Effects of low linolenic soy oil on pre-malignant human mammary epithelial cell progression

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ABSTRACT

Beginning January 1, 2006 the U.S. Food and Drug Administration mandated that the amount of *trans* fats per serving be listed on the Nutrition Facts panel. Consequently new soybean breeds that would no longer be subject to the hydrogenation process, thus reducing *trans* fats, were developed. By traditional plant breeding techniques, plant breeders have developed a low linolenic soybean with 83.36% less alpha-linolenic acid (ALA; omega-3) than conventional soybean. A number of studies have demonstrated that the influence of dietary fats on cancer depends on the quantity as well as the type of lipids and diets with a disproportionately high omega-6 (n-6)/omega-3 (n-3) ratio are thought to contribute to cardiovascular disease, inflammation and cancer. Conventional soybean oil (SO) has an n-6/n-3 ratio of 8/1 while the new low linolenic soy bean oil (LLSO) has an n-6/n-3 ratio of 56/1. In this study, we evaluated the effects of dietary LLSO, SO and lard on the progression of breast cancer (BC). Thirty-five, 6-wk old, ovariectomized, athymic mice received human pre-malignant breast cells (MCF-10AT1 1 x 10^5 cells/40µl/ Matrigel/spot, 4 spots/mouse). Mice were divided into three groups and then fed isocaloric and isonitrogenous diets with disparate fat sources: LLSO (20% of total energy intake), SO (20%) and lard (20%). The dietary treatment lasted 24 weeks upon which the study was terminated and tumors, tissues and blood samples were analyzed. Average tumor surface area of the LLSO group was 45.11 ± 4.46 mm^2, and it was significantly larger that the lard (40.08 ± 4.2 mm^2) and SO (56.63 ± 5.42 mm^2) groups. Messenger RNA (mRNA) expression of HER2/neu, epidermal growth factor receptor (EGFR), H-ras, Bcl-2, cyclooxygenase-2 (COX-2), vascular epidermal growth factor (VEGF), and fatty acid synthase (FAS) in tumors were analyzed using quantitative real time-polymerase chain reaction (qRT-PCR). We found that dietary LLSO supplementation significantly increased tumor expression of oncogenes HER2/neu, EGFR, FAS, and H-ras ($p < 0.05$, Tukey's test), but not in the SO or lard supplemented groups. Tumor morphology was evaluated using histology. Tumors from the LLSO group showed more advanced lesions (grade 2) with areas of four or more layers of epithelial cells and irregularly shaped lumens ($p < 0.05$, Chi Square test). These data suggest that dietary intake of LLSO may accelerate mammary tumor progression at a faster rate than conventional SO or lard.

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The following paper reviews the rationale for conducting further research into the link between low linolenic soybean oil (LLSO) and the development of malignant breast cancer and the historical context for this investigation. Next, the paper discusses the differences between LLSO and previous dietary fatty acids and why this issue is of increasing dietary relevance. A number of possible mechanisms are reviewed to understand LLSO's possible participation in mammary tumorigenesis. Finally, preliminary studies that indicate that LLSO may stimulate the growth of estrogen receptor-postive (ER+) and estrogen receptor-negative (ER-) human breast cancer (BC) cells *in vitro* and human metastatic breast tumors in athymic mice are presented.

1 Chapter1 Introduction

According to the American Cancer Society, BC is the second leading cause of cancer death in women and current trends suggest that BC prevalence is increasing in men [1, 2]. Predictions indicate 179,920 new cases and 40,730 deaths from BC in 2008 [1, 2]. In 1997, the World Cancer Research Fund/American Institute for Cancer Research estimated that between 30-40% of cancer incidence worldwide was the result of unhealthy lifestyle choices [3]. Modification of lifestyle (including diet) could prevent, delay and treat the development and progression of BC. A number of studies have reported on the effects of certain nutrients and their role in the development of BC. Investigations of vitamin E and D [4, 5] and more recently the impact of dietary fat on BC have come under intense scrutiny. Epidemiological, anthropological, nutritional and genetic studies indicate that in the last 150 years humans have significantly altered the amount and type of fat in their diets [6]. Of particular concern is the

replacement of omega-3 (n-3) fatty acids with increased consumption of omega-6 (n-6) fatty acids due to the different metabolic pathways these fats take in the human system.

At the turn of the century, modern agriculture practices and the invention of the continuous screw press and the steam-vacuum deodorization process made possible the industrial size production of vegetable oils and oilseed [6]. Prior to the modernization of agricultural practices, humans received fat from a variety of food sources including lean meats, fish, nuts, dairy and seafood. Monoculture practices along with better technology reduced our sources of dietary fat and intake of n-3 fatty acids and increased our intake of n-6 by increasing the availability of cooking oils from vegetables and oilseeds [6]. The most common of today's vegetable oils used commercially and in households in the U.S. is soybean oil [7].

To increase shelf life and give greater heat stability many of these liquid fats went through the process of hydrogenation to change the liquids oils into more solid plastic fats. Hydrogenated oils are produced at high temperatures with metal catalysts and pressurized hydrogen [8]. In the process of hydrogenation, double bonds between the carbon atoms of the fatty acid are broken and hydrogen is added. It has now become common knowledge that the process of partial hydrogenation, which adds carbon atoms to only some of the double bonds within unsaturated fats acts to weaken the double bonds and causes a large percentage of the natural *cis* double bonds to change to *trans* double bonds [9]. Consumption of these dietary *trans*-fatty acids, however, results in raised levels of low-density lipoproteins (LDL) and total plasma triglycerides (TG), and reduced high-density lipoproteins (HDL), thus increasing the risk of coronary heart diseases [10].

On January 1st of 2006 the United States Food and Drug Administration (FDA) mandated that all food products containing *trans* fats greater than 0.5 grams per serving must be properly

identified on the nutrition facts label [11]. Consequently, this led to a new demand for *trans* fat-free fat sources. Developing out of this demand was a new breed of soybeans that delivered a lower alpha-linolenic acid (ALA) composition, 1-3%, than conventional SO, 7-8% [12]. ALA is an n-3 fatty acid that has three double bonds and, therefore, more labile as a fatty acid than linoleic acid (LA) with just two double bonds. As a result of the reduced ALA composition, LLSO does not require hydrogenation, thereby, reducing the formation of *trans* fats [12]. In addition to the low *trans* fat content, this genetic modification allows for greater heat stability and longer shelf life.

With the belief that LLSO-formulated food would result in decreased consumption of *trans* fats, thereby lowering the risk of cardiovascular disease (CVD), it is likely that consumer consumption of LLSO products will increase. However, the efficacy and safety of LLSO on other human chronic diseases including BC have not been investigated. This review presents some possible mechanisms LLSO may act on cancer progression and to present some preliminary studies that indicate that LLSO stimulates the growth of ER(+) and ER(-) human BC cells *in vitro* and human metastatic breast tumors in athymic mice.

Additionally, while the exact mechanism of fatty acid metabolism and its relationship to cancer remains uncertain, many research findings demonstrate a strong relationship between cancer and the types of fatty acids consumed. Epidemiological studies report increased BC rates when diets of isolated peoples (Alaskan Inuits and Japanese) become infused with LA-rich vegetable oils [13-15]. And xenograft studies using human breast cancer cells in athymic nude mice showed that mammary tumors were stimulated by diets enriched in n-6 polyunsaturated fatty acids (PUFAs), and suppressed by diets containing n-3 PUFAs. High n-3 PUFAs intake has also been reported to inhibit the growth of spontaneous or carcinogen-induced mammary

tumors in mice and rats [16-18]. And *in vitro* studies using mammary cancer cell lines have also shown that n-3 PUFAs [ALA, eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA)] have inhibitory effects and n-6 PUFA (LA) have stimulatory effects on these cell lines[19, 20]. Many of these researchers in their work have suggested possibilities in which fatty acids may be exerting their effect and some even mechanistic studies. However, the complexity that underlie fatty acid metabolism and it relationship to cancer requires much needed and meticulous research before any definitive role (s) can be assigned. Below highlights just a few.

1.1 Justification for Research

Increasingly, the public and private sectors have voiced an interest and need for food products with reduced or no *trans* fats. This *trans* fat-free trend was spurred on by the new food label requirement by U.S. FDA on *trans* fat that began January 1st 2006 [11]. LLSO meets these needs of the public and food manufacturers, and already LLSO is widely used in food products (Kellogg's Pop-Tarts and Cheez-it Crackers and Kentucky Fried Chicken products). It is likely that people consume more LLSO-formulated food products believing that low *trans* fat intake will lower their risks of CVD and other chronic illnesses. However, the efficacy and safety of LLSO on other human chronic disease including BC have not been investigated. In our preliminary studies, we demonstrated that LLSO stimulates the growth of breast cancer cells *in vitro* and in mice. These results are disconcerting given the increase in consumption and demand for LLSO and products that contain the fatty acid. On August 2006, DuPont and Bunge announced the expansion of their soy collaboration: "Acres planted with Pioneer® brand low linolenic soybeans grew from about 35,000 in 2005 to nearly 200,000 in 2006, with plans to significantly increase acres in 2007 to meet growing customer demand. From a targeted base in Iowa in 2005, the geography has expanded each year and is projected to reach into eight states for the 2007 growing season — including Iowa, Illinois, Indiana, Ohio, Michigan, Missouri,

Pennsylvania and Wisconsin" [21]. This increasing demand and consumption of LLSO necessitates further evaluation of LLSO and its impact on human health. The specific objectives presented here will enable us to determine whether the effects of LLSO at concentrations relevant to human fat intake level can accelerate the progression of breast cancer *in vitro* and in animal. This proposed research has very important health implications and will improve our knowledge of the dietary components that influence BC.

1.2 Hypothesis and Specific Aim

Hypothesis

We hypothesize that LLSO accelerates the progression of pre-malignant breast cells to cancerous cells both *in vitro* and *in vivo*.

Specific Aim

Objective 1: To determine if LLSO intake accelerates the progression from pre-malignant human breast cells (MCF-10AT1) to malignant cells in athymic mice.

Objective 2: To evaluate the effect of LLSO on the growth of MCF-10AT1 cells *in vitro*.

2 Literature Review

2.1 Breast Cancer

BC is characterized by progressively unregulated cellular proliferation, the loss of certain epithelial characteristics, genomic instability and loss of normal organization and compartmentalization [22]. Hormonal factors drive the development of normal breast, carcinogenesis and progression of BC. Hormonal factors such as endocrine steroids, peptides and other molecules are produced by secretory cells of the endocrine system including ovaries, pituitary gland, pancreas, thyroid and adrenal cortex [23]. These hormone factors go on to regulate cellular function after their interaction with nuclear or cell surface receptors. Central to understanding how actions take place in mammary tissue are the systemic effects that endocrine hormones have on local production of growth factors in mammary glands. Interaction between these factors (endocrine hormones and growth factors) induce gene products that can regulate

glandular function and sometimes bring about dysfunction resulting in cancer [23]. Polypeptide growth factors continue to be the most studied hormonal modulators, however, fatty acids, prostaglandins and phospholipid degradation products may also behave as growth regulators and modulators. Recent data also indicate that endothelial and immune inflammatory cells are important as regulatory entities [23]. In the context of overall disease progression, however, there are many growth factor pathways and molecular classes that are involved in the disease progression. It is likely that there is no clear pathway that leads to the progression, but a myriad of factors.

Since BC growth is commonly controlled hormonally, measurements of the level of ER in the tumor are currently used to partly classify tumors as either ER(+) or ER(–) which help to assess which patients may benefit from anti-hormonal therapy [23]. Roughly 60% of human BCs are ER positive and two-thirds of these cases will respond to endocrine therapy such as Tamoxifen, which competitively bind to ERs preventing estrogen effects, and aromatase inhibitors, which prevent estrogen production [23]. In both cases of endocrine therapy the long-term use of selective estrogen receptor modulators can result in endocrine therapy resistance and transitioning of ER(+) tumors to ER(-) [24]. Unfortunately, ER(-) are more aggressive and unresponsive to anti-estrogens. Common treatment for ER(-) BC comes in the form of chemotherapy, however, administration of Tamoxifen has also proven beneficial for patients with ER(-) tumors [24].

2.2 Epidermal Growth Factor Receptor (EGFR) and Mitogen-Activated Protein Kinase (MAPK)

EGF is a protein involved in the stimulation of the growth of cells through a cascade of signal transduction. The EGFR pathway, while not completely understood, consists of the EGF protein binding to trans-membrane EGFR-tyrosine kinase, which causes the ligand bound EGFR

to dimerize and activate a series of cascading events that include Ras GTP, Raf-1, MEK, and MAPK [23]. MAPK is a key signal transducing protein involved in both cell proliferation and apoptosis [25]. MAPK is suggested to be the link between the signal transduction of EGFR to transcriptional activation in the nucleus [26, 27]. In human mammary cancers, EGFR and MAPK expression is typically up-regulated and highly active when compared to surrounding normal tissue [28] and much of the research on MAPK supports the concept that MAPK is essential for the stimulated growth of breast cancer cells [25]. Upstream of MAPK, however, is *Ras* an effector protein of MAPK that serves to regulate cell growth, differentiation and even upregulate bcl-2, a suppressor of apoptosis [29]. However, in order for *Ras* to be biologically active it must be localized to the plasma membrane which can then drive tumor development. In a study using colonocytes, treatment with DHA (n-3) when compared to LA (n-6) reduced *Ras* localization to the plasma membrane and suppressed levels of GTP-bound (activated) *Ras* at the plasma membrane ($p < 0.05$) [30]. The same study found that when cells were treated with DHA these exogenous PUFAs could be incorporated into the phospholipid membranes and reduce the presence of arachidonic acid (AA). This served to reduce the levels of GTP-bound (active) *Ras* in colonocytes by decreasing AA's ability to inhibit GTPase activating protein (GAP) activity. GAP serves to stimulate GTPase activity, thereby, terminating a signaling event. Conceivably, diets favoring n-6 PUFA consumption may alter the phospholipid membrane enough that *Ras* is able to localize to the plasma membrane, thereby increasing the potential for tumor development. Furthermore, higher levels of AA within the plasma membrane may allow for a longer signal transduction of EGFR to the nucleus by inhibiting GAP activity [30], ultimately resulting in an increased growth stimulus.

2.3 Angiogensis and Vascular Endothelial Growth Factor (VEGF)

Angiogenesis, the process involving formation of new blood vessels from pre-existing blood vessels, is needed to permit sustained tumor growth [31]. As tumor cells amass and expand, they become deprived on nutrients and oxygen (hypoxic) as they increase their distance form the nearest blood vessel. Under these hypoxic conditions cancer cells will begin to produce VEGF and other growth factors. Consequently, this induces the activation of the receptors of the endothelial cell growth factors and increased matrix metalloproteinase production. Ultimately this allows for vascular permeation and migration of endothelial cells for the formation of a new capillary network [32]. VEGF is believed to have the most critical mitogenic effect on endothelial cells and angiogenesis [33] and critical for *in situ* BC to become invasive [34]. To date, no research exists that has observed the effects of different n-6/n-3 ratios on VEGF regulation. However, several studies show the dietary fat conjugated linoleic acid to have a reducing effect on tumor VEGF mRNA expression and expression in blood plasma [35, 36]. These data support suggest that dietary factors and, specifically, dietary fatty acids may influence the process of angiogenesis. While there is conflicting data on whether VEGF is a suitable biological prognostic factor in BC [33], high tumor VEGF levels appear to correlate with poor prognosis, decreased overall survival and advanced stages of BC [37-40]. VEGF still remains a major target for chemotherapy [41].

2.4 Eicosanoid Production

LA (18:2 n-6) and ALA (18:3 n-3) PUFAs are essential fatty acids that must be obtained from the diet [42]. LA is the most common n-6 in the Western diet and is largely supplied from vegetable oils [43]. ALA is the most common n-3 fatty acid in the diet and its sources are varied, but less ubiquitous within the typical American diet [6, 43, 44]. Cold water oily fish such as wild salmon, mackerel, herring and sardines, but also plant sources such as purslane, flax and

ligonberry are good sources of n-3s [6]. In the body, LA is converted to AA (20:4 n-6) and ALA is converted to EPA (20:5 n-3) and, subsequently to DHA (22:6 n-3). These subsequent PUFAs are synthesized by a variety of desaturase enzymes of which n-3 and n-6 PUFAs compete for [45]. AA and EPA both serve as precursors for eicosanoids [46], however, derived eicosanoids from each fatty acid group (n-3 and n-6) have different biological roles in the body. Eicosanoids are signaling molecules that play an important role in inflammation and the development of human neoplasia [46-48]. Metabolism of AA by the cyclooxygenase (COX) enzymes (COX-1 and COX-2) and 5-lipoxygenase (5-LOX) gives rise to the 2-series prostaglandins (PGE_2) and thromboxanes and the 4-series leukotrienes, respectively. Metabolism, however, of EPA by COX and LOX results in the 3-series prostaglandins and thromboxanes and the 5-series leukotrienes [49, 50]. These EPA-derived eicosanoids have an opposing effect compared to those from AA [45, 46] and are often associated with down regulating inflammation.

Eicosanoids from AA are considered more biologically active than those from EPA [51] and in patients with burns, trauma and various inflammatory disorders these eicosanoids are often present in elevated levels [52]. These AA-derived eicosanoids, if formed in large amounts, can lead to allergic responses, inflammatory disorders and proliferation of cells [6] [45]. Additionally, PGE_2 levels are often elevated in human breast tumors [53] and in rat hepatocytes increased PGE_2 synthesis induced cell proliferation [54, 55]. Therefore, it stands to reason that diets deficient in n-3 fatty acids and consequently deficient in EPA-derived eicosanoids could direct towards an inflammatory response and possibly to early stages of tumorigenesis.

2.5 Fatty Acid Synthase (FAS)

FAS is responsible for *de novo* lipogenesis in mammals. In lipogenic tissues such as the liver, lactating breast and adipocytes the FAS-dependent synthetic pathway functions to store excess energy intake such as fat, synthesize fat from excess carbohydrate or protein when the

diet is low in fat, and synthesize fat for lactation [56]. Under normal physiological conditions FAS expression in human tissues is tightly regulated by environmental, hormonal and nutritional signals and constitutively occurs at low levels as long as dietary intake of fatty acids is sufficient [57]. These signals act to regulate FAS expression through modulation of the SREBP-1c, which is downstream of MAPK/ERK1/2 and phosphoinositide 2-kinase (PI-3'K)/AKT signaling pathways. Immunohistochemical studies reported, however, that FAS protein (OA-519) is overexpressed in the majority of human malignancies including BC. Furthermore, cancer associated FAS appear insensitive to nutritional signals [58]. Menendez et al. have theorized that in response to oncogenic changes such as overexpression of growth factors (e.g. EGF, heregulin) and/or overexpression of growth factor receptors (e.g. Her2/*neu*, EGFR), SREBP-1c may be activated by MAPK/ERK1/2 and PI-3'K/AKT signaling pathways to subvert FAS' ability to respond to dietary fatty acids that serve to downregulate FAS [58, 59] .

In response to reports that FAS-dependent neoplastic lipogenesis is unresponsive to exogenous dietary fats, Menendez et al. [57] assessed the role of n-3 and n-6 PUFAs on FAS in the SK-Br3 human breast cancer cells. SK-Br3 overexpress FAS and the FAS enzyme constitutes up to 28% by weight of the cytosolic proteins [57]. ALA dramatically reduced FAS activity by up to 61%. However, AA and LA, suppressors of FAS expression in adipocytes and hepatocytes in FAS-dependent lipogenesis, failed to inhibit FAS in SK-Br3 BC cells. The data indicate that n-3 PUFA-induced repression of tumor-associated FAS may in part be due to a peroxidative mechanism that is cytotoxic to tumor cells. To determine if the inhibitory effects was due to lipid peroxidation, Menendez et al. supplied both n-3 PUFAs and vitamin E, an inhibitor of lipid peroxidation. As a result, ALA lost its capacity to inhibit FAS activity in the presence of vitamin E [57]. This suggests that decreased levels of n-3, such as that in LSSO, diminishes plasma

membrane uptake of n-3 that could potentially have a protective effect on the development of malignant cells.

2.6 Preliminary studies related to project

2.6.1 LLSO stimulates the growth of ER(+) and ER(-) human BC cells in vitro

LLSO (Asoyia LLC, Winfield, IA), and SO (Welch, Holme & Clark Co., Inc., Newark, NJ) (0.01-5 μl/100 μl media) were tested for their effects on the growth of human ER(+) BC (MCF-7) cells and human ER(-) metastatic BC (MDA-MB-231) cells using WST (water soluble tetrazolium)-1 assay (Roche Diagnostics). Cells (1×10^4 cells/100 μl) were inoculated in 96-well plate. Twenty-four hours after the inoculation, we mixed LLSO or SO in fresh cell culture media, briefly sonicated the mix on ice, then treated the cells for 48 hrs. LLSO treatment increased the growth of MDA-MB-231 cells by 2.5fold at 0.1 μl, 5.4fold at 1 μl, 8.1fold at 2 μl, 11.7fold at 5 μl/100 μl media, respectively ($p < 0.05$) (Figure 1A, gray bars). LLSO increased MCF-7 cell growth by 2.0fold at 1 μl, 2.3fold at 2 μl, 3.1fold at 5 μl/100 μl media, respectively ($p < 0.05$) (Figure 1B, gray bars). SO (≥ 2 μl/100 μl media) increased MDA-MB-231 cell growth by 2.3× and 2.8×, respectively ($p < 0.05$) (Figure 1A, white bars). SO at the selected concentration range, did not stimulate the growth of MCF-7 cells (Figure 1B, white bars).

2.6.2 Dietary LLSO stimulates the growth of human metastatic breast tumors in athymic mice

Effects of dietary LLSO, SO, and lard on the growth of MDA-MB-231 tumors were evaluated. We injected MDA-MB-231 cells (1×10^5 cells/40 μl Matrigel/spot, 4 spots/mouse) into 30 female athymic mice (5-wk old). Mice were divided into three groups; LLSO, SO, and lard (20% of total energy intake; 10 mice/group). American Institute of Nutrition 93G diet was used as a base diet. Dietary treatment directly proceeded cell injection, and lasted for 10 weeks. Tumor size and body weight were measured weekly. After termination of the study, all tumors

were collected and tumor wet weight was measured. Blood samples were analyzed for serum total cholesterol levels.

We observed that dietary LLSO and SO stimulated MDA-MB-231 tumor growth compared to the lard group in athymic mice (Figure 2A). After 10 weeks of dietary treatment, the average tumor sizes were 44.8 ± 6.5 mm^2, 42.6 ± 5.3 mm^2, and 28.9 ± 4.1 mm^2, for LLSO, SO, and lard groups, respectively. Total tumor wet weights were 22.69 g (22 tumors/32 injected spots), 13.14 g (28 tumors/32 injected spots), and 5.34 g (28 tumors/32 injected spots), for LLSO, SO, and lard, respectively (Figure 2B). We also measured total serum cholesterol levels using a commercial kit (Stanbio Laboratory, Boerne, TX) as described in the company protocol. We observed that total serum cholesterol level was lowered by LLSO compared to the lard group (Figure 2C). Total serum cholesterol levels were 103.1 ± 3.3 mg/dL, 85.6 ± 6.3 mg/dL, 84.6 ± 4.8 mg/dL for the lard, SO, and LLSO groups, respectively.

We observed the possible negative effect of LLSO on ER(+) and ER(-) BC cell growth. We also demonstrated that dietary LLSO intake (20% of total calorie intake) stimulated human ER(-) metastatic breast tumor growth in athymic mice. LLSO did lower the serum total cholesterol level as expected, however, its potential detrimental effect on BC is alarming. Possible mechanism(s) by which LLSO can alter breast tumor growth is not known at this point.

2.7 Conclusion

The role of dietary fats and their affects on breast cancer has been a subject of intense debate. However, epidemiological studies do support that PUFA ratios of n-6 to n-3 of 1-4:1 have a protective effect against the growth of BC [14, 15]. The ratio of n-6 to n-3 in Western diets is approximately 15:1, whereas ratios of 2:1 to 4:1 have been associated with reduced mortality from CVD, and decreased risk of BC [6, 60, 61]. Overall the greatest implication when reviewing fatty acid metabolism and its role in cellular proliferation, whether via FAS,

eicosanoid production or EGFR and MAPK, is that not only the quantity but, also the type of fat is an important modulator of BC risk. The ratio of LA to ALA of LLSO is higher than that of SO (by ~7-fold). Therefore, it is conceivable that decreased concentration of ALA in LLSO may alter any of the above or other mechanisms to induce the growth of mammary cancer cells.

The complete role of dietary fatty acids and their influence on BC remains unclear. A systematic study, however, of LLSO and its effects on BC is necessary to ensure minimization or elimination of dietary factors that potentially promote BC. To date no current literature exists on LLSO consumption and its impact on BC. The research proposed here will be used to 1) determine if consumption of LLSO will accelerate the progression of pre-malignant breast cells to cancerous cells and 2) attempt to elucidate mechanisms involved in fatty acid metabolism and the development of BC.

3 Materials and Methods for *In vivo* Study

3.1 Athymic Mouse Xenograft Model

This study utilized an athymic mice xenograft model. The athymic mouse lacks the thymus gland and does not reject human BC cells. The athymic mouse xenograft model has been used extensively for pre-clinical investigations regarding breast cancer studies for many decades and remains a useful tool for viewing human cell interaction with endogenous factors [62]. Athymic mice have similar digestion, absorption, metabolism, and tissue distribution to humans, and urinary excretion of exogenous compounds as do humans [62].

3.2 MCF-10AT1 pre-malignant human breast cells.

The MCF-10AT cell line was developed by investigators at the Karmanos Cancer Institute from the MCF-10A line [63-65]. The MCF-10A line was developed from breast tissue originally taken from a 36-year old woman showing very mild, hyperplastic changes and extensive fibrocystic disease, but no evidence of malignancy [66]. The immortal MCF-10A cells

transfected with T-24 *ras* produced what became known as MCF10AneoT which were thought to be tumorigenic, however, only occasionally produce invasive carcinomas [63, 67]. While point mutations in H-*ras* is not the usual mechanism by which breast cancer is initiated, 50% of human breast cancers express elevated levels of *ras*-related proteins [68]. Cells from a 100 day-old MCF10AneoT lesion produced what became known as the MCF-10AT1 cell line [67]

Breast cancer is a heterogeneous disease and manifold in its disease progression. Lesions produced from the MCF-10AT1 xenograft model show a sequence of progressive histologic changes that include mild hyperplasia, moderate hyperplasia, atypical ductal hyperplasia, carcinoma *in situ*, moderately differentiated and undifferentiated carcinoma and histologically normal ducts [69]. These variations are consistent with observations in the breasts of women at high risk for breast cancer [69]. The MCF-10AT1 xenograft model also possesses the valuable quality of easy manipulability by hormonal agents and lacks a single commitment to a cancer pathway. These attributes combined render the MCF-10AT1 xenograft model the only currently available human model that exhibits histological markings that mimic those of women who are considered high risk for breast cancer and undergo preneoplastic and neoplastic progression *in vivo* [69].

3.3 Animals and Tumor Growth Model.

Female athymic nude mice (4-week old) were purchased from Harlan (Indianapolis, IN). Mice were ovariectomized at 21 days of age by the vendor and allowed a week to recover prior to delivery. An estradiol and cholesterol implant at 1:53 ratio was placed under the skin at the interscapular region of each mouse 1 week prior to the injection of MCF-10AT1 cells. MCF-10AT1 cells (1×10^5 cells/40µl Matrigel/site) were injected into four flanks on the back of each animal. Directly after cell injection the mice were randomized into three diet groups and subject to dietary treatments: 20% LLSO, 20% SO, and 20% lard (Table:1). Twelve to eleven mice were

used for each group in which 25% of the inserted cells would presumably develop carcinomas anywhere from 50 days to 2 years after injection [65]. Tumor area and body weight were measured weekly using a calipers and scale, respectively. Food intake was measured monthly over several 24 hour periods. At the termination of the study, tumors and plasma samples were collected for analysis.

3.4 Animal diets.

American Institute of Nutrition 93 growth semi-purified diet (AIN93G) was used as a base diet that contained 20% lard. LLSO from American Natural Soy Processors (Cherokee, IA) and SO were substituted into the AIN93G diet at 20% of total fat for each substitution. All diets remained isocaloric and water was provided ad libitum. AIN93G has been established as meeting the nutritional requirement of mice [70] .

3.5 E_2 implant preparation.

E_2 implants were placed subcutaneously in the interscapular region of the mice after a one week acclimation period. Each E_2 implant contained a ratio of 1:53 E_2 and cholesterol (total 3 mg in a silastic tube- 0.1 cm inner diameter × 0.06 cm wall, 1.5 cm length), to produce a plasma concentration of 30 pM in the animals [71].

3.6 Tumor implantation.

MCF-10AT1 cells were maintained in Dulbecco minimal essential medium (DMEM/F-12) (Mediatech, Inc, Manassas, VA) containing 10% horse serum, 1% penicillin (100 U/ml), streptomycin (100 U/ml), sodium bicarbonate (30 g/L), epidermal growth factor (EGF) (10 μg/ml), cholera toxin (100 μg/ml) at 37°C in a humidified atmosphere of 5% CO_2. Cells were harvested using trypsin-EDTA, and injected at 1×10^5 cells in 40 μl Matrigel® (Becton Dickinson, Alto, CA) per site into four posterior spots on the back of the athymic mice.

3.7 Tumor measurement.

Tumors were measured weekly, and the tumor cross-sectional area was determined using the formula [length (mm)/2 × width (mm)/2 × π] [72]. The study was terminated when the average tumor size of the LLSO group reached an average cross-sectional area of ~ 33 mm^2.

3.8 Tissue/tumor collection.

At the conclusion of the study, mice were killed by cervical dislocation and tumors and tissues including liver, mammary gland and uterus were collected and weighed. Some tumors (~30 mg) were stored in 1 ml of RNALater (Qiagen, Valencia, CA) at -20°C for qRT-PCR, and some tumors from each mouse were immediately frozen by submersion in liquid nitrogen for immunohistochemical analysis.

3.9 qRT-PCR analysis of gene expression in tumors.

We evaluated genes of interest in MCF-10AT1 cells by qRT-PCR . Major changes in estrogen receptor α (ER), oncogene (Ha-*Ras*), metastasis and invasiveness (growth factors and growth factor receptors, VEGF, EGFR and HER2/neu), inflammation (Cox-2) and apoptosis (Bcl-2) were evaluated. Total RNAs were prepared from cell pellet (≤ 30 mg) using Qiagen reagents (Valencia, CA). Complimentary DNA (cDNA) was generated using 10 ng of RNA and iScript Reverse Transcription Reagents (Bio-Rad, Hercules, CA). The PCR primer pairs were designed using Beacon Designer 4 (Premier Biosoft International). The PCR primer sequences and annealing temperatures are outlines in Table 2. PCR and analysis of qRT-PCR products were performed using the iCycler (Bio-Rad) and SYBR-green detection system. Data was analyzed using a comparative threshold cycle (Ct) method. Samples as well as a control performed with no RNA were run as triplicates in separate tubes to permit quantification of target genes normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

3.10 Hematoxylin and eosin staining and grading of tumors.

Tumors (5 tumors/group, 3 slides/tumor) in 10% formalin were embedded in paraffin blocks, cut into 5-μm sections and placed on microscope slides. Slides were deparaffinized and then stained with hematoxylin and eosin (H&E). Slides were deparaffinized by immersing in xylene 3 times for 4 minutes each and then hydrated by immersing in a series of 100% alcohol twice, 95% alcohol, and then washed in water for 2 minutes. Slides were then immersed in hematoxylin for 3 minutes then washed for 1 minute followed by a clarifier and then washed again. Slides were then subject to blueing for 1 minute followed by a washing and then treated with 95% alcohol for 30 seconds followed by eosin phloxine for 30 seconds. Slides were then dehydrated and fixed by immersing in 95% alcohol followed by 2 washes in 100% alcohol and in xylene 3 times for 1 minute [73].

Using criteria from the Dawson et al. [74] study each 5-6 xenografts were graded according to the categories (0=simple epithelium, 1=mild hyperplasia, 2=moderate hyperplasia, 3=atypical hyperplasia, 4=carcinoma in situ, 5=invasive carcinoma) (See Appendix A). Tumors were graded according to the most advance category present within the sectioned tissue. Categories are intended to correspond to stages of human proliferative breast disease [74].

3.11 Blood lipid, lipid-metabolizing enzyme levels.

Blood was collected by cardiac puncture upon termination of the study. The plasma was separated by centrifuge at 500×g for 20 minutes at 4°C and then stored at -20°C for later analysis. Triglycerides and total cholesterol (TC) were determined using enzymatic reagents designed by Stanbio Laboratories (Boerne, TX).

4 Materials and Methods for *In vitro* Study

4.1 Cell Viability Assay

To evaluate the effects of SO, LLSO, and lard on MCF-10AT1 cell growth, a MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay [75] was used. MCF-10AT1

were grown in 1×DMEM-F12 media prior to treatment with oils. For the assays, MCF-10AT1 cells (2×10^4) were inoculated in triplicate in a 24-well polystyrene culture plate. After 24 h the MCF-10AT1 cells in each plate were treated with various concentrations of LLSO, SO, and lard (0.005-0.1%/mL) (0.05-1 μl/1 ml media). The levels of individual fatty acids added for each percent of oil added to media (0.005-0.1%/mL) are presented in Table 5. Glycerol was subtracted when we determined the molarity of each fatty acid within the oils and fat. In order to make oils available for cellular uptake oils were emulsified in dimethylsulfoxide (DMSO) at 2 parts DMSO with 1 part oil. To ensure accuracy throughout the study a batch of 1 mL of 2:1 DMSO and fat was prepared for all 3 treatments. The DMSO control treatment did not affect the growth of cells (data not shown). After 48 h, the cells were washed with 1 mL of phosphate buffered saline (150mM PBS, pH 7.4) and then treated with MTT for 5 h, followed by treatment with 10% SDS (in 0.01 N HCl) overnight. Optical density (OD) was measured at 570nm. OD values were normalized to numbers of cells based on a standard curve. Each cell viability assay was repeated 3 times.

5 Statistics

Data from the cell viability assay, tumor growth and qRT-PCR were analyzed using Statistical Analysis System (SAS) (Version 9.1, SAS Institute, Inc., Cary, NC). A one-way analysis of variance procedure was used to analyzed the data using a Tukey's Studentized Range (HSD) Test. Histology data was analyzed using a Chi-square test via JMP statistical software (SAS Institute, Inc., Cary, NC). A probability value $p < 0.05$ was considered statistically significant.

6 Animal Study Results

6.1 Animal Model

At the end of the 24-week dietary challenge, 58.33% (7/12), 75% (9/12) and 72.72% (8/11) of the LLSO, SO and lard group, respectively, survived the full length of the study. Deaths occurred for unknown reasons. Seventy-five percent (21 tumors/28 injected sites) of all injected sites formed tumors in the LLSO supplemented group while only 47% (17/36) and 56% (18/32) of injected sites grew tumors for SO and lard supplemented groups, respectively. No differences in mean body weight (Fig. 3) or food intake (Fig. 4 A and B) were observed among treatment groups.

6.2 Effect of LLSO, SO and Lard on Growth of MCF-10AT1 Tumors.

Tumors in the MCF-10AT1 LLSO, SO and lard group were monitored and measured every week for 24 weeks (Figure 5). The average tumor size (surface area) for the LLSO group was 45.11 ± 4.46 mm^2, 40.08 ± 4.2 mm^2 for lard and 56.63 ± 5.42 mm^2 for SO at the end of the study (Figure 6). There were no differences in tumor surface area between the LLSO and SO group, however, differences between lard and LLSO were significant as well as lard and SO ($p < 0.05$). At the termination of the study all tumors were weighed (Figure 7). The total tumor wet weight (mg) of tumors collected for the LLSO, lard and SO was 158 ± 36 mg, 106 ± 51 mg, and 250 ± 79 mg, respectively. No differences were observed in tumor wet weights between groups. Seventy-five percent of all injections in the LLSO group formed lesions; 18.75% greater than the lard group and 27.78% greater than the SO group.

6.3 Total Cholesterol and Triglyceride in Plasma

The levels of total cholesterol (TC) and triglyceride concentrations in fasting plasma are presented in Table 3. Total cholesterol was slightly greater in the LLSO group. No difference, however, was detected between any groups. Total triglyceride within the three groups was greatest in the lard supplemented group, however, no differences were detected among groups.

6.4 Effects of LLSO, SO and lard on mRNA expression in tumors

We examined the relative mRNA concentrations of EGFR and HER2/neu, cell surface receptors in which mutations affecting expression or activity could result in cancer. We found that dietary LLSO supplementation, but not SO increased relative EGFR and HER2/neu mRNA expression levels in tumors relative to lard by 2.60 ± 1.37 fold ($p < 0.0001$) and 3.14 ± 0.44 fold ($p = 0.007$), respectively (Figure 8E & B). Relative mRNA concentrations of VEGF, an important growth factor in angiogenesis and its over-expression may be an early step in the process of metastasis, was significantly greater in LLSO (6.68 ± 0.53; $p = 0.008$) and SO (3.36 ± 0.56; $p = 0.008$) compared to lard (Figure 8D). Relative mRNA expression levels of FAS, a possible oncogene, and H-*ras,* a protein expressed at aberrant levels in 50% of human breast carcinomas, [68] were examined in extracted tumors. Dietary LLSO supplementation, but not SO increased FAS and H-*ras* mRNA expression levels in tumors relative to lard by 2.34 ± 0.51 ($p = 0.0074$) and 2.31 ± 0.01 ($p < 0.001$), respectively (Figure 8A & C). No differences in relative mRNA expression among groups was observed in anti-apoptotic Bcl-2 and Cox-2 (data not shown). MCF10-AT1 has been previously recorded as being ER+ [65], however, we observed that ER-α relative mRNA expression was virtually non-existent.

6.5 Histology

Tumor size and number prevented us from having a larger sample for histological purposes. After RNA extraction, 6 tumors from the LLSO group and 5 from the SO and lard group remained and were stained with H & E and then classified by a certified pathologist. The histological composition of dietary supplemented LLSO, SO and lard are compared in Table 4 and Figure 9. Based on the most advanced category of lesion present on each slide the LLSO treated group had significantly ($p < 0.05$, Chi Square test) more frequent advanced lesions (grade 2) than SO and lard treated groups. Moderate hyperplasia (grade 2) with four or more epithelial

cells and irregularly shaped lumens, representing the most advanced lesion in 5/6 (83%) tumors, were in the LLSO supplemented groups. One slide showed no development at all (grade 0), characterized by simple epithelium and no nuclear enlargement and nucleoli and mitosis. Development beyond grade 1, classified as a mild hyperplasia was not observed in the SO supplemented groups. Two of the 5 lard supplemented grafts contained grade 2 lesions while the remaining 3 failed to develop beyond the mild hyperplasia (grade 1) classification. Tumors showed no advanced development beyond grade 2 classified as moderate hyperplasia.

7 In vitro Study Results

7.1 Cell Viability Assay

LLSO, SO, and lard (0.005-1 μl/1 ml media) were tested for their effects on the growth of human BC (MCF10-AT1) cells using a cell viability MTT assay. No differences among oil types and concentrations were observed among or within groups (Figure 10).

8 Discussion

The FDA has required the listing of saturated fat and dietary cholesterol on food labels since 1993. The addition of *trans* fat to the Nutrition Facts panel on January 1, 2006, now informs consumers about the saturated fat, *trans* fat, and cholesterol content of the foods they choose. The revised label, now including *trans* fat, as well as saturated fat and cholesterol, is of particular interest to people concerned about high blood cholesterol levels and heart disease. The widespread public aversion to foods containing *trans* fats combined with the FDA mandate for adding *trans* fats to the Nutrition Facts labels prompted the development of new soybean breeds that no longer require hydrogenation, the process that leads to *trans* fat formation. Using traditional plant breeding techniques, soybean breeders developed LLSO with 83.36% less n-3 fatty acids than its conventional counterpart. While the increased formulation and consumption of modified soybean oils could produce potential benefits for human health, this growing

acceptance as an alternative to *trans* fat sources should require adequate evaluation of chronic disease impacts, including BC. In this study, we tested whether dietary intake of LLSO, at a dose relevant to human consumption, accelerates the progression of tumorigenesis of MCF-10AT1 cells in athymic mice.

The effects of LLSO on the progression of BC were studied using human premalignant breast cells (MCF-10AT1) on a mouse xenograft model. LLSO- and SO-induced effects on mammary tumorigenesis were compared with lard-induced effects. The MCF-10AT1 cell line, unlike the metastatic MDA-MB-231 cell line, allows for the observation of morphological features that replicate the natural progression of human BC [69]. LLSO intake was observed as a stimulant for MCF-10AT1 tumor growth (Figure 6). Average tumor surface areas (mm^2) of the LLSO and SO groups were significantly greater than lard tumor surface areas ($p < 0.05$).

Previous studies cite EGFR, Her2/neu, H-*ras* and FAS protein levels and activity to be increased in human cancers when compared to normal surrounding tissues [76, 77]. Compared to tumors from the SO and lard-fed groups, we observed that dietary LLSO intake upregulated mRNA expression of EGFR and HER2/neu by as much as 2 to 3 fold. Our results also showed that LLSO intake increased mRNA expression of metastatic biomarkers, EGFR (Figure 8E), HER2/neu (Figure 8B), and H-*ras* (Figure 8C) and oncogene FAS (Figure 8A). These results are consistent with previous findings that EGFR and HER2/neu are often upregulated and highly active in mammary cancers [23, 28]. PGE_2, a prostaglandin derived from LA, has been implicated in the activation of EGFR which preferentially heterodimerizes HER2/neu [23, 78]. The increased concentration of LA in LLSO could potentially result in higher levels of blood plasma PGE_2, thus, increased EGFR activation. In addition, FAS expression is common in many human tumors and high levels of FAS expression correlate with HER2/neu overexpression

[59]. Upregulation of FAS may result from an initial transduction via EGFR which may jumpstart signaling pathways [59]. We observed significantly increased relative mRNA expression levels of FAS and HER2/neu in LLSO supplemented tumors ($p < 0.05$; Figures 8A and 8B). HER2/neu, a known marker of the invasive and metastatic phenotype in human BC, has been shown to upregulate FAS expression in an effort to subvert its ability to respond to dietary fatty acids that serve to downregulate FAS [58]. Tumor enhancement of FAS expression may serve to provide *de novo* lipogenesis for increasing lipid membrane synthesis and allow for tumor expansion; however, as tumors increase in size nutrient and oxygen delivery becomes difficult and can prevent tumor growth. Interestingly, VEGF (the growth factor responsible for angiogenesis) mRNA expression increased 2-fold in SO tumors and 5-fold in LLSO supplemented tumors ($p< 0.05$; Figure 8D). These finding are not surprising since LLSO and SO experienced the largest tumor growth (Figure 6). As tumors enlarge angiogenesis becomes necessary to compensate for insufficiencies in nutrient and oxygen delivery by existing blood vessels. Increased angiogenesis and the ability to induce key enzymes for fatty acid biosynthesis enable membrane lipid synthesis, representing important advantages for the progression of cancer. Furthermore, increased tumor VEGF mRNA expression has been reported when animals were fed diets containing high concentrations of LA [79].

The increase in LLSO tumor progression may also have been the result of an overactive *Ras* protein. For *Ras* to be biologically active, it must be localized to the plasma membrane [30]. Previous research showed that n-3 fatty acids disrupt *Ras*-dependent signal transduction by suppressing plasma membrane localization [30]. Decreased n-3 fatty acid content in LLSO may have allowed for better *Ras* plasma membrane localization which may account for the marked increase in H-*ras* expression in LLSO, but not in SO. These findings suggest a more advanced

LLSO-induced tumor progression than the SO and lard supplemented cells and support previous studies suggesting a link between increased n-6 levels and tumor promotion [16, 44, 80].

The increased expression of these markers reflected morphological features found in the histology examination. Histology sections (Figure 9) from the LLSO supplemented mice xenografts showed a more advanced grade (grade 2; $p < 0.05$) than the other groups. Many of the ducts in the LLSO-induced tumors had irregular lumens, four or more layers of epithelial cells and bridging by cells. None of the tumors progressed beyond grade 2, which suggests that dietary fat alone is not enough to ensure the progression of premalignant cells toward malignant tumors. The correlation between changes in tumor growth and tumor morphology suggests that the consumption of LLSO has a greater chance of accelerating the progression of BC. However, dietary habits in combination with other environmental factors (pollution) or genetic dispositions may have a cumulative effect which advances tumor progression.

Curiously, MCF-10AT1 has been previously documented as being a ER(+) cell line [65]. Our qRT-PCR results, however, show a lack of ERα expression in MCF-10AT1 cells and an inability to induce ERα mRNA expression after a 24-week dietary challenge in tumors (data not shown). Another unexpected result found that lard consumption impacted gene markers important in carcinogenesis, namely the reduction of EGFR, H-*ras*, Her2/neu, VEGF and FAS expression. Lard appears to have a protective effect on the MCF-10AT1 tumor progression in this study as well as human metastatic MDA-MB-231 tumor growth in our unpublished preliminary study. In both the preliminary and current studies the tumor surface areas of the lard group were significantly smaller than both the LLSO and SO groups ($p < 0.05$) (Figures 2A & 5). Interestingly, oleic acid (OA) was found to significantly reduce FAS activity and expression

by 55% in FAS-overexpressing human BC (SK-Br3) cells [81]. Lard's high content of OA (44-47%) may have act to slow down tumor progression.

The consumption of LLSO has been hypothesized to decrease plasma lipoproteins associated with increased risks to CVD due to the low saturated fat content. The dietary intake of LLSO, however, did not lower TC or triglycerides in direct comparison to lard in this study. In examining fasting plasma (Table 3), no difference was identified in average TC and triglycerides. In the latter part of the 20th century, lard consumption sharply declined due in part to its reputation for increasing the risk of CVD. Yet our study does not indicate an association between the consumption of lard at 20% and a rise in plasma TC or triglycerides in athymic mice, but rather the findings raise the intriguing idea that the association of lard with CVD may require a reexamination.

In addition, the differential effects of selected fats on MCF-10AT1 cell growth were tested *in vitro* using an MTT viability assay. A wide range of oil/fat concentrations was used (see Table 5 for μM concentrations of fatty acids). No difference was found among or within the LLSO, SO and lard groups at the selected concentration range (0.05-1 μl/1 ml media). None of the fats appeared to have a stimulatory or inhibitory effect on the growth of MCF-10AT1 cells. The MTT assay remains a crude instrument for assessing effects on cellular viability. It is possible that MCF-10AT1 cells were affected by fat induced metabolites that we have not examined.

In summary, the regular consumption of LLSO may exacerbate the risk of developing BC or accelerate the progression of BC according to our findings of increased BC biomarkers in relative mRNA expression and more advanced BC morphological features in tumors. At present, the possible LLSO mechanisms promoting BC progression are not clear. According to mRNA

expression data, it is possible that HER2/neu, FAS, and/or angiogenesis may be involved. Our data confirm previous research, suggesting an import link not just in the quantity of fats consumed but also in the type of fats consumed [6, 13, 14, 17-19, 82]. This scientific evaluation provides the first study of a new LLSO product and its interaction with BC progression. As enthusiasm for new fatty acid modified soybean oils increases, a proper evaluation of the biological responses of these oils is critical for assessing the safety of these products. While the data presented here identify a number of intriguing associations, further systematic studies regarding the interaction of LLSO and BC and other chronic conditions should be conducted before safe public consumption can be assumed.

Tables

Table 1: Dietary Treatment

Groups	E_2 (E_2: Cholesterol)	LLSO (g/kg diet)	Soy Oil (g/kg diet)	Lard (g/kg diet)	Cells	Mice (n)
20% LLSO	1:53	85.4			MCF-10AT1	12
20% SO	1:53		85.4		MCF-10AT1	12
20% Lard	1:53			85.4	MCF-10AT1	11

Table 2: Oligonucleotide sequences for qRT-PCR

Target Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')	Annealing Temp (°C)
H-ras	GGAAGCAGGTGGTCATTGAT	ATGGCAAACACACACAGGAA	63
VEGF-A	CGAGGGCCTGGAGTGTGT	CCGCATAATCTGCATGGTGAT	
EGFR	CCGTCGCTATCAAGGAATTAAG	GTGGAGGTGAGGCAGATG	53.3
HER2/neu	GGAAACCTGGAACTCACCTACC	TGGGACCTGCCTCACTTGG	58.5
Bcl-2	TGTGGATGACTGAGTACC	TGAGCAGAGTCTTCAGAG	50.7
COX-2	GGTCTGCCTGGTCTG	TGTCTGGAACAACTGCTCATC	56.6
FAS	GACCGCTTCCGAGATTCC	CAGGCTCACAAACGAATGG	54.9

PCR efficiencies between 90% and 105%.

Table 3: Levels of fasting plasma lipids after 24 weeks

Plasma Lipids	LLSO	Lard	SO
Total Cholesterol (mg/dL)	99.75 ± 9.26	91.07 ± 9.29	92.05 ± 7.84
Triglyceride (mg/dL)	101.36 ± 6.24	132.99 ± 21.76	83.81 ± 11.94

*x ± SEM; n=7

Table 4: Histology Data

	Grade					
Group	0	1	2	3	4	5
LLSO (6)*	1	0	5	0	0	0
SO (5)	0	5	0	0	0	0
Lard (5)	0	3	2	0	0	0

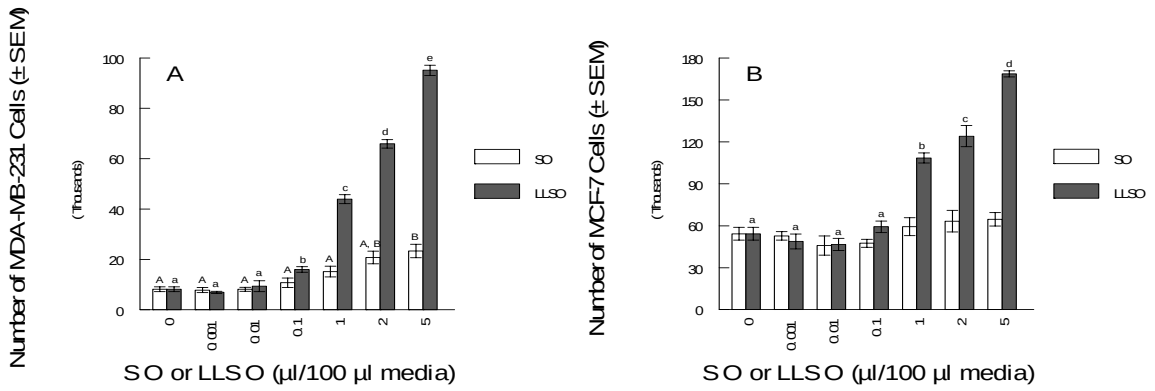
Summary of observed lesions in all three dietary challenges in athymic mice with MCF-10AT1 xenogratfs. *Indicates statistical difference, p-vlaue < 0.05. Grading method: : *Dawson et al.*[74].

Table 5: µM Fatty Acid Concentrations

SO	**0 µl/ml**	**0.05 µl/ml**	**0.1 µl/ml**	**0.5 µl/ml**	**1 µl/ml**
Palmitic	0	15	30	149	298
Stearic	0	7	13	67	133
Oleic	0	35	71	355	710
Linoleic	0	78	156	779	1559
α-Linolenic	0	10	19	95	191
Myristic acid	0	0	0	0	0
Palmitoleic	0	0	0	0	0
LLSO	**0 µl/ml**	**0.05 µl/ml**	**0.1 µl/ml**	**0.5 µl/ml**	**1 µl/ml**
Palmitic	0	15	30	152	305
Stearic	0	7	15	74	147
Oleic	0	40	80	398	795
Linoleic	0	81	161	807	1614
α-Linolenic	0	1	3	14	29
Myristic acid	0	0	0	0	0
Palmitoleic	0	0	0	0	0
Lard	**0 µl/ml**	**0.05 µl/ml**	**0.1 µl/ml**	**0.5 µl/ml**	**1 µl/ml**
Palmitic	0	39	79	393	785
Stearic	0	20	40	198	396
Oleic	0	67	134	670	1340
Linoleic	0	11	23	115	230
α-Linolenic	0	0	0	0	0
Myristic acid	0	2	5	18	35
Palmitoleic	0	5	10	47	95

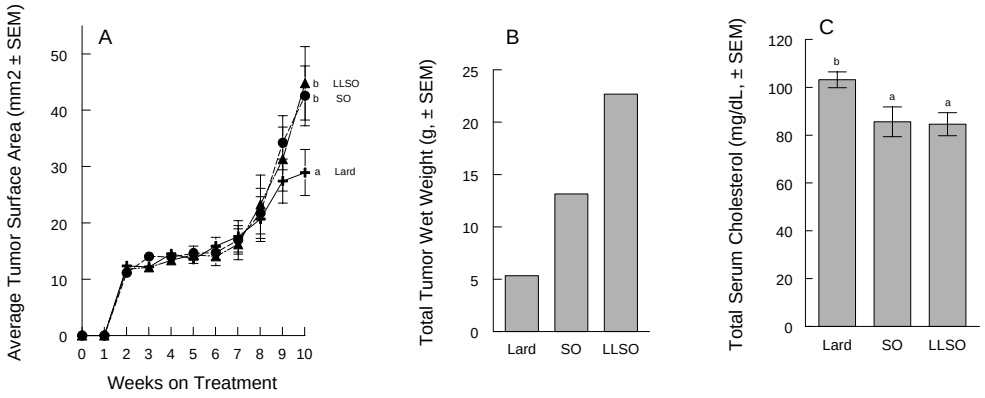
Figures

Figure 1: Effects of SO and LLSO on the growth of ER(-) and ER(+) cell in vitro



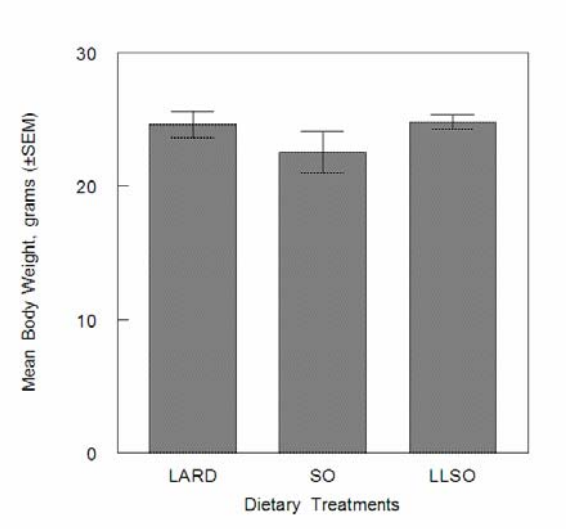
LLSO and SO (0.01-5 µl/100 µl media) were tested for their effects on the growth of ER(+) human BC (MCF-7) cells and ER(-) human metastatic BC (MDA-MB-231) cells using WST (water soluble tetrazorium)-1 assay (Roche Diagnostics). Panel A- LLSO treatment increased the growth of MDA-MB-231 cells ($p < 0.05$) Panel B-LLSO increased MCF-7 cell growth ($p < 0.05$).

Figure 2: Effects of dietary intake of lard, SO and LLSO on the growth of MDA-MB-231 cells in athymic mice



Effects of dietary intake of lard, SO, and LLSO on the growth of MDA-MB-231 in athymic mice. (2A. Tumor growth, 2B. Total tumor wet weight, 2C. Total Serum Cholesterol)

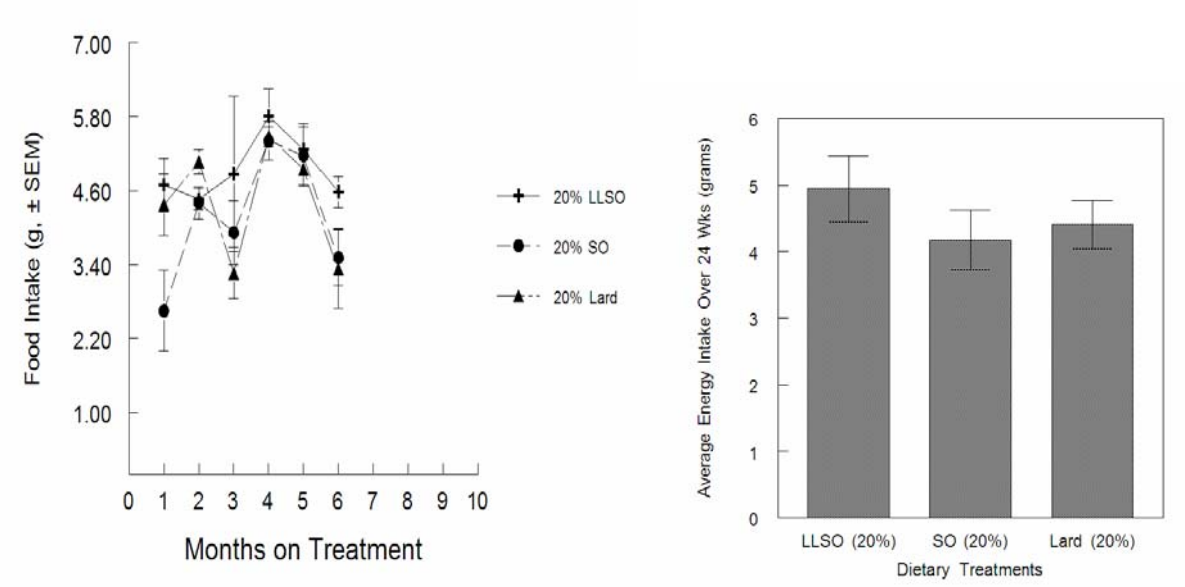
Figure 3: Mean Body Weight at Week 24



Mice in each group were weighed weekly. At the end of the 24 week dietary challenge weights were averaged. There were no statistical differences among groups.

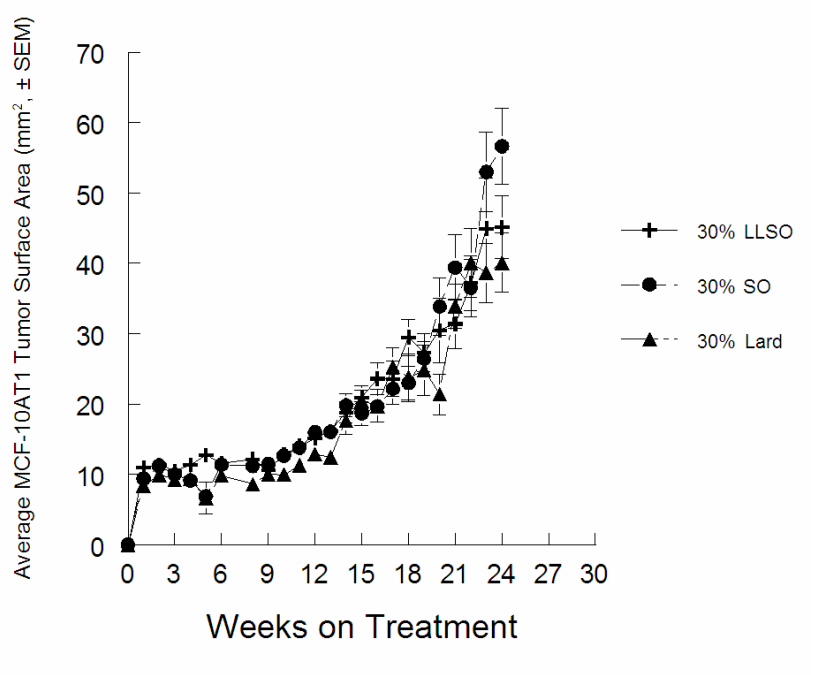
Figure 4: Energy Intake

A. **B.**



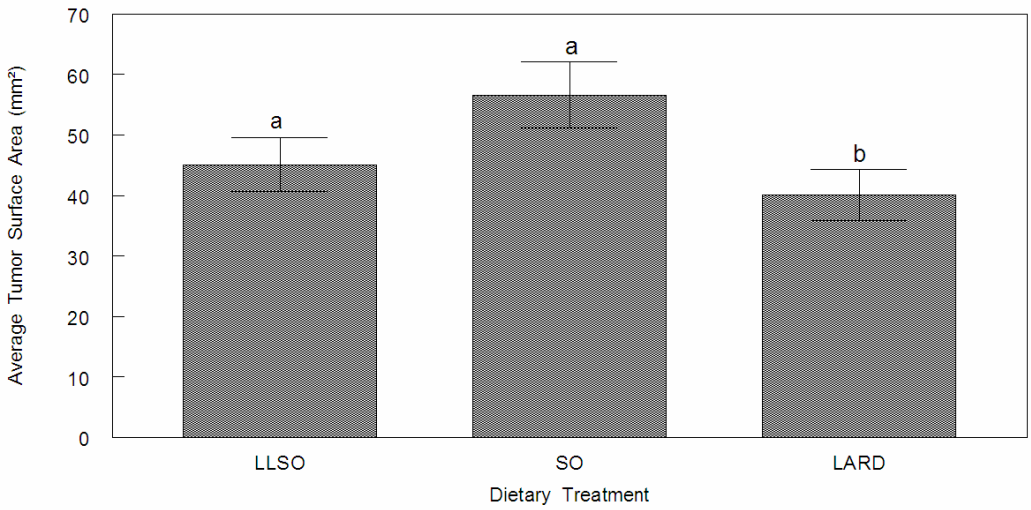
Panel A-Total energy consumption was measured on a monthly basis. **Panel B**-Average energy consumption was calculated at the end of the 24 week treatment. There were no significant differences between energy intake among groups.

Figure 5: Tumor Growth



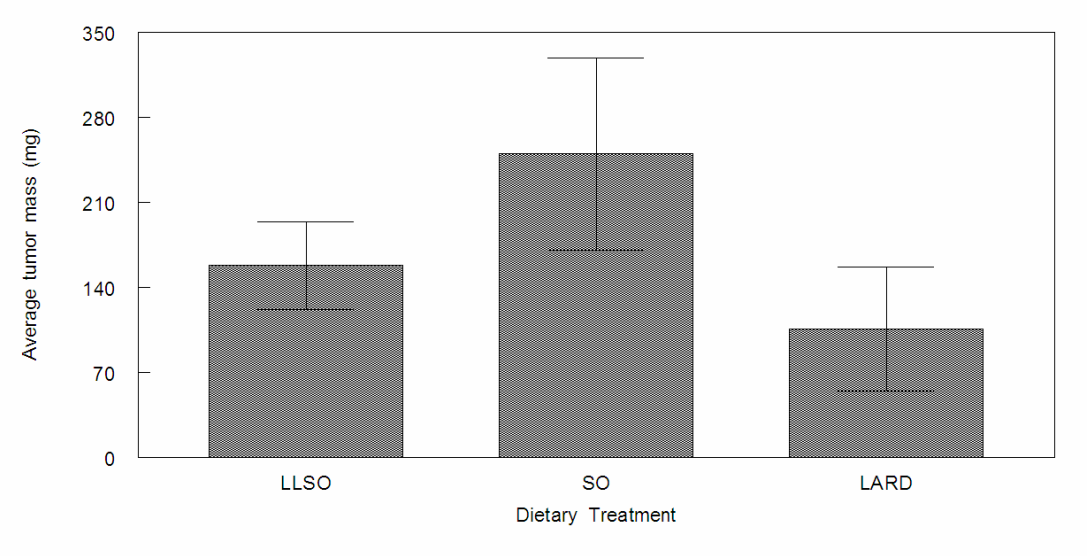
Tumors were measured weekly over a 24 week period, and the tumor cross-sectional area was determined using the formula [length (mm)/2 x width (mm)/2 x π]. Data are expressed as average cross-sectional tumor area (mm^2 ± SEM) for all tumors in each treatment.

Figure 6: Average Tumor Surface Area after 24 Wks



At the end of the 24 weeks, tumor surface area (mm^2) were measured. Data are expressed as average cross-sectional tumor area (mm^2 ± SEM) for all tumors in each treatment. Bars with different letter are significantly different, p-value < 0.05.

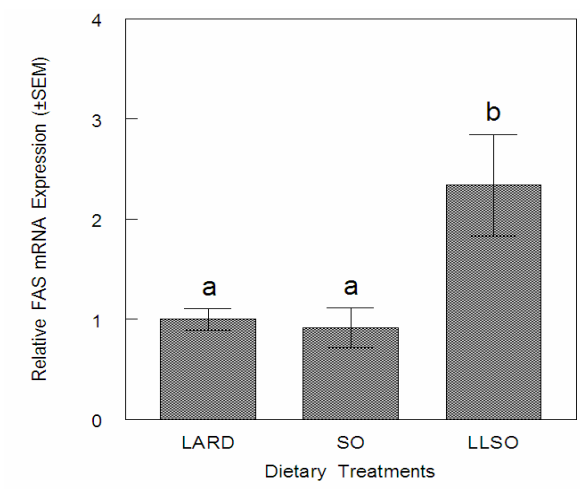
Figure 7: Total Tumor Wet Weight



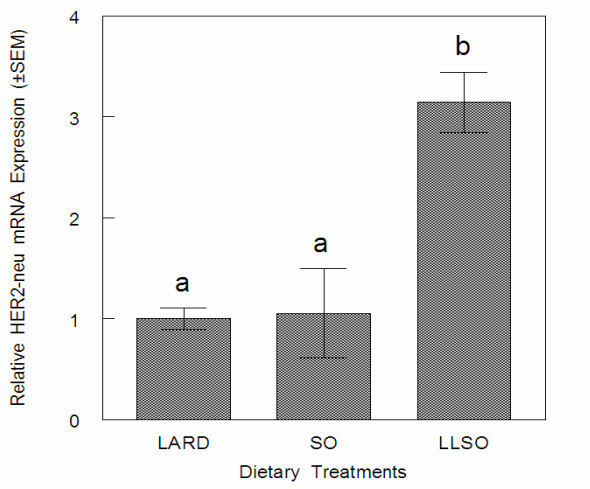
Tumors were weighed (mg) directly after removal from mice after 24 weeks

Figure 8: qRT-PCR oncogene mRNA expression

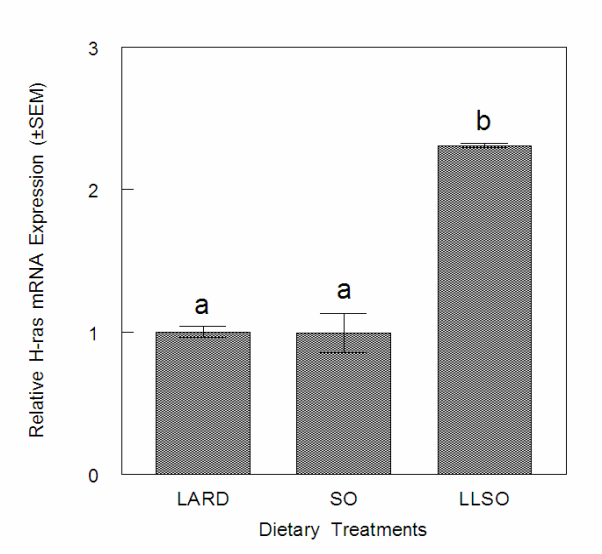
A.



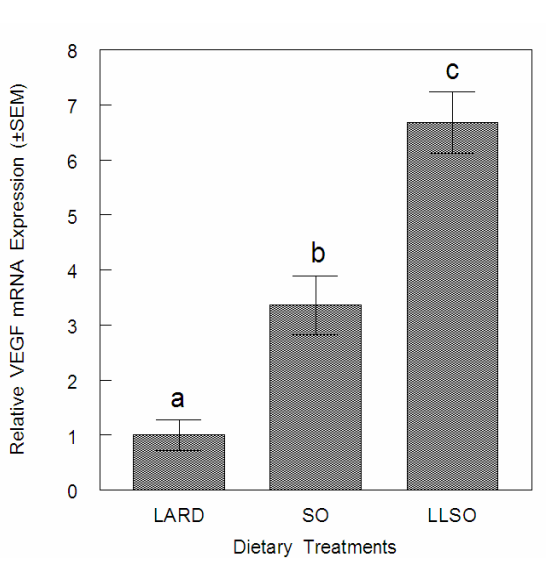
B.



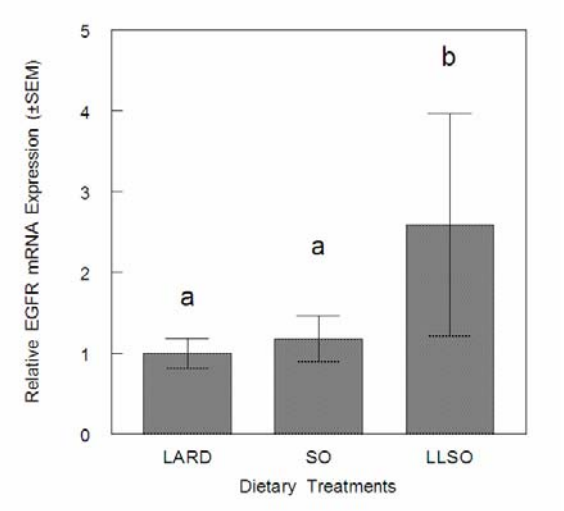
C.



D.

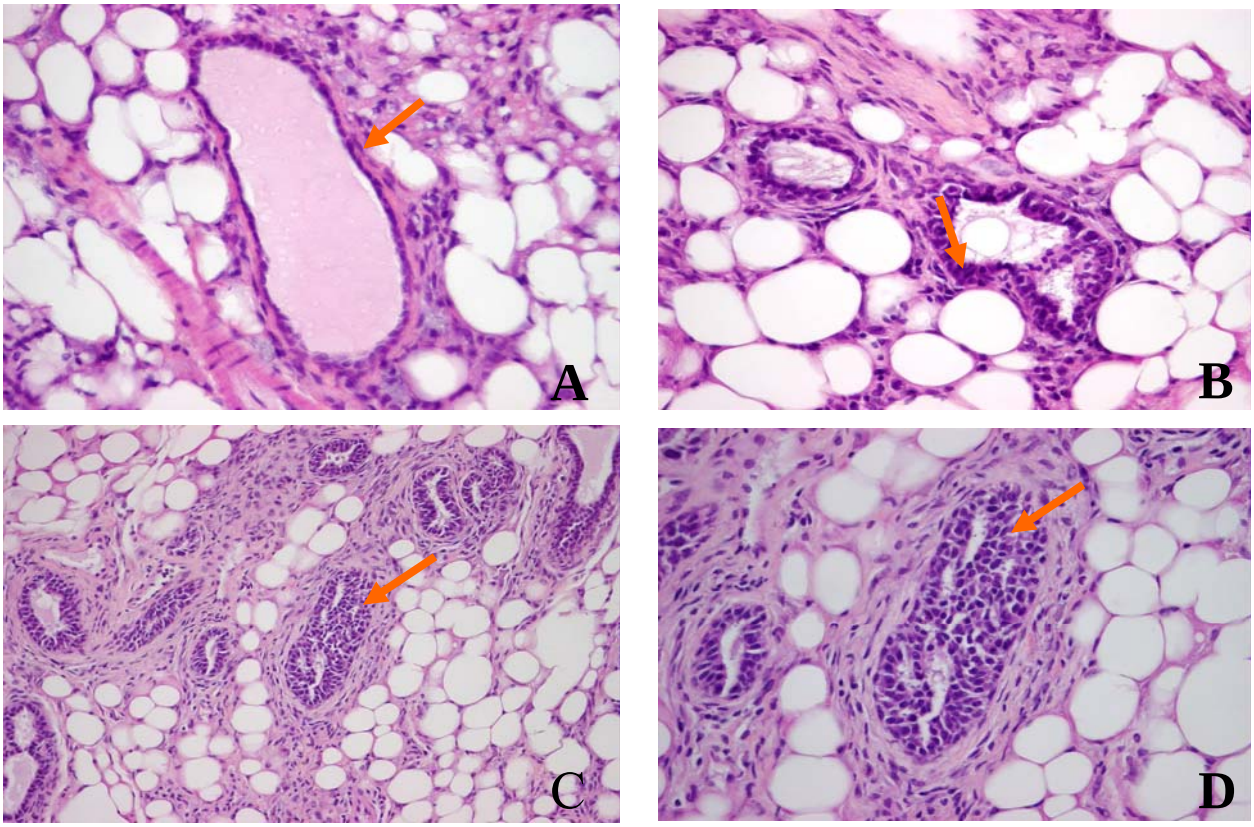


E.



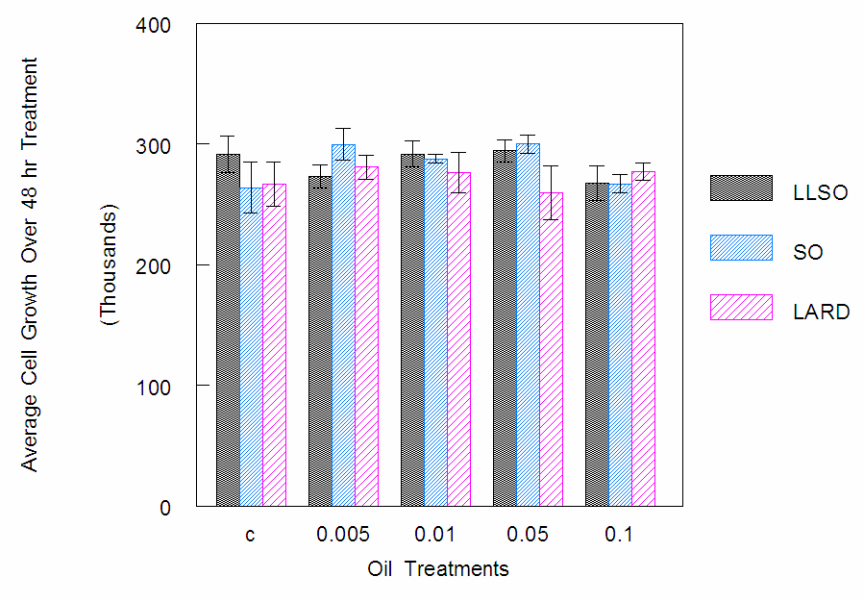
Numbers on the Y-axis represent mRNA levels; bars ± SE. Bars with different letter are significantly different, p-value < 0.05. Data points were obtained using three replicas. GAPDH was used as a standard. Eight tumors from each group were analyzed using qRT-PCR. We observed changes in oncogene (Ha-Ras, FAS) expression and metastasis and invasiveness (including growth factors and growth factor receptors, VEGF, EGFR and HER2/neu) markers to evaluate progression along the mammary cancer spectrum. cDNA was generated using 10 ng of RNA and iScript Reverse Transcription Reagents (Bio-Rad, Hercules, CA). PCR and analysis of PCR products were performed using the iCycler (Bio-Rad) and SYBR-green detection system will be used.

Figure 9: Xenograft Morphology



Panel A – (H&E staining,40 x) Photomicrograph of soy oil supplemented xenograft, grade 0/1 with simple tubules and no nuclear enlargement. Panel B – (H&E staining, 20 x) Lard supplemented xenograft, grade 1 with mild hyperplasia and two or more epithelial cells. Panel C – D (H&E staining, 20 x; H&E staining, 40 x) LLSO supplemented xenograft, grade 2 with moderate hyperplasia, irregularly shaped lumens and four or more layers of epithelial cells.

Figure 10: Cell Viability Assay



LLSO, SO and lard (0.05-1 µl/1 ml media) were tested for their effects on the growth on human BC (MCF-10AT1) cells using a MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay.

9 Appendix A: Criteria for Grading of Proliferative Breast Lesions

Grade	Classification	Description
0	Simple Epithelium	Small ducts Single layer of luminal epithelium No nuclear enlargement No mitoses
1	Mild Hyperplasia	Small ducts Two or more layers of epithelial cells No significant bridging Variable nuclear contours
2	Moderate Hyperplasia	Mild distended ducts Four or more layers of epithelial cells Irregular papillary proliferation Bridging by non-uniform cells Irregularly shaped lumens No solidly filled spaces Indistinct cell boundaries Variable nuclear contours
3	Atypical Hyperplasia	Grossly distended ducts Regular micropapillary configuration Small nucleoli, occasional mitoses Some loss polarity Marked cellular proliferation often forming luminal mass Cells become monotonous
4	Carcinoma *in situ*	Distended ducts filled with uniform cells Distinct cell boundaries Occasional central necrosis Rigid intraluminal bridges forming round spaces Prominent nucleoli, frequent mitoses
5	Invasive Carcinoma	Glandular, squamous or undifferentiated

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