

**Polyunsaturated Fatty Acids and the Epidermal Growth Factor Receptor /
Mitogen- Activated Protein Kinase Signal Transduction Cascade in Mammary
Cancer**

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(ABSTRACT)

Mammary cancer is one of the leading causes of death in both the human and companion animal population. There are many histological and pathophysiological similarities between human and feline mammary cancer, therefore investigating the molecular pathways of disease progression in one species may provide useful information for both. The EGFR/MAPK signal transduction pathway is upregulated in many human breast tumors, and both EGFR and MAPK have been implicated as independent prognostic indicators for decreased survival times in human breast cancer patients. We report here that active MAPK levels can be detected in both peripheral white blood cells (WBC) and mammary adipose tissue in cats. Adipose tissue levels of active MAPK were similar to those observed in peripheral WBC, suggesting that WBC MAPK might serve as a useful biomarker in the diagnosis or follow-up treatment of disease. PUFA have been reported to influence breast cancer risk in humans, and may modulate the EGFR/MAPK pathway through a variety of mechanisms. Dietary PUFA n-6-to-n-3 ratio in cats was reflected in mammary adipose tissue and resulted in altered active MAPK levels in both adipose tissue and peripheral white blood cells, suggesting that PUFA may have similar effects on the feline and human MAPK pathway. In human breast tumor cell line studies, rather than having opposing effects, as was hypothesized, it was demonstrated that n-6 and n-3 PUFA exerted similar effects on EGFR+ breast tumor cell proliferation and activity of the EGFR/MAPK pathway. Slightly more than twice the concentration of n-3 PUFA was needed to elicit the same response as n-6 in cells. These results indicate n-6 and n-3 PUFA can modulate proliferation of EGFR+ tumor cells similarly and may be exerting their effects, in part, through the EGFR/MAPK pathway. Decreasing total PUFA intake, while increasing the n-3-to-n-6 PUFA ratio, may be practical as preventative or adjuvant therapy for breast cancer.

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Chapter 1 : Literature Review

Introduction

Mammary gland neoplasms are common in both the human and companion animal population. According to the American Cancer Society (2004), mammary cancer is the most prevalent cancer diagnosis and the 2nd leading cause of cancer death in women, surpassed only by lung cancer. Mammary gland tumors are a common neoplasm in cats in the United States and other parts of the world (Hahn and Adams, 1997; Viste et al., 2002; Preziosi et al., 2002; Oonuma et al., 2003).

The cat is considered a good model for human mammary growth and tumorigenesis (Hayes et al., 1981; Misdorp et al., 1991; Modiano et al., 1991; Hahn et al., 1994; Vail and MacEwen, 2000). Mammary tumors in both humans and felines occur in middle-age females, occur spontaneously, are histologically similar, and develop from secretory tissues (Hayes et al., 1981; Misdorp et al., 1991; Modiano et al., 1991; Hahn et al., 1994). Determining treatment or prevention modalities for the feline population; therefore, will not only be beneficial to the pet population, but may also prove useful to the human population as well. In felines, early ovariohysterectomy (before 2 years of age) offers significant protection from development of malignancies, but even neutered cats have been diagnosed with mammary cancer (Hayes et al., 1981; Hahn and Adams, 1997; Perez Alenza et al., 2000; Martin de las Mulas et al., 2000). Most feline mammary carcinomas (50 – 90%) are estrogen-receptor-negative (ER-) and are irresponsive to the proliferative effects of estrogen (Hamilton et al., 1976; Misdorp and Weijer, 1980; Hayes et al., 1981; Martin et al., 1984; Martin de las Mulas et al., 2000). Furthermore, ~30% of primary mammary tumors in women are ER-. These ER- tumors, along with feline mammary tumors, are associated with decreased cancer-free survival times, as well as decreased overall survival times. Clearly, determining the factors that play a role in the development and progression of these ER- mammary tumors, and investigating how to modulate those pathways would be beneficial for both humans and felines.

Several nutrients have been reported to alter the growth of human mammary cancer cells *in vitro*. For example, tocotrienols, the form of Vitamin E found largely in palm oils, has been shown to inhibit the growth of human mammary cancer cells, regardless of estrogen-receptor

(ER) status (Nesaretnam et al., 1998), both alone and in combination with tamoxifen (Guthrie et al., 1997). Vitamin D and its analogues have also been reported to inhibit growth, induce apoptosis, and down-regulate ER expression in human mammary cancer cells *in vitro* (Fife et al., 1997; James et al., 1994). Vitamin B₆ was demonstrated to inhibit the growth of human mammary cancer cells *in vitro*, and appeared to be acting irrespective of the cells estrogen-receptor status (Cowing and Davis, 2000) Clearly, nutrients may be important modulators of human mammary cancer cell growth, and further study of their mechanism(s) of action may identify new targets against mammary cancer cell growth and, in turn, could have a significant impact on the treatment and/or prevention of this disease.

Another class of nutrients that may have a promising role in the prevention and/or treatment of mammary cancer are the polyunsaturated fatty acids (PUFA). This review presents the research relating PUFA to mammary cancer progression and prevention, and presents possible mechanisms by which dietary alterations of n-6 and/or n-3 PUFA for both human and companion animals might decrease the prevalence of this disease.

Polyunsaturated Fatty Acids and Mammary Cancer

Polyunsaturated Fatty Acids (PUFA)

Linoleic acid (LA) is the principal n-6 and α -linolenic acid (LNA) is the main n-3 PUFA in the diet. LA is the principal PUFA in the American diet (Jonnalagadda et al., 1995), although there are several fish sources of n-3 PUFA that are widely available in the U.S., namely the Pacific herring, the Greenland halibut, the King mackerel, and the Chinook salmon (Simopoulos, 1991). In the body, LA is converted to arachidonic acid (AA), and LNA is converted to eicosapentanoic acid (EPA) and, subsequently, to docosahexanoic acid (DHA) by a variety of elongation and desaturase isoforms. Both n-3 and n-6 PUFA compete for the desaturase enzymes; therefore, increased consumption of LNA, EPA, and DHA can decrease the production of AA by being preferentially metabolized by those enzymes (Christiansen et al., 1991).

One of the main functions of PUFA in the body is as a precursor for eicosanoids, which are mediators of inflammation and cellular growth. The PUFA are converted to prostaglandins

by the cyclooxygenases (COX) and to leukotrienes by the lipoxygenases (LOX). Arachidonic acid and EPA compete for COX and LOX in the body, resulting in the production of different eicosanoids. In general, AA-derived eicosanoids, such as the 2-series prostanoids and the 4-series leukotrienes, have strong pro-inflammatory effects, whereas the EPA-derived eicosanoids, such as the 3-series prostanoids and the 5-series leukotrienes, have lesser inflammatory effects. Because of the competition of these PUFA in the body, much research has been done to determine the importance of the n-3-to-n-6 PUFA ratio, rather than the absolute level of either class of PUFA, in cancer progression.

Epidemiological studies

Observations made over the past two decades have demonstrated that consumption of fish oil has a protective effect against mammary cancer (Kromann and Green, 1980; Kaizer et al., 1989; Caygill et al., 1996). Because fish oil contains large amounts of n-3 PUFA, whereas vegetable oils (the major fatty acid contributor in the Western diet) contain large amounts of n-6 PUFA (and less n-3 PUFA than fish oil), it has been hypothesized that n-3 and n-6 PUFA may have different effects on the growth and progression of mammary cancer. Epidemiological evidence for 20 years seemed to support that hypothesis. Female breast cancer mortality in Japanese women steadily increased for 40 years (Wynder et al., 1991). The increased breast cancer rates in the Japanese population were accompanied by an increased use of LA-rich vegetable oils and a decreased consumption of high n-3-containing fish, resulting in a decreased n-3-to-n-6 PUFA ratio (Lands et al., 1990). Similarly, Lanier and coworkers (1976) showed that the diet of Alaskan Eskimos, primarily n-3-rich fish, seemed to be a protective factor against mammary cancer in that population. In a follow up study, Lanier and coworkers (1996) showed that breast cancer rates (annual, age-adjusted per 100,000 individuals) in the Alaskan Eskimo population had increased from 0.9 to 86.5 in the 20 years since the previous study, and that, once again, the increased cancer incidence was accompanied by the westernization of this population's culture and dietary habits. It is important to note that life-style factors other than diet changed in these populations as the cultures became more Westernized and may play a role in the increased breast cancer rates. However, the evidence does suggest that diet, specifically a

high ratio of n-3 to n-6 PUFA may have had a protective effect on mammary cancer incidence in those groups.

A study involving 123 patients with non-metastatic invasive breast carcinoma and 59 women with benign breast disease indicated that low α -LNA [n-3 PUFA] in mammary adipose tissue was inversely correlated to increased mammary cancer risk in both pre- and post-menopausal women (Klein et al., 2000). Although α -LNA content appeared in this study to offer a protective effect against breast cancer, long chain n-3 PUFA and n-6 PUFA were not significantly correlated with breast cancer risk.

Although some epidemiological studies have suggested that high intakes of n-3 and low intakes of n-6 may offer protection against the development of mammary cancer, some recent prospective and cohort studies investigating the relationship of breast adipose n-3 and n-6 PUFA and breast cancer, bring that assumption into question. In a case-controlled multi-center study in Europe (Simonsen et al., 1998), “little consistent association” was observed between n-6 or n-3 adipose PUFA and breast cancer. Bagga et al., (2002) reported that total n-3 and n-6 PUFA were higher in breast cancer patients than controls, and the mean n-3-to-n-6 PUFA ratio was similar in both cases and controls. Furthermore, Maillard et al., (2002) also reported that PUFA ratio was comparable in both breast cancer cases and controls. Clearly, further investigation into the role of different n-3 and n-6 PUFA and the development of mammary cancer is warranted, in the light of the seeming discrepancies among these studies.

Cell Line and Rodent Studies

It has been shown that n-3 PUFA (EPA, DHA, and LNA) have inhibitory effects and n-6 PUFA (LA) have stimulatory effects on mammary cancer cell line growth *in vitro*. In 1989, Rose and Connolly demonstrated that human mammary cancer cells incubated with LA for five days had significantly increased growth (measured by cell counts) compared to controls. In 1990, Rose and Connolly examined the effects of several PUFA on cell number and [³H]-thymidine incorporation (a measure of DNA synthesis) of human mammary cancer cells *in vitro*. This study confirmed and expanded the evidence that LA can stimulate the growth of human

mammary cancer cells in culture. This study also demonstrated that n-3 PUFA, specifically DHA and EPA, inhibited cell growth and DNA synthesis ($P < 0.01$) without inducing toxicity (cell viability measured by Trypan blue exclusion was $>90\%$).

Omega-6 fatty acids have been shown to have tumor-promoting effects in rats treated with dimethylbenzyl[a]anthracene (DMBA, a chemical carcinogen commonly used to induce mammary tumors in rats for the study of anti-tumorigenic chemicals or nutrients); whereas n-3 PUFA were shown to have inhibitory effects on the growth of tumors in those same rats (Carroll, 1991). Furthermore, rats on a high n-6 diet (as corn oil) had fewer DMBA-induced mammary tumors when supplemented with the n-3 PUFA found in flaxseed oil, compared to unsupplemented rats on the same corn oil diet ($P < 0.05$). (Thompson et al., 1996).

Xenograft implants of human mammary tumors in athymic nude mice are commonly used to study the effects of different treatments on human mammary cancers. Data from xenograft studies shows that diets rich in n-3 PUFA can inhibit the growth and metastasis of mammary tumors (Borgeson et al., 1989; Gonzalez et al., 1991; Rose and Connolly, 1993; Rose et al., 1995). To determine whether EPA and DHA could affect the growth of mammary cancer, Rose et al., (1995) supplemented the diets of nude mice bearing human mammary tumor xenografts with either 8% or 4% (wt/wt) EPA and DHA, in a diet with 20% of the total calories from fat. Tumor suppression occurred in these mice when compared to the mice fed unsupplemented diets (20% total fat as corn oil). Noguchi et al., (1997) showed that rats fed a diet supplemented with EPA or DHA [to provide an n-3-to-n-6 PUFA ratio of 1 to 1.8 (total fat 20%)], had a 47% and 69% reduction, respectively, in the incidence of mammary gland tumors when challenged with DMBA, compared to rats fed unsupplemented isocaloric diets. Rose et al., (1996) conducted a study to determine if n-3 supplementation before and after excision of mammary tumor xenografts could decrease the local recurrence and metastasis of mammary cancer after the primary tumor was excised. Female nude mice implanted with xenografts were fed a high-fat diet with 8% LA, or 8%, 4%, or 2% EPA or DHA. Seven days after being started on these diets, the tumors were excised, and the animals that were still consuming the LA-diet were either continued on that diet or switched to a diet containing 8%, 4%, or 2% EPA or DHA. Eight weeks, post-excision, animals were necropsied and assessed for local recurrence and

metastases. It was demonstrated that in animals fed a diet containing EPA or DHA before removal of the tumor, the severity of lung metastases was significantly lowered by both n-3 PUFA at all doses ($P < 0.05$). Changing the LA-fed animals to a diet containing DHA post-excision also resulted in significantly less lung involvement ($P < 0.05$). This study provides evidence as to the potential for using n-3 PUFA as an adjuvant therapy to prevent the recurrence and metastases of mammary cancer. These studies appear to support epidemiological evidence concerning the protective role that a high n-3 to n-6 PUFA ratio may have against the progression of mammary cancer.

Epidermal Growth Factor and Mammary Cancer

Steroid hormones, such as estrogen and progesterone, are generally thought to play the greatest role in the modulation of mammary cancers. However a large number of mammary cancers are unresponsive to steroid hormones. These mammary cancers do not respond to hormonal therapies, and tend to be more aggressive, leading to a worse prognosis for the patient. Therefore, it is necessary to determine alternative ways to regulate the growth of these cancers. Furthermore, even in breast cancer patients with estrogen-sensitive tumors (ER+), 40-50% of breast tumors will not respond to anti-hormonal therapy, despite tumor retention of functional ER (Robertson, 1996). An inverse correlation has been observed between EGFR and ER expression in breast cancer cells and tumors (Lee et al., 1990; Koenders et al., 1991; Tsustsui et al., 2002 and 2002), and overexpression of EGFR has been correlated with poor patient response to hormonal therapy (Nicholson et al., 1989 and 1991). It has been suggested that upregulation of the EGFR signaling pathway may provide a mechanism for breast tumors to evade hormonal therapies.

Epidermal Growth Factor (EGF) is a protein that is involved in stimulating the growth of cells through a cascade of signal transduction. Enhanced expression of the epidermal growth factor receptor (EGFR) has been shown to play a role in the growth of normal and neoplastic mammary tissue.

In human mammary cancer, it has been demonstrated that increased expression of EGFR is directly related to the invasiveness of the tumor (Moller et al., 1989), and EGF has been shown

to modulate the expression of *neu* (c-erbB-2) protooncogene in human mammary cancer cell lines *in vitro* (Fernandez-Pol et al., 1989). In 1997, Torregrossa et al., were able to demonstrate that high levels of EGFR expression in tumors of breast cancer patients was associated with a significantly decreased disease-free survival and overall survival compared to patients with low levels of EGFR. Furthermore, it was shown that increased co-expression of EGFR and the *neu* gene product (HER2/neu, which is the preferred heterodimerization partner for EGFR) in mammary tumors was associated with the shortest disease-free survival in breast cancer patients.

Dickson and Lippman (1995, 1998) demonstrated that EGF promoted tumorigenesis in a mouse model, and that overexpression of EGFR led to hormone-unresponsiveness, even in tumors expressing high levels of estrogen receptor. These studies seem to indicate that EGF may be involved in regulating the growth and metastasis of mammary cancer, and may be involved in the acquired resistance of ER+ tumors to anti-hormonal treatments. Recently, it has been shown that hormone-responsive mammary tumor cell lines (ER+) which are grown long-term (~3-6 months) in the presence of anti-hormonal drugs (tamoxifen and Faslodex™) become resistant to the growth-inhibiting properties of the drugs (McClelland et al., 2001), even though the expression levels of ER in the cells remain the same as parental cell lines. Interestingly, these anti-hormone resistant cell lines had increased levels of EGFR mRNA and protein, compared with the anti-hormone susceptible parent cells. Furthermore, inhibitors of the EGFR signal cascade were able to successfully inhibit the growth of the anti-hormone resistant tumor cells. These data suggest that the EGFR pathway may provide a means for ER+ tumors to escape the effects of anti-hormonal therapy and eventually re-establish endocrine-resistant or -insensitive tumor cell growth.

Polyunsaturated Fatty Acids and EGFR/MAPK

Studies suggest that dietary PUFA may regulate the growth and progression of mammary cancer through modulation of the EGFR pathway. The current view of the EGFR pathway is as follows: EGF binds to trans-membrane EGFR-tyrosine kinase, ligand-bound EGFR dimerize, which activates a signal transduction cascade, thus inducing the activity of a variety of kinases including a GTP-bound Ras, Raf-1, MEK, and MAPK (mitogen-activated protein kinase) (Figure 1-1).

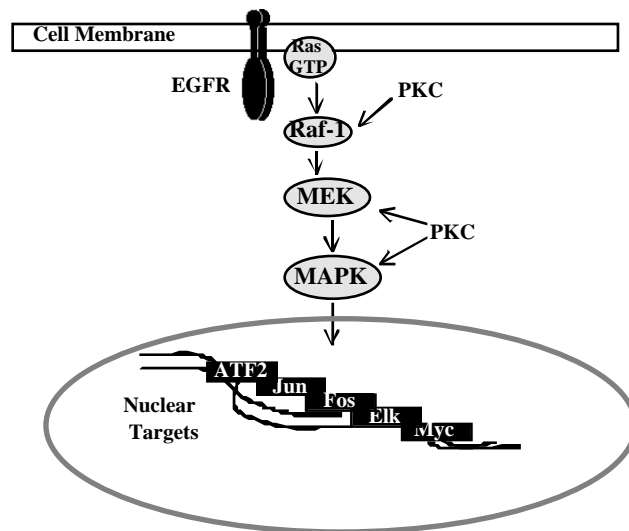


Figure 1-1. **EGFR/MAPK signaling cascade.** Recruitment of signaling molecules to activated EGFR initiates a series of successive phosphorylations and activation of kinases in the MAPK family (Raf-1, MEK, MAPK), which in turn mediate transcription factors (i.e. myc, fos, jun) regulating cell growth and mitogenesis (Figure adapted with permission from Seger, 1996).

Mitogen-activated protein kinase (MAPK)

MAPK is a family of serine/threonine kinases that are regulated by a number of growth factors and oncogenes. It has been suggested that MAPK is the link that connects the signal transduction of EGFR to transcriptional activation in the nucleus. The involvement of MAPK in the progression of mammary cancer is supported by much research. A major role of MAPK is to modulate enzymes and gene transcription involved in cellular proliferation and mitogenesis (Sakakura et al., 1997). Maemura et al., (1999) examined MAPK expression in a total of 20 human mammary cancers, and found an over-expression of MAPK in all of them. Not only was the protein over-expressed, but its activation level was significantly higher in the mammary

carcinomas when compared to the surrounding normal tissue. This indicates that MAPK may be a factor in the enhanced growth of mammary cancer cells. Upstream components of a signal transduction cascade regulate the activation of MAPK. Maemura and colleagues also examined the expression of Raf-1, one of these upstream modulators of MAPK, in mammary cancers, and observed that it was detectable in breast cancer tissues, but not normal surrounding mammary gland.

Cancer cells not only progress because of increased growth, but also because of their ability to avoid apoptosis, or programmed cell death. Activation of Ras, another upstream effector of MAPK, has been shown to up-regulate *bcl-2*, a suppressor of apoptosis, (Kinoshita et al., 1995), suggesting that MAPK may play a role in the progression of mammary cancer through both growth stimulation and decreased apoptosis. Inhibition of MAPK in MCF-7 human mammary cancer cells resulted in an inhibition of estrogen-induced growth in these cells (Lobenhofer et al., 2000). Fiddes et al., (1998) demonstrated that MAPK inhibitors could inhibit the cell-cycle progression of mammary carcinoma cells. Inhibition of MAPK has also been shown to increase the induction of apoptosis in tumor cells (Hovey et al., 1998), implying that MAPK may not only stimulate the growth of mammary cancer cells, but may also prevent cancer cell death through diminished apoptosis.

In 2000, Mueller et al., demonstrated in 131 breast cancer patients that high levels of MAPK activity in primary breast tumors was significantly associated with shortened disease-free survival times. Furthermore, in 2001, Gee et al., was able to confirm those findings in a study of 90 pre- and post-menopausal breast cancer patients, in which it was shown that pMAPK levels were detected in both ER+ and ER- primary breast tumors, but were undetectable in normal breast tissue from control patients (undergoing reduction mammoplasty). In addition, Gee et al., demonstrated that breast cancer patients with high levels of active MAPK also had shortened overall survival and decreased response to hormonal therapy, regardless of the tumor's original ER status. These data provide further insight concerning the means of escape that breast tumors may have in endocrine-resistant cancers, and the role that the EGFR/MAPK pathway may play in these mechanisms.

PUFA and EGFR/MAPK

There is evidence that the mammary fat pad may modulate the growth of mammary epithelial cells through the regulation of EGFR. Hovey et al., (1998) demonstrated that EGF stimulated-growth of mammary epithelial cells was significantly increased by incubation with mammary fat pad, although the fatty acid composition of this fat pad was not described. Dietary PUFA intake has been shown to directly affect the composition of PUFA in feline adipose tissue of growing kittens (van Niel and Beynen, 1997), although there are no studies reporting this same phenomenon in adult cats. Specifically, when kittens were fed diets with different amounts, alone or in combination, of corn, linseed, or fish oil, subcutaneous adipose tissue biopsies showed changes in fatty acid composition that reflected differences in the diets. The study in cats is in agreement with many human studies reporting subcutaneous adipose biopsies as an excellent indicator of dietary fat intake (Beynen and Katan, 1985; van Staveren et al., 1986). It is conceivable that changes in PUFA consumption could influence the growth of mammary tumors by altering the PUFA composition of the surrounding fat pad. There are several proposed mechanisms by which PUFA might regulate the EGFR/MAPK-induced growth of mammary cancer cells (**Figure 1-2**).

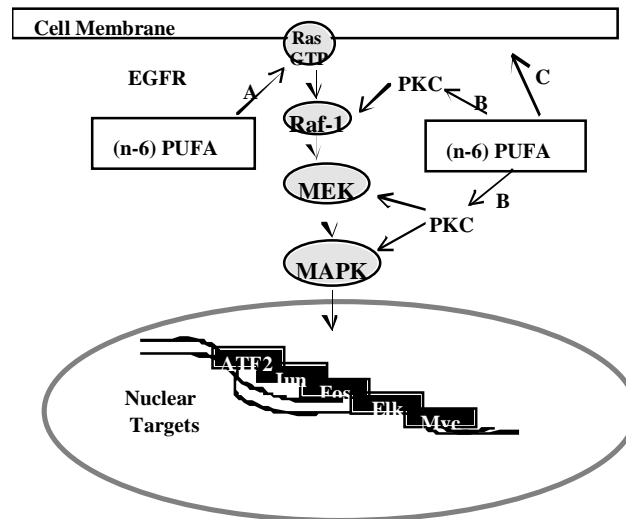


Figure 1-2. **PUFA modulation of EGFR/MAPK cascade.** A) Inactivation of GTPase activating protein by n-6 PUFA leads to retained mitogenic and proliferation activities of MAPK; n-3 PUFA inhibit Ras localization to membrane and binding of GTP to Ras, thus inhibiting mitogenic signal. B) n-6 PUFA stimulates protein kinase-C (PKC), which activates several components of the MAPK signaling cascade. C) PUFA alterations in cell membrane may change membrane fluidity, affecting internalization/localization of EGFR. (Figure adapter with permission from Seger, 1996)

In order for Ras to be biologically active, it must first be localized to the plasma membrane (Schlessinger, 1993; Lowy and Willumsen, 1993) after a series of post-translational events (Lowy and Willumsen, 1993). It is conceivable that alterations in membrane phospholipid fatty acids might influence Ras membrane localization and subsequent signaling in mammary tumor cells. In fact, Collett et al. (2001) recently reported that DHA, compared with LA, decreased 1) membrane localization of Ras, 2) activation of Ras at the membrane, and 3) downstream signal transduction of Ras through MAPK in malignant colonocytes.

Furthermore, researchers have demonstrated that arachidonic acid (AA) can inhibit GTPase activating proteins involved in this cascade (Golubic et al., 1991; Tsai et al., 1989; Yu et al., 1990). This is important because the GTPase-activating protein is involved in the hydrolysis of GTP-bound (active) Ras protein in the EGFR/MAPK cascade. By inhibiting the GTPase-activating protein, AA, in effect, prolongs the signal transduction of EGFR to the nucleus, leading to an increased growth stimulus. Raf-1, a critical regulatory protein in the MAPK signaling cascade, must be recruited to the cell membrane to interact with GTP-bound Ras. The recruitment of Raf-1 is mediated through direct interaction with membrane fatty acids (Rizzo et al., 2000), so this is another pathway which may be modulated through dietary fatty acids.

Also, n-6 PUFA have been implicated in the activation of several isoforms of protein kinase-C (PK-C) (Lester 1990; Fan et al., 1990; Chen and Murakami, 1992). The ability of n-6 PUFA to activate PK-C is important because several PK-C isoforms are effectors of the MAPK signaling cascade. *In vivo*, PK-C_{alpha} and PK-C_{epsilon} have been shown to activate Raf-1, an upstream regulator of MAPK; and PK-C_{zeta} has been shown to activate MEK and, subsequently, MAPK (Toker, 1998; van Dijk et al., 1997). Awad et al., (1996) demonstrated that n-3 supplementation in colon cancer cells, when compared with n-6 or saturated fatty acid supplementation, resulted in decreased activity of phospholipase C (PLC), which is an upstream activator of PKC. This provides evidence that n-6 PUFA may influence the MAPK-mitogenesis of cells through a variety of mechanisms.

These studies demonstrate that n-6 PUFA can regulate cell growth through the EGFR/MAPK cascade at the cellular level, and raise the question as to whether changes in the ratio of dietary n-3 to n-6 PUFA could influence EGFR/MAPK action. AA seems to be implicated in the modulation of the EGFR/MAPK pathways, and it has been demonstrated that increasing dietary n-3 PUFA can decrease the presence of AA and its eicosanoids in the body. Therefore, it seems plausible that altering the dietary ratio of n-3 to n-6 PUFA could alter the EGFR/MAPK signaling cascade, and thereby inhibit the growth of mammary cancer cells.

Conclusion

Since 1987, when the first association was made between EGFR/MAPK and mammary cancer, many drugs that target this pathway have been in development. These drugs include monoclonal antibodies against different domains of the EGFR or HER2/neu receptor, kinase inhibitors to prevent the phosphorylation of EGFR or other components of the signal transduction cascade (Raf, MEK, MAPK), and compounds to block the lipid-mediated component of the EGFR/MAPK pathway (farnesylation inhibitors). Recent data have suggested the role that n-3 PUFA may play in regulating growth, progression, metastasis and postexcision recurrence of mammary tumors in murine models. Furthermore, studies presented here provide evidence that PUFA and their biological metabolites can influence EGFR/MAPK, targeting many of the same components as drugs under development. These data provide convincing evidence that further research into the usefulness of dietary manipulation of PUFA for the prevention and/or adjuvant treatment of mammary cancer, as well as continued research into the mechanisms of PUFA in mammary tumor development is needed.

The data suggest that an important aspect of PUFA in the modulation of mammary cancer growth is the ratio of n-3 to n-6 PUFA rather than the absolute concentration of either. One murine study (Noguchi et al., 1997) has even suggested that a ratio of ~1:1 - 1:2 has the most protective effect against the growth of mammary cancers in rats. If this indeed is the case, a closer look at human and companion animal diets, whose n-3 to n-6 PUFA average ratio is considerably higher (~1:20 and 1:10 - 1:16, respectively) (Simopoulos, 1991; Roudebush et al., 1997), may be warranted.

There is data supporting the modulation of n-3 to n-6 PUFA ratio as a means for suppressing growth in human breast cancer cells (Chamras et al., 1999). However, there are no published reports concerning the optimal range of n-3 to n-6 PUFA ratio by which this growth-suppression occurs. Furthermore, there has been no research concerning the optimal ratio of n-3 to n-6 PUFA for modulating MAPK activity in companion animals. Based on literature presented here, we hypothesized that 1) active MAPK would be detectable in the tissues of felines and influenced by changes in dietary n-3-to-n-6 PUFA ratio, and 2) altering the PUFA ratio in culture medium of EGFR+/ER- breast tumor cells would influence proliferation and EGFR/MAPK signaling in those cells.

Hypotheses

Hypothesis A

We hypothesize that dietary decreases in the n-6 to n-3 PUFA ratio will result in decreased activity of MAPK in peripheral leukocytes and mammary adipose tissue and a decrease in the ratio of n-6 to n-3 PUFA in the mammary adipose tissue of felines.

Hypothesis B

We hypothesize that increasing the ratio of n-3 to n-6 PUFA in the culture media of human cancer cells *in vitro* will result in suppressed growth, and altered EGFR/MAPK expression and/or function in those cells.

Chapter 2: Dietary manipulation of n-6-to-n-3 polyunsaturated fatty acids alters active mitogen-activated protein kinase levels in tissues of cats.

Dietary manipulation of n-6-to-n-3 polyunsaturated fatty acids alters active mitogen-activated protein kinase levels in tissues of cats.

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Running Title:

n-6 AND n-3 PUFA AND MAPK IN CATS

INTRODUCTION

Mammary gland neoplasms are common in both the human and companion animal population. According to the American Cancer Society (2004), mammary cancer is the most prevalent cancer diagnosis and the 2nd leading cause of cancer death in women, surpassed only by lung cancer. Mammary gland tumors are a common neoplasm in cats in the United States and other parts of the world (Hahn and Adams, 1997; Viste et al., 2002; Preziosi et al., 2002; Oonuma et al., 2003).

The cat is considered a good model for human mammary growth and tumorigenesis (Hayes et al., 1981; Misdorp et al., 1991; Modiano et al., 1991; Hahn et al., 1994; Vail and MacEwen 2000). Mammary tumors in both humans and felines occur in middle-age females, occur spontaneously, are histologically similar, and develop from secretory tissues (Hayes et al., 1981; Misdorp et al., 1991; Modiano et al., 1991; Hahn et al., 1994). Determining treatment or prevention modalities for the feline population; therefore, will not only be beneficial to the pet population, but may also prove useful to the human population as well. In felines, early ovariohysterectomy (before 2 years of age) offers significant protection from development of malignancies, but even neutered cats have been diagnosed with mammary cancer (Hayes et al., 1981; Hahn and Adams, 1997; Perez Alenza et al., 2000; Martin de las Mulas et al., 2002). Most feline mammary carcinomas (50 – 90%) are estrogen-receptor-negative (ER-) and are unresponsive to the proliferative effects of estrogen (Hamilton et al., 1976; Misdorp and Weijer, 1980; Hayes et al., 1981; Martin et al., 1984; Martin de las Mulas et al., 2000). Furthermore, ~30% of primary mammary tumors in women are ER-. These ER- tumors, along with feline mammary tumors, are associated with decreased cancer-free survival times, as well as decreased overall survival times. Clearly, determining the factors that play a role in the development and

progression of these ER- mammary tumors, and investigating how to modulate those pathways would be beneficial for both humans and felines.

Polyunsaturated fatty acids (PUFA) have been implicated as a player in the development/progression of breast cancer for the last 30 years. Epidemiological evidence, rodent studies, and *in vitro* experiments have suggested that long chain (LC) n-6 PUFA may stimulate and n-3 PUFA may inhibit the ability of breast tumor cells to proliferate and metastasize through a variety of regulatory pathways. One pathway of particular interest is the epidermal growth factor/mitogen activated protein kinase (EGFR/MAPK) signal transduction cascade (for a more comprehensive review, see J. Nutr., vol. 13, 2001). PUFA have been shown to modulate the EGFR/MAPK pathway in a number of human cells *in vitro* and *in vivo*, including peripheral white blood cells (Denys et al., 2001 and 2002), but no reports to date have confirmed MAPK activity or the ability of PUFA to regulate its action in cats.

Data reported here demonstrate that active MAPK can be detected and measured in the feline, and that altering their dietary n-3 and n-6 PUFA ratio can influence active MAPK levels in both the peripheral white blood cells and mammary adipose tissue in cats.

MATERIALS and METHODS

Reagents and diet. Histopaque 1.077 and 1.119 were purchased from Sigma-Aldrich (St. Louis, MO), and monoclonal antibodies for pMAPK and total MAPK were graciously provided by Andreas Nelsbach (Cell Signalling Technologies (CST), Beverly, MA). All reagents for western blotting applications were purchased from BioRad (Hercules, CA), and nutritionally complete feline diets were formulated and provided by Hill's Pet Nutrition, Inc. (Topeka, KS).

Animals and experimental design. Twenty-eight (4-13 y old) healthy, neutered adult domestic short-haired cats weighing 3-6 kg (females n=24; males n=4) were used for this study. These cats were part of an established colony at the Virginia-Maryland Regional College of Veterinary Medicine (VMRCVM). The study protocol was approved by the Virginia Tech Animal Care Committee. Each cat acted as its own control, and cats were housed in a climate- and environmentally- controlled room. Pre-study diets contained an n-6-to-n-3 PUFA ratio of ~16:1. Cats were blocked by age (young < 4 y, old > 13 y), and randomly allocated into groups (n=7) receiving isocaloric diets with n-6-to-n-3 PUFA ratios of 5, 2.5, 1, or 0.4 for 24 weeks (**Table 2-1**).

Blood was collected from each cat on weeks 0 (pre-study diet), 6, 12, and 24 for MAPK analysis in peripheral white blood cells (WBC). Subcutaneous inguinal mammary adipose biopsies were obtained from anesthetized cats at weeks 0 and 24 for fatty acid and MAPK analysis. Biopsies were immediately placed in liquid nitrogen, and subsequently stored at -70°C until further processing.

Tissue fatty acid analysis. Fatty acid analysis of feline mammary adipose tissue was performed in the laboratory of Dr. J. Herbein (Department of Dairy Science, Virginia Tech,

Blacksburg, VA). Briefly, lipids were extracted with chloroform/methanol, according to Folch et al. (1957) and dried under a stream of nitrogen. Undecenoate (an internal standard, NuCheck Prep, Elysian, MN) was added to the lipid extracts, and the samples were directly methylated using 0.5 N NaOH in methanol, according to Park and Goins (1994). Methyl esters were analyzed by gas chromatography (Hewlett-Packard 5890A, Sunnyvale, CA), using a fused silica capillary column (100 m x 0.25 mm i.d.) (CP-Sil 88, Chrompack, Middleburg, The Netherlands). Fatty acids (FA) were quantified by integration using peak area response factors to known amounts of individual fatty acids, and expressed as microgram FA per milligram tissue.

WBC pMAPK analysis. For flow cytometry, peripheral WBC were isolated from whole blood via a double density histopaque gradient, and analyzed for pMAPK activity according to Chow et al., (2001). Briefly, 5×10^5 WBC were stimulated with 40nM phorbol-13-myristate-15-acetate (PMA), fixed in formaldehyde, permeabilized with methanol, and stained with primary mouse anti-pMAPK monoclonal antibodies (CST) and secondary fluorescein isothiocyanate (FITC)-conjugated goat F(ab')₂ anti-mouse IgG (H+L) antibodies (Caltag, Burlingame, CA). Negative controls included unstimulated cells (no PMA treatment) and cells (stimulated and unstimulated) with no primary antibody. Stained cells were analyzed using a Coulter Epics XL flow cytometer (Beckman Coulter, Miami, FL), and the Overton Subtraction method was used to correct data according to controls.

For western blotting applications, 2×10^6 WBC stimulated with PMA were lysed in sample buffer containing 62.5 mM Tris-HCl (pH 6.8), 2% w/v SDS, 10% glycerol, 50 mM DTT, and 0.01% bromphenol blue, and electrophoresed (20 μ l) on a 10% SDS-PAGE gel. Purified pMAPK and non-phosphorylated MAPK were electrophoresed as positive and negative controls, respectively. Following electrophoresis, gels were electrotransferred to a PVDF membrane via semi-dry method, and membranes were blocked in TBST with 5% non-fat dry milk and stained

with either primary antibody to pMAPK or total MAPK (CST), followed by secondary antibodies conjugated to horseradish peroxidase (HRP, CST). Bands for pMAPK or total MAPK were visualized via chemiluminescence (CST), and quantitated using densitometric analysis (NIH Imager, 1.62). All data is expressed as a ratio of pMAPK to total MAPK, to correct for loading differences.

Adipose tissue pMAPK analysis. For western blotting applications, adipose tissue was processed according to Bendinelli et al., (2000). Briefly, tissue was homogenized in boiling lysis buffer, containing 62.5 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 2mM sodium orthovanadate, 40mM β -glycerophosphate, 10 μ g/ml leupeptin, and 10 μ g/ml aprotinin, boiled for 5 min. at 100°C, and centrifuged at 8000 x g for 10 min. at 4°C. Protein concentrations were quantified using BioRad DC Protein Assay Kit II (Hercules, CA).

Bromphenol blue (0.01%) and mercaptoethanol (5%) were added to each sample, and total protein was electrophoresed on a 10% SDS-PAGE gel. Gel transfer, antibody-staining, and detection and quantification of pMAPK bands were as described for WBC western immunoblotting. Data are expressed as pMAPK signal/ μ g total protein, to account for loading differences.

Statistical Analyses. All statistical analyses were performed using the SAS System (ver. 8.02, SAS Institute, Inc., Cary, NC). To test for effects of treatment and treatment by time, we used Mixed Model Repeated Measures ANOVA with covariate structure. Bonferroni-corrected pairwise comparisons performed, comparing time-zero pMAPK levels to pMAPK levels at each non-zero time point. Adipose tissue data was log transformed prior to statistical analysis. Significance was set at an alpha level of 0.05.

RESULTS

WBC pMAPK response to altered dietary n-6-to-n-3 PUFA ratio. To determine if altering the dietary n-6-to-n-3 PUFA ratio in cats could alter pMAPK activity in peripheral WBC, flow cytometric and western blotting applications were performed. Baseline diets had an n-6-to-n-3 PUFA ratios of ~16, and study diets one through 4 had n-6-to-n-3 PUFA ratios of 5, 2.5, 1, and 0.4, respectively. Across all diets, the time over which the diets were fed had a significant effect on the percent of total WBC cells (**FIGURE 2-1**) staining positively for pMAPK. We observed a trend in all dietary groups for pMAPK-positive cells to decrease over time ($p = 0.0002$); this decrease was statistically significant by week 12 of the study for diet 3 ($p = 0.0102$) and by week six for diet 4 ($p < 0.0001$). When WBC were separated into subgroups (**FIGURE 2-2**), a significant decrease in both pMAPK-positive mononuclear cells ($p = 0.0113$) and granulocytes ($p < 0.0001$) was observed. A statistically significant decrease in both pMAPK-positive mononuclear cells ($p = 0.0199$) and granulocytes ($p = 0.0132$) was observed by study week 12 for diet 4.

Effect of dietary PUFA composition on PUFA composition in mammary adipose tissue. Gas chromatography was used to determine if altering dietary PUFA levels could alter the mammary adipose tissue levels of PUFA in cats. Fatty acid compositions of mammary adipose tissues were obtained at baseline and after cats were fed diets with varying n-6-to-n-3 PUFA ratios for 24 weeks. When compared to pre-study diet levels, total n-3 PUFA in diets 2 through 4 increased 2.8, 3.9, and 4.9 $\mu\text{g FA/mg diet}$, respectively (**FIGURE 2-3**). Likewise, tissue levels of n-3 PUFA increased 1, 4.6, and 8.2 $\mu\text{g FA/mg tissue}$ after 24 weeks of being fed diets 2 through 4, respectively (**FIGURE 2-3**). Complete results from fatty acid analyses can be found in Appendix C – Table 1.

Dietary manipulations of n-6-to-n-3 PUFA ratio results in altered mammary adipose pMAPK levels.

Levels of active-MAPK (pMAPK) were measured via western immunoblotting in mammary adipose tissue at baseline and after 24 weeks of cats being fed diets with varying n-6-to-n-3 PUFA ratios. Across all dietary groups, non-significant decreases in adipose pMAPK were observed ($p = 0.12$) (**FIGURE 2-4**). Although no statistically significant differences were found, there does appear to be a trend for adipose pMAPK to decrease as the n-6-to- n-3 dietary ratio decreases.

DISCUSSION

MAPK activity is increased in human breast cancer cells, when compared to normal surrounding tissue or benign breast hyperplasias. High tumor MAPK activity is associated with shortened disease-free survival (Mueller et al., 2000) and shortened duration of response to anti-hormonal therapies (Gee et al., 2001) in human breast cancer patients. The ability for tumor MAPK activity to serve as a predictor for these parameters may make it useful in the staging and prognosis of breast cancers. Based on the histological and clinico-pathological similarities between human and feline breast cancers, it is possible that MAPK activity might be associated with neoplastic conditions in felines. This study is the first to report the ability to detect and measure active MAPK in the cells and tissue of cats. When electrophoresed via SDS-PAGE and probed with either anti-pMAPK or anti-TOTAL MAPK antibodies (CST), feline pMAPK/TOTAL MAPK was detected as 2 bands (44 and 42 daltons) in the same gel location as human pMAPK/TOTAL MAPK and a purified pMAPK/TOTAL MAPK protein (CST). This suggests that feline pMAPK/TOTAL MAPK has a similar molecular weight and phosphorylation-state homology to its human counter-part (FIGURE A-1).

PUFA have been shown to modulate the MAPK pathway through a variety of mechanisms. Recently, it has been reported that n-6 and n-3 PUFA can differentially regulate the activation of Ras and subsequently MAPK in malignant colonocytes (Collett et al., 2001). In cultured human T cells, Denys et al., (2001) demonstrated that docosahexanoic acid (DHA) and eicosapentanoic acid (EPA), both n-3 PUFAs, decreased PMA and anti-CD2-stimulated MAPK phosphorylation and activity. Arachidonic acid (AA), an n-6 PUFA, has been shown to inhibit GTP-ase activating protein (Golubic et al., 1991), which ultimately translates into sustained mitogenic signalling through MAPK by keeping Ras in an activated state. Furthermore, n-6 and

n-3 PUFA have been shown to differentially modulate protein kinase c isoforms (Mirnikjoo et al., 2001), which are involved in activation of the MAPK cascade (Toker, 1998).

We report evidence directly demonstrating that alterations in dietary n-6-to-n-3 PUFA ratio are reflected in the mammary adipose tissue of cats. This is in agreement with previous reports in humans that subcutaneous fat tissue microbiopsies are a valid index for the dietary fatty acid composition in adults (van Staveren et al., 1986; Beynen and Katan, 1985). There is accumulating evidence that the local environment of the mammary fat pad may play an integral role in modulating the proliferation of neoplastic mammary cells (Hovey et al., 1998; Chamras et al., 1998; Hovey 1999), and may exert their effects directly (through their incorporation into cellular mediators such as diacylglycerol) or indirectly (by serving as precursors for mitogenic effectors). Altering mammary adipose composition via the diet may be an important tool in regulating the growth of breast tumors.

Based on the differential, and seemingly opposing, activities of n-6 and n-3 PUFA, it is possible that altering the n-6-to-n-3 PUFA in tumor cells or adipose tissue surrounding the tumor might influence tumor cell growth via the MAPK pathway. We report that altering the dietary PUFA ratio in the diet, and subsequently the mammary adipose tissue, resulted in decreased MAPK activity in the mammary adipose tissue of cats. This provides evidence that dietary PUFA ratio can directly regulate MAPK activity at the tissue level, and may be a result of PUFA incorporation into the tissue. This is further supported by the decrease in PMA-stimulated MAPK activity observed in feline white blood cells as dietary n-6-to-n-3 PUFA ratio was lowered. Constitutive activity (as determined using non-PMA stimulated controls) remained undetectable in all treatment groups. It is probable that the effect of dietary PUFA ratio on the MAPK activity in WBC was a result of membrane incorporation of PUFA in the cell membrane of the cells. Although we did not investigate the membrane PUFA composition in the WBC, it

has been shown that alterations in dietary PUFA can change immune cell fatty acid composition in rats (Robinson et al., 2001) and likely in cat, as well.

Furthermore, significant decreases in WBC pMAPK were observed by week 12 for diets with n-6-to-n-3 PUFA ratio of 1:1 and 0.4:1, whereas a significant decrease in adipose pMAPK was only observed in cats fed the 0.4:1 diet. These observed differences between WBC and adipose tissue pMAPK may be due to differences in measurement methods (flow cytometry for WBC vs. immunoblotting for adipose tissue). However, immunoblotting was used in preliminary experiments with WBC to confirm the results from flow cytometry, and alterations in pMAPK were similarly measured using both methods, suggesting that tissue differences in pMAPK response to treatment is unrelated to differences in measurement methods. More likely, these differences may suggest that WBC are more susceptible to the effects of dietary PUFA on MAPK activity, or it could simply indicate a difference in the time of PUFA turnover in adipose tissue versus WBC. Adipose tissue in growing kittens have been shown to reflect dietary fat composition in as short a time as 6 weeks (van Niel and Beynen, 1997), but it is conceivable that turnover time in adult animals may be longer than that observed in kittens. If incorporation of dietary PUFA into WBC occurs more quickly than in adipose tissue, and PUFA composition of cells directly or indirectly affects MAPK activity in those cells, then it seems to follow logically that WBC would show alterations in pMAPK more quickly than adipose tissue. This idea of cell PUFA turnover rate affecting time at which we see pMAPK alterations is further supported in the differences in pMAPK response between subgroups of WBC.

The decrease in pMAPK-positive mononuclear cells observed over time was less drastic than that observed in granulocytes, which may be due to the different life cycles of the cell types. Peripheral mononuclear cells, which consist mostly of lymphocytes, have relatively long lives, months and years, compared to the relatively short lifespan of granulocytes (1-3 months).

Because dietary PUFA changes are more likely to be reflected in cells with shorter turnover times, and changes in cellular PUFA may play a role in altering pMAPK activity in cells, it reasonably follows that cells with a shorter turnover time would likely show alterations in pMAPK status than cells with longer lifespans.

Figure 2-5 shows the association between dietary PUFA ratio and MAPK activity in both the WBC and mammary adipose tissue in cats. Although there was no significant correlation between adipose tissue MAPK and WBC MAPK activity, a pattern was observed, indicating that tissue MAPK activity may be reflected in the MAPK activity of peripheral white blood cells. In other forms of cancer, peripheral biomarkers are often used to determine neoplastic tissue activity. For example, serum prostate specific antigen (PSA) or PSA mRNA, detected by RT-PCR can be used to detect, improve the staging of, and provide follow-up information about prostate cancer (Gekmini et al., 2001; Millon et al., 2000). Since MAPK activity is often increased in breast tissue of malignant disease vs. benign conditions, and peripheral WBC MAPK activity appears to reflect that of mammary tissue, peripheral MAPK activity may have potential as a diagnostic biomarker for malignant breast disease.

Although statistically significant decreases were observed in both WBC and adipose tissue pMAPK, variability within dietary groups was larger than expected. This observation may be due to the grouping done for statistical analyses. Group means of outbred animals response variables often contain greater variance than individual changes within an animal, so it is likely that less variance would have been observed had individual responses to dietary treatment been reported rather than group means.

SUMMARY and IMPLICATIONS

In this study, we detected and measured MAPK activity in feline tissues and peripheral cells. This has implications for both the human and companion animal populations. To date, MAPK activity has been associated with several parameters of breast cancer progression in humans, but there are no reports regarding whether MAPK might be associated with cancer in companion animals. Using the methods developed in this study, future studies designed to determine the role that MAPK might play in companion animal malignancies would be useful. Changes in MAPK activity observed across time and treatments were similar in both tissue and peripheral cells. This provides evidence that peripheral MAPK activity may be an indicator of tissue MAPK status, and further studies are necessary to determine its usefulness as a prognostic indicator of disease or tool for treatment follow-up in cancer patients.

We report in this study that decreasing the n-6-to-n-3 PUFA ratio in the diets of cats resulted in decreased MAPK activity in both peripheral WBC and mammary adipose tissue. The ability of dietary PUFA manipulation to influence cellular MAPK activity has important implications for cancer patients. We observed that PUFA-induced alterations in MAPK activity were more pronounced in WBC than adipose tissue, suggesting that turnover rate of tissues may have an effect on the ability of PUFA to modulate MAPK activity. Therefore, it is conceivable that MAPK activity in cancer cells (which are dividing more rapidly, and therefore incorporating dietary PUFA at higher rates, than normal tissues) would be more susceptible to dietary PUFA changes than that observed in this study of normal tissue.

In breast cancer patients, high tumor MAPK levels are correlated with poor prognosis, decreased response to hormonal therapy, and decreased survival in breast cancer patients. Based on data from this study demonstrating the ability of dietary PUFA changes to alter MAPK activity in WBC, it would be useful to determine if MAPK status in cancers involving WBC has

similar correlations to those observed in breast cancer. Nutritional intervention, specifically increasing the dietary n-6-to-n-3 PUFA ratio, may be useful as an adjuvant therapeutic option for breast cancer patients with high tumor MAPK activity as well as other cancer patients with high tumor cell MAPK activity.

Table 2-1. Dietary composition of experimental diets* with differing n-6-to-n-3 PUFA ratios.

Diet	1	2	3	4
Target n-6:n-3 ratio	5:1	2.5:1	1:1	0.4:1
% of total FA as n-3	0.46	0.82	1.45	1.78
% of total FA as n-6	2.10	1.92	1.69	0.83
Crude fat (%)	8.70	9.21	10.13	8.94
Crude protein (%)	12.40	14.26	14.6	13.36
Ash (%)	1.86	2.04	2.09	1.83
Moisture (%)	75.97	71.43	69.91	72.90
Kcal/ kg	1217	1381	1485	1357
Kcal/ 14.25 oz. can	492	558	600	548

***values reported are on an as fed basis.**

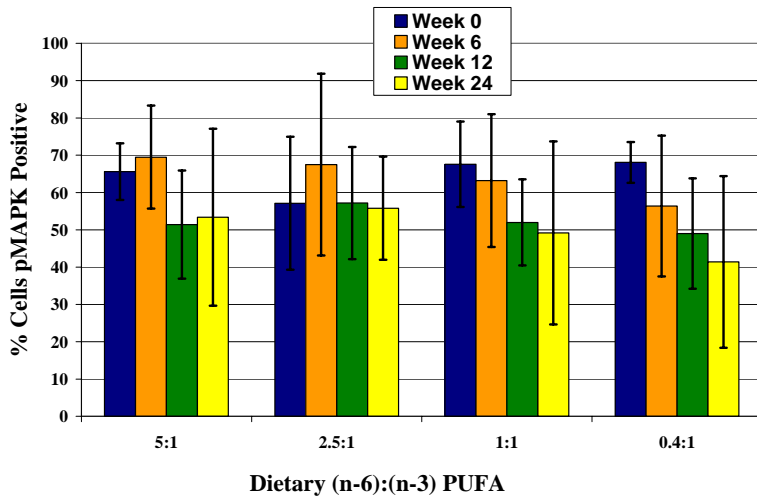


FIGURE 2-1 Effect of dietary PUFA ratio on pMAPK levels in total feline white blood cells. Felines were fed diets with differing (n-6)-to-(n-3) PUFA ratios. WBC isolated from whole feline blood (week 0, 6, 12, & 24 weeks) were stimulated with PMA, fixed, permeabilized, stained with pMAPK antibodies, and pMAPK positive cells were analyzed via flow cytometry. pMAPK levels in total white blood cells decreased from baseline by week 12 in dietary group 3 ($p=0.01$), and by week 6 in dietary group 4 ($p<0.0001$) ($n=7$).

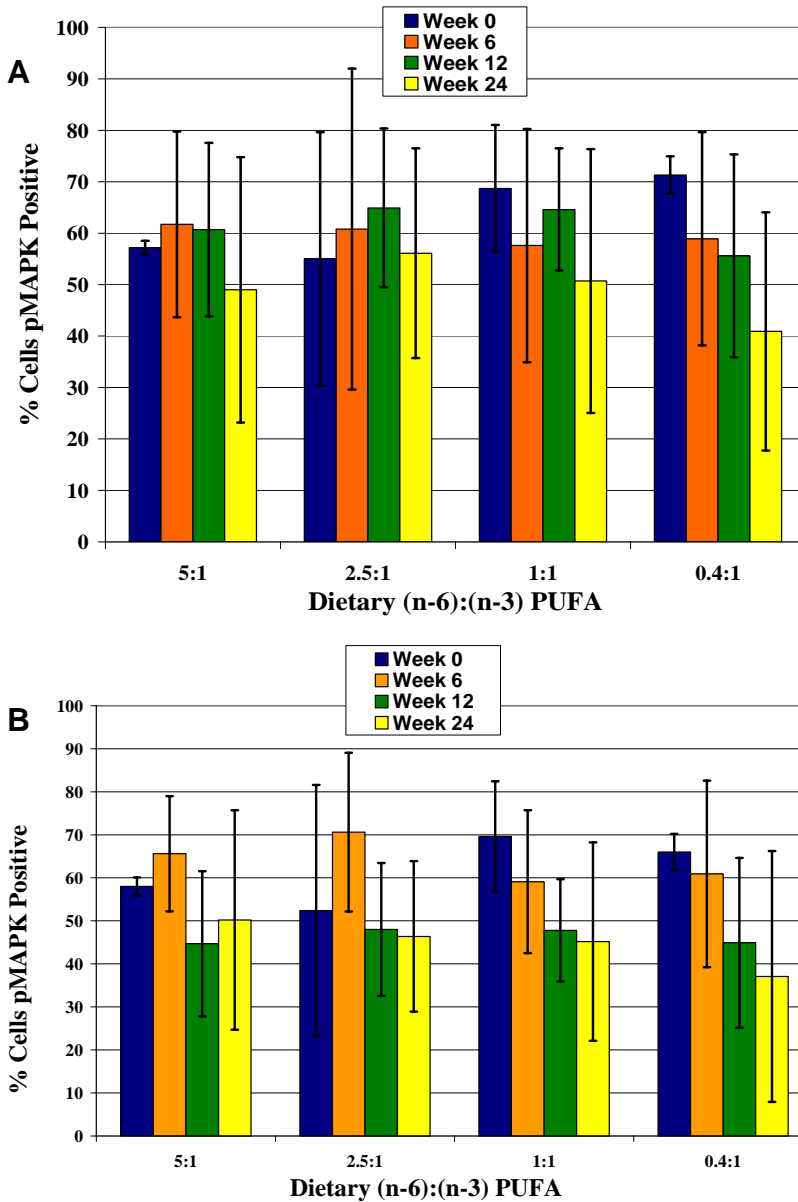


FIGURE 2-2 Effect of dietary PUFA ratio on pMAPK levels in feline mononuclear cells and granulocytes. Felines (n=7) were fed diets with differing (n-6):(n-3) PUFA ratios. Isolated mononuclear cells (A) and granulocytes (B) from whole feline blood (0, 6, 12, & 24 weeks) were stimulated with PMA, fixed, permeabilized, and stained with pMAPK antibodies. pMAPK positive cells were analyzed via flow cytometry. pMAPK levels in both cell types decreased from baseline by week 12 in dietary group 4 (mononuclear cells, p=0.02; granulocytes p=0.01) (n=7).

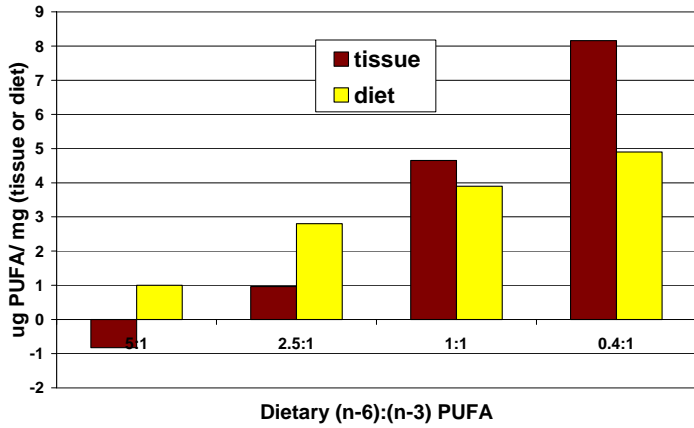


FIGURE 2-3 Effect of dietary n-3 PUFA on mammary adipose n-3 PUFA levels: changes in tissue and diet (n-3) PUFA. Mammary inguinal adipose tissue was obtained from cats before and 24 weeks after being fed diets with differing (n-6):(n-3) PUFA ratios. Adipose and diet PUFA compositions were analyzed via gas chromatography, and data expressed as μg PUFA per mg of tissue (■) or diet (■). A trend was observed in which adipose n-3 PUFA composition increased as dietary n-3 PUFA increased, when compared with baseline diet and tissue values.

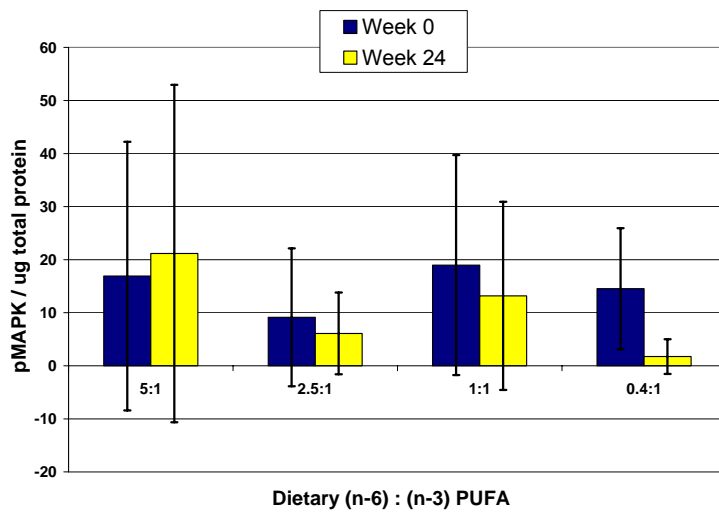


FIGURE 2-4 Effect of dietary PUFA ratio on pMAPK levels in feline adipose tissue. Inguinal mammary adipose tissue was obtained from cats before and 24 weeks after being fed diets with differing PUFA ratios. Total protein extracted from adipose was electrophoresed via SDS-PAGE, transferred to PVDF, stained with anti-pMAPK antibodies. pMAPK bands were visualized via chemiluminescence, quantitated using densitometry and data were expressed as densitometry units per μg total electrophoresed protein. Adipose pMAPK levels decreased across all dietary groups compared to baseline values, although this decrease did not reach statistical significance ($p=0.12$) ($n=7$).

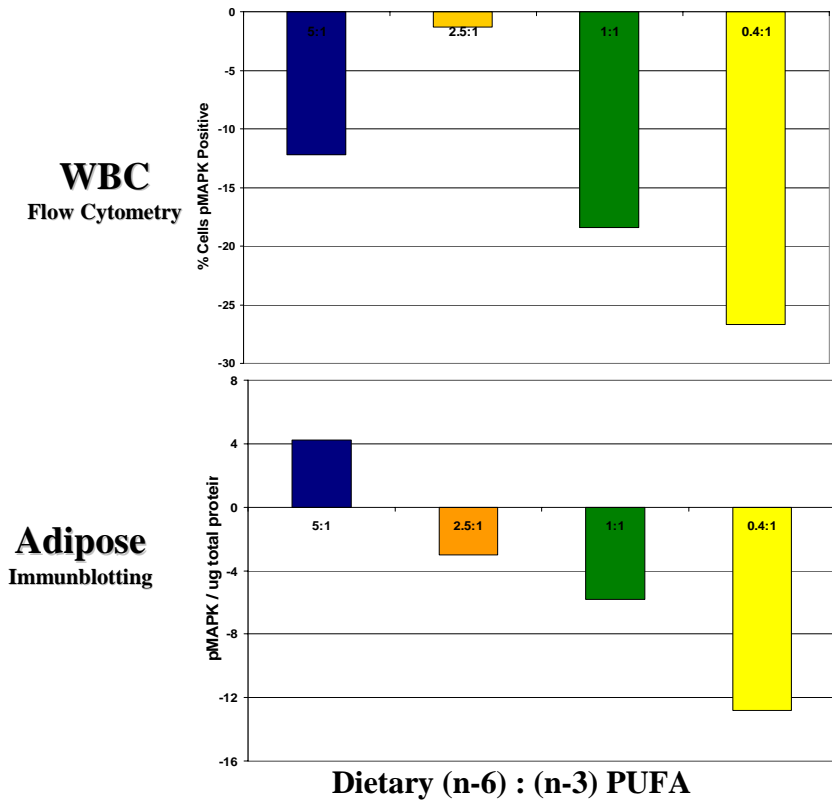


FIGURE 2-5 Effect of dietary PUFA ratio on pMAPK levels is similar in feline adipose tissue and white blood cells. Felines were fed diets with differing (n-6):(n-3) PUFA ratios (5:1, 2.5:1, 1:1, and 0.4:1). White blood cells and mammary adipose tissue were obtained from cats before and 24 weeks after being fed diets with differing PUFA ratios. White blood cell pMAPK was analyzed via flow cytometry and adipose pMAPK via western immunoblotting (as described previously). A non-statistically significant trend was observed in which white blood cell changes in pMAPK reflected adipose tissue pMAPK changes (n=7).

Chapter 3: Effect of Docosahexanoic and Linoleic Acids on the Proliferation of Epidermal Growth Factor Receptor-positive and -negative Breast Tumor Cell Lines

**Effect of Docosahexanoic and Linoleic Acids on the Proliferation of Epidermal Growth
Factor Receptor-positive and -negative Breast Tumor Cell Lines**

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Running Title: DHA, LA, and mammary cancer cell growth

INTRODUCTION

PUFA have been implicated as a player in the development/progression of breast cancer for the last 30 years. Epidemiological evidence, rodent studies, and *in vitro* experiments have suggested that long chain (LC) n-6 PUFA may stimulate and n-3 PUFA may inhibit the ability of breast tumor cells to proliferate and metastasize through a variety of regulatory pathways. One pathway of particular interest is the epidermal growth factor/mitogen activated protein kinase (EGFR/MAPK) signal transduction cascade (for a more comprehensive review, see J. Nutr., vol. 13, 2001).

Activity of this signaling pathway is often enhanced in patients with breast cancers that are initially unresponsive to hormonal treatments, as well as patients who develop resistance after long-term treatment with hormonal therapies. Over the last 3 years, several research groups have reported that high levels of EGFR, c-erbB-2 (a member of the EGFR family), and MAPK are significantly associated with shortened disease-free survival, as well as reduced overall survival and decreased response to hormonal therapy (Suo et al., 2002; Gee et al., 2001; Mueller et al., 2000). These data provide further insight concerning how endocrine-resistant breast tumors evade treatment. If LCPUFA can influence the EGFR/MAPK pathway, then it is possible that adjusting the dietary PUFA intake of breast cancer patients, specifically those patients bearing endocrine-resistant tumors, might be used as an adjuvant treatment along with radiation and chemotherapeutic therapies.

In this report, data are presented from a series of studies investigating the relationship between n-3 and n-6 LCPUFA, proliferation, and EGFR/MAPK signaling in breast tumor cell lines *in vitro*. In particular, we hypothesized that, because of epidemiological, case studies, and rodent study data reported in previous years suggesting opposite effects of n-3 and n-6 PUFA on breast tumor progression, an increased ratio of n-3-to-n-6 LCPUFA available to cultured breast

tumor cell lines would result in decreased proliferation and EGFR/MAPK signaling in cells expressing high levels of EGFR (EGFR+), while having little effect on those cells expressing little or no EGFR (EGFR-). The effects of linoleic acid (LA; 18:2n-6) and docosahexanoic acid (DHA; 22:6n-3) on the proliferation of two EGFR+ and one EFGR- human breast tumor cells lines are reported here.

MATERIALS and METHODS

Reagents. Docosahexanoic and linoleic acids were obtained from ICN Biomedicals (Aurora, OH) as free fatty acids. The proliferation kit used for the tetrazolium (MTS)/ phenazine methosulfate (PMS) assays was purchased from Promega (Madison, WI). For cell culture, media were purchased from Mediatech Inc. (Herndon, VA), fetal bovine serum (FBS) from HyClone (Logan, UT), and all other supplements and reagents from BioWhittaker (Walkersville, MD).

Cell Culture. The human breast cancer cell lines MDA-MB-231 (EGFR+, ER-), BT-20 (EGFR+, ER-), and T-47D (EGFR-, ER+) were obtained from the American Type Culture Collection (Manassas, VA) and maintained at 37°C / 5% CO₂ in complete medium [CM; Eagle's minimum essential medium (EMEM; MDA-MB-231 & BT-20) or RPMI (T-47D) with 10% fetal bovine serum (FBS, Hyclone, Logan, UT), 2mM L-glutamine, 1mM sodium pyruvate, 0.01 mg/ml bovine insulin (Sigma, St. Louis, MO), 0.1 mM nonessential amino acids (EMEM), 4.5 g/L glucose (Sigma, RPMI), and 10 mM HEPES (RPMI)]. Medium was renewed 3 times per week, and cells were passaged as they reached 85 - 90% confluence, as observed by a confocal light microscope. Experimental medium [EM; EMEM or RPMI with 1%FBS, supplements as above, 1% fatty acid free bovine serum albumin (BSA, Sigma)], and different concentrations of DHA (0, 0.5, 1.5, 3, 7.5, 15, 30, or 60 µg/ml) or LA (0, 0.5, 1, 3, 6.25, 12.5, 25 or 58 µg/ml) dissolved in ethanol (EtOH) was used for all proliferation assays, and cells were maintained during experiments in 5% CO₂ at 37°C. All EM had a final EtOH concentration of 1%. For PUFA ratio experiments, DHA and LA were added to experimental wells, such that wells contained a total FA concentration of either 50, 75, 100, or 200µM, with DHA/LA ratios of 0.1, 0.25, 0.5, 2, 4, 8 for each concentration, as well as controls within each concentration that had either DHA or LA alone.

Proliferation Assays. To determine if DHA or LA could alter the growth of EGFR+ or - cells in culture, MDA-MB-231, BT-20, and T-47D breast cancer cells were seeded in 96-well plates (2500 cells/well) and allowed to attach overnight in CM. Cells were then grown in EM for 6 d, with medium renewal on d 3. On d 6, cells were incubated with fresh EM (no FA) and 20 μ l of MTS/PMS reagent for 4 h. MTS bioreduction by cells was measured via SpectraMax250 microplate reader (490 nm, Molecular Devices, Sunnydale, CA) and analyzed with SOFTmax[®]PRO 4.0 software (Molecular Devices). Data were expressed as sample absorbance at 490 nm, an indicator of cell number.

Statistics. All statistical analyses were performed using the SAS System (ver. 8.02, SAS Institute, Inc., Cary, NC). Briefly, data were log transformed and treatments compared using ANOVA. Post hoc tests were performed, using Dunnett Hsu tests to compare each DHA and LA concentration to those samples receiving no PUFA supplementation. Significance was set at an alpha level of 0.05.

RESULTS

Effect of DHA/LA ratio on the proliferation of MDA-MB-231, BT-20, and T-47D breast cancer cells lines. Cell number of each breast cancer line, as determined by MTS/PMS assay, was unaffected by the DHA/LA ratio. However, when observing the ratio data, we discovered that the total amount of fatty acid in the medium (DHA and LA combined) appeared to have an inverse relationship with the cell number in MDA-MB-231 cells (**FIGURE 3-1**). Based on this information, we performed cell growth experiments using different concentrations of either DHA or LA supplements in the medium.

Effect of DHA and LA on the proliferation of MDA-MB-231, BT-20, and T-47D breast cancer cell lines. Both DHA and LA resulted in a biphasic growth response in MDA-MB-231 cells compared to unsupplemented cells, with a peak growth response occurring at 2 $\mu\text{g/ml}$ DHA (6 $\mu\text{mol/L}$) ($P=0.0023$) and 0.75 $\mu\text{g/ml}$ LA (2.7 $\mu\text{mol/L}$) ($P=0.003$) (**FIGURE 3-2**). In BT-20 cells, DHA had a quadratic growth effect ($P=0.008$), and LA resulted in no significant growth response compared to unsupplemented cells at any concentration, although a biphasic trend was observed ($P=0.3383$) (**FIGURE 3-3**). T-47D cells did not respond to LA or DHA at any concentration, when compared to unsupplemented cells (data not shown).

DISCUSSION

Previous epidemiological and murine study data have suggested that omega-3 (n-3) and omega-6 (n-6) PUFA may have opposing effects on the development and growth of breast cancer cells. Specifically, these reports indicated that a diet providing an n-3-to-n-6 PUFA ratio of 1:1.8 may inhibit the incidence and progression of mammary cancer (Noguchi et al., 1997). Based on these data, we hypothesized that supplementing the culture media of breast tumor cells with PUFA would affect the proliferation of the cells *in vitro*. Specifically, it was hypothesized that as the n-3-to-n-6 PUFA ratio (DHA-to-LA ratio) of the cell culture media was increased, proliferation of cells would decrease.

The EGFR/MAPK pathway has been shown to play a role in the development and progression of mammary cancer. There are several targets in this pathway that may be influenced by n-3 and n-6 PUFA. In fact, several research groups (Collett et al., 2001; Toker 1998; Golubic et al., 1991; Tsai et al., 1989; Sermon et al., 1996) have demonstrated the ability of PUFA to modulate effectors of the EGFR/MAPK pathway. Based on this information, it was hypothesized that breast tumor cells expressing EGFR would more likely be influenced by the alteration of n-3-to-n-6 PUFA ratio than those without high EGFR expression.

Cell culture media of EGFR+ and EGFR- cells were supplemented with DHA and LA, such that the n-3-to-n-6 PUFA ratio ranged from 0-10, with a total fatty acid concentration ranging from 25 - 200 μ M. In all cell types, PUFA ratio had no effect on proliferation of the cells, however; it was observed that in EGFR+ MDA-MB-231 cells, cell proliferation decreased as total fatty acid concentration increased. This suggested that, rather than having opposing effects on cell proliferation, DHA and LA were having similar (and potentially additive) effects on cell proliferation.

To further investigate this relationship, cell proliferation experiments were performed, supplementing cell culture media with DHA (0 - 60 $\mu\text{g/ml}$) and LA (0 - 58 $\mu\text{g/ml}$) alone. As expected, based on PUFA ratio results, DHA and LA had no effect on EGFR- T-47D cells, but did appear to influence growth of EGFR+ BT-20 and MDA-MB-231 cells.

When compared to unsupplemented cell growth, LA and DHA supplementation resulted in stimulation of growth in MDA-MB-231 cells, with a peak response occurring at 2 $\mu\text{g/ml}$ for DHA and 0.75 $\mu\text{g/ml}$ for LA. The effect of LA observed in this cell line is in agreement with LA-stimulation at similar concentrations in the literature (Rose and Connolly, 1990; Noguchi et al., 1995). Our results demonstrated that DHA and LA produced a similar growth response in MDA-MB-231 cells, as opposed to the inverse effects reported previously in the literature (Rose and Connolly, 1990; Noguchi et al., 1995). Several factors could account for these discrepancies. In some of these previous experiments, cells were grown in medium which did not contain FBS, whereas our experimental medium was supplemented with 1% FBS. It is possible that growth factors or other compounds in FBS could impede the action of DHA on the cells. Although previous research reported that FA-induced effects on MDA-MB-231 cells were considerably more noticeable in cells grown in serum-free medium, when compared with those grown in serum-supplemented medium (Rose and Connolly, 1989), these studies, as well as those performed in our lab (data not shown) also showed that cells grown in serum-depleted medium had very little basal cell growth compared with cells grown in 1% FBS. We chose to supplement our cells with 1% FBS, insuring that cells had adequate basal cell growth, so that any limiting or stimulating factor on their growth was due to fatty acid supplementation.

BT-20 and MDA-MB-231 cells had drastically different responses to PUFA supplementation, which may be, in part, related to two factors. Both cell lines are considered EGFR+ and ER-, meaning that they stain strongly, immunohistochemically, for EGF and weakly

for ER. Besides these similarities, however, these cell types can have vastly different genomes, resulting in differing responses to various treatments and/or conditions. These dissimilar responses are in agreement with clinical observations that women with tumors expressing similar levels of EGFR and/or ER may have very different responses to treatments. Clearly, there are genetic factors, other than expression of these receptors, that influence tumor cell proliferation in response to various treatment conditions.

Secondly, BT-20 cells and MDA-MB-231 cells are different “ages,” meaning that the time from primary harvesting until these experiments were performed are not similar. Passage number represents the number of times a cell line has been passaged from the time of initial harvesting from a patient. For the experiments reported here, BT-20 cells were between 30 and 40 passages, whereas MDA-MB-231 cells were between 290-300 passages. Certainly, the longer that a cell line has been in culture, the more opportunities for genetic drift to occur. So, even if these cells lines as primary cultures may have had a similar growth response to PUFA supplementation, the vast difference in their “passage age” could drastically alter those response patterns.

These results may indicate that our assumptions regarding the physiological role of n-3 and n-6 PUFA need to be re-evaluated. Specifically, n-6 PUFA are often referred to as pro-inflammatory and n-3 PUFA as anti-inflammatory. Certainly, the inflammatory mediators derived from n-6 PUFA (2 and 4 – series eicosanoids) have greater pro-inflammatory affects than the 3 and 5-series eicosanoids derived from n-3 PUFA (Yammamoto et al., 1996; Lands, 1996); however, we must note that both n-3 and n-6 PUFA are mediators of inflammation. It is conceivable that if n-3 and n-6 PUFA have similar roles in inflammation, albeit at different magnitudes, that they might have similar roles in other cellular and biochemical pathways, including those that regulate cancer progression.

This idea seems to be supported in current case-controlled, prospective studies investigating the relationship of breast adipose n-3 and n-6 PUFA and breast cancer. Bagga et al., (2002) reported that, while total n-3 and n-6 PUFA were significantly higher in breast cancer patients than controls, the mean n-3 to n-6 PUFA ratio was comparable in cases and controls, suggesting that n-3 and n-6 PUFA intake, as reflected in adipose tissue, may not be as strong of a risk factor in breast cancer as previously suggested. Maillard et al., (2002) also reports that the long-chain n-3 to total n-6 PUFA ratio was the same in both breast cancer cases and controls, suggesting that increasing n-3 PUFA and decreasing n-6 in the diet may not be as strong of a risk modifier as earlier epidemiological reports claimed. In a case controlled multi-center study in Europe (Simonsen et al., 1998), “little consistent association” was observed between n-6 or n-3 adipose PUFA and breast cancer. Therefore, claims made by early epidemiological studies (Lands et al., 1996; Lanier et al., 1996) do not appear to be supported by data from recent, case-controlled studies investigating either PUFA ratio or individual PUFA classes.

Data reported here seem to be in agreement with these recent case-controlled studies. We hypothesized, based on the assumption that n-3 and n-6 PUFA would have opposing effects on the growth of tumor cells, that increasing the n-3 to n-6 PUFA ratio in the culture medium of tumor cells would result in a decrease in the proliferation of those cells. What we observed was that PUFA ratio within a constant amount of total fatty acids provided had no effect on the proliferation of either cell type, but that total proliferation was inversely related to total PUFA supplementation. This seemed to imply, not that the different classes of PUFA were opposing each other, but rather having similar effects on the growth of cells. To further investigate this possibility, we evaluated growth of cells when supplemented with either LA n-6 or DHA n-3, and found that, in fact, LA and DHA were promoting similar growth responses in both cell types. Interestingly, peak growth promotion in cells was obtained at a concentration of DHA that was

slightly more than twice the concentration of LA needed for the same response. This seems to support the idea that n-3 and n-6 PUFA have similar roles physiologically, but that n-3 must be present at higher levels to elicit a physiological response than that required for n-6.

The changes that can occur as a cell line is immortalized for long-term culture use are important to consider when attempting to extrapolate results to a “whole animal” conclusion. While cell culture is an invaluable tool for investigating the molecular mechanisms behind certain diseases and the treatments that may be useful in dealing with them, response differences and genetic variation between immortalized cell lines and primary tumor cells must be considered.

SUMMARY and IMPLICATIONS

Considering the vital role of nutrients in the cellular physiology of both normal and diseased tissue, determining the role of nutrients in the prevention and progression of chronic diseases is a necessity. This area of study is complicated, however, because of the complex interactions that nutrients have with each other and other compounds in the body. Data reported here suggest that n-3 and n-6 PUFA may have similar, rather than opposing, effects on the proliferation of breast tumor cells, and that these PUFA may be exerting these effects, in part, through the EGFR pathway. Further investigation into the EGFR pathway and others should be done to determine where these PUFA may be exerting their effects in tumor cells, and whether n-3 and n-6 have similar effects in these pathways.

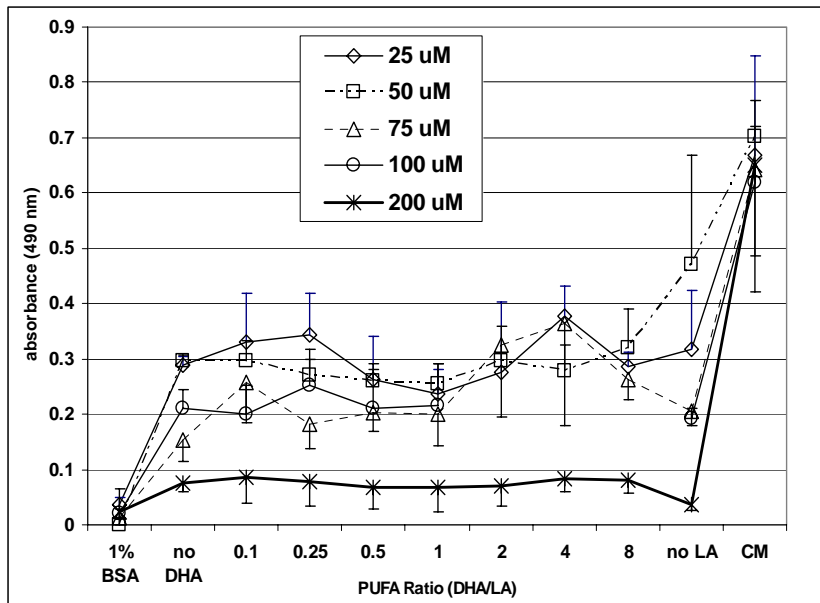


FIGURE 3-1 Effect of PUFA ratio on proliferation of MDA-MB-231 cells. Cells were cultured in medium containing 1%FBS/ 1% BSA and supplemented with docosahexanoic (DHA; 22:6 n-3) and linoleic (LA; 18:2 n-6) acid, such that the DHA/LA ratio ranged from 0.1 – 8, as well as samples with no LA or no DHA added. Total PUFA concentrations ranged from 0-200 $\mu\text{mol/L}$. Negative control cells were cultured in medium with 1% BSA only (no PUFA) and positive control cells in complete medium (CM, 10% FBS). At d 6, cells were analyzed via PMS/MTS assay; cell number represented as absorbance at 490 nm. PUFA ratio had no effect on proliferation at any concentration. Total concentration of PUFA had an inverse relationship with cell growth ($p < 0.001$).

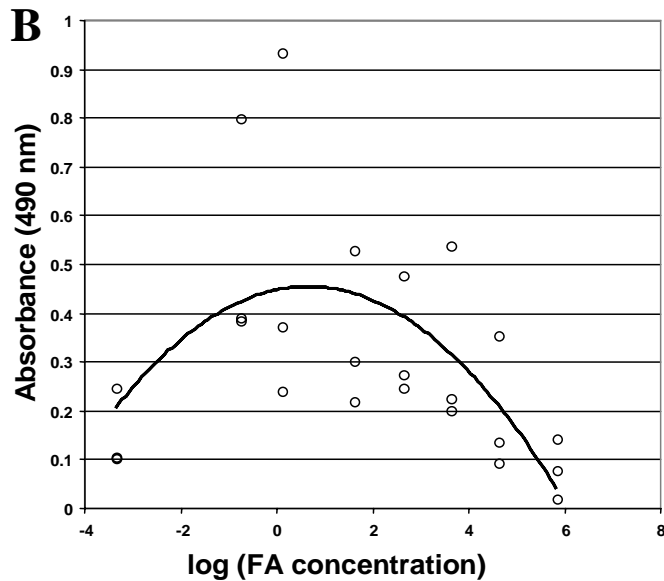
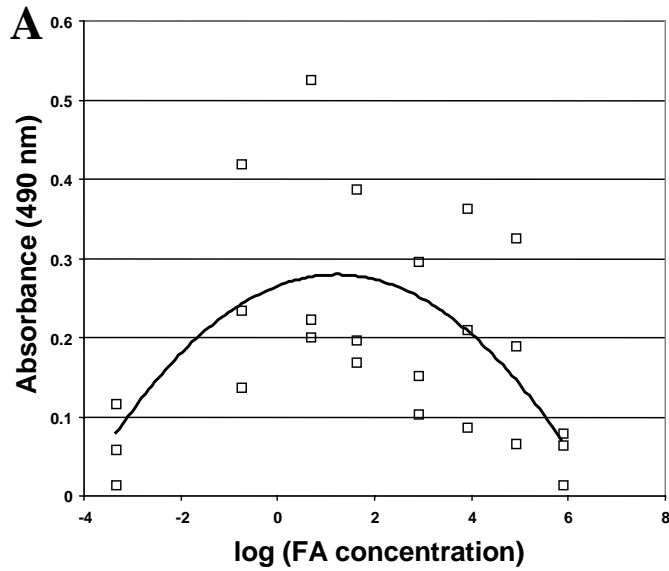


FIGURE 3-2 Effect of PUFA on MDA-MB-231 cell proliferation. Cells were cultured in medium containing 1% FBS and supplemented with concentrations from (A) 0-60 $\mu\text{g/ml}$ of docosahexanoic acid (DHA, 22:6 n-3) or (B) 0-58 $\mu\text{g/ml}$ linoleic (LA, 18:2 n-6). At day 6, cells were analyzed via PMS/MTS assay; cell number represented by absorbance at 490 nm. Both DHA and LA affected cell proliferation quadratically, with peak stimulation occurring at 2 $\mu\text{g/ml}$ (DHA; $p=0.0023$) and 0.75 $\mu\text{g/ml}$ (LA; $p=0.0030$). Each point represents the mean of 3 triplicate experiments ($n=9$). Quadratic curves fitted to the graphs were found to significantly explain the data (DHA, $y=0.0097x^2 + 0.041x + 0.2627$, $R^2=0.3658$; and LA, $y=0.0155x^2 + 0.0212x + 0.4445$, $R^2 = 0.3941$).

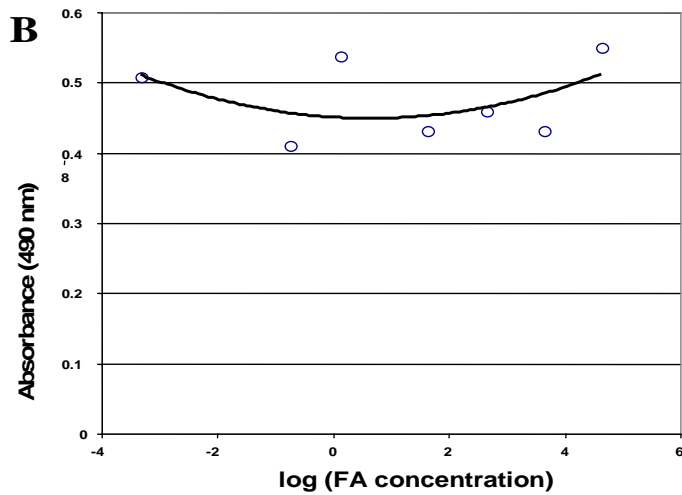
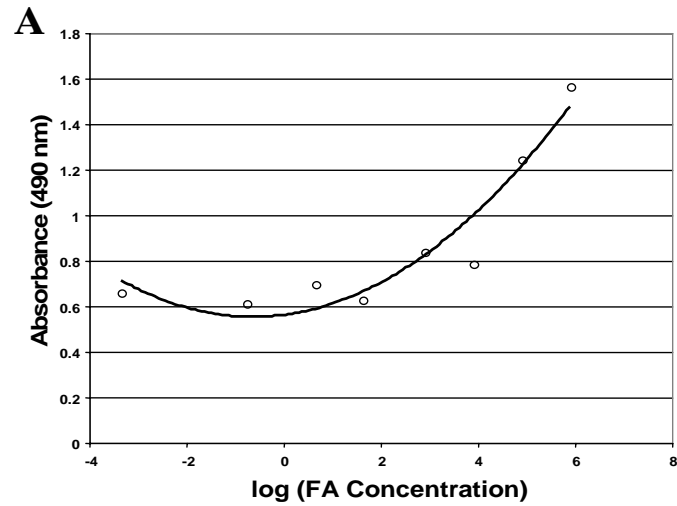


FIGURE 3-3 Effect of PUFA on BT-20 cell proliferation. Cells were cultured in medium containing 1% FBS and supplemented with concentrations from (A) 0-60 $\mu\text{g/ml}$ of docosahexanoic acid (DHA, 22:6 n-3) or (B) 0-58 $\mu\text{g/ml}$ linoleic (LA, 18:2 n-6). At day 6, cells were analyzed via PMS/MTS assay; cell number represented by absorbance at 490 nm. DHA supplementation stimulated cell growth at concentrations of 6.25 $\mu\text{g/ml}$ and higher ($p=0.009$); LA supplementation had no significant effect on cell proliferation ($p=0.3383$). Each point represents the mean of 3 triplicate experiments ($n=9$). Quadratic curves fitted to the graphs were found to significantly explain the data (DHA, $y=0.0127x^2 + 0.0266x + 0.559$, $R^2=0.9115$).

Chapter 4: Docohosahexanoic and Linoleic Acid modulate epidermal growth factor/ mitogen-activated protein kinase pathway in MDA-MB-231 and BT-20 human breast cancer cells lines

Docohosahexanoic and Linoleic Acid modulate epidermal growth factor/ mitogen-activated protein kinase pathway in MDA-MB-231 and BT-20 human breast cancer cells lines

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Running Title: PUFA and EGFR/MAPK in ER- Breast Cancer Cells

INTRODUCTION

The role of dietary fat in the progression of mammary cancer has been a source of much debate over the past 30 years. In recent years, it has been shown that specific types of dietary fat consumed seem to be more indicative of cancer risk than the amount of overall dietary fat consumed. However, a quick review of the literature shows that there is a plethora of evidence that seemingly contradicts itself. For example, it has been reported that omega-3 fatty acids (n-3) inhibit, whereas omega-6 (n-6) fatty acids stimulate the growth of xenografted mammary tumor cells in murine models (Borgeson et al., 1989; Gonzales et al., 1991; Rose and Connolly, 1993; Rose et al., 1995, 1996; Noguchi et al., 1997). Epidemiological evidence at first seemed to support the animal studies (Kromann and Green, 1980; Kaizer et al., 1989; Lands et al., 1990; Wynder et al., 1991; Caygill et al., 1996; Lanier et al., 1996), but prospective and cohort studies now seem to suggest only weak evidence, if any, of a protective effect of n-3 PUFA against breast cancer (Kohlmeier et al., 1997), although a relationship still seems to be present between high intakes of n-6 PUFA and risk of breast cancer (Kohlmeier et al., 1997; Wirflat et al., 2002; Rissanen et al., 2003). Clearly, the effect of n-6 and n-3 PUFA on breast cancer risk, development, and progression is complex, and if a relationship exists, it likely involves numerous mechanistic pathways. Our lab was particularly interested in the relationship between n-6 and n-3 PUFA and signaling pathways that may be modulators of mammary cancer progression.

The epidermal growth factor receptor (EGFR), a 170 kD transmembrane glycoprotein, is a member of a group of receptors known as the EGFR family. EGFR has many known ligands, two of which are transforming growth factor alpha (TGF- α) and epidermal growth factor (EGF). It has been suggested that aberrant expression of EGFR and its ligands may play a role in the development of many neoplasms, including mammary cancer (for a review, see Salomon et al.,

1995). One of the methods by which EGFR may stimulate the growth of mammary cancer cells is through the mitogen-activated protein kinase (MAPK) signal transduction cascade. EGFR and MAPK have both been implicated as independent prognostic indicators for decreased relapse time and overall survival time, and it has been suggested that overexpression and increased activity of this pathway's components may be responsible, in part, for the progression of certain highly aggressive, non-hormone responsive breast cancers (Seshari, et al., 1996; Mueller et al., 2000; Gee et al., 2001).

PUFA have been shown to modulate the action of the EGFR/MAPK pathway in certain cell types (Denys et al., 2002 and 2001), however very little research exists examining the effect of n-3 and n-6 PUFA on the action of EGFR/MAPK in breast cancer cells. Most studies investigating the potential role of PUFA in EGFR/MAPK signaling concentrate on the activity levels of downstream pathway effectors (pMAPK, pMEK, pRaf). Another way that PUFA might influence this signaling, however, is through altering the endocytosis of activated EGFR in the cells. Regulation of receptor-mediated endocytosis (internalization) of ligand-bound EGFR has been suggested as an important way to control cell signalling (Martin-Fernandez et al., 2000; Bao et al., 2000; Pennock and Wang, 2003). Cell-mediated endocytosis of ligand-bound membrane receptors can result in degradation of ligand and receptor – resulting in an abolishment of mitogenic signal – or in recycling of ligand- bound or -unbound receptor to the surface (for complete review, see Wiley and Burke, 2001). Regulation of the endocytic recycling of ligand-bound EGFR through chemo- or radiation-treatment of cells has been demonstrated (Caraglia et al., 1999; Oksvold et al., 2002). Considering the growing role that EGFR/MAPK seems to have in the progression of hormone-resistant breast cancer, and the possibility that targeting this pathway may inhibit tumor growth, we examined the effect of linoleic (LA, 18:2 n-6) and docosahexanoic (DHA; 22:6 n-3) acid on the activity of the

EGFR/MAPK pathway by measuring changes in receptor internalization, and phosphorylation (activity) of downstream components of this pathway (Raf, MEK1/2, and MAPK).

MATERIALS and METHODS

Cell Culture

Estrogen receptor positive [ER+, T-47D (HTB-133)] and –negative [ER-, MDA-MB-231 (HTB-26), BT-20 (HTB-19)] were obtained from the American Type Culture Collection (ATCC, Manassas, VA). ER- cells lines were maintained in Minimal Essential Medium (MEM, Mediatech, Herndon, VA) supplemented with 10% fetal bovine serum (FBS, HyClone, Logan, UT), 2mM L-glutamine (BioWhittaker, Walkersville, MD), 1.0 mM sodium pyruvate (BioWhittaker), 0.1 mM non-essential amino acids (BioWhittaker), and 0.01 mg/ml bovine insulin (Sigma). ER+ cells were maintained in RPMI 1640 medium (Mediatech) supplemented with FBS, glutamine, sodium pyruvate, and insulin as above, as well as 10 mM HEPES (BioWhittaker) and 2.5g/L d-glucose (Sigma). All cells were maintained in T-75 cm² flasks at 37°C with 5% CO₂ according to the recommendations of the supplier, with media renewal 3 times per week, and passaging when cells reached 85-90% confluence as observed using a confocal light microscope. For experiments, cells were maintained in experimental medium [EM, MEM or RPMI containing 1% FBS, 1% fatty acid-free bovine serum albumin (BSA, Sigma), and supplements as above].

Analysis of pRaf, pMEK, and pMAPK in cell lines

Cells were seeded into 35 mm plates (1-3 x 10⁵ cells/well), allowed to attach in culture medium for 24 h, and then maintained for 6 d in EM supplemented with LA (0, 0.75, 12.5, or 58 µg/ml) or DHA (0, 2, 15, or 60 µg/ml). On d 7, cells were incubated for 2 h in serum-free EM

(no fatty acids), stimulated with human-recombinant epidermal growth factor (EGF, R&D Systems, Minneapolis, MN) (32 ng/ml, 8 min), lysed in sample buffer containing 62.5 mM Tris-HCl (pH 6.8), 2% w/v SDS, and 10% glycerol, and processed via Western immunoblotting. Protein concentrations were quantified using BioRad DC Protein Assay Kit II (Hercules, CA). Bromphenol blue (0.01%) and β -mercaptoethanol (5%) were added to each sample, and total protein was electrophoresed on a 10% SDS-PAGE gel (BioRad). Following electrophoresis, gels were electrotransferred to a PVDF membrane via semi-dry method, and membranes were blocked in TBST with 5% non-fat dry milk (pMAPK) or 5% BSA (pRaf, pMEK) and stained with appropriate primary antibodies (CST), followed by secondary antibodies conjugated to horseradish peroxidase (CST). Bands for pMAPK, pRaf, or pMEK were visualized via chemiluminescence (CST), and quantitated using densitometric analysis (NIH Imager 1.63). All data is expressed as densitometric units/ μ g total protein. Time course experiments were performed to determine the appropriate length of time for EGF incubation to observed changes in pRaf, pMEK, and pMAPK. For time course experiments, cells were incubated with 100 ng/ml EGF as described above for 0, 1, 2, 4, 8, 15, 30, 60, and 90 min, as well as 30 sec. Positive controls were incubated in 10% FBS and no EGF. Subsequently, concentration curve experiments were performed to determine the appropriate amount of EGF in which to incubate cells. Cells were incubated for 8 min in 0, 0.1, 0.32, 1, 3, 10, 32, 100, or 320 ng/ml EGF. For both time course and concentration curve experiments, cells were harvested as described above.

Receptor internalization assays

Cells were seeded into 12-well plates ($1-3 \times 10^5$ cells/well), allowed to attach for 24 h in culture medium, and then maintained in EM as described above for 6 d. On d 6, cells were incubated with 0.5 ml serum-free medium containing 0.1% BSA for 1 h, washed with PBS, and

incubated in HEPES binding medium (7.4 pH MEM, 0.1% BSA, 0.04 M HEPES), containing (¹²⁵I)-epidermal growth factor (EGF, ~70,000 cpm/ml, 1150 Ci/mmol) and 5 nM non-labeled EGF at 37°C for 5, 15, 30, 90, or 180 min. To determine internalized radioactivity, cells were washed with acid (50 mM glycine, 100 mM NaCl, pH 3.0), solubilized (1 M NaOH) and radioactivity measured via an auto-gamma counter (Cobra II, Packard Instruments, Meriden, CT). To determine total (internal plus external) radioactivity, additional cells from the same plate receiving no acid wash, were also processed. Radioactivity was measured via an auto-gamma counter and expressed as counts per minute (cpm). Excess unlabeled EGF (100 nM) was added to additional cells to determine non-specific binding. Data were expressed as corrected cpm (internalized or total counts minus non-specific binding).

Fatty acid analysis of cell lines

Cells were seeded in triplicate in 35 mm plates (1-2 x 10⁵ cells/ plate), allowed to attach for 24 h in culture medium, and then maintained in EM as described above. On d 6, cells were washed with PBS, trypsinized, and resuspended in PBS, and stored at -20°C until fatty acid analysis. Fatty acid analysis was performed in the laboratory of Dr. J.H. Herbein (Department of Dairy Science, Virginia Tech, Blacksburg, VA). Briefly, lipids were extracted with chloroform/methanol, according to Folch et al. (1957) and dried under a stream of nitrogen. Undecenoate (an internal standard, NuCheck Prep, Elysian, MN) was added to the lipid extracts, and the samples were directly methylated using 0.5 N NaOH in methanol, according to Park and Goins (1994). Methyl esters were analyzed by gas chromatography (Hewlett-Packard 5890 A), Sunnyvale, CA), using a fused silica capillary column (100 m x 0.25 mm i.d.) (CP-Sil 88, Chrompack, Middleburg, The Netherlands). Fatty acids were quantified by integration using

peak area response factors to known amounts of individual fatty acids. Cellular LA and DHA were expressed as a percentage of total cellular FA present.

Statistics

All data were statistically analyzed using SPSS 12.0 (Chicago, IL). To determine the effect of DHA or LA concentration on pRaf, pMEK, pMAPK levels, as well as internalization of EGFR, ANOVA was used. Bonferroni-corrected pairwise comparisons were performed when comparing specific times or concentrations within a fatty acid. Statistical significance was set at a P=0.05 level.

RESULTS

Effect of EGF and PUFA supplementation on pRaf, pMEK, and pMAPK levels in EGF-stimulated BT-20 and MDA-MB-231 cells.

pRaf, pMEK, and pMAPK were undetectable in T-47D (EGFR-/- ER+) cells stimulated with EGF (data not shown). EGF stimulation resulted in increased levels of pRaf in BT-20 cells at all concentrations of DHA (p <0.01) and LA (p<0.01) supplementation (**FIGURE 4-1**). In contrast, pRaf levels remained unchanged by EGF stimulation in both LA and DHA supplemented MDA-MB-231 cells (**FIGURE 4-2**). In both BT-20 (**FIGURE 4-1**) and MDA-MB-231 cells (**FIGURE 4-2**), EGF stimulation resulted in increased levels of pMEK in both DHA (BT-20, p<0.05) and LA (BT-20, p<0.05) supplemented cells, although this increase did not reach our level of statistical significance in MDA-MB-231 cells (DHA, p=0.06; LA, p=0.08). EGF stimulation resulted in increased pMAPK levels in both BT-20 (**FIGURE 4-1**) and MDA-MB-231 cells (**FIGURE 4-2**). This increase was observed in cells supplemented with either DHA (p<0.05) or LA (p<0.01).

Supplementation with DHA or LA did not have any observable effect on the level of pRaf in BT-20 cells, compared to unsupplemented cells (**FIGURE 4-1**). A trend was observed in which pMAPK levels in BT-20 cells increased as DHA supplementation increased and in which LA supplementation produced a biphasic response in pMAPK levels, but this trend did not reach statistical significance (**FIGURE 4-1**). Cells supplemented with LA showed biphasic changes in pMEK levels ($p < 0.01$), whereas levels of pMEK in cells supplemented with DHA increased ($p < 0.01$) as the concentration of DHA increased (**FIGURE 4-1**).

pRaf levels were unaffected by DHA or LA supplementation in the culture medium of MDA-MB-231 cells (**FIGURE 4-2**). A trend in pMAPK levels was observed in cells supplemented with either DHA or LA, with higher pMAPK levels in cells supplemented with 0 or the highest concentration of FA and lower pMAPK levels at moderate concentrations of the FA (**FIGURE 4-2**). Although this trend did not reach statistical significance ($p = 0.2$), a similar, statistically significant pattern was observed in pMEK levels ($p < 0.05$) (**FIGURE 4-2**).

Effect of DHA and LA supplementation on EGFR internalization in BT-20 and MDA-MB-231 cells.

At every concentration of DHA or LA supplementation, internalization of EGFR in BT-20 cells (measured as acid-stable radioactivity) increased with time to 90 min, and decreased from 90 min to 180 min ($p < 0.001$) (**TABLE 4-1**). This same curve was observed in the total EGFR (radioactivity measured in non-acid treated cells) ($p < 0.001$) of cells at every level of DHA supplementation (**TABLE 4-1**). Furthermore, at every time point, within each concentration of DHA and LA supplemented, total EGFR measured in BT-20 cells was higher than internalized

EGFR (**FIGURE 4-5**) ($p < 0.05$). At every concentration of LA and DHA supplemented, the percent of total EGFR that internalized increased over time (**FIGURE 4-5**).

In MDA-MB-231 cells, at every concentration of DHA and LA supplemented, internalized and total EGFR increased over time (DHA, $p < 0.005$; LA, $p < 0.05$) (**TABLE 4-2**). Total EGFR was consistently higher than internalized EGFR at each time point for each concentration of supplemented DHA and LA ($p < 0.05$) (**TABLE 4-2**). At every concentration of LA and DHA, the percent of total EGFR that was internalized increased over time (**FIGURE 4-6**).

Effect of LA and DHA supplementation on cellular fatty acid composition of BT-20 and MDA-MB-231 cells.

Cellular LA and DHA in BT-20 and MDA-MB-231 cells were directly related to LA and DHA provided in the culture medium (**FIGURE 4-7, 4-8**). As DHA supplementation in culture medium increased, the percentage of cellular DHA increased ($p < 0.001$) in both cell types. Similarly, as LA supplementation was increased in the culture medium, the percentage of cellular LA increased ($p < 0.0001$). A decrease ($p < 0.02$) in cellular DHA in both BT-20 and MDA-MB-231 cells as LA supplementation increased was observed.

DISCUSSION

There have been many studies suggesting the role of PUFA in the development and progression of breast cancer. Furthermore, EGFR/MAPK have both been shown to be independent prognostic indicators of breast cancer risk and decreased survival time. PUFA have been shown to alter MAPK activation in certain types of cells, but little evidence has been provided concerning the role of PUFA in modulating EGFR/MAPK in breast cancer cells.

Previous data from our lab (data not shown) demonstrated that supplementation with DHA and LA resulted in alterations in the proliferation of EGFR+/ER- breast tumor cell lines, while exerting no effect in EGFR-/ER+ breast tumor cell lines. Based on those results, we hypothesized that n-3 and n-6 PUFA might modulate the growth of steroid hormone-insensitive tumors, in part, through the EGFR/MAPK pathway.

We report here that n-3 and n-6 PUFA do, in fact, modulate the EGFR/MAPK pathway in a manner consistent with cell growth. We observed that cellular concentrations of LA and DHA are influenced by supplementation of the same in the culture medium of both BT-20 and MDA-MB-231 cells. Interestingly, supplementation with LA resulted in an observable and significant decrease in cellular DHA; DHA supplementation did not have the same influence on cellular LA. This may suggest a cellular preference in LA incorporation into cell membranes, at the expense of DHA. In BT-20 cells, supplementation with DHA resulted in a dose-dependent decrease in acid-stable radioligand-bound EGFR, suggesting either a decrease in ligand-bound EGFR internalization or increased time of recycling to the cell membrane. This decrease in “internalized EGFR” was accompanied by dose-dependent increases in both pMEK and pMAPK levels (although not significant for pMAPK). These results are consistent with cellular conditions that would promote proliferation. Receptor internalization as a means of regulating tyrosine kinase signaling is well-established (reviewed in Wiley and Burke, 2001), and decreases in a cell’s ability to internalize (and subsequently degrade) ligand-bound EGFR would be expected to result in increased downstream signaling to pMEK and pMAPK. Interestingly, no affect was observed in pRaf levels in the experimental conditions for which the above changes in pMAPK, and pMEK were observed. A possible explanation for this, and the non-significant trend observed in pMAPK, is the time of EGF stimulation. pRaf is an upstream effector for pMEK and pMAPK, so any changes in pRaf would likely be seen at a time point prior to

changes observed in pMEK or pMAPK. Furthermore, an influence of treatment on pMAPK stimulation would likely occur at a time later than that observed for pMEK, which is consistent with our observed, but non-statistically significant, changes in pMAPK levels.

In BT-20 cells, supplemented with LA, similar internalization patterns for EGFR were observed. However, pMEK and pMAPK exhibited biphasic responses to increases in LA concentrations. This suggests that LA influence on pMAPK and pMEK in BT-20 cells may be exerted independently of EGFR. LA and the product of its metabolism, arachidonic acid, have been shown to alter the activity of protein kinase C isoforms, which can directly influence the activity of pMEK and pMAPK downstream of EGFR (Lester, 1990; Fan et al., 1990; Chen and Murakami, 1992). Our data are consistent with the idea that LA may be influencing pMEK and pMAPK in BT-20 cells through the protein lipase/ diacylglycerol (DAG) mediated protein kinase c pathway, or another EGFR-independent pathway.

In MDA-MB-231 cells, supplementation with DHA or LA resulted in biphasic responses in “internalized EGFR,” pMEK, and pMAPK levels. Specifically, unsupplemented cells or cells supplemented with high concentrations (DHA – 183 $\mu\text{mol/L}$; LA – 207 $\mu\text{mol/L}$) had lower levels of “internalized EGFR,” pMAPK, and pMEK than cells supplemented with moderate levels of LA or DHA (peak at DHA – 6 $\mu\text{mol/L}$; LA – 2.67 $\mu\text{mol/L}$). This pattern of behavior is consistent with the pattern of LA-influenced cell growth observed in previous studies (Rose and Connolly, 1990), in which growth was biphasic in response to LA supplementation, with peak stimulation at LA concentration of 0.75 $\mu\text{g/ml}$. The biphasic response observed may be explained, in part, by the essential nature of LA (and DHA). Some level of LA and DHA are required for the cells to grow normally, but a concentration in excess of that which is essential seems to result in aberrant growth patterns (either excess stimulation or inhibition of growth).

We report here that in BT-20 cells alterations in downstream effectors of EGFR (specifically pMEK and pMAPK) by LA and DHA supplementation are similar to the patterns of altered growth observed under the same conditions. In contrast, MDA-MB-231 cells had alterations in pMEK and pMAPK that were the opposite of observed growth patterns. In preliminary studies with BT-20 and MDA-MB-231 cells, BT-20 cells had much higher EGFR expression than MDA-MB-231 cells (data not shown), which is in agreement with previously reported data in the literature (Koga et al., 1990). It is conceivable that proliferation in cell lines with an amplified EGFR pathway might be regulated more strongly through that EGFR pathway than in cells with fewer expressed EGFR. Therefore, even though BT-20 and MDA-MB-231 cells both had proliferation alterations in response to PUFA supplementation, BT-20 alterations in EGFR pathway components would be expected to more strongly correlate to growth patterns than would MDA-MB-231 cells, where pathways other than EGFR may have a stronger role in proliferation.

The most interesting results observed were the similarities in action of LA and DHA. Historically, LA and DHA have been suggested to have opposing effects in breast tumor cells based on assumptions regarding their biological roles. For example, n-6 PUFA are often referred to as pro-inflammatory and n-3 PUFA as anti-inflammatory. Certainly, the inflammatory mediators derived from n-6 PUFA (2 and 4 – series eicosanoids) have greater pro-inflammatory effects than the 3 and 5-series eicosanoids derived from n-3 PUFA (Yamamoto et al., 1996; Lands, 1996); however, we must note that both n-3 and n-6 PUFA are mediators of inflammation. It is conceivable that if n-3 and n-6 PUFA have similar roles in inflammation, albeit at different magnitudes, that they might have similar roles in other cellular and biochemical pathways, including those that regulate cancer progression.

This idea seems to be supported in current case-controlled, prospective studies investigating the relationship of breast adipose n-3 and n-6 PUFA and breast cancer. Bagga et al., (2002) reported that, while total n-3 and n-6 PUFA were significantly higher in breast cancer patients than controls, the mean n-3 to n-6 ratio PUFA ratio was comparable in cases and controls. Maillard et al., (2002) also reports that the long-chain n-3 to total n-6 PUFA ratio was the same in both breast cancer cases and controls. In a case controlled multi-center study in Europe (Simonsen et al., 1998), “little consistent association” was observed between n-6 or n-3 adipose PUFA and breast cancer. These recent studies seem to contradict earlier reports (Lands et al., 1996; Lanier et al. 1996) implicating dietary n-3 and n-6 PUFA intakes in the prevention or development of breast cancer.

Data reported here seem to be in agreement with these recent case-controlled studies, as well as previous growth studies performed in our lab (data not shown). LA and DHA were observed to promote similar growth responses in both cell types. Interestingly, peak growth promotion in cells was obtained at a concentration of DHA that was slightly more than twice the concentration of LA needed for the same response. This seems to support the idea that n-3 and n-6 PUFA have similar roles physiologically, but that a higher level of n-3 concentration, compared to n-6, is required to elicit a physiological response. Perhaps both DHA and LA have similar mitogenic effects on breast cancer cells *in vivo*, but that greater dietary intakes of n-3 PUFA are necessary to elicit these effects, compared to n-6 PUFA.

SUMMARY and IMPLICATIONS

Nutrients play a vital role in the physiology of both normal and malignant tissues. Elucidating the specific influence that nutrients have on cellular pathways of tumor cells can provide us with information useful in drug development, insight regarding the use of nutrients as adjuvant therapy for cancer patients, as well as information useful in making dietary

recommendations for the prevention of neoplasia. Explicating the exact effect of a nutrient in these processes can be difficult, considering the complex interactions that nutrients have with each other and other biological compounds. This difficulty can be observed in the plethora of data regarding the role of n-3 and n-6 PUFA in breast tumor cell growth, much of which appears to be conflicting. Data reported here suggest that, rather than having opposing effects on breast tumor cells, n-3 and n-6 PUFA may influence growth of steroid hormone-resistant breast tumor cells comparably, and that these effects may be exerted, in part, through the EGFR and its downstream effectors. Further research regarding the effects of PUFA on the EGFR/MAPK pathway should be performed to illuminate the relationship between specific dietary fat classes and progression of steroid hormone-resistant breast tumors through signal transduction pathways. Investigating n-3 and n-6 PUFA regulation of the many pathways that are involved in cross-talk with the EGFR/MAPK cascade is warranted, to determine whether n-3 and n-6 PUFA exert their effects on EGFR/MAPK signaling through similar or different pathways.

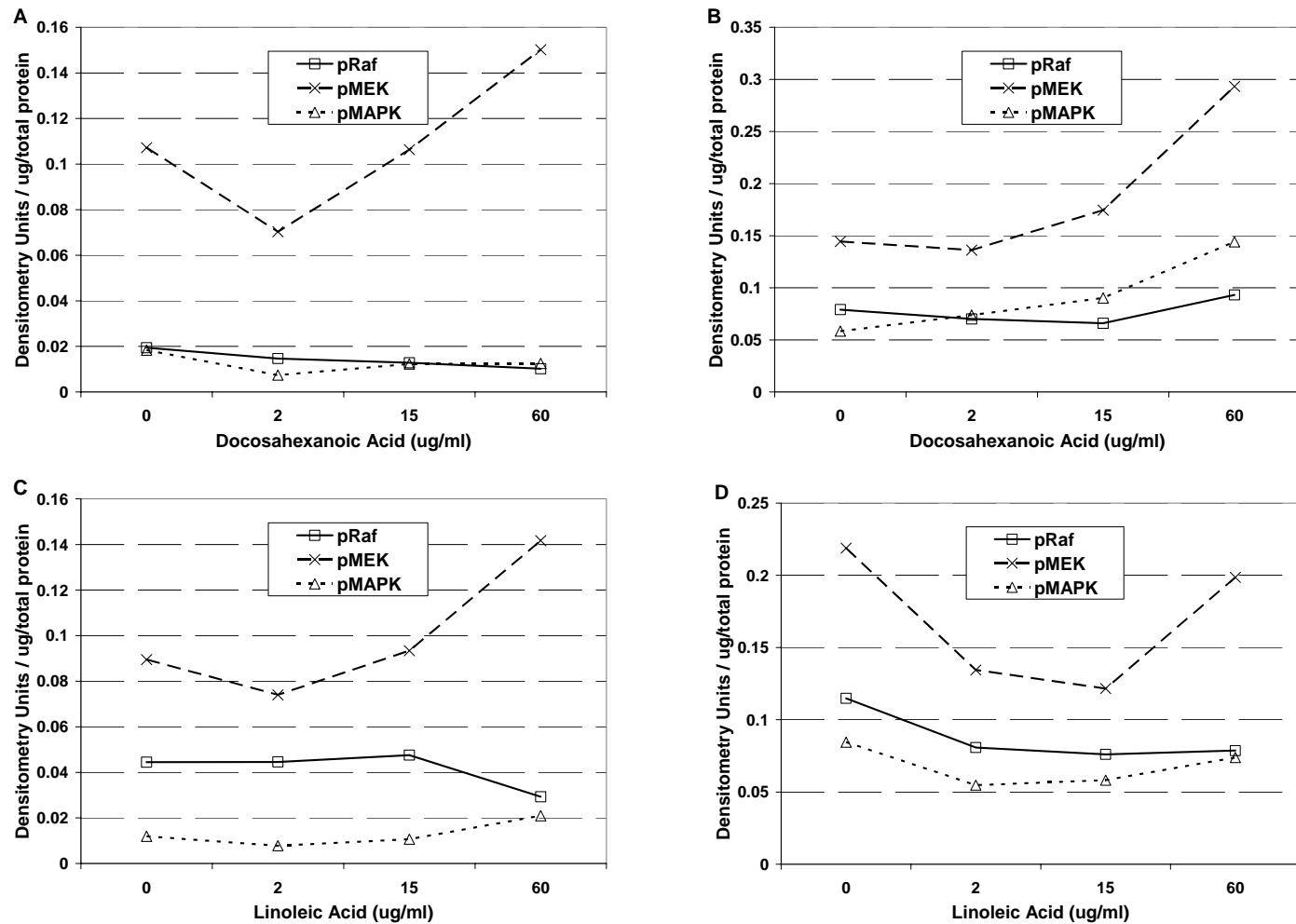


FIGURE 4-1 Effect of EGF & PUFA on pRaf, pMEK, & pMAPK in BT-20 cells. Cells were cultured in medium containing 1%FBS/1%BSA and supplemented with 0 to 60 μ g/ml docosahexanoic (A & B, DHA, 22:6n-3) or linoleic (C & D, LA, 18:2n-6) acid. At d 6, cells were stimulated with 0 or 32 ng/ml EGF, and pRaf, pMEK, or pMAPK levels were analyzed using immunoblotting / chemiluminescence assays. EGF (B & D, 32 ng/ml) stimulation resulted in significant increases in pRaf, pMEK, and pMAPK in both DHA & LA supplemented cells vs. EGF-unstimulated cells (A & C). (A & B) pMEK ($p < 0.01$) & pMAPK exhibited dose-responsive increases to DHA supplementation, although changes in pMAPK levels did not reach statistical significance ($p = 0.3$). (C & D) LA supplementation resulted in biphasic changes in pMEK ($p < 0.01$) and pMAPK levels, although changes in pMAPK did not reach statistical significance ($p = 0.4$). pRaf was unaffected by either DHA or LA supplementation. Bars represent means of 2 experiments ($n = 4$).

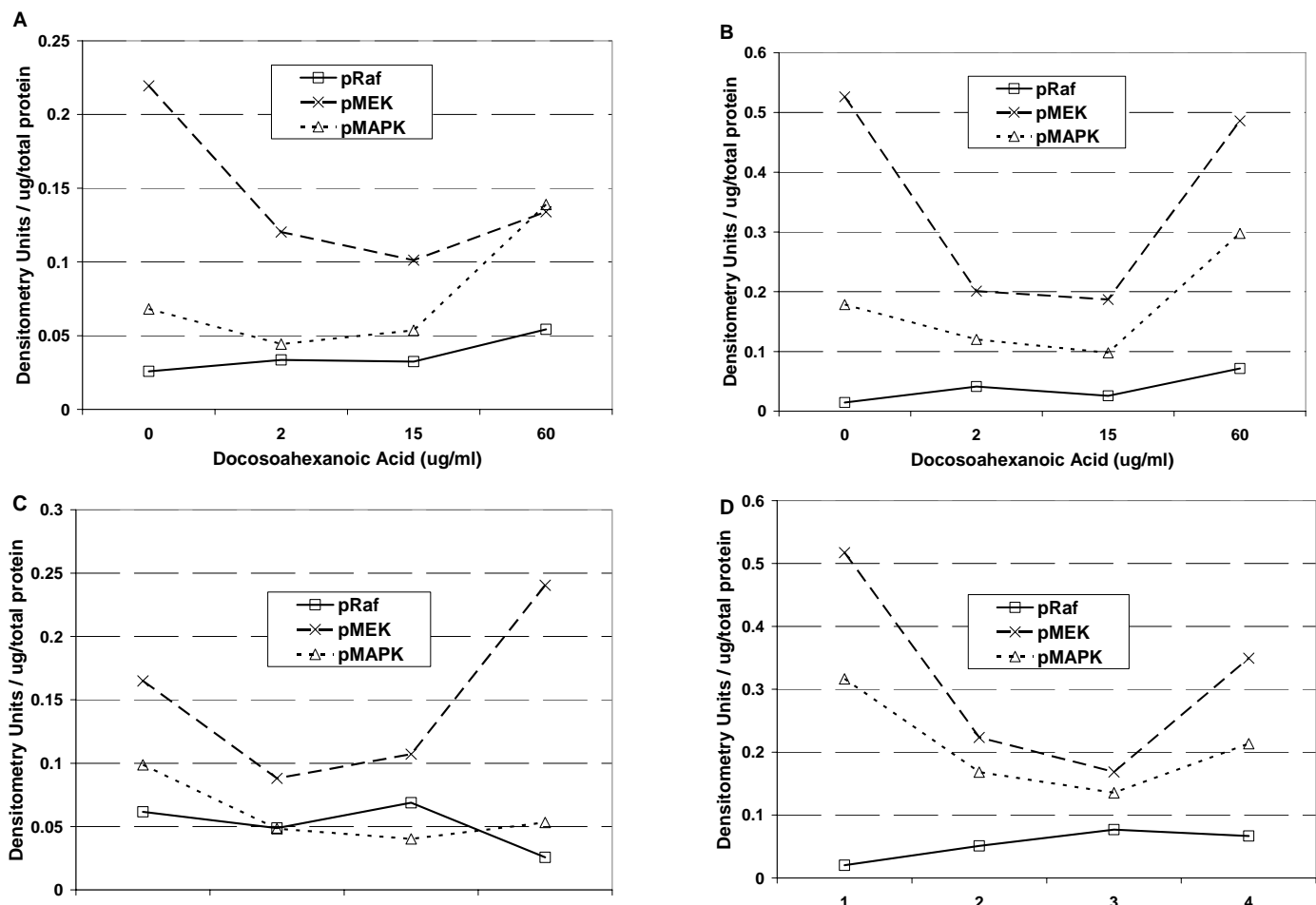


FIGURE 4-2 Effect of EGF & PUFA on pRaf, pMEK, & pMAPK in MDA-MB-231 cells. Cells were cultured in medium containing 1%FBS/1%BSA and supplemented with 0 to 60 $\mu\text{g/ml}$ docosahexanoic (A & B, DHA, 22:6n-3) or linoleic (B, LA, 18:2n-6) acid. At d 6, cells were stimulated with 0 or 32 ng/ml EGF, and pRaf, pMEK, or pMAPK levels were analyzed using immunoblotting / chemiluminescence assays. EGF (B & D, 32 ng/ml) stimulation resulted in significant increases in pRaf, pMEK, and pMAPK in both DHA & LA supplemented cells vs. EGF unstimulated cells (A & C). pMEK ($p < 0.05$) & pMAPK exhibited quadratic responses to both DHA & LA supplementation, although changes in pMAPK levels did not reach statistical significance ($p = 0.25$). pRaf was unaffected by either DHA or LA supplementation. Bars represent means of 2 experiments ($n = 4$).

Table 4-1 Effect of DHA and LA on EGFR internalization* in BT-20 cells. *acid-stable radioactivity represents internalized EGFR. (All data have been corrected for non-specific binding).

DHA concentration (ug/ml)		0					2				
Time (min)		5	15	30	90	180	5	15	30	90	180
Acid-Stable Radioactivity (cpm)		59.1	1141	1796	3543	2960.5	72	840	1668.5	2149.5	2007.5
		74	987	1797	3076	2660.5	112	789	1469.5		1181.5
Total Radioactivity (cpm)		96	2613.5	3477	4743	3184.5	1569.5	2593	4035	3752.5	2970.5
Ave. % Internalized Radioactivity (cpm)		70%	38%	46%	69%	78%	6%	34%	42%	57%	62%

DHA concentration (ug/ml)		15					60				
Time (min)		5	15	30	90	180	5	15	30	90	180
Acid-Stable Radioactivity (cpm)		54	661.5	1337	2478	1017	22	106	117	358.5	205.5
		78	688.5	1114	2323	516	15	131	326	410.5	216.5
Total Radioactivity (cpm)		1243	1960.5	2851.5	3057.5	1250.5	169		532.5	703.5	419.5
Ave. % Internalized Radioactivity (cpm)		5%	35%	43%	73%	58%	7%	44%	51%	53%	56%

LA concentration (ug/ml)		0					0.75				
Time (min)		5	15	30	90	180	5	15	30	90	180
Acid-Stable Radioactivity (cpm)		59.1	1141	1796	3543	2960.5	79.5	138.5	1418	3162	2820
		74	987	1797	3076	2660.5	99.5	97.5	1219	1936	2195
Total Radioactivity (cpm)		96	2613.5	3477	4743	3184.5	1468	434.5	3926.5	4155.5	2417.5
Ave. % Internalized Radioactivity (cpm)		70%	38%	46%	69%	78%	6%	32%	36%	64%	79%

LA concentration (ug/ml)		12.5					58				
Time (min)		5	15	30	90	180	5	15	30	90	180
Acid-Stable Radioactivity (cpm)		137	1025	1341	3657.5	1965	168	867.5	1200	1598	1091.5
		97	707	939		1248	148	684.5	918	665	848.5
Total Radioactivity (cpm)		1647.5	2280.5	3324.5	4278	1942	1612.5	1278.5	1703	1854.5	1358.5
Ave. % Internalized Radioactivity (cpm)		7%	38%	34%	86%	76%	10%	43%	45%	48%	64%

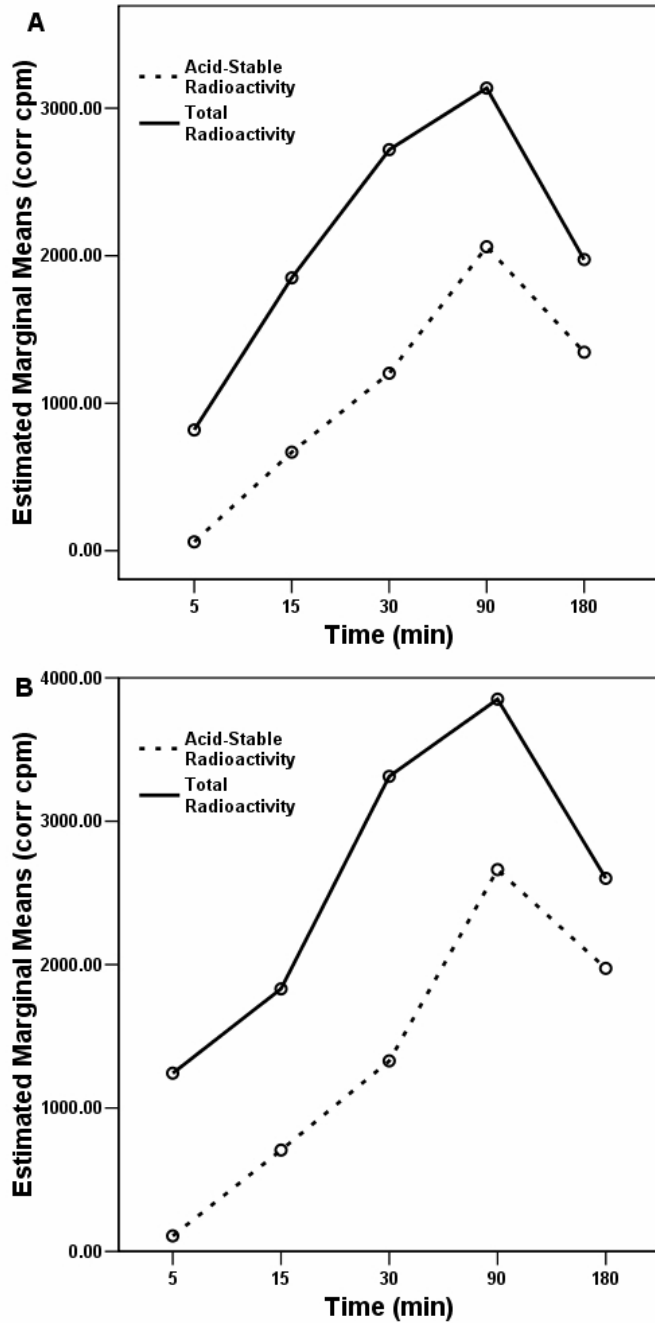


FIGURE 4-3 Internalized EGFR vs. total EGFR in DHA- & LA-supplemented BT-20 cells. Cells were cultured in medium containing 1%FBS/1%BSA, supplemented with 0-60 $\mu\text{g/ml}$ DHA (A) or LA (B). On d 6, cells were incubated in HEPES binding medium, containing (^{125}I)-epidermal growth factor (EGF, $\sim 70,000$ cpm/ml, 1150 Ci/mmol) and 5 nM non-labeled EGF at 37°C for 5, 15, 30, 90, or 180 min. Cells receiving an acid wash were analyzed for internalized radioactivity. Total radioactivity was determined from additional cells receiving no acid wash. Background radioactivity was determined in cells receiving excess (100 nM) EGF, and adjusted radioactivity was expressed as corrected counts per minute. At every time point, within each concentration of DHA or LA, total EGFR measured in BT-20 cells was higher than internalized EGFR ($p < 0.05$). Points represent mean of 2 experiments ($n=4$).

Table 4-2 Effect of DHA and LA on EGFR internalization* in MDA-MB-231 cells. *acid-stable radioactivity represents internalized EGFR. (All data have been corrected for non-specific binding).

DHA concentration (ug/ml)	0					2					
	Time (min)	5	15	30	90	180	5	15	30	90	180
Acid-Stable Radioactivity (cpm)		47.5	167	229.5	251.5	241	68.5	222.5	327	320	348.5
		65.5	137	145.5	70.5	242	72.5	176.5	218	285	306.5
Total Radioactivity (cpm)		187.5	230.5	267.5	206.5	256.5	217	362	353.5	392	468.5
Ave. % Internalized Radioactivity (cpm)		206.5	202.5	226.5	246.5	310.5	312	295	326.5	386	378.5
		29%	70%	76%	71%	85%	27%	61%	80%	78%	77%

DHA concentration (ug/ml)	15					60					
	Time (min)	5	15	30	90	180	5	15	30	90	180
Acid-Stable Radioactivity (cpm)		50	190.5	212.5	270.5	339	27	138.5	68	217.5	207
		39	176.5	164.5	196.5	328	37	103.5	49	169.5	226
Total Radioactivity (cpm)		281.5	287.5	335	363.5	443	164.5	174.5	84	236	246
Ave. % Internalized Radioactivity (cpm)		267.5	201.5	313	293.5	413	108.5	139.5	64	220	280
		16%	75%	58%	71%	78%	23%	77%	79%	85%	82%

LA concentration (ug/ml)	0					0.75					
	Time (min)	5	15	30	90	180	5	15	30	90	180
Acid-Stable Radioactivity (cpm)		47.5	137	145.5	251.5	241	72	251.5	314	281.5	342
		65.5	167	229.5	70.5	242	57	206.5	257	184.5	244
Total Radioactivity (cpm)		187.5	230.5	226.5	206.5	265.5	280	329.5	327.5	413.5	425
Ave. % Internalized Radioactivity (cpm)		206.5	202.5	267.5	246.5	310.5	311	181.5	284.5	424.5	386
		29%	70%	76%	71%	84%	22%	90%	93%	56%	72%

LA concentration (ug/ml)	12.5					58					
	Time (min)	5	15	30	90	180	5	15	30	90	180
Acid-Stable Radioactivity (cpm)		54.5	204.5	238.5	257	211.5	85	196.5	133	230.5	272
		50.5	159.5	187.5	284	307.5	81	171.5	150	219.5	223
Total Radioactivity (cpm)		318	314.5	269	352	349	186	259.5	155	256	312
Ave. % Internalized Radioactivity (cpm)		323	264.5	270	367	398	254	275.5	198	318	333
		16%	63%	79%	75%	69%	38%	69%	80%	78%	77%

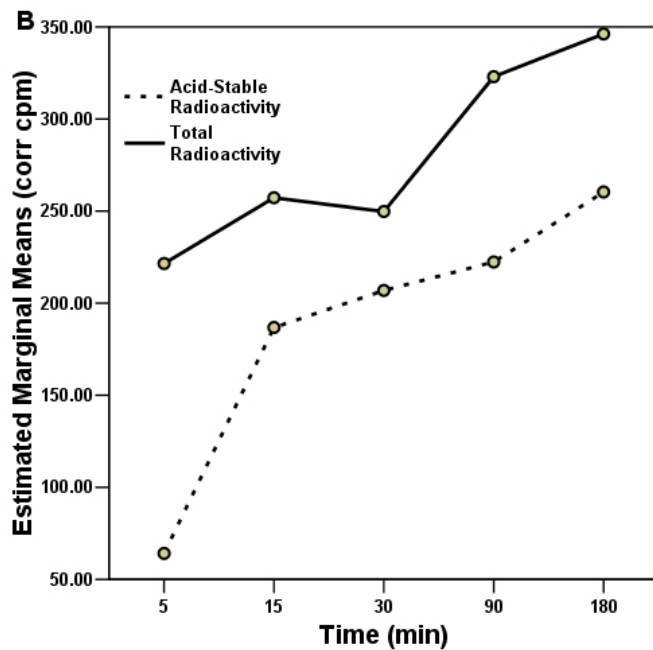
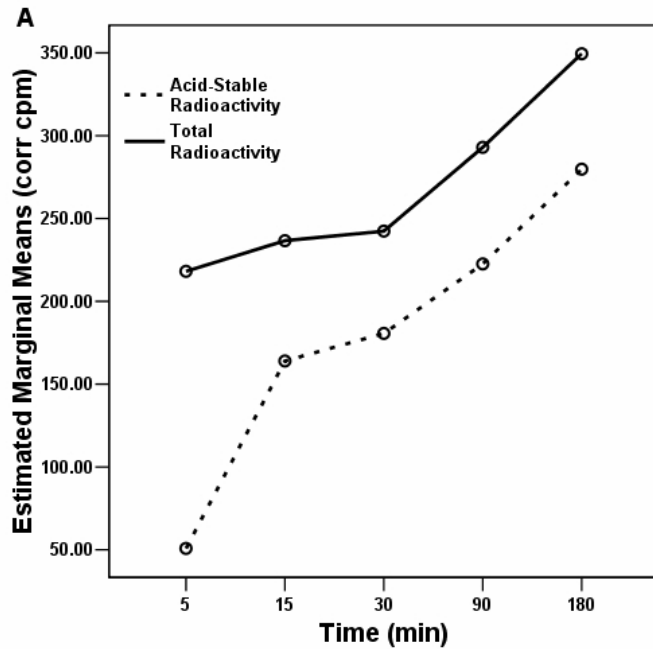


FIGURE 4-4 Internalized EGFR vs. total EGFR in DHA- & LA-supplemented MDA-MB-231 cells. Cells were cultured in medium containing 1%FBS/1%BSA, supplemented with 0-60 $\mu\text{g/ml}$ DHA (A) or LA (B). On d 6, cells were incubated in HEPES binding medium, containing (^{125}I)-epidermal growth factor (EGF, $\sim 70,000$ cpm/ml, 1150 Ci/mmol) and 5 nM non-labeled EGF at 37°C for 5, 15, 30, 90, or 180 min. Cells receiving an acid wash were analyzed for internalized radioactivity. Total radioactivity was determined from additional cells receiving no acid wash. Background radioactivity was determined in cells receiving excess (100 nM) EGF, and adjusted radioactivity was expressed as corrected counts per minute (corr cpm). Total EGFR was consistently higher than internalized EGFR at each time point for each concentration of DHA & LA ($p < 0.05$). Points represent mean of 2 experiments ($n=4$).

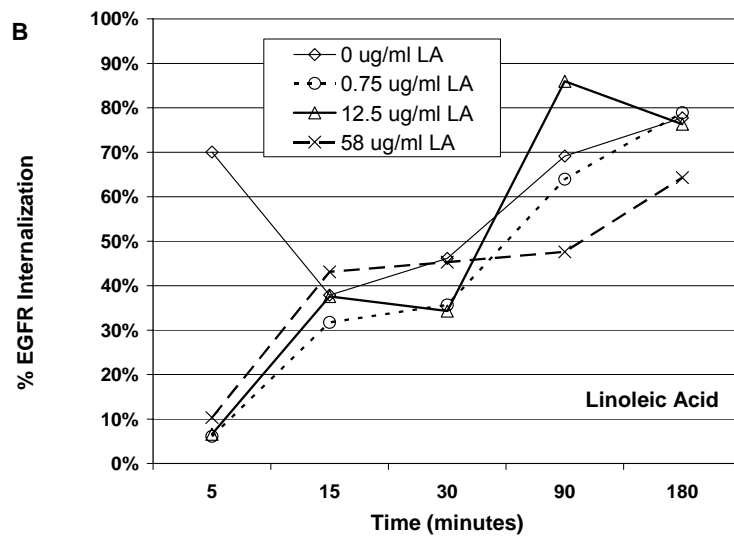
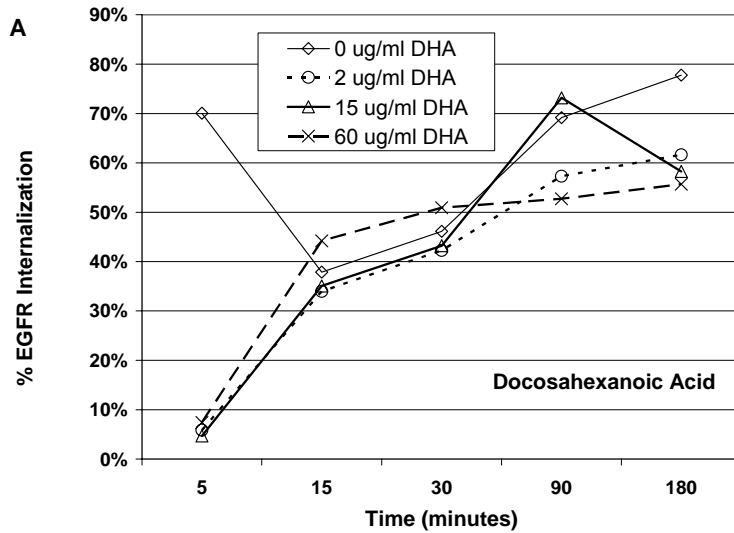


FIGURE 4-5 Effect of PUFA concentration on EGFR internalization in BT-20 cells. Cells were cultured in medium containing 1%FBS/1%BSA, supplemented with 0-60 $\mu\text{g/ml}$ DHA. On d 6, cells were incubated in HEPES binding medium, containing (^{125}I)-epidermal growth factor (EGF, $\sim 70,000$ cpm/ml, 1150 Ci/mmol) and 5 nM non-labeled EGF at 37°C for 5, 15, 30, 90, or 180 min. Cells receiving an acid wash were analyzed for internalized radioactivity. Total radioactivity was determined from additional cells receiving no acid wash. Background radioactivity was determined in cells receiving excess (100 nM) EGF, and adjusted radioactivity was expressed as corrected counts per minute (corr cpm). % Internalization was calculated by dividing total corrected radioactivity by acid-stable corrected radioactivity. At every concentration of DHA or LA supplementation beyond 0 $\mu\text{g/ml}$, internalization of EGFR increased over time.

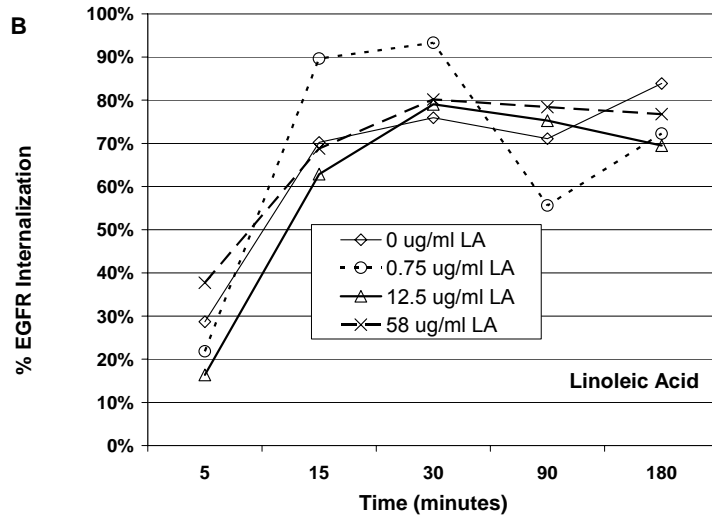
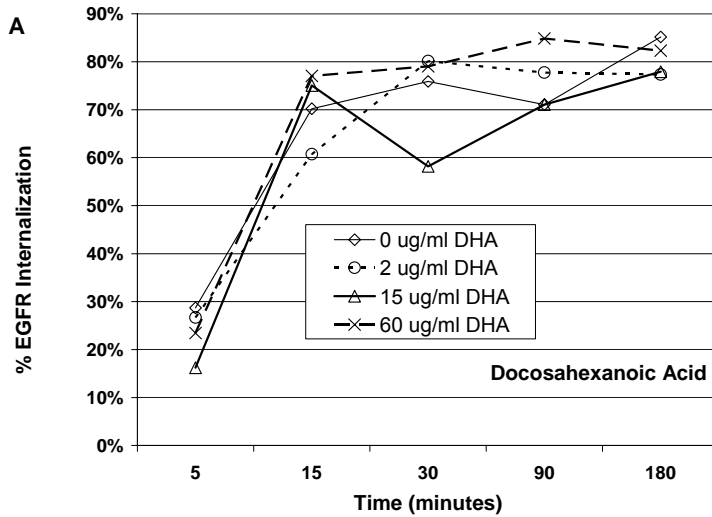


FIGURE 4-6 Effect of PUFA concentration on EGFR internalization MDA-MB-231 cells. Cells were cultured in medium containing 1%FBS/1%BSA, supplemented with 0-60 $\mu\text{g/ml}$ DHA. On d 6, cells were incubated in HEPES binding medium, containing (^{125}I) -epidermal growth factor (EGF, $\sim 70,000$ cpm/ml, 1150 Ci/mmol) and 5 nM non-labeled EGF at 37°C for 5, 15, 30, 90, or 180 min. Cells receiving an acid wash were analyzed for internalized radioactivity. Total radioactivity was determined from additional cells receiving no acid wash. Background radioactivity was determined in cells receiving excess (100 nM) EGF, and adjusted radioactivity was expressed as corrected counts per minute (corr cpm). % Internalization was calculated by dividing total corrected radioactivity by acid-stable corrected radioactivity.

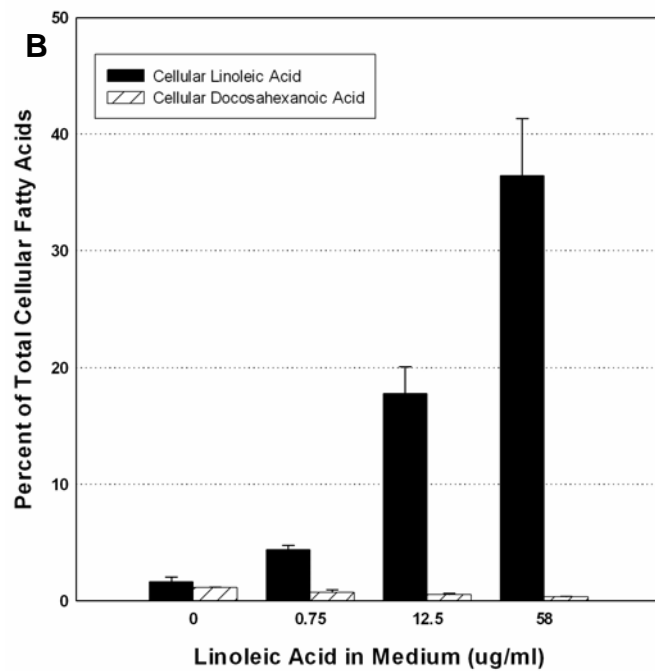
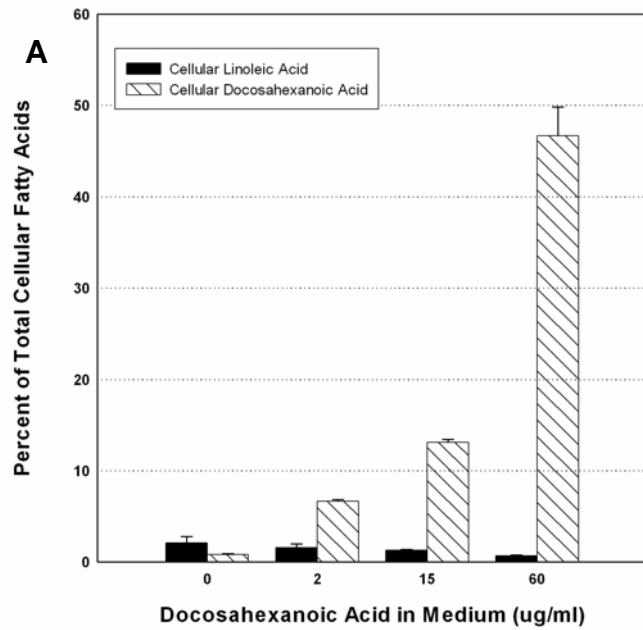


FIGURE 4-7 Effect of DHA & LA supplementation on cellular DHA & LA composition in BT-20 cells. Cells were maintained in medium containing 1% FBS/1%BSA and 0 – 60 $\mu\text{g/ml}$ DHA (A) or LA (B). On d 6, cells were harvested and total cellular fatty acids were analyzed via gas chromatography. Cellular DHA & LA increased as DHA & LA in culture medium increased, respectively ($p < 0.001$). LA supplementation resulted in decreases in cellular DHA ($p < 0.02$). Bars represent means of 2 experiments (N=4)

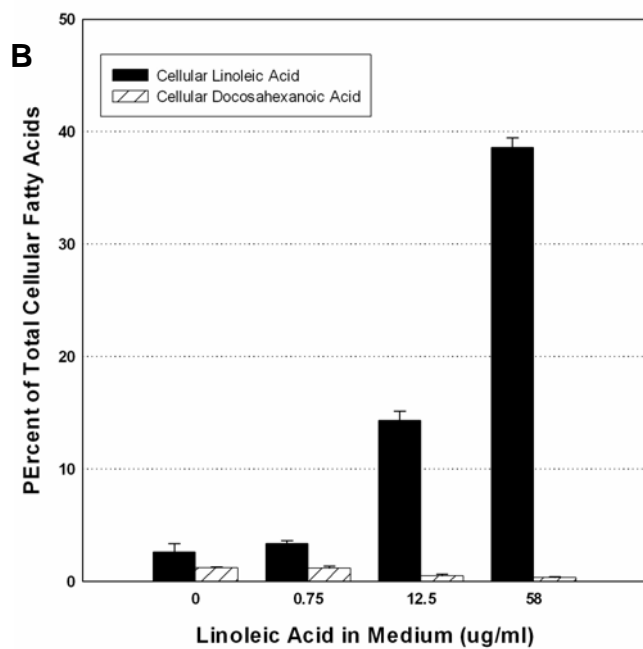
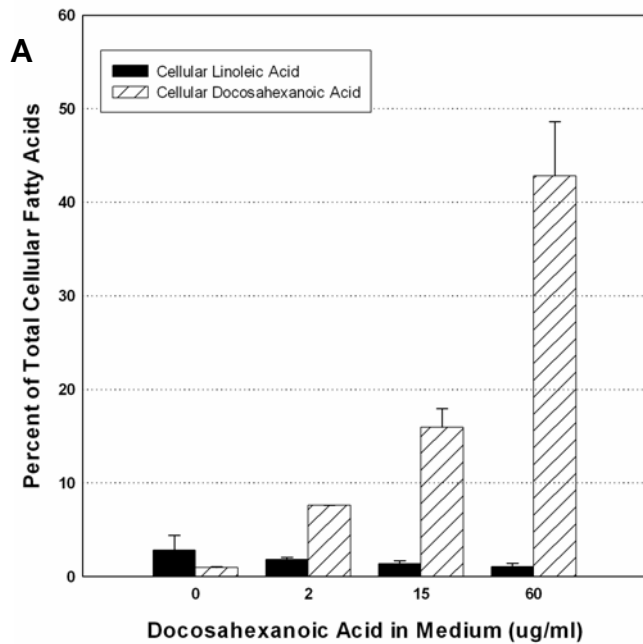


FIGURE 4-8 Effect of DHA & LA supplementation on cellular DHA & LA composition in MDA-MB-231 cells. Cells were maintained in medium containing 1% FBS/1%BSA and 0 – 60 $\mu\text{g/ml}$ DHA (A) or LA (B). On d 6, cells were harvested and total cellular fatty acids were analyzed via gas chromatography. Cellular DHA & LA increased as DHA & LA in culture medium increased, respectively ($p < 0.001$). LA supplementation resulted in decreases in cellular DHA ($p < 0.02$). Bars represent means of 2 experiments (N=4)

Chapter 5: Summary and Implications

There are no reports in the literature regarding the role of MAPK in feline mammary cancer, but we have shown that MAPK can be detected in the tissues of cats. Considering the similarities in histology and pathophysiology that exist between human and feline mammary cancer, it may be useful to measure active MAPK levels in feline tumor tissues to determine its usefulness as a prognostic indicator of disease.

Alterations in dietary PUFA ratio were reflected in the mammary adipose tissue of cats. Furthermore, increasing the dietary n-3-to-n-6 PUFA ratio for 24 weeks in cats reduced active MAPK levels in the WBC and mammary adipose tissue, a reduction which was statistically significant for WBC. There is accumulating evidence that the local environment of the mammary fat pad may play an integral role in modulating the proliferation of neoplastic mammary cells. Those reports, along with these data, may indicate that altering mammary adipose composition via the diet may be an important tool in regulating the growth of breast tumors, and that the action of PUFA in breast cells may be, in part, through its effects on the EGFR/MAPK pathway.

In contrast, alterations of n-3-to-n-6 PUFA ratio in the culture medium of human breast tumor cells had no effect on proliferation when total fatty acids ranged between 25 and 200 $\mu\text{mol/L}$. When evaluated separately, both DHA and LA were shown to influence the growth and EGFR/MAPK signaling in EGFR+ tumor cells lines, while no observable effects on growth or EGF-stimulated signaling were seen in EGFR- tumor cells. Differences in cell response to PUFA in EGFR+ and EGFR-, suggest that PUFA are exerting their effects in these cells through the EGFR/MAPK pathway or another pathway not highly expressed in ER+/EGFR- cell lines. Collectively, epidemiological reports over the past 20 years, utilizing food frequency questionnaires and dietary recall, have suggested that n-3 PUFA inhibit and n-6 PUFA stimulate the growth of breast tumor cells. Data reported here are in conflict with those data, but in agreement with recent case-controlled studies, in which “little consistent association” was observed between n-6 or n-3 adipose PUFA and breast cancer (Bagga et al, 2002; Maillard et al., 2002; Simonsen et al., 1998).

We report that n-3 and n-6 PUFA have similar effects on the proliferation and EGFR/MAPK signaling in EGFR+ breast tumor cells. Interestingly, peak promotion of growth and signaling was obtained at a molar concentration of DHA that was slightly more than twice that needed of LA to elicit the same responses. This seems to support the concept that n-6 and n-3 PUFA may have similar roles in the pathophysiology of breast cancer, but that a higher concentration of n-3 is needed to elicit the same physiological response. This could mean that at low dietary intakes of total fat, n-3 PUFA would seemingly have a protective effect against breast tumor development, not due to some innate inhibitory action, but rather because of the requirement of its presence at a higher concentration to elicit the same stimulatory effects as n-6 PUFA.

It is important to note, however, that data reported here are from cells in culture. Changing the culture media in a controlled system may result in physiological cell changes that would not necessarily be observed *in vivo*, where many biological factors can influence the mechanism(s) by which n-3 and n-6 PUFA (as well as other nutrients or synthetic compounds) may act. While it is important to utilize controlled, *in vitro* systems to elucidate possible mechanism(s) by which potential anti-neoplastic agents exert their effects, we must use caution in extrapolating our results to the “whole animal.”

With that in mind, based on the data reported here, adjusting dietary intake of n-3 and n-6 PUFA as a means of adjuvant breast cancer therapy, or simply as a means to lower one’s risk factors for disease, may still be a reasonable suggestion. Rather than merely changing the ratio of n-3-to-n-6 in the diet, however, an alternate suggestion may be more beneficial. Reducing the total n-3 and n-6 PUFA in the diet, while simultaneously increasing the n-3-to-n-6 ratio, may provide the most plausible protection against the development and progression of mammary cancer in the human and pet populations.

Appendix A: Materials and Methods

Analysis of pMAPK in felines

Animal Care

Twenty-eight healthy, neutered, domestic short-hair cats (in a pre-existing colony; females n=24; males n=4), ranging in age from 4-13 years (weighing 3-6 kg) were housed in a climate- and environmentally-controlled room at the Virginia-Maryland Regional College of Veterinary Medicine (VMRCVM), Blacksburg, VA. The study protocol was approved by the Virginia Tech Animal Care Committee. Cats were randomly allocated into each of four treatment groups (n=7 per group). Due to the wide age-range, cats were blocked according to age and randomized into groups from those blocks, each treatment group having the same average age. Each cat served as its own control, having received the pre-study diet [n-6-to-n-3 PUFA ratio of ~16). Cats received isocaloric diets with n-6-to-n-3 PUFA ratios of 5, 2.5, 1, or 0.4 for 24 weeks. Cats were fed once daily, and provided water *ad libitum*. Body weights were assessed weekly, and cats were monitored daily for adequate veterinary health. Any cat found to be unhealthy at any point during the study was removed from the study and provided with proper veterinary care. Whole blood was obtained from each cat on weeks 0 (pre-study diet), 6, 12, and 24 for pMAPK analysis in peripheral white blood cells (WBC). Subcutaneous inguinal mammary adipose biopsies were obtained from anesthetized animals at weeks 0 and 24 for fatty acid and pMAPK analysis.

Cell Isolation and Flow cytometric analysis of pMAPK

For flow cytometry, peripheral WBC were isolated from whole blood via a double density histopaque gradient, and analyzed for pMAPK activity according to Chow et al., (2001). Specifically, 3ml of 1.119 histopaque (Sigma, St. Louis, MO) was placed into a 15 ml conical centrifuge tube, and 3 ml of 1.007 histopaque (Sigma) was carefully layered over it. 3 ml of whole blood was slowly layered over the histopaque 1.007 and centrifuged at 18°C for 30 min (700 x g, no brake). Plasma was discarded, and the PBMC (layer of mononuclear cells between plasma and 1.007 histopaque) and PMNC (layer of granulocytes between the 2 histopaque

layers) were removed to separate 15 ml conical tubes containing 12 ml room temperature PBS. Cell suspensions were centrifuged for 10 min (200 x g, 18°C), and washed again with 12 ml PBS. Cells were then resuspended in 10 ml RPMI containing 5% fetal bovine serum (FBS, Sigma) and centrifuged for 20 min (400 x g) to remove platelets. Cells were resuspended in 3 ml PBS and counted via hemocytometer. 2.5×10^5 of each cell layer were placed into each of 6 (15 ml) conical centrifuge tubes in 1 ml of RPMI containing 10 % FBS and stimulated with 40 nM phorbol-13-myristate-15-acetate (PMA, Sigma) for 15 min in a 37°C water bath. Following PMA stimulation, cells were fixed in a total of 2% formaldehyde (60 µl of 37% formaldehyde per tube) for 30 min in a 37°C water bath, and permeabilized in a 90 % methanol solution (10 ml of 100% methanol added per tube). Cells were stored at -20°C until antibody processing.

Methanol-permeabilized cells were centrifuged for 10 min (200 x g, 18°C), removed to flow cytometry vials, and washed with 2 ml PBS containing 4% FBS (PBS/FBS). Cells were then resuspended in 100 µl PBS/FBS and incubated with 0.5 µg mouse anti-pMAPK mAb (Cell Signaling Technology, CST, Beverly, MA) at room temperature for 15 min. Cells were washed with 2 ml PBS/FBS and then incubated in 100 µl PBS/FBS containing 0.25 µg secondary fluorescein isothiocyanate (FITC)-conjugated goat F(ab')₂ anti-mouse IgG (H+L) antibodies (Caltag, Burlingame, CA). Negative controls included unstimulated cells (no PMA treatment) and cells (stimulated and unstimulated) with no primary antibody. Stained cells were analyzed using a Coulter Epics XL flow cytometer (Beckman Coulter, Miami, FL), and the Overton Subtraction method was used to correct data according to controls.

Western analysis of pMAPK in white blood cells

For western blotting applications, 2×10^6 WBC (obtained as described above for flow cytometry) were stimulated with 40 nM PMA in a 37°C water bath for 15 min, washed with 5 ml ice-cold PBS/FBS (centrifuged at 200 x g, 4°C), and lysed in 100 µl sample buffer (62.5 mM Tris-HCl pH 6.8, 2% w/v SDS, 10% glycerol, 50 mM DTT, and 0.1% bromphenol blue). Lysed samples were sonicated for 30 sec, heated for 5 min at 95°C, and electrophoresed (20 µl) on a 10% SDS-PAGE Tris-HCl gel (BioRad, Hercules, CA) in electrophoresis running buffer. Purified pMAPK and non-phosphorylated MAPK proteins (CST) were electrophoresed as positive and negative controls, respectively. Following electrophoresis, gels were

electrotransferred to a PVDF membrane (BioRad) via semi-dry method (2 gels, 45 min, 150 V) in transfer buffer (25 mM Tris Base, 0.2 M glycine, 20% methanol), and membranes were blocked in TTBS with 5% non-fat dry milk (pMAPK) or 5% bovine serum albumin (BSA, Sigma) overnight at 4°C. Following blocking, membranes were washed 3 times for 5 min with 15 ml wash buffer (0.01M Tris Base, 0.15 M NaCl, 0.1 % Tween-20), and then incubated with either mouse anti-pMAPK primary antibody (1:2000 dilution) or rabbit anti-total MAPK (1:1000 dilution) (CST) in appropriate blocking buffer at room temperature for 1 h. Membranes were then washed as described previously, and incubated for 1 h at room temperature with either goat anti-mouse IgG or goat anti-rabbit IgG secondary antibodies conjugated to horseradish peroxidase (HRP, CST). Membranes were then washed as above, incubated with chemiluminescent substrate (LumiGLO®, CST) for 1 min, and exposed to autoradiograph film (Kodak BioMax Light, Fisher Scientific, Middletown, VA). Bands were quantified using densitometric analysis (NIH Imager, 1.63), and data expressed as a ratio of pMAPK to total MAPK, to correct for loading differences.

Western analysis of pMAPK in feline adipose tissue

For western blotting applications, frozen samples (~40 mg) were homogenized with a tissue grinder in boiling lysis buffer (62.5 mM Tris HCl pH6.8, 2% SDS, 10% glycerol, 2 mM sodium orthovanadate, 10 µg/ml leupeptin, 10 µg/ml aprotinin). Homogenates were then boiled at 100°C for 5 min and centrifuged for 10 min (8000 x g, 4°C). Supernatants were removed to a fresh 1.5 ml microtube.

The concentration of each sample was determined using a colorimetric assay (BioRad), in which the protein in the sample reacts with a copper-alkaline solution, and subsequently reduces an added Folin reagent, resulting in a color change, measured at 750 nm. Absorbance of each sample was measured via SpectraMax250 microplate reader (Molecular Devices, Sunnydale, CA) and analyzed with SOFTmax[®]PRO 4.0 software (Molecular Devices). Known amounts of BSA were prepared in lysis buffer and used as a standard curve for calculating protein concentrations.

Bromphenol blue and β-mercaptoethanol were added to each sample for final concentration of 0.1% and 5%, respectively. Samples were then heated at 100°C for 5 min, and

electrophoresed (20 μ l/lane) on a 10% SDS-PAGE Tris HCl gel (Biorad) (2 gels, 45 min, 150 V) in electrophoresis running buffer (pH 8.5, 0.025 mM Tris Base, 0.19 M glycine, 0.1% SDS). Purified phosphorylated and non-phosphorylated MAPK proteins (CST) were run on each gel as controls, and prestained molecular weight markers (CST) were run to visualize that electrophoresis and transfer methods worked properly.

Following electrophoresis, gels were electrotransferred to a PVDF membrane via semi-dry method (2 gels, 45 min, 150 V) in transfer buffer (25 mM Tris Base, 0.2 M glycine, 20% methanol), and membranes were blocked in TTBS with 5% non-fat dry milk overnight at 4°C. Following blocking, membranes were washed 3 times for 5 min with 15 ml wash buffer (0.01M Tris Base, 0.15 M NaCl, 0.1 % Tween-20), and then incubated with mouse anti-pMAPK primary antibody (CST, 1:2000 dilution) in blocking buffer at room temperature for 1 h. Membranes were washed as described previously, and incubated for 1 h at room temperature with goat anti-mouse IgG secondary antibody conjugated to horseradish peroxidase (HRP, CST). Membranes were washed as above, incubated with chemiluminescent substrate (LumiGLO®, CST) for 1 min, and exposed to autoradiograph film (Kodak BioMax Light). Bands were quantified using densitometric analysis (NIH Imager, 1.63), and data expressed as densitometry units / μ g protein.

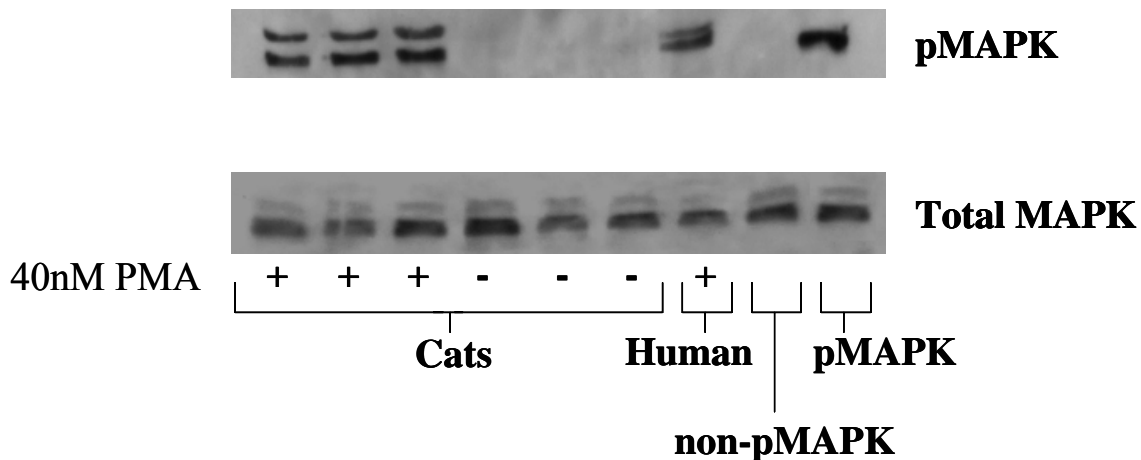


Figure A-1. Western Blot illustrating homology between human and feline phosphorylated and non-phosphorylated proteins. Using mouse anti-pMAPK and anti-MAPK monoclonal antibodies (Cell Signaling Technology, Beverly, MA), both human and feline proteins were detected at similar locations on SDS-PAGE gels, indicating similar size and sequence homology.

Fatty acid analysis of feline adipose tissue

Fatty acid analysis was performed in the laboratory of Dr. J. Herbein (Department of Dairy Science, Virginia Tech, Blacksburg, VA). Lipids were extracted with chloroform/methanol, according to Folch et al. (1957) and dried under a stream of nitrogen. Approximately 40 mg of tissue was homogenized (Polytron PCU-2-110, Brinkman Instruments, Westbury, NY) in a 50 ml glass tube containing 15 ml of a 2:1 chloroform:methanol solution. Homogenates were incubated at room temperature for 1 h, and then filtered into a new glass tube through Whatman 54 or 52 filter paper. Original homogenate tubes were washed with 7 ml chloroform:methanol solution, which was added to the first filtered solution. 5 ml of 0.88% KCl was added and homogenate was vigorously mixed on a horizontal shaker for 10 min. Homogenates were then centrifuged for 5 min (room temperature, 3000 rpm), and the non-lipid layer was discarded. Tube containing the remaining lipid layer was placed under a stream of N₂ and dried until 2 ml remained (N-EVAP, model 112, Organomation, South Bend, MA). Sample was transferred to a 16x100 mm glass tube, along with 2 rinses of 2:1 chloroform:methanol (2 ml each rinse), and then evaporated under N₂ to dryness. Undecenoate (an internal standard, NuCheck Prep, Elysian, MN) was added to the lipid extracts, and the samples were directly methylated according to Park and Goins (1994). Dichloromethane (200 µl), hexane (500 µl, 120 µg/ml free acid), and 0.5 N NaOH in methanol (2 ml) were added to each tube; tubes were capped tightly, vortexed, and heated at 95°C for 10-20 min. Samples were cooled to room temperature, 14% BF₃ in methanol (2 ml) was added, and samples were heated and cooled as above. Deionized water and hexane (1 ml each) were added to samples, which were then mixed for 10 min on a platform shaker, and centrifuged for 5 min at 1500 rpm. Top layer (hexane layer) of sample was transferred to a crimp vial containing a small amount of dry sodium sulfate. Methyl esters were analyzed by gas chromatography (Hewlett-Packard 5890A, Sunnyvale, CA), using a fused silica capillary column (100 m x 0.25 mm i.d.) (CP-Sil 88, Chrompack, Middleburg, The Netherlands). Fatty acids (FA) were quantified by integration using peak area response factors to known amounts of individual fatty acids, and expressed as microgram FA per milligram tissue.

Cell lines and culture conditions

Estrogen receptor positive [ER+, T-47D (HTB-133)] and –negative [ER-, MDA-MB-231 (HTB-26), BT-20 (HTB-19)] were obtained from the American Type Culture Collection (ATCC, Manassas, VA). ER- cells lines were maintained in Minimal Essential Medium (MEM, Mediatech, Herndon, VA) supplemented with 10% fetal bovine serum (FBS, HyClone, Logan, UT), 2mM L-glutamine (BioWhittaker, Walkersville, MD), 1.0 mM sodium pyruvate (BioWhittaker), 0.1 mM non-essential amino acids (BioWhittaker), and 0.01 mg/ml bovine insulin (Sigma). ER+ cells were maintained in RPMI 1640 medium (Mediatech) supplemented with FBS, glutamine, sodium pyruvate, and insulin as above, as well as 10 mM HEPES (BioWhittaker) and 2.5g/L d-glucose (Sigma). All cells were maintained in T-75 cm² flasks at 37°C with 5% CO₂ according to the recommendations of the supplier, with media renewal 3 times per week, and passaging when cells reached 85-90% confluence as observed with an inverted microscope using phase contrast. For experiments, cells were maintained in experimental medium [EM, MEM or RPMI containing 1% FBS, 1% fatty acid-free bovine serum albumin (BSA, Sigma), and supplements as above].

Counting experiments

Cells were seeded into 24-well plates (2-4 x 10⁴ cells/well), allowed to attach for 24 h in culture medium, and then maintained for 6 d in EM (0.75ml) supplemented with either linoleic acid (LA, 18:2 n-6, 0, 0.5, 1, 3, 6.25, 12.5, 25, or 58 µg/ml) or docosahexanoic acid (DHA, 22:6 n-3, 0, 0.5, 1.5, 3, 7.5, 15, 30, or 60 µg/ml), with media renewal on d 3. On d 6, cells were incubated with 100 µl of 0.05%-trypsin-0.02% EDTA (BioWhittaker) for 2-5 min, until cells began to detach. Culture medium (200 µl) was then added to each well to inactivate the trypsin; the cell suspension was removed to a 500 µl microtube. 50 µl of cell suspension was placed in a microtube and diluted 1:1 with Trypan Blue Dye (TPB, GIBCO BRL, Gaithersburg, MD) (50 µl cell suspension and 50 µl TPB). Approximately 10 µl of the cell/TPB suspension was loaded into each chamber of a hemocytometer, and cells in the middle and four corner squares of each chamber were counted. Cell counts from the two chambers were averaged and divided by 5 to provide the average cell/mm; this number was then multiplied by 20,000 to render the average cell/ml, which was then multiplied by the total original cell suspension amount (300 µl) to obtain the total cells per sample.

Proliferation experiments

Ratio experiments

Cells were seeded into 96-well plates ($2-4 \times 10^3$ cells/well), allowed to attach in culture medium for 24 h, and then maintained as above in EM (200 μ l) supplemented with LA and DHA (NuChek Prep), such that total fatty acid concentrations (DHA plus LA) were 0, 25, 50, 75, 100, or 200 μ M and the ratio of DHA-to-LA within each fatty acid concentration was as follows: 0 DHA, 0.1, 0.25, 0.5, 1, 2, 4, 8, or 0 LA). Cell numbers following treatment were evaluated via colorometric assay (CellTiter 96[®] AQueous, Promega, Madison, WI) utilizing a tetrazolium compound (MTS), which is bio-reduced to a formazan product by living cells and can be measured at 490 nm, and an electron coupling reagent (phenazine methosulfate, PMS). On d 6, EM was replaced with fresh EM (100 μ l/well, no fatty acids) and MTS/PMS solution (20 μ l/well), and cells were incubated at 37°C/ 5% CO₂ for 4 h. MTS bio-reduction by cells (as a direct indicator of cell number) was measured via SpectraMax250 microplate reader (490 nm) and analyzed with SOFTmax[®]PRO 4.0 software.

Range-finding experiments

Cells were seeded into 96-well plates ($2-4 \times 10^3$ cells/well), allowed to attach for 24 h in culture medium, and then maintained as above in EM (200 μ l) with supplements as described for counting experiments. On d 6, cell numbers were evaluated via MTS/PMS colorimetric assay as described previously.

Analysis of pRaf, pMEK, and pMAPK in cell lines

Protein extraction

Cells were seeded into 35 mm plates ($1-3 \times 10^5$ cells/well), allowed to attach in culture medium for 24 h, and then maintained for 6 d in EM (1ml) supplemented with LA (0, 0.75, 12.5, or 58 μ g/ml) or DHA (0, 2, 15, or 60 μ g/ml). On d 6, cells were incubated overnight with EM-B (1 ml EM with all supplements as previously, except 1% FBS and 0.1% BSA). On d 7, cells

were incubated for 2 h in EM-C (1 ml, same as EM, except 0% FBS and 0.1% BSA). Cells were then incubated for 8 min with fresh EM-C (1 ml) was added to each plate, along with 32 ng/ml of human-recombinant epidermal growth factor (EGF, R&D Systems, Minneapolis, MN). Following incubation, cells were rinsed PBS (1 ml), lysed with 100 μ l lysis buffer (65 mM Tris HCl, pH 6.5, 10% glycerol, 2%SDS), and stored in 1.5 ml microtubes at -70°C until processed via Western immunoblotting.

The concentration of each sample was determined using a colorimetric assay, as described previously for adipose tissue. Known amounts of BSA were prepared in lysis buffer and used as a standard curve for calculating protein concentrations.

Gel electrophoresis and transfer

Bromphenol blue and β -mercaptoethanol were added to each sample to give a final concentration of 0.01% and 5%, respectively; samples were then sonicated for 30 sec, heated for 5 min at 95°C, and electrophoresed (25 μ l/ lane) on 10% SDS-PAGE Tris-HCl gels (BioRad) for 45 min (2 gels) at 150 V (constant voltage) in electrophoresis running buffer. Purified pMAPK proteins (CST) were run on each gel as controls (positive controls for pMAPK and negative controls for pRaf and pMEK), and prestained molecular weight markers (CST) were run to visualize that electrophoresis and transfer methods worked properly.

Following gel electrophoresis, gels were soaked in transfer buffer (25 mM Tris Base, 0.2 M glycine, 20% methanol) for 15 min. Separated proteins were transferred to a PVDF membrane (BioRad) using a semi-dry blotter (C.B.S. Scientific, Del Mar, CA) as follows: extra-thick filter paper (BioRad), pre-wet with transfer buffer was placed on top of cutouts in a Mylar mask on the bottom plate of the semi-dry blotter. Transfer-buffer soaked PVDF (pre-wetted with methanol prior to soaking in transfer buffer) was then placed directly on the filter paper, followed by the pre-soaked gel. Another layer of pre-soaked filter paper was placed on top of the gel, and the top plate of the semi-dry blotter was placed on top and secured. Two gels were transferred at a time at a constant voltage of 25 V for 60 min. Completion of transfer was verified visually by pre-stained markers run on the gel along with samples.

Antibody staining

Following semi-dry transfer, PVDF membranes were incubated in blocking buffer at room temperature (RT) for 60 min or overnight (OV) at 4°C, and then stained with appropriate primary and horseradish peroxidase-linked-secondary polyclonal antibodies (Ab) in the same blocking buffer. The blocking solutions, times and temperatures of incubations, and antibodies can be seen in **Table 1**. Between primary and secondary antibody incubations, membranes were washed 3 times (5 min/ wash) in 15 ml wash buffer (TTBS, 0.01 M Tris Base, 0.15 M NaCl, 0.1% Tween-20).

Table 1: Description of blocking buffers, primary and secondary antibodies, and incubation temperatures and times for immunoblotting of pRaf, pMEK, and pMAPK.

Protein	Blocking Buffer (temp, time)	Primary Ab (temp, time)	Secondary Ab (temp, time)
pRaf	5% BSA in TTBS (RT, 60 min)	Rabbit, anti-pRaf (4°C,OV)	Goat, anti-rabbit IgG (H&L), HRP-linked (RT, 60 min)
pMEK	5% BSA in TTBS (RT, 60 min)	Rabbit, anti-pMEK1/2 (4°C,OV)	Goat, anti-rabbit IgG (H&L), HRP-linked (RT, 60 min)
pMAPK	5% milk in TTBS (4°C, OV)	Rabbit, anti-pMAPK (RT,60 min)	Goat, anti-rabbit IgG (H&L), HRP-linked (RT, 60 min)

(OV= overnight; RT = room temp)

Chemiluminescence and Densitometry

Following antibody staining, protein bands were visualized using chemiluminescence. Membranes were washed as described above, incubated with chemiluminescent substrate (LumiGLO®, CST) for 1 min, and exposed to autoradiograph film (Kodak BioMax Light). pRaf, pMEK, and pMAPK bands were quantified using densitometry software (NIH Imager 1.63). Bands were expressed as densitometry units per µg protein loaded onto gel.

Receptor internalization assays

Cells were seeded into 12-well plates ($1-3 \times 10^5$ cells/well), allowed to attach for 24 h in culture medium, and then maintained in EM (1 ml) as described above for 6 d. On d 6, cells were incubated with 0.5 ml serum-free medium containing 0.1% BSA for 1 h and then washed three times with 2 ml room temperature PBS. Cells were then incubated in HEPES binding medium (7.4 pH MEM, 0.1% BSA, 0.04 M HEPES), containing (^{125}I)-epidermal growth factor (EGF, $\sim 70,000$ cpm/ml, 1150 Ci/mmol) and either 5 nM or 100 nM non-labeled EGF for varying periods of time at 37°C (5 min, 15 min, 30 min, 90 min, 180 min). To determine internalized radioactivity, cells were washed twice with 2ml acid wash (50 mM glycine, 100 mM NaCl, pH 3.0), once with PBS, then solubilized in 1 M NaOH for 1 h, and transferred to counting vials. To determine total (internal plus external) radioactivity, additional cells from the same plate receiving no acid wash, were also processed. Radioactivity was measured via an auto-gamma counter (Cobra II, Packard Instruments, Meriden, CT), and expressed as counts per minute (cpm). Excess unlabeled EGF (100 nM) was added to additional cells (with and without acid wash) on the same plate, in order to determine non-specific binding. Data were expressed as corrected cpm (internalized or total counts minus non-specific binding).

Fatty acid analysis of cell lines

Cells were seeded in triplicate in 35 mm plates ($1-2 \times 10^5$ cells/ plate), allowed to attach for 24 h in culture medium, and then maintained in EM as described above for analysis of pRaf, pMEK, and pMAPK. On d 6, EM was removed, and cells were washed with 1 ml room temperature PBS. Cells were trypsinized with 200 μl 0.05%-trypsin-0.02% EDTA, and 400 μl of PBS was added to inhibit the action of trypsin. Cell suspensions from triplicate plates were removed to a 2 ml microtube, and samples were stored at -20°C until fatty acid analysis. Fatty acid analysis was performed as described previously for adipose tissue. Cellular LA and DHA were expressed as a percentage of total cellular FA present.

Statistical Analyses

Cat MAPK study

Prior to experimentation, cats were blocked by age and randomly selected into groups receiving different experimental diets. Our experimental design was completely randomized with a repeated measurements sampling structure. For WBC pMAPK, we used the Mixed Model ANOVA to determine if there was a time by treatment interaction. Pair wise comparisons were then made comparing time zero pMAPK levels to pMAPK levels at each non-zero time point. Bonferroni tests were used for pair wise comparisons, in order to correct for multiplicity.

For adipose pMAPK, residual plots revealed that as the response variable value increased, variability increased. This phenomenon is commonly observed in biological systems. Based on this, the data was log transformed. Using the log transformed data, Mixed Model ANOVA was used to determine if a ratio by time effect existed.

Cell proliferation study

Prior to statistical analysis, experiments were designed to be randomized. Specifically, cells were randomly allocated to wells in 96-well plates, but treatments were not. Following data collection, residual plots revealed increasing variability as response variable values increased. The data was log transformed, and Lowess smoothing was then utilized to visualize curvature of the response, as an aid to selecting a functional for the model. Quadratic equations across concentrations was fit to the data using Mixed Model ANOVA.

Cell Line pRaf, pMEK, and pMAPK western immunoblotting

Experimental design consisted of a completely randomized block, with a 2 x 4 x 2 structure, consisting of two fatty acids (FA), 4 concentrations within each fatty acid, and 2 cells lines. To determine if EGF or DHA and LA affected levels of pRaf, pMEK, and pMAPK, General ANOVA was used. Specifically, to determine if EGF stimulation resulted in alterations in pRaf, pMEK, and pMAPK levels, data was split by cell type and fatty acid, and an ANOVA testing EGF by FA concentration was performed. To determine if concentration affected these same response variables, data was split by cell type and EGF status, and an ANOVA testing concentration by fatty acid (DHA or LA) was performed.

Radioligand Binding Assays

To determine the effect of DHA or LA concentration on internalization of EGFR, ANOVA was used to determine if a time, location, concentration, time by location, time by concentration, or location by concentration effects existed. When significant effects were observed, Bonferroni-corrected pair wise comparisons comparing specific times or concentrations within a fatty acid were performed.

Appendix B – Statistics Tables

Chapter 2

*Table 2-1 Effect of dietary PUFA on pMAPK levels over time in feline total white blood cells.
ANOVA Results – Tests of Between Subject Effects.*

Effect	Num DF	Den DF	F Value	Pr > F
Age	1	20	0.41	0.5304
Ratio	3	20	0.92	0.4503
Age * Ratio	3	20	0.09	0.9621
Time	2	38	4.47	0.0180
Age * Time	2	38	0.38	0.6889
Ratio * Time	6	38	0.35	0.9066
Age * Ratio * Time	6	38	0.21	0.9726

*Table 2-2 Effect of dietary PUFA on pMAPK levels over time in feline mononuclear cells.
ANOVA Results – Tests of Between Subject Effects*

Effect	Num DF	Den DF	F Value	Pr > F
Age	1	20	0.06	0.8057
Ratio	3	20	0.54	0.6598
Age * Ratio	3	20	0.09	0.9630
Time	2	38	5.69	0.0069
Age * Time	2	38	0.28	0.7601
Ratio * Time	6	38	0.26	0.9536
Age * Ratio * Time	6	38	0.31	0.9294

*Table 2-3 Effect of dietary PUFA on pMAPK levels over time in isolated feline granulocytes.
ANOVA Result – Tests of Between Subjects Effects.*

Effect	Num DF	Den DF	F Value	Pr > F
Age	1	20	0.31	0.5853
Ratio	3	20	0.23	0.8755
Age * Ratio	3	20	0.11	0.9533
Time	2	38	9.52	0.0004
Age * Time	2	38	0.63	0.5372
Ratio * Time	6	38	0.29	0.9385
Age * Ratio * Time	6	38	0.29	0.9376

Table 2-3 Effect of dietary PUFA on pMAPK levels in feline mammary adipose tissue.

ANOVA Results – Tests of Between Subjects Effects.

Effect	Num DF	Den DF	F Value	Pr > F
Age	1	17	1.05	0.3190
Ratio	3	17	2.20	0.1253
Age * Ratio	3	17	0.92	0.4534
Time	1	6	0.82	0.4011
Age * Time	1	6	0.00	0.9890
Ratio * Time	3	6	1.74	0.2580
Age * Ratio * Time	2	6	0.04	0.9611

Chapter 4

Table 4-1 Effect of EGF stimulation on pRaf levels in DHA-supplemented BT-20 cells.

ANOVA Results – Tests of Between Subjects Effects

Effect	Num DF	Mean Square	F Value	Pr > F
Model	5	0.005	52.267	0.004
EGF Treatment	1	0.008	83.178	0.003
Concentration	3	0.000	0.700	0.612
Error	3	0.000		
Total	8			

Table 4-2 Effect of EGF stimulation on pRaf levels in LA-supplemented BT-20 cells.

ANOVA Results – Test of Between Subjects Effects

Effect	Num DF	Mean Square	F Value	Pr > F
Model	5	0.008	45.337	0.005
EGF Treatment	1	0.004	25.187	0.015
Concentration	3	0.000	1.385	0.398
Error	3	0.000		
Total	8			

Table 4-3 Effect of EGF stimulation on pRaf in DHA-supplemented MDA-MB-231 cells.

ANOVA Results – Tests of Between Subjects Effects.

Effect	Num DF	Mean Square	F Value	Pr > F
Model	5	0.003	30.932	0.009
EGF Treatment	1	0.000	0.076	0.801
Concentration	3	0.001	7.903	0.062
Error	3	0.000		
Total	8			

Table 4-4 Effect of EGF stimulation on pRaf in LA-supplemented MDA-MB-231 cells.

ANOVA Results – Tests of Between Subjects Effects.

Effect	Num DF	Mean Square	F Value	Pr > F
Model	5	0.005	8.062	0.058
EGF Treatment	1	0.000	0.020	0.896
Concentration	3	0.000	0.688	0.617
Error	3	0.001		
Total	8			

Table 4-5 Effect of EGF stimulation on pMEK in DHA-supplemented BT-20 cells.

ANOVA Results – Tests of Between Subjects Effects.

Effect	Num DF	Mean Square	F Value	Pr > F
Model	5	0.041	39.759	0.006
EGF Treatment	1	0.012	12.090	0.040
Concentration	3	0.005	5.210	0.104
Error	3	0.001		
Total	8			

Table 4-6 Effect of EGF stimulation on pMEK in LA-supplemented BT-20 cells.

ANOVA Results – Tests of Between Subjects Effects.

Effect	Num DF	Mean Square	F Value	Pr > F
Model	5	0.032	34.748	0.007
EGF Treatment	1	0.009	10.285	0.049
Concentration	3	0.002	2.394	0.246
Error	3	0.001		
Total	8			

Table 4-7 Effect of EGF stimulation on pMEK in DHA-supplemented MDA-MB-231 cells.

ANOVA Results – Tests of Between Subjects Effects.

Effect	Num DF	Mean Square	F Value	Pr > F
Model	5	0.130	12.632	0.031
EGF Treatment	1	0.085	8.290	0.064
Concentration	3	0.025	2.458	0.240
Error	3	0.010		
Total	8			

Table 4-8 Effect of EGF stimulation on pMEK in LA-supplemented MDA-MB-231 cells.

ANOVA Results – Tests of Between Subjects Effects.

Effect	Num DF	Mean Square	F Value	Pr > F
Model	5	0.109	13.179	0.030
EGF Treatment	1	0.054	6.519	0.084
Concentration	3	0.020	2.451	0.240
Error	3	0.008		
Total	8			

Table 4-9 Effect of EGF stimulation on pMAPK in DHA-supplemented BT-20 cells.

ANOVA Results – Tests of Between Subjects Effects.

Effect	Num DF	Mean Square	F Value	Pr > F
Model	5	0.007	9.768	0.045
EGF Treatment	1	0.012	16.811	0.026
Concentration	3	0.001	0.907	0.531
Error	3	0.001		
Total	8			

Table 4-10 Effect of EGF stimulation on pMAPK in LA-supplemented BT-20 cells.

ANOVA Results – Tests of Between Subjects Effects.

Effect	Num DF	Mean Square	F Value	Pr > F
Model	5	0.004	54.009	0.004
EGF Treatment	1	0.006	83.634	0.003
Concentration	3	0.000	2.122	0.276
Error	3	0.000		
Total	8			

Table 4-11 Effect of EGF stimulation on pMAPK in DHA-supplemented MDA-MB-231 cells.

ANOVA Results – Tests of Between Subjects Effects.

Effect	Num DF	Mean Square	F Value	Pr > F
Model	5	0.034	28.208	0.010
EGF Treatment	1	0.019	15.742	0.029
Concentration	3	0.009	7.188	0.070
Error	3	0.001		
Total	8			

Table 4-12 Effect of EGF stimulation on pMAPK in LA-supplemented MDA-MB-231 cells.

ANOVA Results – Tests of Between Subjects Effects.

Effect	Num DF	Mean Square	F Value	Pr > F
Model	5	0.041	28.475	0.010
EGF Treatment	1	0.044	30.611	0.012
Concentration	3	0.005	3.812	0.150
Error	3	0.001		
Total	8			

Table 4-13 Effect of PUFA concentration on pRaf in BT-20 cells.

ANOVA Results – Tests of Between Subjects Effects.

Effect	Num DF	Mean Square	F Value	Pr > F
Model	5	0.011	52.197	0.004
Concentration	3	0.000	1.271	0.424
Fatty Acid	1	0.000	1.043	0.382
Error	3	0.000		
Total	8			

Table 4-14 Effect of PUFA concentration on pRaf in MDA-MB-231 cells.

ANOVA Results – Tests of Between Subjects Effects.

Effect	Num DF	Mean Square	F Value	Pr > F
Model	5	0.004	13.330	0.029
Concentration	3	0.001	3.043	0.193
Fatty Acid	1	0.000	1.556	0.301
Error	3	0.000		
Total	8			

Table 4-15 Effect of PUFA concentration on pMEK in BT-20 cells.

ANOVA Results – Tests of Between Subjects Effects.

Effect	Num DF	Mean Square	F Value	Pr > F
Model	5	0.018	437.042	0.000
Concentration	3	0.002	44.479	0.005
Fatty Acid	1	0.000	3.730	0.149
Error	3	0.000		
Total	8			

Table 4-16 Effect of PUFA concentration on pMEK in MDA-MB-231 cells.

ANOVA Results – Tests of Between Subjects Effects.

Effect	Num DF	Mean Square	F Value	Pr > F
Model	5	0.210	86.250	0.002
Concentration	3	0.054	22.326	0.015
Fatty Acid	1	0.002	1.027	0.386
Error	3	0.002		
Total	8			

Table 4-17 Effect of PUFA concentration on pMAPK in BT-20 cells.

ANOVA Results – Tests of Between Subjects Effects.

Effect	Num DF	Mean Square	F Value	Pr > F
Model	5	0.011	13.776	0.028
Concentration	3	0.001	1.012	0.496
Fatty Acid	1	0.001	1.431	0.317
Error	3	0.001		
Total	8			

Table 4-18 Effect of PUFA concentration on pMAPK in MDA-MB-231 cells.

ANOVA Results – Tests of Between Subjects Effects.

Effect	Num DF	Mean Square	F Value	Pr > F
Model	5	0.065	15.539	0.024
Concentration	3	0.010	2.408	0.245
Fatty Acid	1	0.002	0.585	0.500
Error	3	0.002		
Total	8			

Table 4-19 Effect of DHA supplementation on cellular DHA and LA in BT-20 cells.

ANOVA Results

Effect	Num DF	Mean Square	F Value	Pr > F
Cellular LA				
Between Groups	3	0.700	3.888	0.111
Within Groups	4	0.180		
Total	7			
Cellular DHA				
Between Groups	3	843.574	338.494	0.000
Within Groups	4	2.492		
Total	7			

Table 4-20 Effect of LA supplementation on cellular DHA and LA in BT-20 cells.

ANOVA Results

Effect	Num DF	Mean Square	F Value	Pr > F
Cellular LA				
Between Groups	3	505.473	68.601	0.001
Within Groups	4	7.368		
Total	7			
Cellular DHA				
Between Groups	3	0.230	13.110	0.015
Within Groups	4	0.018		
Total	7			

Table 4-21 Effect of DHA supplementation on DHA and LA in MDA-MB-231 cells.

ANOVA Results

Effect	Num DF	Mean Square	F Value	Pr > F
Cellular LA				
Between Groups	3	1.167	1.672	0.309
Within Groups	4	0.698		
Total	7			
Cellular DHA				
Between Groups	3	674.988	72.486	0.001
Within Groups	4	9.312		
Total	7			

Table 4-22 Effect of LA supplementation on DHA and LA in MDA-MB-231 cells.

ANOVA Results

Effect	Num DF	Mean Square	F Value	Pr > F
Cellular LA				
Between Groups	3	563.778	1108.789	0.000
Within Groups	4	0.508		
Total	7			
Cellular DHA				
Between Groups	3	0.401	25.366	0.005
Within Groups	4	0.016		
Total	7			

Table 4-23 Effect of DHA supplementation on EGFR internalization in BT-20 cells.

ANOVA Results

Effect	Num DF	Mean Square	F Value	Pr > F
Corrected Model	39	3574925.725	57.590	0.000
Intercept	1	186641181.251	3006.695	0.000
Time	4	9280392.091	149.502	0.000
Location	1	19811424.112	319.152	0.000
Concentration	3	15698935.752	252.902	0.000
Time * Location	4	484869.250	7.811	0.000
Time * Concentration	12	1564977.384	25.211	0.000
Location * Concentration	3	1456767.381	23.468	0.000
Time * Location * Concentration	12	181461.910	2.923	0.006
Error	37	62075.203		
Total	77			
Corrected Total	76			

Table 4-24 Effect of LA supplementation on EGFR internalization in BT-20 cells.

ANOVA Results

Effect	Num DF	Mean Square	F Value	Pr > F
Corrected Model	39	3351281.178	19.991	0.000
Intercept	1	286350171.098	1708.142	0.000
Time	4	14649028.936	87.385	0.000
Location	1	27332460.377	163.044	0.000
Concentration	3	4002193.264	23.874	0.000
Time * Location	4	883859.314	5.272	0.002
Time * Concentration	12	1777315.243	10.602	0.000
Location * Concentration	3	32716.881	0.195	0.899

Time * Location * Concentration	12	296416.377	1.768	0.091
Error	37	167638.351		
Total	77			
Corrected Total	76			

Table 4-25 Effect of DHA supplementation on EGFR internalization in MDA-MB-231 cells.

ANOVA Results

Effect	Num DF	Mean Square	F Value	Pr > F
Corrected Model	39	20762.923	13.798	0.000
Intercept	1	4005125.000	2661.588	0.000
Time	4	72333.844	48.069	0.000
Location	1	156114.450	103.745	0.000
Concentration	3	81889.933	54.420	0.000
Time * Location	4	7849.106	5.216	0.002
Time * Concentration	12	4748.069	3.155	0.003
Location * Concentration	3	7202.183	4.786	0.006
Time * Location * Concentration	12	721.215	0.479	0.915
Error	40	1504.787		
Total	80			
Corrected Total	79			

Table 4-26 Effect of LA supplementation on EGFR internalization in MDA-MB-231 cells.

ANOVA Results

Effect	Num DF	Mean Square	F Value	Pr > F
Corrected Model	39	16669.681	9.421	0.000
Intercept	1	4373930.450	2471.845	0.000
Time	4	59169.200	33.438	0.000
Location	1	167262.050	94.525	0.000
Concentration	3	37638.950	21.271	0.000
Time * Location	4	7259.800	4.103	0.007
Time * Concentration	12	3422.200	1.934	0.059
Location * Concentration	3	6929.883	3.916	0.015
Time * Location * Concentration	12	3530.550	1.995	0.051
Error	40	1769.500		
Total	80			
Corrected Total	79			

Appendix C – Fatty Acid Analyses

Table 1: Pre-study (time 0) and 24 week fatty acid analysis of adipose tissue from felines fed diets with differing n-6 to n-3 PUFA ratios. Fatty acids are labeled using common scientific nomenclature*, and are presented as total micrograms of fatty acid per milligram of tissue analyzed.

Cat ID	Time (week)	6:0	8:0	10:0	12:0	14:0	14:1	15:0	16:0	t9 16:1	c9 18:1	17:0	18:0	t9 18:1	t10 18:1	t11 18:1	c/t 18:1	t12&c7 18:1	t13&c6 18:1	c9 18:1	c11 18:1	c12 18:1	c13 18:1	t16 18:1	c15 18:1
1	0	0.000	0.000	0.076	0.594	13.411	0.262	0.592	43.937	0.112	7.847	0.771	20.699	0.098	0.708	0.264	0.288	0.000	0.145	53.899	3.058	0.346	0.216	0.048	0.216
2	0	0.000	0.000	0.024	0.313	1.696	0.161	0.704	42.737	0.080	6.643	0.942	38.148	0.108	0.706	0.223	0.315	0.000	0.285	83.634	4.892	0.437	0.329	0.033	0.583
7	0	0.000	0.000	0.022	0.359	3.450	0.107	0.481	32.485	0.112	5.249	0.887	24.027	0.096	0.637	0.205	0.370	0.000	0.249	56.793	3.287	0.356	0.180	0.052	0.192
8	0	0.016	0.026	0.090	0.451	3.528	0.082	0.346	33.420	0.078	4.886	0.718	25.638	0.092	0.584	0.275	0.347	0.000	0.130	51.368	3.155	0.299	0.201	0.039	0.251
12	0	0.014	0.020	0.124	0.603	10.411	0.537	0.914	66.103	0.185	16.262	1.337	41.521	0.273	1.198	0.429	0.454	0.000	0.341	118.928	6.113	0.734	0.466	0.125	0.525
22	0	0.011	0.021	0.119	0.593	8.192	0.198	0.863	55.631	0.123	8.550	1.188	44.475	0.182	0.951	0.446	0.528	0.000	0.377	96.693	5.511	0.629	0.402	0.060	0.555
23	0	0.000	0.000	0.125	0.648	10.364	0.311	0.791	56.644	0.208	9.420	1.146	35.987	0.170	0.966	0.427	0.482	0.000	0.260	90.948	5.138	0.539	0.322	0.072	0.381
27	0	0.020	0.020	0.146	0.677	13.945	0.461	0.990	72.062	0.174	14.277	1.550	49.253	0.301	1.053	0.474	0.562	0.000	0.306	122.661	7.074	0.714	0.443	0.080	0.556
28	0	0.036	0.030	0.253	0.815	9.440	0.421	1.086	68.840	0.129	15.647	1.104	40.382	0.255	0.955	0.375	0.389	0.000	0.250	105.744	6.746	0.635	0.473	0.071	0.471
50	0	0.017	0.031	0.256	0.907	19.925	2.550	0.826	58.987	0.206	21.174	1.194	34.210	0.253	1.210	0.800	0.641	0.000	0.348	105.372	5.354	0.661	0.356	0.090	0.446
51	0	0.012	0.000	0.046	0.639	8.380	0.433	0.864	53.834	0.190	13.401	1.156	34.046	0.149	1.258	0.610	0.617	0.000	0.313	96.108	5.399	0.650	0.379	0.099	0.438
53	0	0.011	0.016	0.062	0.626	14.002	3.450	0.966	41.635	0.261	34.730	0.830	17.832	0.200	1.150	0.486	0.340	0.000	0.287	110.326	5.454	0.704	0.381	0.066	0.331
54	0	0.000	0.000	0.081	0.643	11.685	2.095	0.886	41.474	0.248	36.075	0.823	18.514	0.185	1.077	0.327	0.263	0.000	0.265	115.734	5.659	0.635	0.346	0.053	0.412
57	0	0.019	0.031	0.205	0.796	17.536	1.950	0.818	52.432	0.151	16.534	0.877	24.550	0.155	0.743	0.399	0.375	0.000	0.237	69.242	3.990	0.403	0.290	0.072	0.360
58	0	0.012	0.025	0.150	0.797	17.621	3.200	0.913	50.182	0.263	27.807	1.049	23.997	0.205	0.960	0.481	0.413	0.000	0.342	92.728	4.826	0.535	0.346	0.079	0.390
68	0	0.023	0.024	0.146	0.571	7.891	0.321	0.846	68.611	0.177	11.437	1.594	51.710	0.296	1.014	0.552	0.736	0.000	0.358	115.970	6.880	0.673	0.449	0.096	0.449
106	0	0.014	0.019	0.108	0.541	5.124	0.256	0.672	57.620	0.124	9.786	1.046	39.538	0.157	0.906	0.358	0.447	0.000	0.324	89.202	5.341	0.542	0.418	0.066	0.354
113	0	0.008	0.013	0.034	0.307	4.239	0.138	0.821	58.251	0.205	8.918	1.577	46.246	0.290	0.934	0.423	0.497	0.000	0.220	110.624	6.184	0.637	0.439	0.057	0.491
AA3	0	0.042	0.047	0.481	1.140	13.792	0.435	0.884	62.658	0.115	12.521	0.935	33.733	0.174	0.907	0.378	0.389	0.000	0.305	87.366	5.567	0.516	0.431	0.061	0.401
AL15	0	0.025	0.025	0.108	0.548	4.949	0.154	0.998	54.647	0.254	8.738	1.475	53.111	0.168	0.822	0.538	0.706	0.451	0.000	91.940	5.700	0.512	0.420	0.082	0.626
IAD4	0	0.032	0.042	0.263	0.674	8.891	0.427	1.258	75.423	0.184	15.270	1.449	51.264	0.358	1.124	0.460	0.585	0.000	0.263	134.259	8.010	0.767	0.549	0.093	0.667
IKE3	0	0.052	0.032	0.374	1.080	9.665	0.392	0.972	75.745	0.129	14.680	1.210	49.939	0.229	1.245	0.496	0.548	0.000	0.380	123.247	7.305	0.695	0.482	0.083	0.535
QAB7	0	0.000	0.000	0.052	0.413	3.139	0.098	0.837	57.634	0.159	9.900	1.177	43.740	0.191	0.889	0.409	0.504	0.000	0.459	99.413	6.016	0.604	0.425	0.069	0.458
QAF5	0	0.019	0.000	0.150	0.546	5.241	0.150	0.869	52.280	0.178	7.570	1.265	44.908	0.176	0.827	0.567	0.612	0.000	0.407	88.143	5.068	0.517	0.392	0.082	0.468
QAGA	0	0.000	0.000	0.141	0.733	4.088	0.115	0.796	44.946	0.191	5.363	1.249	45.293	0.143	0.800	0.626	0.779	0.246	0.234	76.032	4.279	0.414	0.318	0.087	0.480
QBX7	0	0.010	0.014	0.084	0.507	3.515	0.102	0.774	52.396	0.129	8.151	1.058	37.024	0.156	1.068	0.350	0.483	0.000	0.313	96.832	5.363	0.525	0.285	0.053	0.366
RL05	0	0.018	0.026	0.174	0.562	8.470	0.270	0.862	59.110	0.210	10.539	1.265	41.371	0.216	1.016	0.502	0.566	0.000	0.321	102.024	5.560	0.615	0.409	0.078	0.412
RPU5	0	0.019	0.015	0.066	0.609	2.363	0.113	0.715	0.000	0.143	7.818	1.099	44.010	0.164	1.005	0.504	0.638	0.171	0.000	88.658	5.028	0.503	0.324	0.093	0.451

Cat ID	Time (week)	t9,12 18:2	c9,t12 18:2	t9,c12 18:2	t11,c15 18:1	18:2n6	20:0	t18:3n3	t11 18:2	t11,c13 18:2	t10,c12 18:2	c9,c11 18:2	t11,t13 18:2	other t/t 18:2	20:3n3	22:1	20:4n6	24:1	20:5n3	24:0	22:4n6	22:5n3	22:6n3
1	0	0.063	0.262	0.180	0.017	39.085	0.128	0.943	0.172	0.028	0.047	0.041	0.045	0.062	0.256	0.013	0.912	0.030	0.010	0.010	0.628	0.371	0.341
2	0	0.078	0.439	0.272	0.020	89.289	0.892	2.375	0.062	0.000	0.018	0.012	0.155	0.155	0.653	0.048	1.057	0.330	0.083	0.072	1.267	0.429	0.593
7	0	0.077	0.257	0.142	0.017	41.974	0.102	0.804	0.221	0.000	0.024	0.029	0.053	0.068	0.268	0.010	0.746	0.031	0.006	0.009	0.703	0.336	0.191
8	0	0.082	0.237	0.147	0.022	38.455	0.226	1.194	0.067	0.007	0.009	0.017	0.047	0.077	0.239	0.020	0.977	0.090	0.019	0.015	0.724	0.254	0.206
12	0	0.111	0.477	0.256	0.027	84.364	0.443	1.980	0.590	0.000	0.000	0.061	0.102	0.143	0.556	0.027	1.714	0.066	0.032	0.019	1.378	0.537	0.273
22	0	0.081	0.379	0.227	0.040	73.642	0.610	1.833	0.252	0.067	0.094	0.031	0.133	0.232	0.645	0.140	1.338	0.326	0.058	0.057	1.634	0.772	0.623
23	0	0.075	0.338	0.213	0.024	61.755	0.279	1.516	0.254	0.072	0.125	0.074	0.082	0.121	0.442	0.025	1.338	0.082	0.023	0.022	1.237	0.434	0.226
27	0	0.191	0.473	0.172	0.021	87.908	0.441	2.103	0.743	0.005	0.040	0.077	0.120	0.166	0.772	0.032	2.103	0.086	0.025	0.034	1.979	0.645	0.247
28	0	0.103	0.517	0.354	0.037	86.751	0.616	3.008	0.224	0.000	0.023	0.047	0.104	0.199	0.720	0.038	2.087	0.292	0.074	0.054	0.804	0.433	0.546
50	0	0.139	0.518	0.352	0.040	69.350	0.400	2.015	0.217	0.000	0.000	0.063	0.067	0.111	0.571	0.022	2.213	0.094	0.032	0.029	1.308	0.547	0.261
51	0	0.134	0.498	0.318	0.035	68.088	0.310	1.432	0.145	0.000	0.000	0.065	0.091	0.076	0.641	0.032	1.924	0.060	0.019	0.022	1.810	0.595	0.249
53	0	0.104	0.512	0.280	0.033	72.179	0.312	1.477	0.303	0.000	0.000	0.060	0.067	0.087	0.477	0.030	1.044	0.057	0.018	0.023	1.087	0.366	0.159
54	0	0.129	0.476	0.232	0.041	75.922	0.452	1.652	0.423	0.000	0.072	0.078	0.096	0.132	0.476	0.035	1.151	0.088	0.032	0.033	1.189	0.565	0.366
57	0	0.061	0.322	0.212	0.032	50.706	0.260	1.525	0.219	0.046	0.066	0.029	0.053	0.090	0.352	0.026	1.429	0.073	0.026	0.015	0.725	0.293	0.206
58	0	0.091	0.412	0.243	0.024	61.830	0.252	1.584	0.360	0.037	0.113	0.034	0.083	0.097	0.514	0.024	1.879	0.051	0.019	0.017	1.073	0.535	0.274
68	0	0.170	0.471	0.277	0.037	84.868	0.393	4.561	0.461	0.123	0.293	0.040	0.152	0.233	0.895	0.038	2.438	0.331	0.024	0.028	2.090	0.995	0.533
106	0	0.080	0.364	0.233	0.036	65.273	0.297	2.127	0.551	0.000	0.014	0.071	0.096	0.195	0.601	0.024	2.020	0.330	0.024	0.020	1.298	0.657	0.544
113	0	0.131	0.372	0.197	0.017	70.717	0.385	1.931	0.464	0.128	0.227	0.028	0.139	0.217	0.644	0.033	1.710	0.267	0.030	0.032	2.308	0.897	0.517
AAX3	0	0.079	0.449	0.313	0.042	70.855	0.454	2.523	0.170	0.000	0.021	0.036	0.084	0.202	0.507	0.035	1.763	0.440	0.050	0.039	0.644	0.375	0.614
ALI5	0	0.091	0.433	0.099	0.142	80.092	1.233	3.054	0.158	0.000	0.018	0.000	0.206	0.752	0.701	0.117	2.405	2.210	0.208	0.153	1.516	1.620	3.394
IAD4	0	0.085	0.522	0.316	0.018	101.151	0.724	3.312	0.294	0.000	0.024	0.049	0.160	0.222	0.817	0.057	2.335	0.417	0.071	0.052	1.466	0.574	0.667
IKE3	0	0.096	0.602	0.396	0.047	96.522	0.652	3.423	0.191	0.000	0.023	0.046	0.097	0.216	0.689	0.041	2.480	0.353	0.065	0.042	0.870	0.411	0.531
QAB7	0	0.074	0.441	0.255	0.063	79.351	0.647	2.605	0.163	0.000	0.017	0.027	0.115	0.322	0.601	0.050	2.066	1.003	0.085	0.073	0.885	0.712	1.211
QAF5	0	0.070	0.378	0.199	0.062	73.567	0.709	2.638	0.160	0.000	0.016	0.022	0.112	0.370	0.614	0.051	2.299	0.925	0.115	0.091	1.051	0.878	1.608
QAGA	0	0.103	0.355	0.217	0.064	68.318	0.938	2.545	0.111	0.000	0.013	0.022	0.121	0.440	0.606	0.073	1.943	0.859	0.160	0.119	1.150	0.993	1.755
QBX7	0	0.069	0.474	0.318	0.030	67.721	0.350	1.917	0.118	0.000	0.018	0.026	0.082	0.108	0.445	0.021	1.309	0.128	0.028	0.023	0.848	0.328	0.259
RL05	0	0.090	0.413	0.269	0.029	67.545	0.420	2.328	0.218	0.000	0.019	0.031	0.100	0.144	0.523	0.042	1.923	0.277	0.031	0.038	0.958	0.417	0.419
RPU5	0	0.061	0.474	0.319	0.053	71.474	0.632	2.725	0.130	0.000	0.020	0.012	0.087	0.183	0.578	0.044	2.483	0.451	0.091	0.067	0.950	0.588	0.702

Cat ID	Time (week)	4:0	6:0	8:0	10:0	12:0	14:0	14:1	15:0	16:0	t9 16:1	c9 18:1	17:0	18:0	t9 18:1	t10 18:1	t11 18:1	c/t 18:1	t12&c7 18:1	t13&c6 18:1	c9 18:1	c11 18:1	c12 18:1	c13 18:1	t16 18:1
1	24	0.008	0.006	0.011	0.048	0.458	8.850	0.237	1.020	57.181	0.251	12.686	1.317	34.902	0.167	0.971	0.398	0.421	0.206	0.238	84.789	4.819	0.513	0.351	0.062
2	24	0.000	0.006	0.010	0.023	0.345	8.775	0.231	1.241	39.264	0.452	15.001	1.472	34.295	0.141	0.518	0.436	0.536	1.065	0.000	45.626	4.829	0.332	0.000	0.091
7	24	0.012	0.005	0.009	0.032	0.416	5.995	0.291	0.989	56.103	0.361	11.921	1.648	46.510	0.256	0.996	0.783	0.814	0.146	0.330	99.293	5.292	0.585	0.420	0.097
8	24	0.030	0.012	0.023	0.074	0.440	4.803	0.209	1.034	67.347	0.370	8.652	1.824	65.358	0.293	1.047	1.073	1.145	0.105	0.314	110.569	5.874	0.594	0.421	0.122
12	24	0.027	0.006	0.012	0.029	0.343	4.769	0.095	0.973	55.027	0.251	9.408	1.541	45.151	0.197	0.970	0.358	0.492	0.208	0.292	92.505	5.135	0.562	0.374	0.052
22	24	0.023	0.018	0.012	0.083	0.416	14.880	0.304	1.758	59.346	0.626	20.198	2.171	49.800	0.242	0.705	0.623	0.691	1.362	0.000	68.898	6.713	0.547	0.588	0.111
23	24	0.008	0.006	0.012	0.038	0.473	5.057	0.100	0.942	59.195	0.240	9.149	1.530	46.235	0.193	0.930	0.491	0.602	0.317	0.000	97.631	5.751	0.533	0.359	0.071
27	24	0.013	0.008	0.010	0.059	0.569	8.820	0.558	1.194	56.772	0.172	13.849	1.254	38.441	0.178	0.993	0.485	0.589	0.000	0.367	104.920	5.530	0.553	0.338	0.079
28	24	0.011	0.005	0.010	0.080	0.383	5.706	0.265	1.115	53.667	0.317	9.499	1.457	43.987	0.225	0.740	0.747	0.820	0.164	0.193	81.576	4.960	0.456	0.393	0.099
50	24	0.012	0.005	0.019	0.138	0.664	18.457	1.501	1.148	68.275	0.385	22.582	1.768	45.580	0.000	0.821	0.699	1.304	0.532	0.146	125.559	6.517	0.892	0.530	0.099
51	24	0.009	0.011	0.017	0.072	0.636	10.722	0.643	0.882	50.039	0.266	15.385	1.196	29.767	0.140	0.909	0.518	0.558	0.445	0.000	76.392	4.991	0.579	0.408	0.077
53	24	0.000	0.015	0.000	0.066	0.625	12.866	1.588	1.070	50.373	0.388	30.049	1.244	28.906	0.209	1.147	0.625	0.505	0.356	0.245	113.238	6.239	0.651	0.442	0.082
58	24	0.000	0.007	0.020	0.046	0.593	7.042	0.553	0.823	41.801	0.244	14.464	1.046	26.775	0.123	0.933	0.339	0.391	0.000	0.444	80.989	3.874	0.376	0.261	0.041
68	24	0.000	0.000	0.000	0.048	0.619	3.967	0.073	0.592	48.213	0.134	5.061	1.375	47.405	0.155	0.923	0.457	0.818	0.246	0.000	90.916	5.114	0.563	0.309	0.060
106	24	0.016	0.000	0.014	0.040	0.339	4.503	0.162	0.826	58.595	0.276	9.559	1.472	46.046	0.235	0.847	0.596	0.703	0.082	0.243	91.262	5.445	0.533	0.429	0.090
113	24	0.011	0.006	0.009	0.028	0.332	5.441	0.223	1.006	53.317	0.336	11.699	1.655	44.722	0.216	0.712	0.491	0.561	0.405	0.213	82.923	5.844	0.530	0.510	0.095
AAX3	24	0.009	0.005	0.015	0.143	0.476	10.883	0.232	1.253	51.791	0.364	14.187	1.448	35.003	0.186	0.559	0.379	0.420	0.633	0.000	65.118	5.028	0.441	0.405	0.055
ALI5	24	0.060	0.017	0.011	0.047	0.411	8.447	0.245	1.369	47.489	0.557	13.743	1.875	47.631	0.179	0.476	0.522	0.693	1.166	0.000	54.636	5.412	0.366	0.437	0.092
IAD4	24	0.035	0.010	0.000	0.084	0.485	8.800	0.222	1.474	59.500	0.412	15.008	1.855	53.656	0.251	0.860	0.511	0.650	0.859	0.000	94.441	6.840	0.619	0.533	0.083
IKE3	24	0.028	0.007	0.022	0.083	0.593	4.247	0.106	0.743	43.951	0.214	6.222	1.183	38.319	0.143	0.717	0.553	0.703	0.203	0.000	71.821	4.193	0.392	0.301	0.096
QAB7	24	0.000	0.000	0.000	0.021	0.484	6.410	0.139	0.962	41.501	0.348	8.743	1.461	41.770	0.122	0.534	0.396	0.580	0.534	0.000	61.874	4.689	0.341	0.000	0.060
QAG4	24	0.046	0.013	0.010	0.034	0.367	4.927	0.298	1.040	46.876	0.473	8.671	1.751	55.569	0.241	0.670	1.099	1.148	0.585	0.000	66.848	4.505	0.473	0.525	0.149
QBX7	24	0.000	0.000	0.000	0.030	0.308	4.976	0.181	1.059	65.890	0.394	9.261	1.828	59.956	0.353	1.071	1.016	1.134	0.000	0.265	114.434	6.008	0.611	0.377	0.141
RLO5	24	0.000	0.000	0.000	0.095	0.624	4.949	0.179	0.681	54.733	0.223	8.144	1.343	45.364	0.173	0.944	0.686	0.844	0.198	0.000	85.628	4.932	0.562	0.359	0.084
RPU5	24	0.031	0.000	0.021	0.022	0.550	3.826	0.097	0.811	52.950	0.311	6.336	1.557	55.060	0.227	0.910	1.019	1.253	0.284	0.000	83.894	4.535	0.446	0.322	0.150

Cat ID	Time (week)	c15 18:1	t9,12 18:2	c9,t12 18:2	t9,c12 18:2	t11,c15 18:2	18:2n6	20:0	18:3n3	c9,t11 18:2	c11,c13 18:2	t10,c12 18:2	c9,c11 18:2	c10,c12 18:2	t11,t13 18:2	other t/ 18:2	20:3n3	22:1	20:4n6	24:1	20:5n3	24:0	22:4n6	22:5n3	22:6n3
1	24	0.439	0.078	0.389	0.200	0.128	57.913	0.387	1.623	0.308	0.054	0.114	0.033	0.036	0.184	0.652	0.496	0.047	1.632	1.844	0.031	0.037	1.375	1.381	3.090
2	24	0.440	0.129	0.448	0.210	0.721	34.798	0.532	2.386	0.177	0.000	0.004	0.000	0.000	0.000	2.654	0.636	0.103	3.928	9.156	0.089	0.115	1.054	3.983	14.548
7	24	0.437	0.120	0.432	0.168	0.057	81.914	0.363	3.704	0.500	0.000	0.012	0.038	0.000	0.146	0.573	0.477	0.036	2.036	1.491	0.027	0.037	0.990	1.235	2.995
8	24	0.603	0.118	0.445	0.251	0.066	97.165	0.673	4.546	0.246	0.000	0.006	0.042	0.000	0.112	0.287	0.591	0.049	3.328	0.698	0.074	0.074	1.525	1.229	1.840
12	24	0.538	0.155	0.375	0.153	0.115	61.270	0.535	1.400	0.583	0.000	0.018	0.040	0.016	0.249	0.606	0.525	0.063	1.498	1.768	0.039	0.064	1.757	1.503	3.374
22	24	0.621	0.185	0.572	0.262	0.986	48.765	0.661	3.097	0.277	0.008	0.020	0.000	0.000	0.516	3.276	0.793	0.162	4.581	11.022	0.095	0.130	1.559	4.633	16.171
23	24	0.468	0.092	0.383	0.240	0.082	73.631	0.435	2.450	0.285	0.062	0.101	0.062	0.020	0.190	0.452	0.595	0.041	1.647	1.238	0.030	0.031	1.987	1.522	2.603
27	24	0.435	0.094	0.387	0.193	0.032	75.646	0.361	2.203	0.428	0.000	0.031	0.037	0.011	0.104	0.185	0.527	0.028	1.800	0.239	0.030	0.036	1.521	0.641	0.635
28	24	0.470	0.068	0.389	0.167	0.073	77.363	0.619	4.132	0.248	0.000	0.014	0.038	0.000	0.132	0.544	0.501	0.052	2.071	1.400	0.080	0.080	0.901	1.233	2.867
50	24	0.633	0.189	0.513	0.201	0.191	75.516	0.609	2.128	0.354	0.000	0.011	0.070	0.028	0.256	0.709	0.730	0.068	2.538	2.305	0.048	0.060	2.218	1.821	3.650
51	24	0.345	0.128	0.415	0.243	0.157	59.940	0.222	2.489	0.220	0.000	0.009	0.025	0.000	0.136	0.817	0.549	0.035	2.623	2.942	0.022	0.018	1.453	1.319	3.526
53	24	0.408	0.101	0.550	0.265	0.223	85.354	0.387	3.360	0.290	0.000	0.055	0.052	0.022	0.185	1.005	0.587	0.041	2.262	3.295	0.029	0.038	1.326	1.621	4.361
58	24	0.423	0.070	0.360	0.238	0.056	54.407	0.392	1.544	0.221	0.024	0.054	0.018	0.000	0.141	0.262	0.361	0.038	1.173	0.708	0.036	0.031	1.101	0.848	1.421
68	24	0.425	0.166	0.363	0.220	0.029	69.061	0.419	1.764	0.263	0.062	0.149	0.058	0.022	0.164	0.186	0.762	0.053	1.724	0.184	0.026	0.034	2.806	1.295	0.833
106	24	0.375	0.089	0.377	0.213	0.050	72.983	0.308	3.192	0.637	0.000	0.021	0.055	0.017	0.125	0.386	0.580	0.035	2.332	1.080	0.023	0.027	1.341	1.150	2.087
113	24	0.436	0.106	0.396	0.195	0.298	68.666	0.364	3.860	0.316	0.059	0.091	0.022	0.015	0.226	1.243	0.587	0.060	2.994	4.335	0.033	0.047	1.667	2.178	6.318
AAX3	24	0.447	0.085	0.401	0.177	0.369	47.121	0.531	2.207	0.190	0.000	0.009	0.010	0.025	0.305	1.390	0.495	0.081	2.374	4.381	0.080	0.092	0.854	2.223	7.198
ALI5	24	0.457	0.116	0.425	0.168	0.797	68.866	0.699	5.740	0.195	0.000	0.009	0.000	0.000	0.343	3.025	0.671	0.147	6.007	9.425	0.134	0.186	1.194	4.479	17.040
IAD4	24	0.732	0.117	0.521	0.227	0.393	65.410	0.939	2.532	0.200	0.000	0.034	0.012	0.039	0.455	1.799	0.832	0.139	3.310	6.107	0.126	0.171	1.974	3.738	11.053
IKE3	24	0.404	0.064	0.356	0.173	0.053	62.971	0.509	2.706	0.126	0.000	0.012	0.040	0.000	0.097	0.374	0.331	0.046	1.723	0.948	0.065	0.063	0.714	0.831	2.012
QAB7	24	0.415	0.064	0.344	0.153	0.191	62.144	0.621	3.267	0.112	0.000	0.006	0.006	0.000	0.260	1.094	0.465	0.094	2.594	2.918	0.095	0.140	0.991	2.555	7.124
QAG4	24	0.457	0.074	0.348	0.143	0.268	78.782	0.736	5.508	0.284	0.000	0.014	0.042	0.000	0.167	1.258	0.541	0.076	4.010	3.333	0.121	0.122	1.009	2.546	7.294
QBX7	24	0.487	0.055	0.516	0.231	0.070	105.566	0.537	5.577	0.306	0.000	0.019	0.035	0.023	0.120	0.368	0.603	0.045	3.278	1.051	0.047	0.049	1.290	1.262	1.993
RLO5	24	0.375	0.083	0.401	0.262	0.047	70.910	0.379	3.585	0.197	0.000	0.015	0.025	0.000	0.096	0.255	0.453	0.030	2.626	0.682	0.038	0.031	0.881	0.737	1.251
RPUS	24	0.436	0.073	0.458	0.251	0.088	86.101	0.565	5.205	0.215	0.000	0.007	0.018	0.000	0.096	0.407	0.502	0.036	4.401	1.308	0.079	0.074	1.075	1.339	2.355

*Example (c9 18:1 = 18 carbons; 1 cis double bond at the 9th carbon)

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Vita

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