *Lef1*, *Myc (c-myc)*, Pparg, *Tcf7*, Vegfa, *Wisp1*.

Hedgehog Pathway: *Bmp2, Bmp4, En1 (engrailed), Foxa2 (forkhead box A2 / HNF3B), Hhip, Ptch1 (patched 1), Wnt1, Wnt2.*

TGF- β Pathway: Cdkn1a (p21^{Waf1}, p21^{Cip1}), Cdkn1b (p27), *Cdkn2a (p16^{Ink4})*, Cdkn2b (p15^{Ink2b}).

Survival Pathway:

PI3 Kinase / AKT Pathway: *Bcl2 (Bcl-2)*, Ccnd1, Fn1 (fibronectin), *Jun (c-jun)*, *Mmp7 (matrilysin)*, *Myc (c-myc)*.

Jak / Src Pathway: *Bcl2 (Bcl-2)*, *Bcl2l1 (Bcl-XL)*.

p53 Pathway: *Bax*, Cdkn1a (p21^{Waf1}, p21^{Cip1}), Ei24 (Pig3), Fas (Tnfrsf6), *Gadd45a (gadd45)*, Igfbp3, *Mdm2*.

Stress Pathway: *Atf2*, *Fos (c-fos)*, Hsf1 (tcf5), Hspb1 (Hsp25), *Myc (c-myc)*, Trp53 (p53).

NF κ B Pathway: *Birc1a, Birc2 (c-IAP2), Birc3 (c-IAP1), Ccl20, Cxcl1, Icam1, Ikbkb, Il1a, Il2, Lta (TNFb), Nfkbia, Nos2 (iNOS), Tank, Tnf (TNFa), Tert, Vcam1.*

NFAT Pathway: *Cd5*, Fasl (Tnfsf6), *Il2*.

CREB Pathway: *Cyp19a1, Egr1 (egr-1), Fos (c-fos).*

Jak-Stat Pathway: Cxcl9 (MIG), *Il4ra*, Irf1, *Mmp10 (stromelysin-2)*, *Nos2 (iNOS)*.

Estrogen Pathway: *Bcl2 (Bcl-2)*, Brca1, *Greb1, Igfbp4*, Nrip1.

Androgen Pathway: Cdk2, Cdkn1a (p21^{Waf1}, p21^{Cip1}), *Tmepai*.

Calcium and Protein Kinase C Pathway: Csf2 (GM-CSF), *Fos (c-fos), Il2, Il2ra (IL-2 R?), Jun (c-jun), Myc (c-myc)*, Odc1, Tfrc.

Phospholipase C Pathway: *Bcl2 (Bcl-2), Egr1, Fos (c-fos), Icam1, Jun (c-jun), Nos2 (iNOS)*, Ptgs2 (cox-2), *Vcam1*.

Insulin Pathway: *Cebpb (C/EBP- β), Fasn (fatty acid synthase), Gys1 (GS, glycogen synthase), Hk2 (hexokinase II), Lep (Ob).*

LDL Pathway: Ccl2, Csf2 (GM-CSF), *Sele*, Selp (P-selectin), *Vcam1*.

Retinoic Acid Pathway: *En1 (engrailed)*, Hoxa1, *Rbp1 (CRBPI)*.

The data indicate that walnut consumption influences pathways involved in cell proliferation, cell death and differentiation of the mammary glands and that would be expected to alter carcinogenesis.

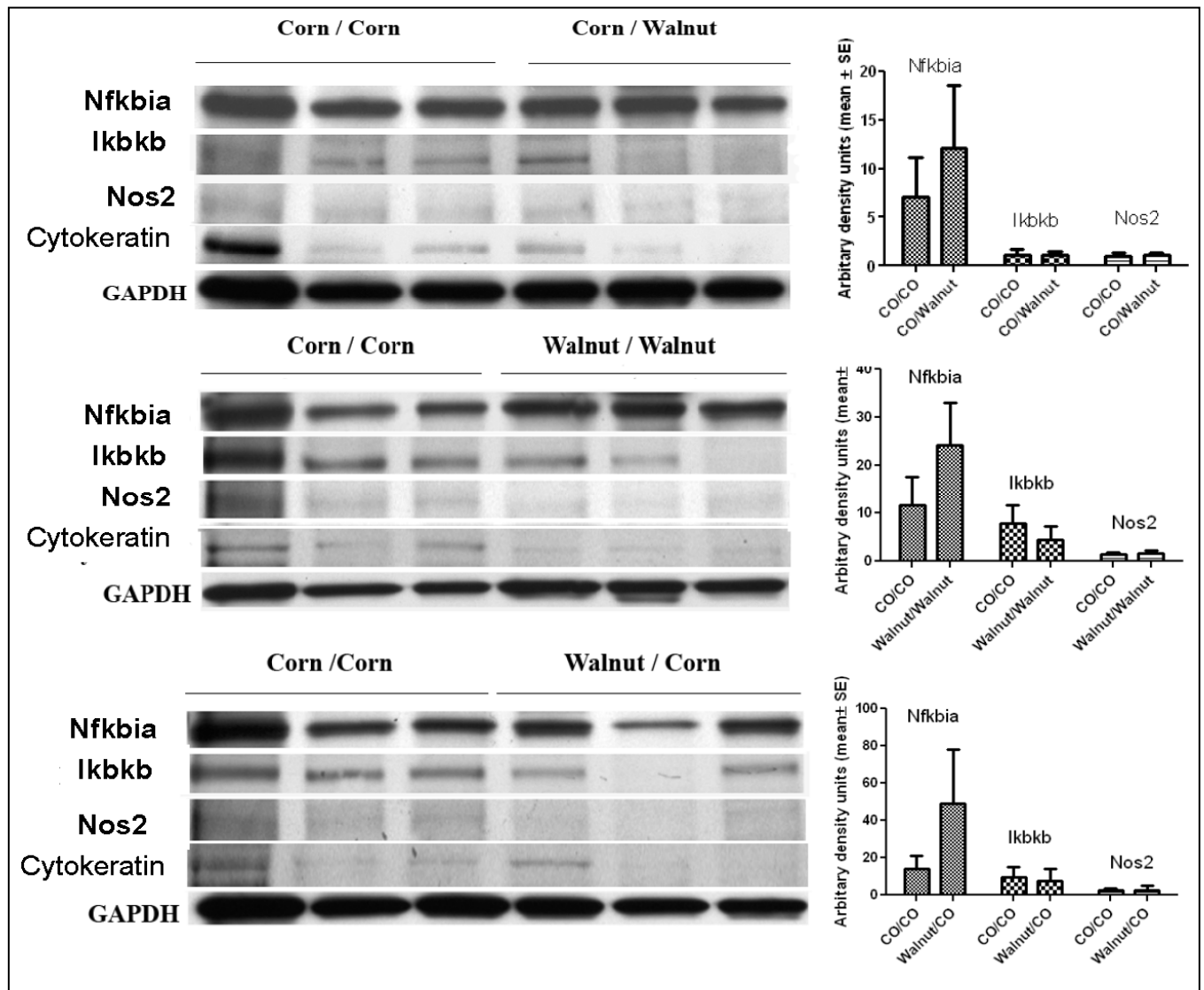


Fig. 3.3 Protein quantification for genes of interest in NFκB pathway. Increased NFκB activity has been associated with increased carcinogenesis. Western blot followed by densitometry was used to quantify NFκB inhibitor alpha (Nfkbia), inhibitor of kappa B kinase beta (Ikbkb) and inducible nitric oxide synthase. Nfkbia binds to and keeps NFκB inactive, Ikbkb cleaves Nfkbia for activation of NFκB thus increased Nfkbia and decreased Ikbkb would be associated with less activation of NFκB. Nos2 is downstream of NFκB but protein was not changed. N=3 mice per group. Individual protein changes are not significantly different. GAPDH bands are shown. Because we were interested in gene expression in the epithelial cells, columns in graphs show protein normalized for cytokeratin which is present in epithelial cells of the mammary gland.

Comparison of tumor multiplicity with a diet containing the same omega 3 amount as the walnut diet

We have previously reported partial results of a study in which incorporation of canola oil instead of corn oil was the dietary intervention (*Ion et al.*, 2010). The experimental design was the same as above except that 10% canola oil was incorporated into the diet of some mother mice and offspring instead of 10% corn oil. The canola oil diet contained 1% α -linolenic acid (10g/kg dry weight), the same amount of α -linolenic acid as in the walnut diet of this study. The canola

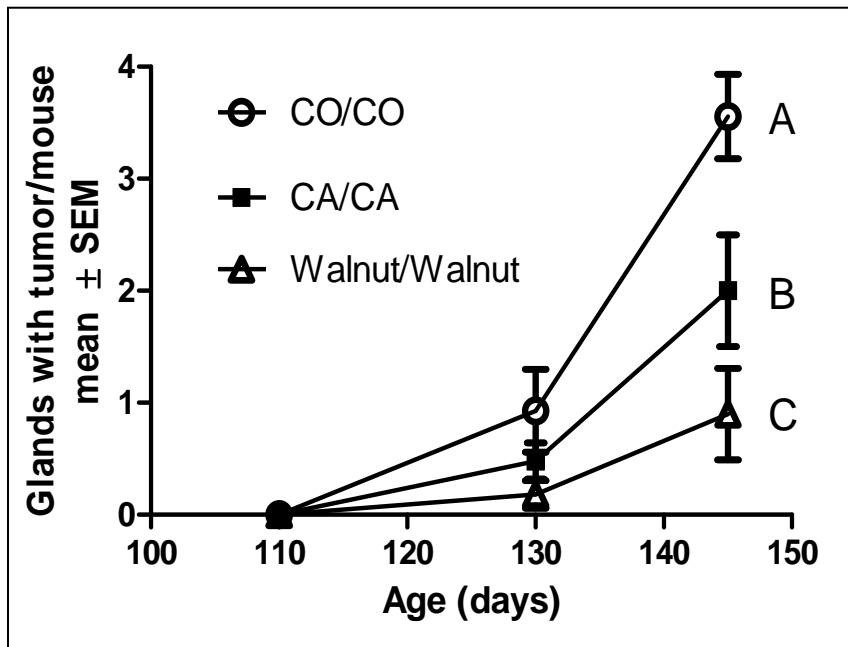


Fig. 3.4 Glands with tumor per mouse for corn oil/corn oil (CO/CO), canola oil/canola oil (CA/CA) and walnut/walnut groups. This experimental design represents dietary exposures for a population that routinely consumes these components in the diet. Tumor multiplicity for each group is significantly different ($p < 0.05$, two-way ANOVA, Bonferroni post test) from the two other groups, $CO/CO > CA/CA > walnut/walnut$ as indicated by different letters for each data set.

oil study (with different funding) was run in parallel and at the same time as the walnut study using the same control group. Figure 3.4 shows the number of glands with tumor per mouse with time, from 110 to 145 days of age. It is clear that fastest tumor development was when the diets of mother and offspring contained only corn oil (CO/CO), providing canola oil in the diet significantly suppressed tumor development (CA/CA), yet there was additional significant suppression of tumor development when walnut was included in the diet (Walnut/Walnut). The number of glands with tumor per mouse of each group are each significantly different from the other two groups (two way ANOVA followed by Bonferroni post-test, n=11-13 mice per time point).

Discussion

Epidemiology studies indicate that some populations have lower incidences of cancer (World Cancer Research Fund, 2007; Mitrou *et al.*, 2007), the challenge is to identify the foods that contribute to reducing cancer risk given the complex compositions of whole foods. Another challenge in all dietary studies is to determine whether it is the addition of a beneficial component or the subtraction of a detrimental component that provided the benefit against cancer.

The primary aim of this study was to assess the effects of walnut consumption on mammary gland cancer risk. The results presented herein indicate that walnut consumption could significantly alter expression of multiple genes and mammary gland cancer development. Walnut consumption by both mother and offspring, as would naturally occur when walnuts are

consumed as part of the usual diet of a population, did significantly reduce mammary gland cancer development and reduced multiplicity in this transgenic mouse. Consumption of walnut after weaning, as might occur when children migrate to a different environment or choose to add walnut to their diet, also slowed mammary gland cancer development; showing a 40% reduction in tumor incidence and a 44% reduction in multiplicity compared to mice not exposed to walnut. The next question is: What component of walnut was effective at slowing carcinogenesis?

Our first hypothesis was that the increased omega-3 content and decreased omega-6 content of the diet reduced carcinogenesis. Long chain omega-3 fatty acids have been shown to slow breast cancer growth in multiple animal studies (examples Karmali *et al.*, 1984; Karmali *et al.*, 1987; Rose and Connolly, 1993; Cave, 1997; Rose and Connolly, 1999; Petrik *et al.*, 2000) and have been proposed for cancer prevention (Rose and Connolly, 1999). Conversely, omega-6 fatty acids, especially linoleic acid as found in corn oil, have been shown to increase carcinogenesis (Rose *et al.*, 1994; Hilakivi-Clarke *et al.*, 1997; Rose *et al.*, 1999). In order to maintain balanced fat in the diet, if one species is increased (α -linolenic) then another (linoleic) has to decrease. We had data from another recently completed study [partially reported (Ion *et al.*, 2010)] which provided information that indicated that increasing the α -linolenic content contributed to reduced mammary gland cancer risk. After doing the calculations we realized that the canola oil and walnut diets contained the same amount of omega-3 in the form of α -linolenic acid. The canola oil containing diet did significantly suppress tumorigenesis compared to the corn oil containing diet. However, the walnut diet resulted in an additional significant suppression of tumorigenesis. Thus, some component of the diet, in addition to the increased α -linolenic content (or reduced linoleic acid), was functioning to suppress carcinogenesis.

There are reports that β -sitosterol can suppress cancer cell growth (Moon *et al.*, 2007; Moon *et al.*, 2008; Zhao *et al.*, 2009) and walnuts contain a significant amount of β -sitosterol (Maguire *et al.*, 2004; Pereira *et al.*, 2008). However, in assessing the β -sitosterol composition of the diets [using composition of the diet and (USDA, 2009)] we found that the β -sitosterol content was : corn oil diet - 968 mg/kg; canola oil diet – 413 mg/kg; walnut diet – 71 mg/kg thus the total β -sitosterol content of the walnut diet was less than that of the corn oil diet. Most of the references for benefit of β -sitosterol against cancer do NOT use breast cancer cells, which might be stimulated by binding of β -sitosterol to the estrogen receptor, but use other cell types that are not usually considered estrogen dependent. Interestingly, we did find one paper (Ju *et al.*, 2004) indicating that β -sitosterol may increase breast cancer cell growth in estrogen responsive cells; this would agree with our data. The tumors formed by this C3(1)/SV40 TAg model have been well characterized (Green *et al.*, 2000; Maroulakou *et al.*, 2001). Green *et al.*, (2000) report that: “atypia of the mammary ductal epithelium develops at about 8 weeks of age, progressing to mammary intraepithelial neoplasia (resembling human ductal carcinoma *in situ*) at about 12 weeks of age with invasive carcinomas at about 16 weeks in 100% of female mice. The tumors appear hormone responsive at early stages, invasive carcinomas are hormone independent, which corresponds to loss of ER α during progression.” Tumors were evident sooner in the mice that consumed the corn oil diet, perhaps in response to the β -sitosterol stimulation of ER α . At later times, when tumors became invasive and should be ER α -, the growth of tumors of mice that consumed the corn oil diet was not as influenced by corn oil diet. More research will be needed to determine the role of β -sitosterol in mammary carcinogenesis.

Assessment of vitamin E in the diets did provide a lead to an additional active component that was common to the diets. We found the gamma tocopherol is associated with slowing cancer cell growth (Yu et al., 2009; Lu et al., 2010; Pierpaoli et al., 2010) whereas alpha tocopherol did not have benefit against cancer and may block some of the activity of gamma tocopherol.

Assessing the tocopherol content of the diets revealed: corn oil diet – alpha tocopherol -14.3 mg/kg, gamma tocopherol – 0; canola oil diet – alpha tocopherol -17.46 mg/kg diet, gamma tocopherol – 27.34 mg/kg diet; walnut – alpha tocopherol 1.77 mg/kg diet, gamma tocopherol – 22.9 mg/kg diet. The changes of alpha and gamma tocopherol are clearly in the direction that according to the work of others would indicate benefit against cancer. Studies could be devised to test this question.

Conclusion

These data indicate that exposure to a small amount of walnut in the diet of this transgenic mouse slowed the development and reduced the multiplicity of mammary gland cancers but does not define the mechanism of action for the walnut nor an “active ingredient” of the walnut.

Walnut in the diet was associated with alterations in cell signaling pathways involved in proliferation, cell differentiation, and apoptosis. The signaling pathways altered in mammary glands of these mice have been identified as important in the development of human breast cancer thus this study should be relevant to humans. The fatty acid composition of the mammary glands was altered but comparison to another study with the same amount of omega-3 fatty acids in the diet indicates that increased omega-3 fatty acids in the mammary gland alone does not

explain the altered tumor incidence. However, alterations in dietary gamma tocopherol were inversely associated with tumorigenesis.

More work will need to be done to determine the components of walnut and the mechanisms associated with tumor suppression. However, humans eat the whole nut, not specific components. It seems likely that incorporation of walnuts as part of a healthy diet could reduce the risk for breast cancer in humans.

Acknowledgements:

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In the previous two chapters, we investigated the maternal consumption of canola oil diet on breast cancer risk in the offspring, and the regular consumption of walnut diet on the risk for breast cancer. Mammary gland tumorigenesis was suppressed due to consumption of canola oil or walnut diet. Studies have reported that diet also plays a role in the development of prostate cancer. The next chapter presents my investigation on the effects of the consumption of high omega-3 fatty acid diet from fish oil concentrate on prostate tumorigenesis. Fish oil is a good source of longer chain omega-3 fatty acids namely, EPA and DHA.

CHAPTER FOUR

Consumption of high ω -3 fatty acid diet suppressed prostate tumorigenesis in C3(1) TAg mice

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Abstract

Prostate cancer incidence and mortality are high in the Western world and high omega-6/omega-3 PUFA ratio in the Western diet may be a contributing factor. We investigated whether changing from a diet that approximates the omega-6 fat content of the Western diet to a high omega-3 fat diet at adulthood might reduce prostate cancer risk.

Female SV129 mice that had consumed a high omega-6 diet containing corn oil for 2wks were bred with homozygous C3(1)TA_g transgenic male mice. All male offspring were weaned to the corn oil diet until post puberty when half of the male offspring were transferred to a high omega-3 diet containing canola oil and fish oil concentrate.

High omega-3 diet increased omega-3 and decreased omega-6 fat content of mice tissues. The average weights of prostate and genitourinary bloc were significantly lower in mice consuming high omega-3 diet at adulthood (CO-FS) than mice fed a life-time high omega-6 diet (CO-CO). There was slower progression of tumorigenesis in dorsolateral prostate of CO-FS than in CO-CO mice. CO-FS mice had lower plasma testosterone level at 24wks and 40wks, significantly lower estradiol level at 40wks, and significantly less expressed androgen receptor in the dorsolateral prostate at 40wks than CO-CO mice. Consumption of high omega-3 diet decreased the expression of genes expected to increase proliferation and decrease apoptosis in the dorsolateral prostate.

Our results suggest that consumption of high omega-3 fatty acid diet slows down prostate tumorigenesis by promoting apoptosis and suppressing cell proliferation, in association with lowering estradiol, testosterone, and androgen receptor levels in C3(1)TA_g mice.

Introduction

Prostate cancer has remained the most frequently diagnosed cancer and the second leading cause of cancer related death among men in the US, accounting for 28% of total expected cancer incidence in men of US in 2010 (Jemal *et al.*, 2010). In general, the incidence and mortality of prostate cancer are high in North America and Northern Europe, but much lower in Japan and other Asian countries (Jemal *et al.*, 2011). Migration studies show that Asian men living in the US have a lower risk of prostate cancer than the Caucasians, but have a higher risk than their counterparts living in Asia (Platz and Giovannucci, 2006). Japanese men that immigrate to the US die of prostate cancer with increasing frequency as a function of the number of years of their residency (Astorg, 2004). The major factor for this increased frequency in prostate cancer death is thought to be the Western diet.

Polyunsaturated fatty acids (PUFA) are a component of dietary fat reported from several investigations to influence the development of prostate cancer (Rose and Connolly, 1991; Pandalai *et al.*, 1996; Berquin *et al.*, 2007). In the past 100 years, the fatty acid composition of Western diets has undergone a dramatic change, largely due to a high increase in the consumption of omega-6 (ω -6) PUFA from vegetable oils and red meat, and less consumption of omega-3 (ω -3) PUFA (Weaver *et al.*, 2009). This has resulted in an ω -6/ ω -3 ratio of 25-40:1 in the US diets rather than near the ideal 1:1 ratio. Asian diets are lower in ω -6 and higher in ω -3 than the Western diet due to more consumption of fish and other sea products and low consumption of plant oils and red meat.

Several studies have investigated the effects of ω -3 and ω -6 fat on prostate tumor cells. Omega-3 PUFA (eicosapentaenoic acid - EPA and docosahexaenoic acid - DHA) inhibit tumor

cell growth in animal models and human prostate cell lines (Rose and Connolly, 1991; Rose and Connolly, 1999), whereas ω -6 PUFA (linoleic acid - LA and arachidonic acid - AA) increase growth of human prostate tumor cell lines (Rose and Connolly, 1991; Connolly *et al.*, 1997; Rose and Connolly, 1999). Epidemiologic studies also reported a decrease of metastatic prostate cancer risk and prostate cancer death in men who consume the most fish (Tzonou *et al.*, 1999; Terry *et al.*, 2001; Hedelin *et al.*, 2007, Chavarro *et al.*, 2008), a good source of EPA and DHA. Linoleic acid is the most abundant ω -6 PUFA in the human diet. It is abundant in many plant oils such as corn oil, safflower oil and sunflower oil. Linoleic acid is the precursor for the synthesis of AA, which is abundant in red meat and meat fat.

Prostate cancer burden continues to increase because of the aging and growing population as well as nutritional patterns that tend to increase the risk for the disease. There is the need to identify and establish factors that might prevent or slow the progression of prostate cancer. If beneficial, a dietary change that includes the reduction in the intake of ω -6 fatty acids and increase the proportion of dietary ω -3 fatty acids may therefore be a powerful tool for prevention of mortality from prostate cancer.

In this study, we tested the hypothesis that compared to a diet that approximates the ω -6 fatty acid content of the Western diet, exposure to a diet with more ω -3 fatty acids during adulthood will slow the progression of prostate cancer, and to identify the underlying molecular factors. The C3(1)TA_g mouse model was used for the study because it develops prostate cancer slowly and has a well characterized disease progression making it suitable for prevention studies (Shibata *et al.*, 1996). We show that compared to a diet that is high in ω -6/ ω -3 fat, consumption of low ω -6/ ω -3 diet slows prostate carcinogenesis.

Materials and methods

Mice

Twenty female SV 129 mice, at 6wks of age, were obtained from Charles River Laboratories (Wilmington, MA), quarantined for 2wks, and transferred to a study room. Mice that bear a transgene for the SV40 large T antigen with a C3(1) rat prostatic steroid binding protein promoter were obtained from Dr. Jeffrey Green for breeding. The male transgenic mice develop prostatic intraepithelial neoplasia (PIN) that progresses to prostate carcinoma due to expression of the large T antigen in the prostate (Shibata *et al.*, 1996). All mice were genotyped to confirm the presence of the transgene.

Study design and feeding

All female SV 129 mice were placed on a diet containing 10% w/w corn oil (high ω -6, control diet, ratio of ω -6/ ω -3 was 50:1). After 2wks they were bred with homozygous C3(1) TAg transgenic male mice. All male hemizygous offspring were weaned to the corn oil diet and consumed this diet until the post puberty age of 7wks. Half of the offspring were retained on the corn oil diet to generate the high ω -6 CO-CO (mother - pup diet) group, while the remaining half were transferred to a higher ω -3 diet (test diet) to generate the CO-FS (mother - pup diet) group. The high ω -3 diet contained 5% canola oil (to provide adequate essential ω -6 fatty acid) and 5% fish oil concentrate (ratio of ω -6/ ω -3 in the diet was 1:3). The offspring were housed not more than 4 in a cage, numbered for individual identification and weighed weekly. All animals were maintained in an isolated environment in barrier cages and fed the specified diet. Mice were fed *ad libitum* with fresh food 6 days per week, and any leftover food in the cages was discarded.

The animal protocol was approved by the Marshall University school of Medicine Institutional Animal Care and Use Committee.

Diet

Diets were prepared in the animal diet prep room of Marshall University School of Medicine, using an AIN-76-A rodent diet modified to contain 10% total fat (Table 4.1). The AIN-76-A diet is adequate for the supply of nutrition and growth of the mice (American Institute of Nutrition, 1977). The control and test diets were isocaloric and isonutrient, and are relevant to human consumption. Casein, corn starch and other dry ingredients for the diets (except sugar) were obtained from MP Biomedicals (Solon, Ohio), omega-3 concentrates from fish oil (containing 60% EPA + DHA) were obtained from Zone Labs Inc. (Danvers, MA) while sugar and oil (100% canola oil or 100% corn oil) were obtained locally. Diets were prepared in batches as needed and stored in sealed containers at -20°C to prevent bacterial growth and fat oxidation in the food.

Assessment of transgene copy number

Ear punches stored at -20°C were digested as previously described (Ion *et al.*, 2010) and assessed for the presence of SV40 TAg transgene by real time PCR method, using an ABI Prism 7000 instrument (Applied Biosystems, Foster City, CA).

Body weights

To assess the effect of diet on body weight, mouse body weights were recorded weekly and terminally during dissection.

Dissection and tissue collection

Mice were euthanized at 24wks and 40 wks of age. A 24wks time point was chosen as adequate time for early stage disease progression in our heterozygote mice because previous studies indicated that C3(1)Tag male mice develop high-grade prostatic intraepithelial lesion (HGPIN) at 20wks (Shibata *et al.*, 1996). PIN is the primary precursor of prostate cancer. The high-grade PIN in C3(1)Tag male mice progresses to prostate carcinoma by 28wks (Shibata *et al.*, 1996). The 40wks time point therefore ensures advanced disease progression and provides for adequate time for intervention in our prevention study. After macroscopic evaluation during necropsy, the genitourinary (GU) bloc (consisting of the anterior prostate (AP), dorsolateral prostate (DL), ventral prostate (VP), seminal vesicle, bladder, proximal ductal deferens, and proximal urethra) was dissected and weighed to assess the effect of diet on GU weight. The AP, VP and DL prostate lobes were dissected from the GU apparatus with the aid of a dissecting microscope, and weighed to assess the effect of diet on prostate weight. GU blocs were fixed in phosphate-buffered 10% paraformaldehyde for 4 hours and transferred to 70% ethanol until ready for histopathological analyses. Mouse inguinal fat, liver and prostate were collected and stored at -80°C for other analyses.

Blood collection

Blood (about 600 μ l) was collected from each animal into EDTA vials by cardiac puncture at necropsy to assess testosterone and estrogen levels. Plasma was separated from blood samples by centrifugation and stored at -80 °C until assayed.

Plasma measures

Total testosterone and estrogen were measured in plasma obtained from individual mice from CO-CO and CO-FS groups at 24wks and 40wks, using the Testosterone EIA Kit or Estradiol EIA Kit respectively (Cayman Chemical Company, Ann Arbor, MI). Measurements were done in triplicate following the protocol provided in the kit.

Histopathological assessment

To assess cancer progression due to diet, GU apparatuses previously fixed in paraformaldehyde and thereafter in ethanol were sent to the Rodent Histopathology Core of Dana-Farber/Harvard Cancer Center for processing and pathology diagnosis. Briefly, the prostatic complex was paraffin embedded, sectioned on a slide (6 sections per prostate), and stained with hematoxylin and eosin (H&E). The animal identities were coded to avoid observer bias in the pathological diagnosis. Two independent evaluators analyzed all specimens. Observations were done for the presence and degree of epithelial hyperplasia and the presence of nuclear atypia in the epithelial cells in dorsolateral lobes of the prostate. Specimens with nuclear atypia were classified as LGPIN or HGPIN lesions following the guideline established by the Mouse Models of Human Cancer Consortium Prostate cancer Committee (Shappell *et al.*, 2004). Scores from two evaluators were averaged.

Fatty acid composition analysis

The fatty acid compositions of prostate, liver and fat at 24wks were analyzed by gas chromatography. Extraction of fatty acids was done as previously described (Ion *et al.*, 2010). Fatty acids suspended in iso-octane were separated and identified using a PerkinElmer Clarus 500 Gas Chromatography (Shelton, CT) with a Elite MWAX 30m x 0.53mm x 1.0 μ m crosslinked polyethylene glycol Capillary Column, in the following conditions: 220°C for 100 min, injector and flame ion detector at 240°C, helium carrier gas flow rate of 2.0ml/min. Peak identification was done using a customized standard (GLC #704) (Nu-Chek-Prep, Elysian, MN), which contains 10 fatty acids as methyl esters of stearate, oleate, linoleate, alpha linolenate, gamma linolenate, homogamma linolenate, arachidonate, eicosapentaenoate, docosapentaenoate, and docosahexaenoate. Fatty acid methyl esters were estimated as the percentage of the methylated fatty acids (area under the curve).

Gene expression RT² PCR assay

Gene expression analyses of DL tissues from 24wks old mice on different diets (3 tissues per group) were carried out, using the Mouse Signal Transduction Pathway Finder™ RT² profiler™ PCR Array (PAMM-014) (SuperArray Bioscience Corporation, Frederick, MD). This array was chosen to identify genes in signal transduction pathways that are influenced by high ω -3 fat diet and that may be applicable to cancer development. Briefly, DL prostates were dissected in RNA stabilization reagent (Omega Bio-tek, Inc., Norcross, GA) and frozen at -80°C until ready to use. Total RNA was isolated from frozen DL prostate tissue with RNeasy Fibrous Tissue Mini Kit (Qiagen, Valencia, CA), according to the manufacturer's protocol. RNA yield, purity and integrity were determined using an Agilent 2100 Bioanalyzer (Santa Clara, CA). Further quality

control was performed on all RNA samples with the RT² RNA QC PCR Arrays (SuperArray Bioscience Corporation, Frederick, MD). cDNA was prepared from RNA using RT² First Strand Kit and amplified by real time PCR using SuperArray RT² qPCR Master Mix on ABI Prism 700 (Applied Biosystems, Foster City, CA), following the protocol provided by the manufacturer. Differential gene expression and statistical analysis of data were done using the SuperArray software.

Immunoblot analysis

Frozen DL prostate tissues (3 to 4 per group) were homogenized in Tri Reagent (Sigma-Aldrich, St. Louis, Mo), followed by protein extraction according to protocol from the manufacturer. Protein concentration was determined by BCA protein assay kit (EMD Biosciences, Inc. Darmstad Germany). Fifteen micrograms of protein were loaded onto a 4-20% Tris-HCL polyacrylamide gradient gel (Bio-Rad Laboratories, Hercules, CA), separated by electrophoresis and transferred onto a nitrocellulose membrane. Blots were blocked using 5% BSA in TBST for 1 hour at room temperature and thereafter probed with primary antibodies in blocking buffer overnight at 4°C against: inhibitor of NFkappa-B kinase beta (IKKβ), B-cell leukemia/lymphoma 2 (BCL2) (Cell Signaling Technology, Inc.); Nitric oxide synthase Type II (NOS2) (BD Biosciences), NFkappa-B(p65) (Santa Cruz Biotechnology, Inc.); or NF kappa-B inhibitor alpha (IKα) (Abcam Inc.) for 1 hour at room temperature. Beta-actin (Santa Cruz Biotechnology, Inc.) was used to normalize protein loading. Membranes were then incubated with either horseradish peroxidase (HRP) conjugated antimouse (Santa Cruz Biotechnology, Inc.) or antirabbit (PIERCE Inc.) secondary antibody as appropriate. A Chemiluminescence ECL Kit (PIERCE Inc.) was used for band detection. Densitometric analyses were carried out using ChemDoc XRS system

(Bio-Rad Laboratories, Hercules, CA) to acquire the image, followed by image analysis with “Quantity One” software, V.4.5.2 (Bio-Rad Laboratories, Hercules, CA).

Immunohistochemistry

Expressions of androgen receptor (AR) and proliferating cell nuclear antigen (PCNA) were assessed in mouse DL prostate by immunohistochemistry. Paraffin sections (6 prostate sections per slide) were deparaffinized in xylene, hydrated in a series of graded ethanol, and rinsed in distilled water. Antigen retrieval was done in a microwave oven using Vector Antigen Unmasking solution (Vector Laboratories, Inc., Burlingame, CA). This was followed by blocking endogenous peroxidase activity using 3% hydrogen peroxide and Avidin/Biotin activity using Vector Avidin/Biotin blocking kit (Vector Laboratories, Inc., Burlingame, CA) following the manufacturers protocols. After blocking, sections were treated with rabbit anti-AR (PG21)(Millipore, Temecula CA) or mouse anti-PCNA (Biogenex, San Ramon, CA) and thereafter with secondary antibodies and vectastain Elite ABC kit (Vector Laboratories, Inc., Burlingame, CA) for rabbit antibody or Vector M.O.M kit (Vector Laboratories, Inc., Burlingame, CA) for mouse primary antibodies. Sections were incubated with 3, 3'-diaminobenzidine (Vector Laboratories, Inc., Burlingame, CA) and counterstained using hematoxylin. The levels of proliferation or AR expression were compared between the two diet groups by counting the number of cells with PCNA or AR positively stained nucleus respectively per 1000 cells within viable areas of lesions. Apoptosis was assayed by morphological identification (nuclear and cellular condensation, fragmentation of nuclei, and/or membrane blebbing and fragmentation of the cell into apoptotic bodies) of apoptotic cells (Narayanan et al,

2004). The numbers of cells undergoing apoptosis based on morphological indicators in 1000 epithelial cells of viable areas of lesions were counted to estimate an apoptotic index.

Statistics

Western blot protein expression levels, apoptosis, AR and PCNA levels, fatty acid composition, mean body weight gain, prostate and GU weight between diet groups and across time, were statistically analyzed by t-test. Testosterone and estradiol levels between diet groups and across time were analyzed by Mann Whitney test. The differences in prostate lesion incidence between diet groups were assessed by Fisher exact test. All statistical tests were performed using Prism software.

Results

Effect of diets on tissue lipid composition

The lipid compositions of the prostate, liver and fat at 24wks of age were analyzed. Mice at this age were on either corn oil diet or fish oil diet. As expected, the lipid composition of tissues reflected the lipid content of the diet (Supplementary figure 4.6). The prostate, liver and fat of mice on corn oil diet contained significantly more (t-test at $p < 0.05$) LA than the prostate, liver and fat of mice on fish oil diet. AA was significantly higher (t-test at $p < 0.05$) in the liver and prostate but not in the fat of mice on the corn oil diet than mice on fish oil diet. The liver, prostate and fat of mice on the fish oil diet contained significantly more (t-test at $p < 0.05$) ALA, EPA and DHA than the liver, fat and prostate of mice on corn oil diet.

Effect of diets on body weight gain

To assess the effect of diet on body weight of mice, we weighed the mice weekly from the time of weaning to time of sacrifice (40wks). Control and test diets were designed to be nutritionally balanced and with the same percentage of energy from fat. There was no significant difference in body weight gain between mice on the high ω -6 control diet (CO-CO group) and mice on the high ω -3 test diet (CO-FS group) (Figure 4.1A).

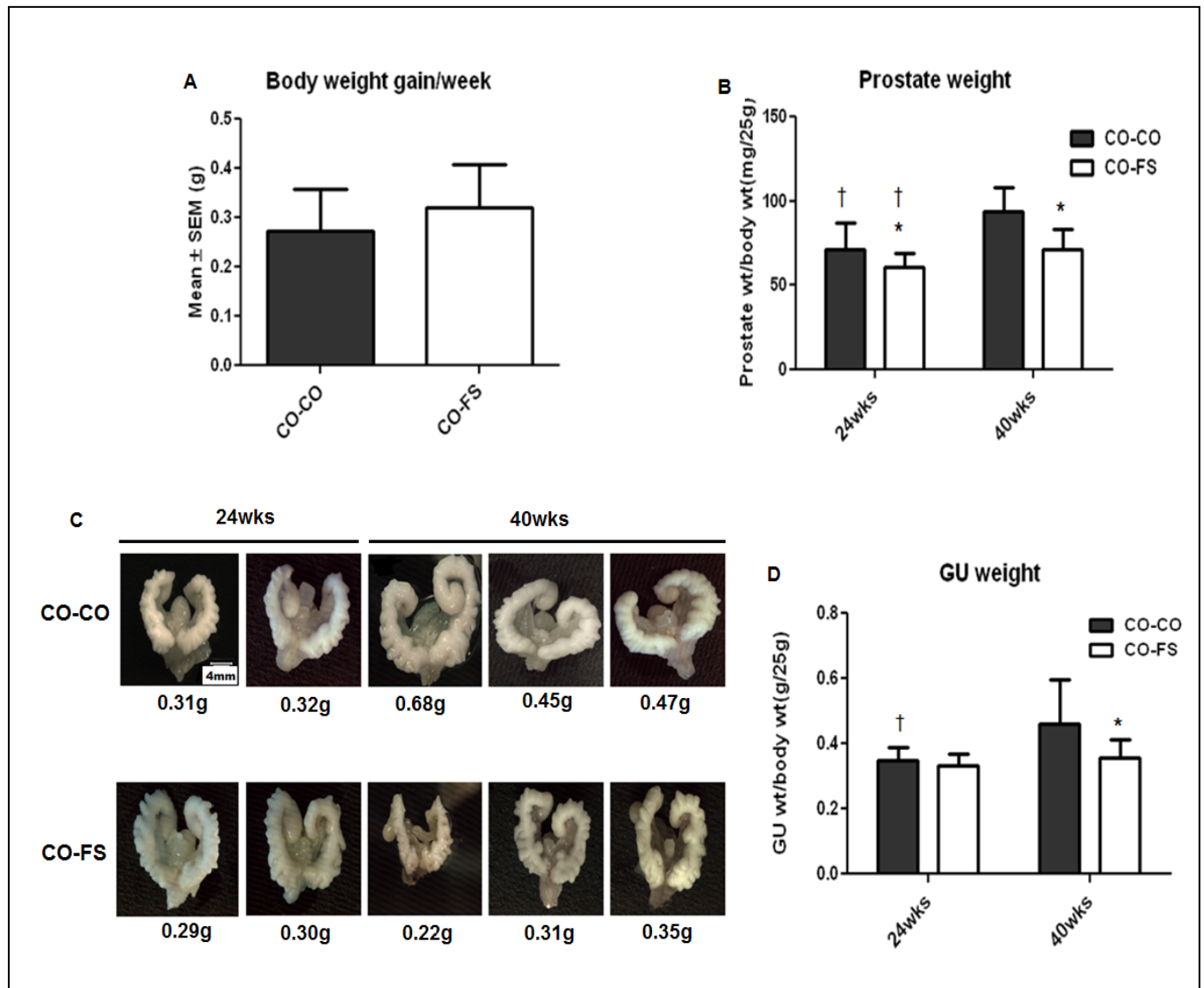


Fig. 4.1 Effect of diets on average body weight gain, prostate weight and GU weight between CO-CO and CO-FS mice. A) Average body weight gain. Data are from measurements of body weight gain for individual mice from the time of weaning to 40wks of age. Analysis by t-test showed no significant difference in body weight gain between the two groups at $p < 0.05$. $n = 9 / \text{group}$. B) Prostate weight at 24wks and 40wks of age. Mouse DL, AP, and VP lobes were dissected and weighed, and the sums were expressed as milligrams per 25 grams body weight. The average weight was significantly different between the two groups at 24wks ($n = 15 - 19 / \text{group}$) and 40wks ($n = 12 / \text{group}$) by t-test and at $p < 0.05$. Average prostate weight significantly increased in both groups with time by t-test at $p < 0.05$. C & D) Gross appearance of some GU and GU weight at 24wks and 40wks of age respectively. Mouse GU were dissected at euthanasia and weighed. The weights were expressed as milligrams per 25 grams body weight. The average GU weight was not significantly different between the two groups at 24wks but significantly lower at 40wks in the CO-FS group than in the CO-CO group by t-test and at $p < 0.05$, ($n = 19 / \text{group}$). Average GU weight increased significantly in CO-CO group with time.* Significantly different between groups, † significantly different across time at $p < 0.05$.

Effect of diets on prostate weight and GU weight

We assessed the effect of diet on prostate and GU weights as an indicator of progression of prostate tumorigenesis. Two time points, 24wks and 40wks, were selected for assessment of disease progression. The prostate of mice on the high ω -3 diet weighed significantly less than prostate of mice on the high ω -6 diet at 24wks and 40wks (by t-test at $p < 0.05$) (Figure 4.1B). Mice on high ω -3 diet had an increase of 10.21mg in prostate weight between 24wks and 40wks, while mice on high ω -6 diet had an increase of 22.49mg. Diet did not affect the GU weight significantly at 24wks (Figure 4.1C & D). The gross appearance of the GU bloc (Figure 4.1C) was similar between the two diet groups at 24wks. In contrast, the gross appearance of the GU bloc of mice on high ω -3 diet appeared smaller and the average weight was significantly lower than GU bloc of mice on high ω -6 diet at 40wks (Figure 4.1D).

Effect of diets on prostate histopathology

The histology of the prostate in C3(1) TAg mice of various ages has previously been documented, and it is known that on a usual diet, mice develop HGPIN in the prostate beginning about 20wks of age, which subsequently progresses to adenocarcinoma with advancing age (Shibata et al., 1996). Dorsolateral prostates of C3(1) TAg mice were evaluated in CO-CO and CO-FS groups at 24wks and 40wks of age. The degree and progression of epithelial hyperplasia and atypia were compared between the two diet groups (Figure 4.2). There was a slower progression of disease in mice on the high ω -3 diet than in mice on the high ω -6 diet at each time point (Figure 4.2). At 24wks, there was a significantly low proportion of LGPIN incidence (45% in CO-CO vs 64% in CO-FS) and significantly high proportion of HGPIN incidence (55% in

CO-CO vs 36% in CO-FS) in CO-CO group compared to CO-FS group (by Fischer exact test at

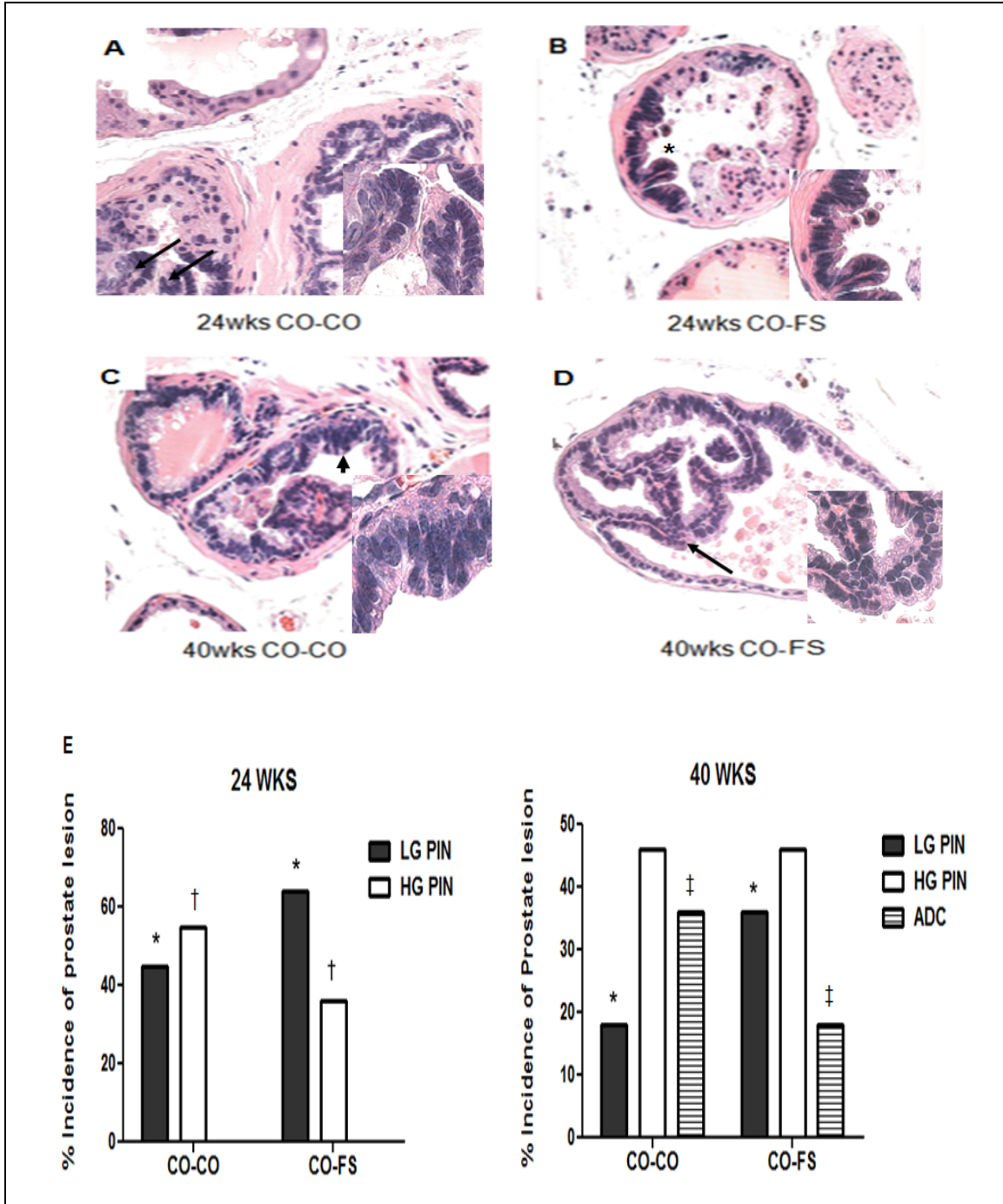


Fig. 4.2 Effect of diets on the incidence of PIN and adenocarcinoma lesions in the DL prostate. A-D) Representative H & E stained sections from 24wks and 40wks old mice. A & C) illustrate the presence of LG- and HG-grade PIN and ADC lesions as demonstrated by epithelial cells proliferation, hyperchromasia, nuclear enlargement and membrane irregularity. B & D) Consumption of high ω -3 diet resulted in marked reduction of epithelial cells proliferation, hyperchromasia, nuclear enlargement and membrane irregularity. E) incidence of PIN lesions and adenocarcinoma in CO-CO and CO-FS groups at 24wks and 40wks of age. Prostate specimens were histologically examined for lesions by two independent evaluators and scores were averaged (n=11 – 12 / group). Pictures were taken at X40 magnification. (Star indicates *LGPIN*, low-grade prostatic intraepithelial neoplasia; arrow indicates *HGPIN*, high-grade prostatic intraepithelial neoplasia; arrow head indicates *ADC*, adenocarcinoma. *, †, ‡ represent significant difference in *LGPIN*, *HGPIN* and *ADC* respectively between CO-CO and CO-FS group at $p < 0.05$).

$p < 0.05$). No adenocarcinomas (*ADC*) were observed in mice at 24wks. There was a significantly lower proportion of *LGPIN* incidence and higher proportion of *ADC* incidence in CO-CO group compared to CO-FS group at 40wks (by Fischer exact test at $p < 0.05$). At 40wks, the CO-CO group had 18% *LGPIN*, 46% *HGPIN* and 36% *ADC* while the CO-FS group had 36% *LGPIN*, 46% *HGPIN* and 18% *ADC*.

Effect of diets on plasma estrogen and testosterone levels and expression of AR

Plasma testosterone and estradiol levels were measured by enzyme immunoassays. Testosterone levels were not significantly higher in mice on the high ω -6 diet than in mice on the high ω -3 diet, or as mice progressed in age in mice on the high ω -6 diet (Figure 4.3A). To further test the effect of diet on testosterone action, androgen receptor expression in the dorsolateral prostate was analyzed by immunohistochemistry. The expression level of AR in dorsolateral prostate was significantly higher in mice on the high ω -6 diet than in mice on the high ω -3 diet at 40wks but not at 24wks of age (Figure 4.3B). There were significant differences in the levels of estradiol in mice

exposed to high ω -6 diet compared to mice on high ω -3 diet at 24wks and 40wks of age (Figure 4.3C). Estradiol level was significantly increased in mice that consumed high ω -3 diet compared to mice that consumed high ω -6 diet at 24wks. However at 40wks, estradiol level was significantly less in mice that consumed high ω -3 diet (10.45pg/ml) compared to mice that consumed high ω -6 diet (15.89pg/ml). Consumption of high ω -3 diet resulted in a significant decrease in estradiol level as mice progressed in age while consumption of high ω -6 diet resulted in no significant change in estradiol level as mice progressed in age.

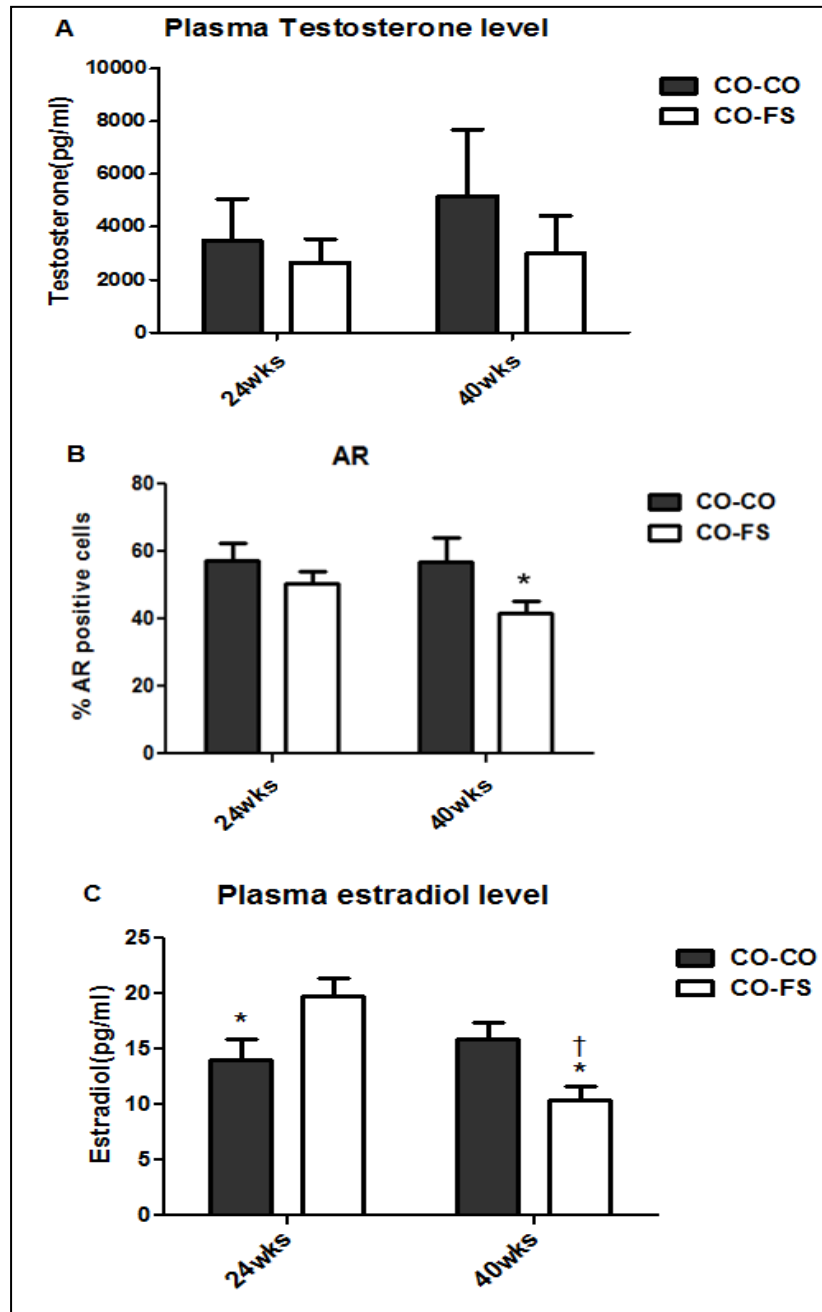


Fig. 4.3 Effect of diets on plasma testosterone and estradiol levels. Mice on both diets were sacrificed at 24wks and 40kws of age. Plasma was obtained for individual assay from blood collected from mice. A) The level of testosterone was less in mice from CO-FS group than mice from CO-CO group at 24wks and 40wks ($n = 12 - 19 / \text{group}$), and increased as mice progressed in age in the CO-CO group. These differences were not significant by Mann Whitney test at $p < 0.05$. B) Expression of AR was lower significantly in the CO-FS group than the CO-CO group at 40wks by t- test, $n = 5 / \text{group}$. A total of 1000 epithelial cells within viable areas of lesions per slide were counted for AR expression assay. C) Estradiol levels were significantly different between the two groups at 24wks and 40wks by Mann Whitney test at $p < 0.05$ ($n = 15 - 20 /$

group). Estradiol level was higher but not significantly as mice progressed in age in CO-CO group, and decreased significantly in CO-FS group than CO-CO group at 40wks and as mice progressed in age in CO-FS group. * Significantly different at $p < 0.05$, † significantly different across time at $p < 0.05$.

Effect of diets on gene and protein expression in the DL prostate at 24wks of age

To investigate the molecular mechanism by which high ω -3 diet may be slowing down the progression of prostate tumorigenesis in the DL prostate, we analyzed the expression of several genes involved in signal transduction pathways. We chose the 24wks time point because mice are in the early stages of prostate carcinogenesis, and, therefore, gene expression difference due to the diet and not to the tumor can be assessed. A total of 84 genes were quantitatively assayed using the Mouse Signal Transduction Pathway Finder™ RT² profiler™ PCR Array. Table 4.2 presents 31 genes with more than 2 fold differences (using SuperArray statistical analyses software) between CO-FS and CO-CO group at 24wks of age. We further analyzed the genes using the Ingenuity Systems software (IPA 8.0), and this showed that a number of the altered genes are associated with NF κ B and apoptosis pathways (data not shown). We chose NF κ B inhibitor alpha (IK α), I κ B kinase beta (IKK β), NF κ Bp65 and nitric oxide synthase 2 (NOS2) from the NF κ B pathway, and B-cell leukemia/lymphoma 2 (BCL2) from the apoptosis pathway for Western blot analysis. Western blot results showed I κ α protein (Figure 4.4A) to be increased but not significantly and BCL2 protein (Figure 4.4B) to be significantly decreased due to consumption of high ω -3 diet. NF κ Bp65, IKK β and NOS2 proteins were also significantly decreased due to consumption of high ω -3 diet (Figure 4.4A).

Table 4.2 Gene expression in dorsolateral prostate at 24wks of age

Gene name	Symbol	Fold difference CO-FS vs CO-CO
Activating transcription factor 2	Atf2	4.91
B-cell leukemia/lymphoma 2	Bcl2	-9.94
Bone morphogenetic protein 2	Bmp2	-2.81
Chemokine (C-C motif) ligand 2	Ccl2	-3.56
Cyclin-dependent kinase 2	Cdk2	2.37
Chemokine (C-X-C motif) ligand 9	Cxcl9	-3.64
Cytochrome P450, family 19, subfamily a, polypeptide	Cyp19a1	-2.26
Early growth response 1	Egr1	-2.45
Etoposide induced 2.4 mRNA	Ei24	3.91
Engrailed 1	En1	-3.18
Fas ligand (TNF superfamily, member 6)	Fasl	-2.98
Fatty acid synthase	Fasn	-2.15
Fibroblast growth factor 4	Fgf4	-4.44
Forkhead box A2	Foxa2	-3.87
Glycogen synthase 1, muscle	Gys1	2.11
Hexokinase 2	Hk2	2.25
Homeo box A1	Hoxa1	-3.31
Insulin-like growth factor binding protein 3	Igfbp3	-2.15
Interleukin 1 alpha	Il1a	-21.61
Jun oncogene	Jun	2.54
Lymphoid enhancer binding factor 1	Lef1	-3.1
Matrix metalloproteinase 10	Mmp10	-2.5
Ornithine decarboxylase, structural 1	Odc1	2.19
Peroxisome proliferator activated receptor gamma	Pparg	-7.6
Retinol binding protein 1, cellular	Rbp1	-2.08
Transcription factor 7, T-cell specific	Tcf7	-2.51
Telomerase reverse transcriptase	Tert	-3.35
Prostate transmembrane protein, androgen induced 1	Pmepa1	-3.41
Vascular cell adhesion molecule 1	Vcam1	-2.35
WNT1 inducible signaling pathway protein 1	Wisp1	-9.67
Wingless-related MMTV integration site 2	Wnt2	-2.66

Mice were placed on either corn oil diet or transferred to fish oil diet at 7wks of age. Gene expression in the DL prostate of mice at 24wks of age was performed by real time reverse transcriptase PCR. Genes with more than 2-fold differences in mRNA expression are shown. Results are from three different samples for each group.

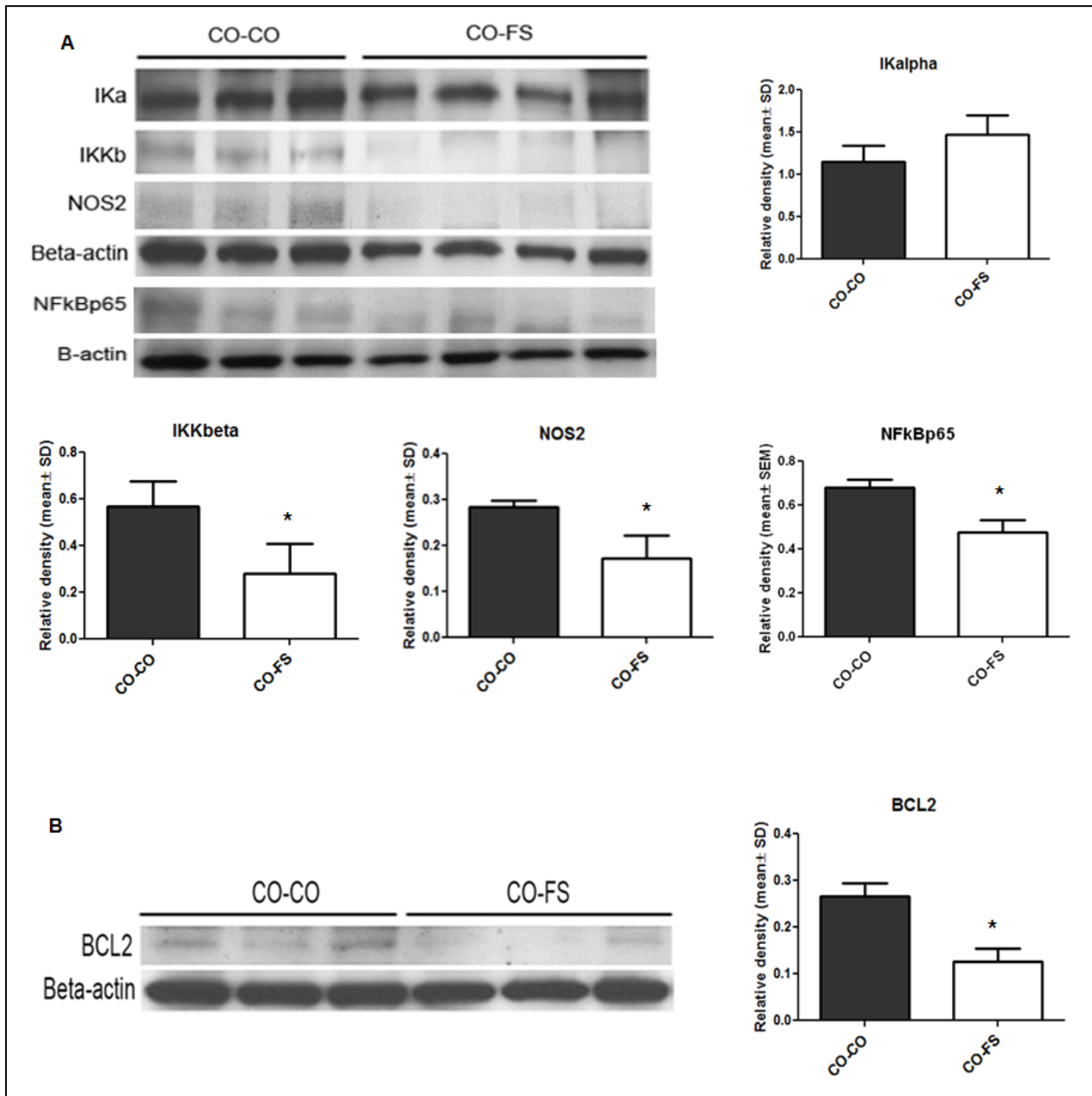
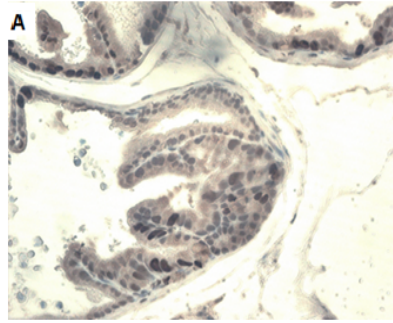


Fig. 4.4 Effect of diets on proteins in NFκB and apoptosis pathways in DL prostate. A) Protein expression of NFκBp65, IKα, IKKβ and NOS2 in the DL prostate and their corresponding densitometric analyses in 24wks old mice. Expression of IKα protein was increased but not significantly, while the expressions of NFκBp65, IKKβ and NOS2 proteins were significantly decreased in DL prostate of mice on high ω-3 fat diet than mice on high ω-6 fat diet. B) Protein expression of BCL2 in the DL prostate and the densitometric analysis in 24wks old mice. Expression of BCL2 protein was decreased significantly in the DL prostate of

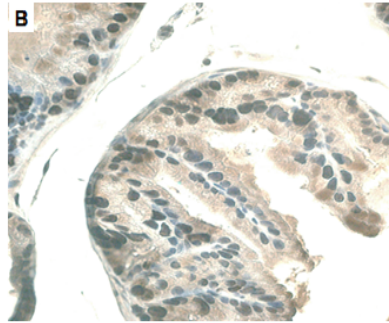
mice on high ω -3 fat diet compared to mice on high ω -6 fat diet. Equal loading of protein in the lanes was confirmed by β -actin. Values are relative density corrected for β -actin. * Significantly different at $p < 0.05$, $n = 3 - 5$ / group.

Effect of diets on cell proliferation and apoptosis in the dorsolateral prostates

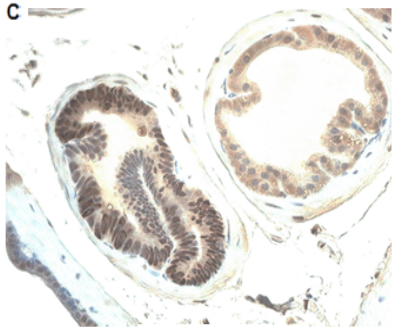
We further tested the molecular mechanism by which diet may be influencing the progression of prostate tumorigenesis by comparing the proliferation and apoptosis in the areas of lesions in DL prostate of mice from the two different diet groups. Proliferation was significantly decreased (Figure 4.5A) and apoptosis was significantly increased (Figure 4.5B) in prostate epithelial cells in mice that consumed high ω -3 diet compared to mice that consumed high ω -6 diet.



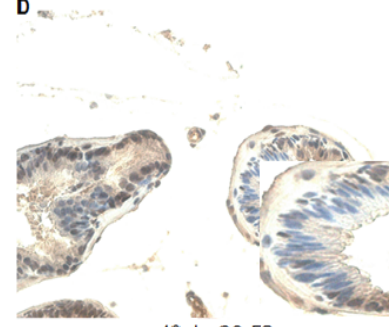
24wks CO-CO



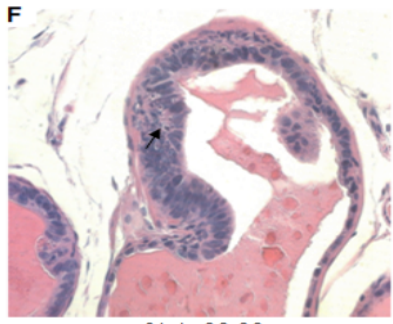
24wks CO-FS



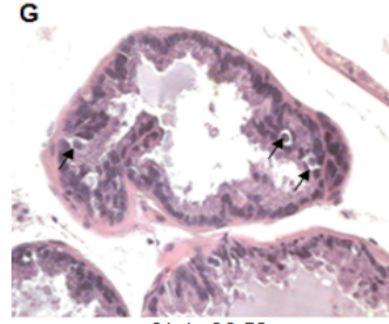
40wks CO-CO



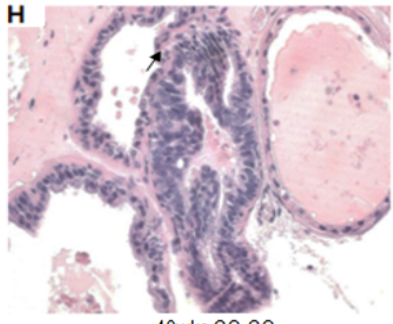
40wks CO-FS



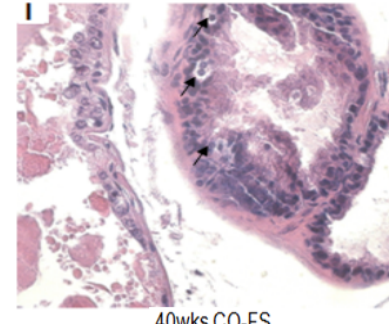
24wks CO-CO



24wks CO-FS



40wks CO-CO



40wks CO-FS

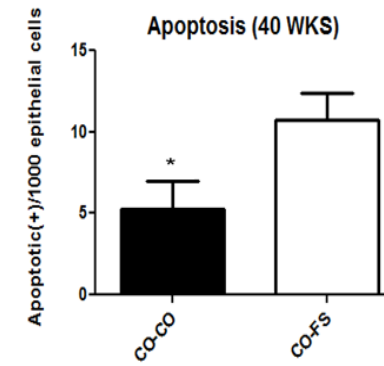
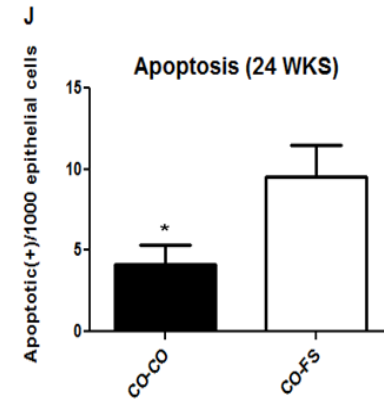
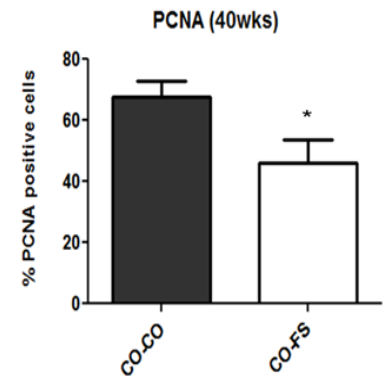
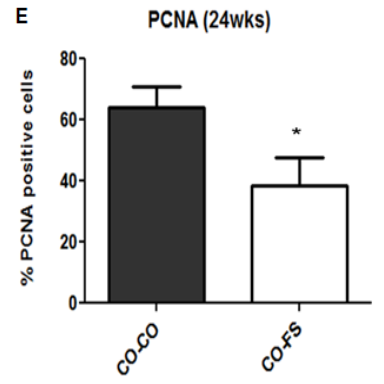


Fig. 4.5 Effect of diets on the extent of proliferation and apoptosis in DL prostate. DL prostate sections from the two groups were assessed for proliferation by immunohistochemistry analysis of PCNA at 24wks and 40wks (Fig 4A,B,C,D). Proliferation was significantly decreased in mice on high ω -3 diet compared to mice on high ω -6 diet by t-test at $p < 0.05$ (Fig 4E). Apoptosis was assessed by morphological identification of apoptotic cells in H & E stained sections (Fig 4F,G,H,I), and was significantly increased in mice on high ω -3 diet compared to mice on high ω -6 diet by t-test at $p < 0.05$ (Fig 4J). A total of 1000 epithelial cells within viable areas of lesions per slide were counted for proliferation and apoptosis assays. $n = 5$ mice / group for proliferation assay, $n = 7$ mice / group for apoptosis assay. Pictures were taken at 40X magnification. Arrow indicates apoptotic cells.

Discussion

One obvious characteristic of the Western diet is the high intake of ω -6 fat which has been associated with high prostate cancer risk in epidemiologic studies (Lanier *et al.*, 1980; Giovannucci *et al.*, 1993; Augustsson *et al.*, 2003). These studies also suggest that consumption of fish or fish oil, which are very high in ω -3 fatty acids, may reduce prostate cancer risk (Terry *et al.*, 2001; Augustsson *et al.*, 2003). We investigated the effect of high ω -3 and low ω -6 diet in the development of prostate cancer and the molecular mechanism that might be involved. The 24wks and 40wks intervention time points were chosen as adequate time for early stage disease progression and advanced disease progression since studies indicate that C3(1)TA_g male mice on a usual diet, develop HGPIN beginning about 20wks and progressing to prostate carcinoma from 28 wks (Shibata *et al.*, 1996; Maroulakou *et al.*, 1994). We chose the C3(1) TA_g model for this study for its well characterized disease progression from normal to PIN to adenocarcinoma (Shibata *et al.*, 1996).

A switch from high ω -6 diet to a high ω -3 diet at post-puberty was chosen for this study to model a change in diet initiated in adulthood. Post-pubertal consumption of a high ω -3 fat diet

significantly slowed the progression of prostate tumorigenesis in mice as observed by the reduced number of high grade prostate lesions in mice on high ω -3 diet compared to mice on high ω -6 diet. The ability of high the ω -3 diet to slow promotion of tumorigenesis in our mouse model was also observed as decreased GU bloc and prostate weights in mice that consumed high ω -3 diet. At the molecular level, mice on high ω -3 diet showed increased apoptosis and decreased proliferation in epithelial cells of the prostate as shown by decreased expression of the antiapoptotic gene BCL2 at mRNA and protein levels, and less staining for PCNA compared to mice on the high ω -6 diet. The EPA and DHA in the high ω -3 diet were accumulated in the prostate of mice fed with the high ω -3 diet, which likely slowed of disease progression. This is supported by Rose and Connolly, 1991, indicating that EPA and DHA suppress proliferation and promote apoptosis in prostate cancer cells.

To identify the underlying molecular factors by which the high ω -3 diet may be slowing down the progression of prostate tumorigenesis, we analyzed for the expression of some genes involved in NF κ B pathway because further analysis of the gene array into pathway-focused array (IPA 8.0) showed the NF κ B pathway to be differentially activated. NF κ B is a key mediator of survival, and is upregulated during prostate cancer progression (Suh and Rabson., 2004; Huang *et al.*, 2001). While in an inactive state, NF κ B is located in the cytosol complexed with the inhibitor of NF κ B (IK). A variety of extracellular signals can activate the inhibitor of NF κ B kinase (IKK) which in turn, phosphorylates the IK protein resulting in the dissociation of IK from NF κ B, and its eventual degradation. The activated NF κ B is then translocated into the nucleus to regulate the expression of downstream genes. Consumption of high ω -3 fat diet did not increase the expression of IK significantly however, it did significantly decrease the expression of IKK protein (Figure 4A). The mRNA expression fold for IK and IKK was 1.2 and

-1.97 (CO-FS vs CO-CO) respectively. Consumption of high ω -3 fat diet also significantly decreased the expression of NF κ Bp65 subunit and NOS2, a downstream gene in the NF κ B pathway. NOS2 is highly expressed in prostate tumors (Uotila *et al.*, 2001).

We also investigated the effect that high ω -3 diet may have on testosterone and estradiol levels in circulation. Testosterone has been vigorously studied because of its role in promoting prostate cancer, but the role of estrogen in prostate disease has received very little attention, despite reports that estrogen may promote prostate tumorigenesis (DeKlerk *et al.*, 1979; Henderson *et al.*, 1988; Thomas and Keenan, 1994; Ho, 2004). Serum testosterone level declines in the aging male whereas estrogen level increases and remains constant (Vermuelen *et al.*, 2002; Kaufman and Vermeulen, 2005). However, rising estradiol level may increase prostate sensitivity to testosterone via upregulation of AR (Moore *et al.*, 1979; Trachtenberg *et al.*, 1980). We showed that consumption of high ω -3 diet resulted in significantly lower estradiol level and lower testosterone level, with a concomitant significant decrease in the expression level of androgen receptor, suggesting a decrease in androgen stimulation due to consumption of high ω -3 diet.

We conclude that a high ω -3 diet slowed prostate tumorigenesis in the C3(1) Tag mouse model, and the mechanisms involved may include lowering of testosterone and estradiol levels, resulting in lower expression of androgen receptor, which in turn leads to a decrease in the expression levels of genes in the NF κ B and the antiapoptotic BCL2 pathways. These molecular changes could account for the suppressed proliferation and enhanced apoptosis that we observed within the lesions in the dorsolateral prostates of the mice. Therefore, a dietary change by humans that involves consumption of more ω -3 fat such as obtained from fish and fish oil,

instead of corn oil with little or no ω -3 fat, may be helpful in the suppression and prevention of prostate cancer.

Acknowledgements

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Supplementary Materials

Table 4.1. Diet Composition

Ingredient	% of weight	High ω -6 diet	High ω -3 diet
		Amount / 100g	Amount / 100g
Casein (protein)	20%	20g	20g
Sucrose	45%	45g	45g
Corn starch (carbohydrate)	15%	15g	15g
Alphacel (fiber)	5%	5g	5g
Choline bitartrate	0.20%	0.2g	0.2g
DL-methionine	0.30%	0.3g	0.3g
Mineral mix	3.50%	3.5g	3.5g
Vitamin mix	1.00%	1g	1g
Corn oil (fat)	10%	10g	
Canola oil (fat)	5%		5g
Fish oil (fat)	5%		5g
Total	100%	100g	100g
Total fat		10g	10g
Total protein		20g	20g
Total carbohydrate		60g	60g

The AIN-76A mouse recipe was modified to contain 10% w/w corn oil (high ω -6, low ω -3 control diet) with an ω -6/ ω -3 ratio of 50 or 5% canola oil and 5% fish oil concentrate (low ω -6, high ω -3 test diet) with an ω -6/ ω -3 ratio of 0.3. Both diets were isocaloric and isonutrients and are adequate for growth and nutrition of mice.

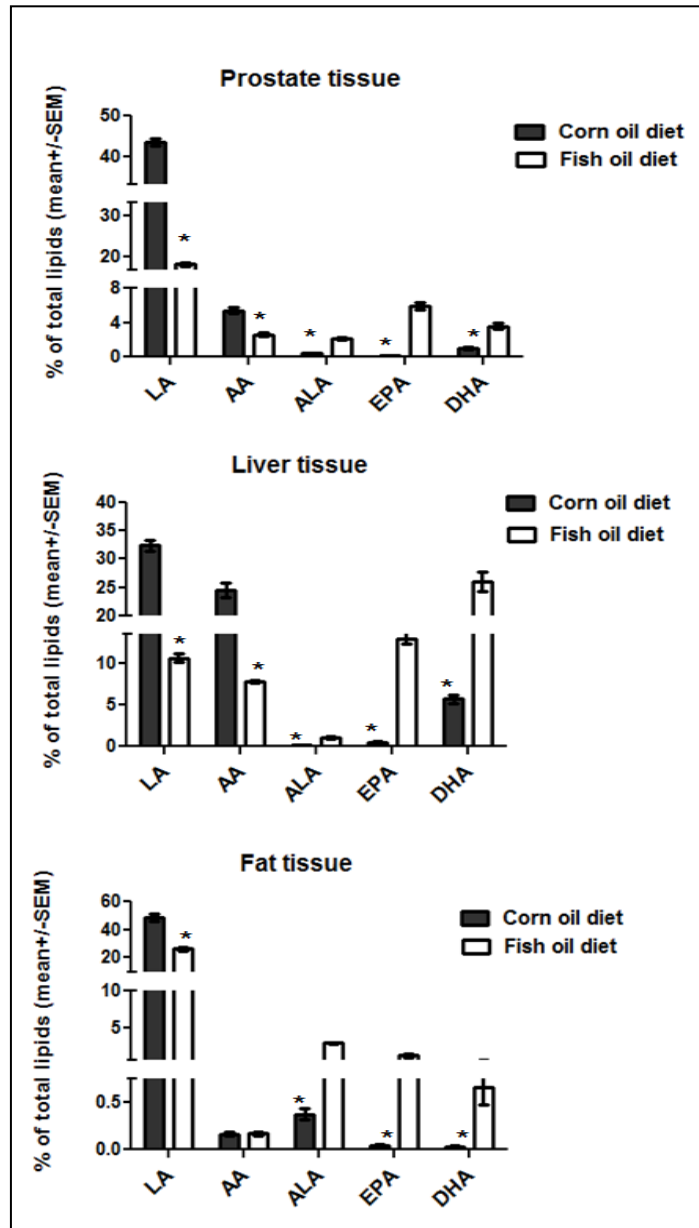


Fig. 4.6. Effect of diets on major ω -3 and ω -6 fatty acid levels in mice tissues. The results of t-tests showed significant differences in fatty acid levels in mouse tissues due to diet. ω -6 fatty acids – LA (Linoleic acid), AA (Arachidonic acid). ω -3 fatty acids – ALA (α -Linolenic acid), EPA (Eicosapentaenoic), DHA (Docosahexaenoic acid). * Significantly different at $p < 0.05$, $n = 5$ to 6 mice / diet group.

CHAPTER FIVE

GENERAL DISCUSSION AND FUTURE DIRECTIONS

The patterns of human food production and consumption have changed greatly due to industrialization and urbanization, contributing to increasing rates of diseases including cancer (Simopoulos, 2008). Such changes include an increase in omega-6 fatty acids and a decrease in omega-3 fatty acids in the Western diet (Simopoulos, 2002). This is in contrast to an omega-6/omega-3 ratio of about 1:1 found in wild animals and on which humans evolved (Simopoulos *et al.*, 1999; Simopoulos, 2008). The balance of omega-6 and omega-3 fatty acids is important and required for homeostasis and normal development (Simopoulos, 2006). Although cancer is a disease of mutated genes and can be inherited, environmental factors such as diet play important roles in influencing the disease (Doll and Peto, 1981). Migration studies show that cancer risk of adults who migrate to the USA from regions with low rates of cancer incidence such as Asia, increases to that of the new country (Cook *et al.*, 1999). Asian diets are lower in omega-6 and higher in omega-3 than the Western diets. These reports thus imply that cancer may be delayed or prevented by increasing the dietary omega-3/omega-6 ratio.

Although epidemiology and experimental studies have been carried out on the benefits of consuming high omega-3 fat and lowering the intake of omega-6 fat (in contrast to high omega-6 and very low omega-3 in the Western diet) in reducing the risk for some diseases, reports are inconsistent for cancer. There is also a growing interest in the use of natural products to suppress cancer growth (Bennett *et al.*, 2009) and to prevent it. Thus, there is need for more knowledge about dietary components with potential benefits in suppressing cancer growth. To address this problem, we fed the C3(1) TAg transgenic mice with diet modified to contain more

omega-3 and less omega-6 fatty acids than the levels in Western diet, using (1) canola oil, (2) walnut or, (3) fish oil concentrate. Our dietary modifications (canola oil, walnut and fish oil diets) did not significantly affect body weight compared to the control diet (corn oil diet). The benefits of such diets against breast and prostate cancers were investigated. The C3(1) TAg mice bear a transgene for the SV40 large T antigen with a rat prostatic steroid binding protein promoter, which drives the expression of the transgene in the epithelium of the mammary and prostate glands thereby inducing transformation and tumorigenesis (Yoshidome *et al.*, 1998). We chose C3(1) TAg mice for the studies because they develop mammary and prostate tumors that are histologically similar to human breast and prostate cancers (Green *et al.*, 2000).

In our first study we placed female mother mice on a diet containing 10% w/w corn oil (control diet) or 10% w/w canola oil (test diet), and during gestation and lactation of the offspring. All female offspring from these mother mice were weaned to corn oil diet and identified as corn / corn (CO/CO) or canola / corn (CA/CO) groups. In utero and lactation exposure to the canola oil diet caused a significant delay in mammary gland tumor formation in the offspring. As mice advanced in age, almost as many tumors, but significantly less tumor burden, developed in the CA/CO mice compared to the CO/CO mice. Differences in the expression of genes involved in proliferation, differentiation and apoptosis were observed in the mammary gland of adult mice, supporting the notion that *in utero* diet exposure may be influencing gene imprinting. There was a higher expression of CEBP β (a transcription factor involved in the transcription of multiple factors involved in proliferation, differentiation and apoptosis in the mammary gland, (Grimm and Rosen, 2003)), and lower expression of fatty acid synthase at mRNA and protein levels in CA/CO group than in CO/CO group. Fatty acid synthase is associated with human mammary carcinogenesis (Esslimani-Sahla *et al.*, 2007). The

expression of *Egr1*, a tumor suppressor, was increased in CA/CO group. The inhibitor of κ B kinase β (IKK β) and genes downstream in the NF κ B pathway, namely inducible nitric oxide synthase (iNOS), tumor necrosis factor (TNF) and vascular cell adhesion molecule 1 (Vcam1), were less expressed in CA/CO mice, suggesting the inactivation of the NF κ B pathway by the canola oil diet. Genes in the apoptotic pathway were also differentially expressed in the mammary gland of adult mice. The pro-apoptotic gene, Bcl-2 associated X (BAX), was more expressed and the anti-apoptotic gene, BCL-2-like-1(Bcl2l1) was less expressed in the CA/CO group.

In our second study, we investigated the regular consumption of walnut and its influence on breast cancer risk. The regular consumption of walnut in a population means that offspring in such a population are exposed to the nutrients from walnut during gestation, lactation and at adulthood. Thus, in our investigation, we considered four experimental groups namely; corn oil/corn oil (CO/CO), corn oil/walnut (CO/walnut), walnut/walnut, or walnut/corn oil (walnut/CO); where the first diet was the maternal diet and the second diet was the offspring's diet.

Consumption of walnut by mother and offspring (walnut/walnut) as expected in a population that consumes walnut regularly, significantly reduced tumor size, tumor multiplicity and tumor incidence in mice mammary glands compared to in the mammary glands of mice not exposed to walnut (CO/CO). Similarly, consumption of walnut after weaning (CO/walnut) as would occur when children move to a different environment or choose to make walnut part of the diet, reduced tumor size, tumor incidence and significantly reduced tumor multiplicity in mice mammary glands compared to in the mammary glands of mice not exposed to walnut, but not as

much as was observed in the mammary glands of the walnut/walnut group. Offspring who were exposed to walnut via gestation and lactation alone (walnut/CO) showed less reduced tumor mass and multiplicity compared to the CO/walnut group, while tumor incidence was similar to the control group. Exposure to dietary walnut altered several genes associated with mitogenesis, NF κ B among others. Western blot analyses of genes in the NF κ B pathway showed that walnut consumption resulted in increased expression of NF κ B inhibitor alpha (Nfkbia) while a longer exposure to walnut (walnut/walnut) also resulted in decreased expression of inhibitor of NF κ B kinase (IKK). These results suggest that walnut consumption may be suppressing mammary gland tumorigenesis. A comparison between the walnut diet and the canola diet with the same amount of α -linolenic acid showed that, walnut consumption (Walnut/Walnut) provided additional significant suppression of tumor development, compared to the canola oil diet.

In our third study, fish oil concentrate was used along with canola oil to obtain a high omega-3- low omega-6 diet that included EPA and DHA (test diet), and investigated the effects of such a diet on the risk for prostate cancer and the molecular mechanism that might be involved. EPA and DHA are abundant in fish oil (Petrik *et al.*, 2000) while canola oil contains high amounts of LA and ALA. Hemizygous male pups from mothers on control corn oil diet (high omega-6/omega-3) were weaned on corn oil diet until a post puberty time point. This model equates to adulthood in the human and a time at which diet choices can be made. Half of the offspring were transferred to the high omega-3 fish oil diet while the remaining half were retained on the corn oil diet. Two experimental groups were therefore considered for this study namely; corn oil/corn oil (CO/CO) and corn oil/fish oil (CO/FS); where the first diet was the maternal/weaning diet and the second diet was the offspring's diet. Twenty four weeks and forty weeks were considered for tumor progression because on a usual diet, C3(1) TAg male mice

develop HGPIN beginning about 20wks and progressing to prostate carcinoma from 28wks (Maroulakou *et al.*, 1994; Shibata *et al.*, 1996).

Post pubertal consumption of high omega-3 fat diet resulted in decreased GU and prostate weights, and slower development of high grade prostate lesions, with a concomitant increase in apoptosis and decrease in proliferation in the epithelial cells of the prostate of mice. Pathway-focused array (IPA 8.0) analysis of the gene array results showed genes in the NF κ B pathway to be differentially expressed due to diet. Consumption of high omega-3 fat diet increased the expression of inhibitor of NF κ B (IK) and significantly decreased the expression of inhibitor of NF κ B kinase (IKK). Consumption of high omega-3 fat diet also significantly decreased the expression of NF κ Bp65 subunit and NOS2. High omega-3 diet consumption resulted in significantly lower estradiol level and lower testosterone level with a concomitant significant decrease in the expression level of androgen receptor, suggesting a decrease in androgen stimulation due to consumption of high omega-3 diet.

Our findings have shown that reducing the omega-6/omega-3 ratio in the diet can have a suppressive effect on mammary and prostate tumorigenesis in the transgenic mice. The additional significant suppression of tumorigenesis in the mammary gland via consumption of walnut diet compared to canola oil diet suggests that omega-3 fatty acids may interact with some other dietary components for greater effects. This is not surprising because studies have also shown that the anticarcinogenic and antitumorigenic benefits of garlic likely arise from a wide variety of components, possibly working synergistically (Krest *et al.*, 2000; Amagase *et al.*, 2001; Don *et al.*, 2001; Lind, 2004). A study by Krishnan *et al.*, (2007) showed that combination of calcitriol

and genistein was more effective in retarding prostate cancer cell growth than either agent alone. Testing every possible combination and fraction before recommendation is obviously a daunting task. Therefore, consumption of diets that contain a variety and balance of food may be more beneficial in the prevention of cancer because multiple food substances that may work both synergistically and independently are supplied to the body.

The suppression of mammary gland tumorigenesis in the offspring of mothers that consumed canola oil could be a pointer to an epigenetic modification due to mother's diet. Interestingly *in utero* exposure to the walnut diet did not suppress mammary gland tumorigenesis in the offspring. In addition to some other genes, the tumor suppressor gene Early growth response 1, was more expressed in mammary gland of offspring of mothers consuming canola oil diet or walnut diet. However, the expression of Fatty acid synthase (Fas) was suppressed in the mammary gland of offspring of mothers consuming canola oil diet, and increased in the mammary gland of offspring of mothers consuming walnut diet. Expression of Fatty acid synthase is associated with the early stages of mammary tumorigenesis, and its inhibition is associated with apoptosis in breast cancer cells (Esslimani-Sahla *et al.*, 2007; Bandyopadhyay *et al.*, 2006). One mechanism by which the consumption of canola oil diet by mothers suppressed mammary gland tumorigenesis in the offspring may be via the epigenetic modification resulting in the suppression of expression of Fas and some other genes. The hypothesis that "maternal consumption of diet high in omega-3 fatty acid during gestation and lactation will suppress mammary gland tumorigenesis in the offspring via the inhibition of histone deacetylase (HDAC) activity" may test this notion. Some dietary components from gallic and cruciferous vegetables are reported to inhibit the activity of HDAC, inhibiting cell proliferation and stimulating apoptosis in cancer cells (Ross, 2007). Also, because *in utero* exposure to the walnut diet did not

suppress mammary gland tumorigenesis in the offspring as observed in offspring of mothers that consumed canola oil diet, it is suggestive that variations in response to different diets may occur due to differences in the diet, the timing, duration or the genetics of the individual.

Furthermore, the gene-diet interactions observed when corn oil was substituted with canola oil, walnut or fish oil concentrate were interesting. Several of the genes differentially expressed at mRNA and/or at protein levels due to diet in our studies, are involved in proliferation, apoptosis and differentiation. Our studies showed that NF κ B and apoptosis pathways may be targets of our diet interventions to suppress tumorigenesis in mammary gland as well as in the prostate of the mice. NF κ B is a key mediator of survival, and is upregulated during breast and prostate cancer progressions (Huang *et al.*, 2001; Helbig *et al.*, 2003; Suh and Rabson, 2004). NF κ B is activated in inflammatory process, and its activation inhibits apoptosis and increase proliferation in cancer cells (Escárcega *et al.*, 2007). The inflammatory genes Interleukin 1 alpha and Interleukin 2 receptor alpha, were less expressed due to consumption of diet high in omega-3 and low in omega-6 fatty acids in our studies. Interestingly, the expressions of these genes as well as androgen receptor (AR was less expressed in the DL prostate due to consumption of high omega-3 diet) are modulated by NF κ B (Ballard *et al.*; 1988; Mori and Prager, 1996; Zhang *et al.*; 2004). However, while Early growth factor 1 gene, a target gene of NF κ B (Zhou *et al.*, 2003), was more expressed in the mammary gland, its expression was suppressed in the dorsolateral prostate of the mice consuming diet high in omega-3 and low in omega-6 fatty acids, suggesting that different genes may be targets of NF κ B in different organs. Other studies have shown that diet components can slow proliferation and induce apoptosis in tumors (Martin, 2007; Meeran and Katiyar, 2008). More work will be needed to elucidate the underlying molecular mechanisms of the effect of our high omega-3 diets in suppressing

mammary gland and prostate tumorigenesis. The differential gene expression levels observed in the mammary gland of offspring due to the mother's diet suggest epigenetic effects of the diet. Food components such as selenium, retinoic acid and sulforaphane influence DNA methylation patterns (Ross, 2003; Dashwood and Ho, 2007). A dietary change from high omega-6/omega-3 diet to a low omega-6/omega-3 by expectant mothers may be helpful in decreasing the risk for breast cancer in their offspring by suppressing mammary tumor incidence in the offspring.

The future directions of these studies will be to determine if consumption of canola oil diet and our control corn oil diet by normal mice will amount to similar morphological and gene expression changes seen in the transgenic mice. Such a study may yield biomarkers of reduced risk of mammary gland cancer that may be useful in clinical trials. Analysis of our results from the walnut study suggested that vitamin E, an antioxidant, in the walnut may be contributing to the additional benefit against mammary gland tumorigenesis observed in our mouse model. A further study that provides a combination of omega-3 fatty acid and vitamin E in diet versus diet with omega-3 or vitamin E alone would shed more light on this notion.

The *in utero* exposure to high omega-3 diet compared to high omega-6 diet, and the effect on life time risk of prostate cancer in offspring should be investigated. This is desirable because our female study showed that mother's diet may be imprinting genes in the offspring to affect later cancer development in the mammary gland. A clinical study of placing normal male individuals on omega-3 supplement to check on prostate cancer biomarkers as influenced by omega-3 fatty acid consumption should also be investigated.

In summary, substituting corn oil with canola oil in the maternal diet reduced the risk for mammary gland tumorigenesis in the offspring, and the consumption of walnut also reduced the

risk for mammary gland tumorigenesis compared to consumption of corn oil diet in transgenic mice. Substituting corn oil with fish oil reduced the risk for prostate tumorigenesis in transgenic mice. Thus, a dietary change that involves consumption of diets with higher omega-3 and lower omega-6 fatty acids than currently obtained in the Western diets could be beneficial in decreasing the risk for breast and prostate cancers.

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