University of Alberta

Establishing the anti-cancer effects of unsaturated fatty acids and a novel oil on human breast cancer cells

by

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Abstract

N-3 and n-6 fatty acids and conjugated linoleic (CLA) acid have been known to inhibit breast cancer cell growth, however, effects on normal cells and of single and mixtures of n-3 and n-6 pathway intermediates have not been thoroughly investigated. The objective of this thesis was to determine the effects of fatty acids with potential anti-cancer activity and a fatty acid mixture, representing a new plant source, on breast cell viability and cell membrane composition. Human tumorigenic MDA-MB-231 and MCF-7 cells lines and a non-tumorigenic MCF-12A cell line were studied. All fatty acids, including the mixture representing a stearidonic acid (SDA) enriched flax oil (SO) reduced the viability of tumorigenic but not non-tumorigenic cells, compared to cells without fatty acids ($p<0.05$). α-linolenic acid, docosahexaenoic acid (DHA), linoleic acid, and arachidonic acid (AA) from treatments were minimally converted down their respective pathways and incorporated into membranes as the original fatty acid. Intermediates SDA, eicosatetraenoic acid and eicosapentaenoic acid showed significant conversion down the n-3 pathway, but not to DHA. N-6 γ-linolenic acid and dihomo-γ-linolenic acid (DGLA) treatments accumulated DGLA in the phospholipids, but not AA. SO treatment showed conversion to docosapentaenoic acid and DGLA in tumorigenic cell lines, but was not well converted in MCF-12A cells. C9, t11-CLA decreased tumorigenic growth ($p<0.05$) and was isomerized to a more potent isomer on cell viability. Overall, our data demonstrates that *in vitro,* n-3 and n-6 fatty acid intermediates, CLA and a n-3 and n-6 mixture decrease tumorigenic cell viability and are incorporated into

membranes with significant conversion down their pathways. Inhibitory effects of n-3 and n-6 fatty acid intermediates seem to be independent of conversion to biological endpoints, and c9, t11-CLA's inhibitory effects may be due to its isomerization.

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Abbreviations

AA	arachidonic acid
ALA	α-linolenic acid
BSA	bovine serum albumin
CLA	conjugated linoleic acid
DGLA	dihomo-γ-linolenic acid
DHA	docosahexaenoic acid
DPA	docosapentaenoic acid
EPA	eicosapentaenoic acid
ER	estrogen receptor
ETA	eicosatetraenoic acid
GLA	γ-linolenic acid
LA	linoleic acid
MUFA	monounsaturated fatty acids
OA	oleic acid
PL	phospholipids
PUFA	polyunsaturated fatty acids
PR	progesterone receptor
SDA	stearidonic acid
SFA	saturated fatty acids
SO	stearidonic acid-enriched flax oil mixture

Chapter One – Study Rationale

I. Rationale

N-3 polyunsaturated fatty acids (PUFA) eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), n-6 PUFA arachidonic acid (AA), and conjugated linoleic acid (CLA) have been reported to inhibit the growth of human breast cancer cells, however intakes of these fatty acids are low in the North American diet (1-5).

Long chain PUFA EPA, DHA and AA, can be produced in the body from their short chained family member α-linolenic acid (ALA) and linoleic acid (LA) (6). Despite this ability, synthesis is very low in humans due to the low catalytic activity of a Δ6-desaturase enzyme as well as the competition between the substrates ALA and LA for this enzyme (7-11). Still, a number of metabolic intermediates exist in the n-3 and n-6 pathways, and it may be possible to supply pathway intermediates after the step involving a Δ6-desaturase enzyme to increase the content of endogenously synthesized long chain PUFA. There is also some support that n-3 and n-6 intermediates stearidonic acid (SDA) and γ-linolenic acid (GLA), respectively, may possess anti-carcinogenic properties; however, whether their anti-carcinogenic properties are independent of their conversion to their downstream intermediates eicosatetraenoic acid (ETA) and dihomo-γ-linolenic acid (DGLA) or further to DHA and AA have yet to be elucidated (12, 13). As changes in the membranes of tumours have not been thoroughly investigated, changes in membrane fatty acid composition due to dietary lipid supplementation are of a high interest in relation to the lipids' anti-carcinogenic properties.

N-3 and n-6 pathway intermediates are present in trace amounts in the diet, and significant sources of these intermediates, if any, are not commonly eaten (14-17). Therefore, new commercial crops enriched with fatty acid intermediates could become a possible source of these fatty acids. Although novel oils have yet to be tested for their efficacies in breast cancer, the efficacy of fish oils seem to have similar effects to their individual components EPA and

DHA, and fatty acid mixtures with n-3 fatty acids have been shown to be converted through their metabolic pathway (18). Therefore, the oil from these plants could have anti-carcinogenic properties in breast cancer, and be a new source of lipids in the diet for the population.

Whether these n-3 and n-6 fatty acids have effects on non-tumorigenic cells have also not been thoroughly investigated in terms of viability and changes in the phospholipid (PL) composition. Previous studies have shown that fatty acids are incorporated into tumorigenic breast cells much more readily than non-tumorigenic cells(19, 20). These fatty acids seem to reduce the n-6 fatty acid content in tumours, and it has been suggested that this tumour membrane enrichment may be a mechanism through which n-3 and n-6 fatty acids work (19, 20).

CLA can also be produced from ALA and LA by rumen anaerobes in the production of stearic acid, however, the predominate isomer, c9, t11-CLA, can only be produced from LA (21-23). Although the human gut microflora have bacterial species with enzymes that can catalyze the conversion of ALA and LA to CLA, minimal conversion is observed, possibly due to the triglyceride form of LA that humans typically consume (24-26). It has been reported that mammalian cells can, however, reverse part of this pathway, converting vaccenic acid to c9, t11-CLA (27). Whether this reaction can go further, in that CLA can be retroconverted back to LA or ALA, or turn into vaccenic acid and stearic acid, has not been confirmed in transformed cells. Although the c9, t11-CLA isomer has been found to reduce growth in human breast cancer cells, it is not well established if this effect is specific to tumorigenic breast cell lines. It has also been shown that this isomer may exhibit anti-tumorigenic effects through its incorporation into the cell membrane lipids as a possible mechanism (29). However, whether this effect is specific to cancer cells and if it influences n-6 fatty acid content in the membrane similarly to anti-tumorigenic n-3 and n-6 fatty acid treatments is unknown. These points will be explored in the current thesis.

II. Objectives and Hypotheses

The objective of this research is to determine whether the n-3 and n-6 pathway intermediates, an SDA-enriched flax oil mixture, and c9, t11-CLA affect the viability of tumorigenic MDA-MB-231 and MCF-7 and non-tumorigenic MCF-12A breast cells, and whether these fatty acids are incorporated into PLs as the original fatty acids or are converted into other fatty acids.

We hypothesized that:

1. n-3 and n-6 fatty acids (n-3: ALA, SDA, ETA, EPA and DHA; n-6: LA, GLA, DGLA and AA) will decrease the growth of tumorigenic cells without affecting the growth of non-tumorigenic cells.
2. individual n-3 and n-6 fatty acids will be incorporated into the PLs of all cells. The proportion of n-3 and n-6 fatty acids incorporated into membrane PLs will be greater in tumorigenic cells than non-tumorigenic cells after incubations
3. n-3 and n-6 fatty acid intermediate supplementation (SDA, ETA, GLA, and DGLA) will result in their conversion to their longer-chained counterparts and will be reflected in the fatty acid content of the cell PLs
4. a mixture of fatty acids representative of the composition of an SDA-enriched flax seed oil will reduce cell growth in tumorigenic but not non-tumorigenic human breast cells
5. fatty acids within an oil that represents the composition of an SDA-enriched flax seed oil will exhibit fatty acid conversion to their longer-chained counterparts and be incorporated into the PLs to a greater extent in tumorigenic cells than non-tumorigenic cells.
6. c9, t11-CLA will not negatively affect the growth of non-tumorigenic breast cells
7. c9, t11-CLA will alter fatty acid tumour PL composition, replacing n-6 fatty acids in the PL

III. Chapter Format

The above hypotheses were tested in separate experiments and the results were divided into separate chapters.

Chapter two provides an introduction and literature review on the topics investigated in this thesis.

Chapter three provides details on the methods used to test the hypotheses.

Chapter four investigates the effects of n-3 and n-6 pathway fatty acids on the viability of the breast cancer cell lines and cell membrane PLs. This experiment tests the hypothesis that n-3 and n-6 fatty acids decrease the viability of tumorigenic cells without affecting non-tumorigenic cells. We also test the hypothesis that n-3 and n-6 fatty acids past the step requiring the catalytic activity of Δ6-desaturase will be converted to DHA and AA, respectively, and incorporated into the membrane PLs as DHA and AA.

Chapter five reports the effects of a fatty acid mixture that represents the oil composition of an SDA-enriched flax seed oil on the viability and membrane PLs of breast cancer cells. These effects will be compared to those of the individual fatty acids that make up the oil. It is hypothesized that this oil will have similar effects to the individual fatty acids in both viability and membrane PLs composition.

Chapter 6 examines the effects of CLA on the viability and cell PLs of breast cells compared to the effects of its precursors on the cells. Through these results, we want to determine the effects on normal breast cells and compare the results to the PL effects of n-3 and n-6 treatment effects.

Chapter 7 summarizes the results of the thesis and discusses the implications and future directions for determining the anti-carcinogenic mechanisms of bioactive fatty acids and mixtures.

IV. References

1. Macronutrients,A Report of the Panel on, Intakes,Subcommittees on Upper Reference Levels of Nutrients and Interpretation and Uses of Dietary Reference, Intakes,Standing Committee on the Scientific Evaluation of Dietary Reference. Dietary reference intakes for energy, carbohydrate, fiber, fat, fatty acids, cholesterol, protein, and amino acids (macronutrients). The National Academies Press; 2005.

2. Schley PD, Brindley DN, Field CJ. (n-3) PUFA alter raft lipid composition and decrease epidermal growth factor receptor levels in lipid rafts of human breast cancer cells. J Nutr. 2007 Mar;137(3):548-53.

3. Schonberg S, Krokan HE. The inhibitory effect of conjugated dienoic derivatives (CLA) of linoleic-acid on the growth of human tumor-cell lines is in part due to increased lipid-peroxidation. Anticancer Res. 1995 Jul-Aug 1995;15(4):1241-6.

4. Bocca C, Bozzo F, Martinasso G, Canuto RA, Miglietta A. Involvement of PPAR alpha in the growth inhibitory effect of arachidonic acid on breast cancer cells. Br J Nutr. 2008 Oct;100(4):739-50.

5. Ritzenthaler KL, McGuire MK, Falen R, Shultz TD, Dasgupta N, McGuire MA. Estimation of conjugated linoleic acid intake by written dietary assessment methodologies underestimates actual intake evaluated by food duplicate methodology. J Nutr. 2001 May;131(5):1548-54.

6. Sprecher H. Metabolism of highly unsaturated n-3 and n-6 fatty acids. Biochim Biophys Acta. 2000 Jul 19;1486(2-3):219-31.

7. Burdge GC, Wootton SA. Conversion of alpha-linolenic acid to eicosapentaenoic, docosapentaenoic and docosahexaenoic acids in young women. Br J Nutr. 2002 Oct;88(4):411-20.

8. Pawlosky RJ, Hibbeln JR, Novotny JA, Salem N,Jr. Physiological compartmental analysis of alpha-linolenic acid metabolism in adult humans. J Lipid Res. 2001 Aug;42(8):1257-65.

9. Sauerwald T, Hachey D, Jensen C, Chen H, Anderson R, Heird W. Effect of dietary alpha-linolenic acid intake on incorporation of docosahexaenoic and arachidonic acids into plasma phospholipids of term infants. Lipids. 1996 Mar;31:S131-5.

10. Chen Q, Nilsson A. Desaturation and chain elongation of N-3 and N-6 polyunsaturated fatty-acids in the human caco-2 cell-line. Biochim Biophys Acta. 1993 Feb 24;1166(2-3):193-201.

11. Emken E, Adlof R, Gulley R. Dietary linoleic-acid influences desaturation and acylation of deuterium-labeled linoleic and linolenic acids in young-adult males. Biochimica Et Biophysica Acta-Lipids and Lipid Metabolism. 1994 Aug 4;1213(3):277-88.

12. Horia E, Watkins B. Comparison of stearidonic acid and alpha-linolenic acid on PGE(2) production and COX-2 protein levels in MDA-MB-231 breast cancer cell cultures. J Nutr Biochem. 2005 Mar;16(3):184-92.

13. Jiang W, Hiscox S, Horrobin D, Bryce R, Mansel R. Gamma linolenic acid regulates expression of maspin and the motility of cancer cells. Biochem Biophys Res Commun. 1997 Aug 28;237(3):639-44.

14. Passi S, Cataudella S, Di Marco P, De Simone F, Rastrelli L. Fatty acid composition and antioxidant levels in muscle tissue of different mediterranean marine species of fish and shellfish. J Agric Food Chem. 2002 Dec 4;50(25):7314-22.

15. Ghasemnezhad A, Honermeier B. Yield, oil constituents, and protein content of evening primrose (oenothera biennis L.) seeds depending on harvest time, harvest method and nitrogen application. Industrial Crops and Products. 2008 Jul;28(1):17-23.

16. Sewon P, Tyystjarvi E. Stearidonic and gamma-linolenic acid contents of common borage leaves. Phytochemistry. 1993 Jul;33(5):1029-32.

17. Surette ME, Edens M, Chilton FH, Tramposch KM. Dietary echium oil increases plasma and neutrophil long-chain (n-3) fatty acids and lowers serum triacylglycerols in hypertriglyceridemic humans. J Nutr. 2004 Jun;134(6):1406-11.

18. Yuri T, Danbara N, Tsujita-Kyutoku M, Fukunaga K, Takada H, Inoue Y, et al. Dietary docosahexaenoic acid suppresses N-methyl-N-nitrosourea-induced mammary carcinogenesis in rats more effectively than eicosapentaenoic acid. Nutrition and Cancer-an International Journal. 2003;45(2):211-7.

19. Grammatikos S, Subbaiah P, Victor T, Miller W. N-3 and N-6 fatty-acid processing and growth effects in neoplastic and non-cancerous human mammary epithelial-cell lines. Br J Cancer. 1994 Aug;70(2):219-27.

20. Larsson SC, Kumlin M, Ingelman-Sundberg M, Wolk A. Dietary long-chain n-3 fatty acids for the prevention of cancer: A review of potential mechanisms. Am J Clin Nutr. 2004 Jun;79(6):935-45.

21. Kepler CR, Hirons KP, Mcneill JJ, Tove SB. Intermediates and products of biohydrogenation of linoleic acid by butyrivibrio fibrisolvens. J Biol Chem. 1966 1966;241(6):1350,&.

22. Schmid A, Collomb M, Sieber R, Bee G. Conjugated linoleic acid in meat and meat products: A review. Meat Sci. 2006 May;73(1):29-41.

23. Palmquist DL, Lock AL, Shingfield KJ, Bauman DE. Biosynthesis of conjugated linoleic acid in ruminants and humans. Adv Food Nutr Res. 2005;50:179-217.

24. Herbel BK, McGuire MK, McGuire MA, Shultz TD. Safflower oil consumption does not increase plasma conjugated linoleic acid concentrations in humans. Am J Clin Nutr. 1998 Feb;67(2):332-7.

25. Rosberg-Cody E, Ross RP, Hussey S, Ryan CA, Murphy BP, Fitzgerald GF, et al. Mining the microbiota of the neonatal gastrointestinal tract for conjugated linoleic acid-producing bifidobacteria. Appl Environ Microbiol. 2004 Aug 2004;70(8):4635-41.

26. Coakley M, Ross RP, Nordgren M, Fitzgerald G, Devery R, Stanton C. Conjugated linoleic acid biosynthesis by human-derived bifidobacterium species. J Appl Microbiol. 2003 2003;94(1):138-45.

27. Turpeinen AM, Mutanen M, Aro A, Salminen I, Basu S, Palmquist DL, et al. Bioconversion of vaccenic acid to conjugated linoleic acid in humans. Am J Clin Nutr. 2002 Sep 2002;76(3):504-10.

28. Mahfouz MM, Valicenti AJ, Holman RT. Desaturation of isomeric trans-octadecenoic acids by rat liver microsomes. Biochim Biophys Acta. 1980 Apr 18;618(1):1-12.

29. Field CJ, Schley PD. Evidence for potential mechanisms for the effect of conjugated linoleic acid on tumor metabolism and immune function: Lessons from n-3 fatty acids. Am J Clin Nutr. 2004 Jun;79(6 Suppl):1190S-8S.

Chapter Two – Introduction & Literature Review

I. Breast Cancer

In Canada, cancer is the leading cause of death, causing 30% of all deaths (1). Currently, it is estimated that 40% of all women and 45% of all men will develop cancer during their lifetimes, of which 43% of new cancer cases and 61% of deaths will occur in people over 70 years of age (1). Due to the aging Canadian population, the number of new cancer cases is only expected to increase, increasing the burden on the health care system. Currently, there is a 64% survival rate 5-years after diagnosis (1).

Of all types of cancer, breast cancer is the type of cancer most commonly diagnosed in Canadian women, making up 28% of all new cancer cases and 15% of all cancer deaths (1). Breast cancer occurs when cells in the breast tissue become malignant. These cells are usually ductal cells, which make up about 85% of breast cancer cases, or milk-producing lobule cells that make up about 15% of breast cancer cases (2). Despite the different histology types, overall breast cancer survival in populations of German, Australian, Italian and Dutch women have been found to be similar (3-6). Instead, survival may be more dependent on other characteristics. Breast tissue contains a large lymphatic system. Through this lymphatic system, tumour cells can be easily transported to other parts of the body, leading to a higher degree of tumour metastasis and reoccurrence in comparison to other types of cancers (2). Breast tumours are usually graded depending on their size, lymph node status and metastatic status, and are assigned a stage from I to IV, where IV indicates the most serious tumour (2). The mortality rates of patients with stage IV cancer are over twice that of stage I cancer patients (7).

Another unique characteristic of breast cancer is that it can be sensitive to circulating hormones in the body depending on which receptors it expresses—or its receptor status. For breast cancer, the receptor status is determined by the expression of three receptors: the estrogen receptor (ER), the progesterone receptor (PR), and the human epidermal growth factor receptor 2 (HER2). Cells that have either ER or PR increase cell proliferation in response to high estrogen

and progesterone levels, respectively, which may be of particular concern in women who are using hormone therapies. A cohort of breast cancer patients have found that 63% of the cohort had an ER+PR+ breast cancer, while about 20% had ER-PR- breast cancer, and that women with cancers of the ER-PR- phenotype had a mortality risk over twice that of ER+PR+ breast cancers (7). Breast cancers that have HER2 are associated with a more aggressive cancer that is more likely to reoccur (8-10). By characterizing breast cancers, better predictors of cancer progression and better treatments can be determined in order to improve breast cancer outcomes. Unlike tumour histology, hormone receptor status has been found to be an independent factor for cancer survival, where Fortunato et al. (2012) found a hazard ratio of 0.57 (95% confidence interval (CI) 0.40-0.81) when comparing positive to negative receptor status (6).

Many other factors have been found to negatively affect cancer risk, progression and mortality. 15% of all breast cancer cases are due to heredity, mainly in the form of having specific mutation in the breast cancer genes BRCA1 and BRCA2 (2). Other non-modifiable risk factors for breast cancer include being female, age, age of menarche, and menopausal status (1, 2, 11-14).

Modifiable factors have also been found to negatively impact the risk of breast cancer, as seen in Table 2.1.

Table 2.1 Modifiable Risk Factors for Developing Breast Cancer

Factor	***Reference***
Obesity/Weight Gain	(15, 16)
Lack of Exercise	(17, 18)
Alcohol	(19)
Smoking	(20, 21)
Radiation	(22, 23)
Hormone Replacement Therapy	(24, 25)
High Fat Diet	(18, 26-29)

Of the identified risk factors, one of the most easily modifiable is a change in the diet, which will be further discussed.

II. Unsaturated Fatty Acids

ω-fatty acids are unsaturated fatty acids differentiated by the location of their final carbon-carbon double bond, in a cis-conformation, counted from the methyl-end of the fatty acid's carbon-chain. They can vary in length and degrees of unsaturation. The most commonly recognized groups of ω-fatty acids are the ω-3 (n-3), ω-6 (n-6), and ω-9 (n-9) fatty acids, each of which can be obtained from the diet, and play a variety of important roles in the human body. Although there are some monounsaturated fatty acids (MUFA) in these groups (mainly in the n-9 group), most of the common ω-fatty acids are polyunsaturated fatty acids (PUFAs). Of the different families of ω-fatty acids, two of them have gained much attention: the n-3 fatty acid and the n-6 fatty acids. Two of the fatty acids within these families are considered dietary essential fatty acids: α-linoleic acid (ALA, C18:3 n-3) and linoleic acid (LA, C18:2 n-6), as they cannot be produced in the body. A source of these fatty acids is required in the diet to form the longer chained PUFAs that act as structural components of cells (lipid rafts and membrane phospholipids (PL)) and are part of signaling molecules like eicosanoids (30). After ingestion, these fatty acids are then metabolized according to their respective pathways outlined in Figure 2.1 (31). In these pathways, attention is typically drawn towards the longer fatty acids outside of their essential precursors: eicosapentaenoic acid (EPA, C20:5 n-3), docosahexaenoic acid (DHA, C22:6 n-3), and arachidonic acid (AA, C20:4 n-6). These fatty acids are precursors to a wide variety of eicosanoids that are considered either anti-inflammatory or pro-inflammatory, where AA-derived eicosanoids are generally considered more pro-inflammatory than EPA and DHA-derived eicosanoids. Although functions of the intermediate fatty acids in these pathways are less studied, they are gaining more attention due to their potential to have biological roles and function as precursors of their longer-chained counterparts.

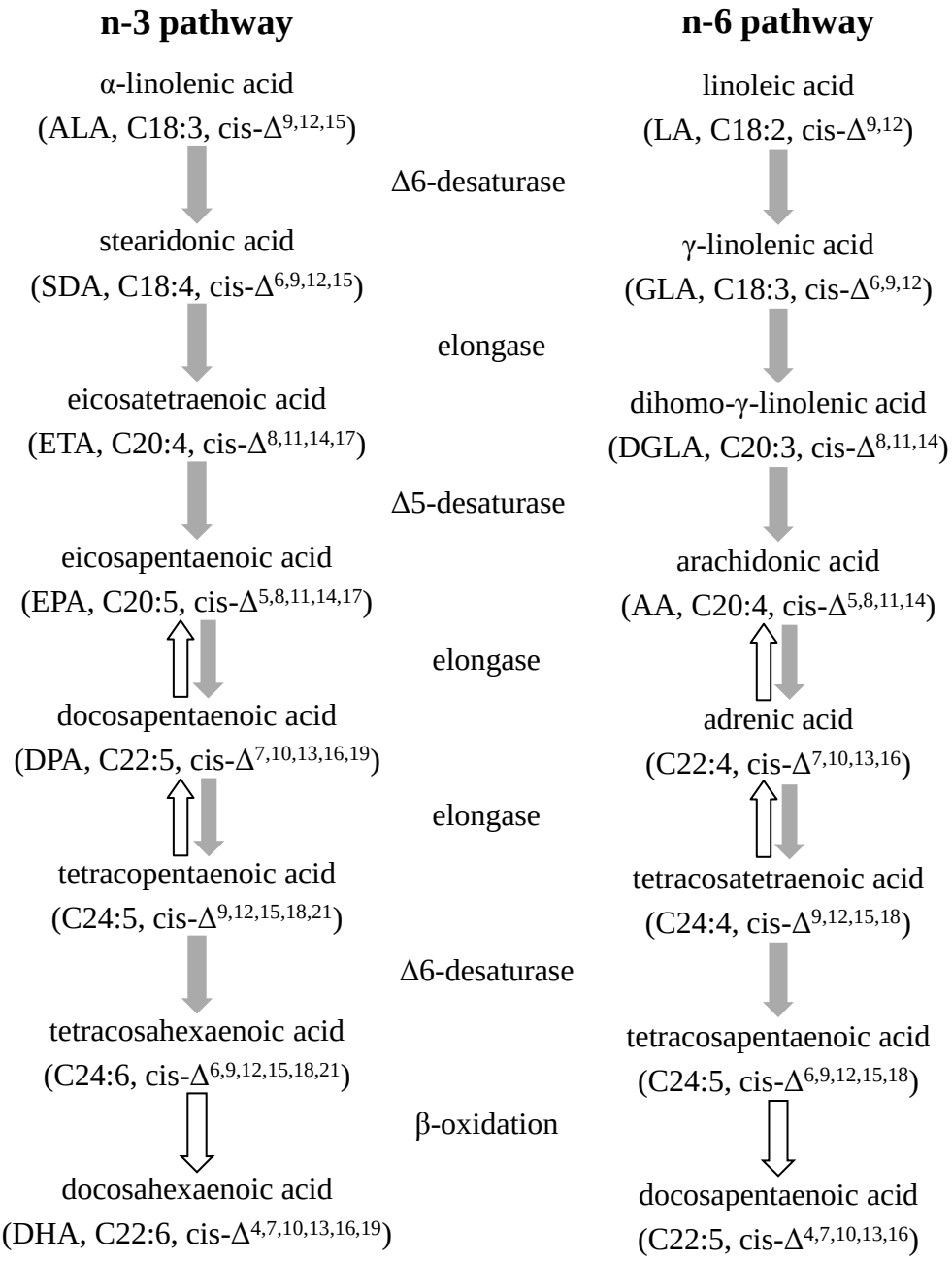

Figure 2.1 Main N-3 and N-6 Metabolic Pathways in Humans. Gray arrows indicate activity in the endoplasmic reticulum and white arrows indicate activity in the proxisome.

A. N-3 Fatty Acids

N-3 fatty acids are PUFAs with a cis-carbon-carbon double bond on the third carbon from the methyl end of the fatty acid. Although there are many different fatty acids in this family, compared to the other fatty acids in the

pathway ALA, EPA and DHA are found in highest quantity in both the diet and body (31). As mentioned before, of all the n-3 fatty acids in the n-3 metabolic pathway, the shortest one, ALA, is considered to be a dietary essential fatty acid. Humans lack the Δ12- and Δ15-desaturase enzymes required to desaturate fatty acid chains beyond the Δ9 position, and therefore need to be obtained through an exogenous source (30). From ALA, its longer-chained n-3 fatty acids can be produced through the catalytic activities of a series of alternating desaturase and elongase enzymes to its biological end-product DHA, as seen in Figure 2.1. ALA is desaturated by the catalytic activity of a Δ6-desaturase enzyme to stearidonic acid (SDA, C18:4 n-3), which is then elongated to eicosatetraenoic acid (ETA, C20:4 n-3), desaturated to EPA catalyzed by a Δ5-desaturase enzyme and elongated to docosapentaenoic acid (DPA, C22:4 n-3), before being further elongated, desaturated and oxidized to DHA. In humans, however, this process is inefficient, where the conversion efficiency from ALA to DHA has been found to be 9% in women and 0.05% in men (32, 33). Discrepancies between the sexes may be due to differences in hormonal factors and lower levels of β-oxidation in women, but the overall pathway inefficiency is suggested to be due to the first enzyme's catalytic activity in this conversion—the Δ6-desaturase enzyme which has been found to have a low catalytic activity for substrates in the n-3 and n-6 fatty acid pathways (32-34). Due to this low conversion rate, EPA and DHA are sometimes considered conditionally essential fatty acids. There is interest in providing pathway fatty acid intermediates to bypass the need for the Δ6-desaturase enzyme.

Despite the low conversion rates, humans do not need to depend on endogenous synthesis to obtain all of these longer chained n-3 fatty acids, as they can be obtained from our diet. Shorter n-3 fatty acid ALA is commonly found in plant oils, where flax oil can contain 53% ALA, while walnut and canola oil contain about 10% and 9% ALA, respectively (35). Longer-chained counterparts EPA and DHA are found in oily cold water fish like sardines, salmon and mackerel which can contain 1.0%, 1.4% and 2.0% of EPA and DHA (35). Unlike ALA, EPA and DHA, SDA and ETA are less common in the diet. Various

species of Mediterranean fish have been found to have between 0-2% SDA or 0-2% ETA in their muscle tissue, while a type of brown seaweed has been found to have between 0.0007-0.0017% SDA (36, 37). Non-marine sources of these fatty acids include echium oil, with 12.5% SDA and 0.3% ETA, black currant seeds with 2.5-4.5% SDA, and borage leave with 0.57-0.90% SDA (38-40).

Although n-3 fatty acids are required for the production of eicosanoids, the intake of these n-3 fatty acids are reported to have additional beneficial effects on human health (41). ALA has been found to have cardioprotective and anti-carcinogenic effects, and intake is associated with a decreased the risk of depression (42-44). EPA and DHA have been reported to have cardioprotective, anti-inflammatory and anti-carcinogenic properties, as well as possible neuroprotective and insulin sensitizing effects in animal and cell models of human disease (45-49). Additionally, EPA has been tested as an anti-cachectic therapy in pancreactic cancer patients, where it improved quality of life (50). As a pro-EPA, in that it increases levels of EPA, SDA supplementation has been associated with similar effects as EPA, including anti-inflammatory and anti-carcinogenic properties in mice (51, 52). The studies providing SDA have used borage or echium oils which also provide other ω-fatty acids ALA and LA, which make up a significant portion of the oil and may also affect the oil's bioactive properties. Because SDA is not abundant in the diet, there have been few studies that have explored the biological activity of this n-3 intermediate. Even less is known about the biological activity of ETA. Only a single paper showing that low ETA levels were associated with chronic heart failure mortality was found (53). Despite the lack of literature concerning the intermediates, generally, the existing literature concludes that increasing the intake of fatty acids in the n-3 family are associated with positive biological effects.

Health Canada recommends that people consume 2 servings of fish high in n-3 fatty acids each week to obtain about 500mg EPA and DHA/day (0.06-0.12% of energy), and set an adequate intake of total n-3 fatty acids as 1.6g/day for men and 1.1g/day for women 19-50 years old (about 0.6-1.2% energy) (54-57). Deficiencies in n-3 fatty acids have been associated with scaly and hemorrhagic

dermatitis, hemorrhagic folliculitis of the scalp, impaired wound healing and growth retardation (58). Canadians generally meet total n-3 intake by consuming ALA, however, typically they do not meet the recommendation for fish intake to obtain the longer chained n-3 fatty acids (56). Studies of an adult population in Quebec, long-term care facility patients in Waterloo, and cancer patients across Canada reported that the intake of fish, that provides EPA and DHA, fall well below 2 servings a week (59-62). Even in pregnant women, where DHA is considered important for fetal development, most women fail to meet this recommendation (63). Although Health Canada has not set an upper limit in regards to n-3 intake specifically, they have noted that this was due to the lack of definitive data and that adverse reactions have been associated with high n-3 intake (57, 64). For oil sources available in Canada, Health Canada has recommended that dosages of 5g (about 890mg GLA), 3g (about 1.2g EPA and DHA), and 32g (about 16g ALA)/day of borage, fish/seal oil and flaxseed oil not to be exceeded (65-68). The evidence used for this statement is that there are reports of high consumption of long chain n-3 fatty acids being associated with impairment of the immune system, increased bleeding, and increased oxidative damage in various tissues (56, 69-72).

Since most people have low intakes of long chain n-3 fatty acids, some researchers have been investing the enhancement of SDA content in commercial crops to provide an alternate dietary source of n-3 fatty acids or a precursor that does not need the Δ6-desaturase enzyme for endogenous synthesis of EPA and DHA. Novel flax with 13.4% of its triacylglycerol being SDA, canola with 16-23% SDA and soybeans with up to 4.2-28.2% SDA have all been produced (73-76). Despite the production of these novel plants, only one study has tested the efficacy of novel oils on chronic disease, where a 28.2% SDA-enriched soybean oil was evaluated in a clinical study on cardiovascular risk. This study found that this oil significantly increased the ω-3 index that estimates cardiovascular disease risk, as well as increase EPA and DPA content in erythrocyte membranes (76). Using these crops, it may be possible to increase the intake of n-3 fatty acids of Canadians.

B. N-6 Fatty Acids

N-6 fatty acids are characterized by their final carbon-carbon double bond located at the sixth carbon from the methyl end in a cis-conformation. As with ALA, LA is also considered an essential fatty acid, due to the same absence of desaturase enzymes in the human body, and can be used by the body to produce its longer-chained counterparts. LA can be desaturated by the catalytic activity of Δ6-desaturase enzyme to γ-linolenic acid (GLA, C18:3 n-6), which can then be elongated to dihomo-γ-linolenic acid (DGLA, C20:3 n-6), and then desaturated by the catalytic activity of a Δ5-desaturase to AA, the biological endpoint of this pathway (Figure 2.1) (31). From this pathway, it can be seen that the enzymes required for the metabolism of n-6 fatty acids are the same as those in the n-3 pathway. Like in the n-3 pathway, conversions from LA to AA are also limited by the catalytic activity of desaturase enzymes (77). The conversion from LA to AA was reported to be 1.5% in T-lymphocytes and 6.05% in fetal liver, suggesting that this rate varies between tissues, but are still not very high (78, 79)

Like n-3 fatty acids, n-6 fatty acids found in the metabolic pathway can be obtained from the diet. LA is mainly found in plant oils of sunflowers, corn, soybeans and peanuts, where they make up 71%, 57%, 54% and 33% of the oils, respectively (35). AA is found in the diet primarily in animal products like meat, eggs and dairy, however, levels are not very high (80). Intermediates GLA and DGLA are not as readily available in the diet. Plant oils provide the only known dietary source of GLA and include evening primrose oil, black currant, echium and borage oil, where the GLA content is 8-10%, 11.1-22.7%, 11% and 1.7-25% respectively (38-40, 81). DGLA is only found in trace amounts in some animal products, and at present, there are no commercial sources available.

Adequate intakes for LA have been set to 17g/d for men and 12g/day for women 19-50 years old (about 5-10% of energy) (57). With extreme LA deficiency, people can develop rough scaly skin and dermatitis, as well as have an increased plasma eicosatrienoic acid: AA ratio (82). However, such an extreme deficiency is not usually seen in the Canadian population, as intake is estimated to be around 12-17g/day for men and 9-11g/day for women (56). Upper limits for

LA intake have not been established, however, elevated intake levels have been associated with a pro-oxidant state that can aggravate diseases like coronary heart disease and cancer (80).

Like the n-3 pathway fatty acids, most of the n-6 pathway fatty acids can be found in the diet, albeit some in very small amounts. LA is the most commonly ingested PUFA, typically comprising 5-10% of energy in the diet (57). They are important for cell membrane structure (making up over 10% of cell membranes), cell signaling, as well as eicosanoid production from its conversion to AA (83, 84). The longer chained AA, like its n-3 counterparts, is also a precursor to eicosanoids, however, some of the n-6-related eicosanoids are more strongly associated with pro-inflammatory responses in the body (85). Despite n-6 fatty acids' often being considered pro-inflammatory, they play essential roles in the body. Consumption of n-6 fatty acids are associated with a reduced risk of cardiovascular disease, lower total and LDL cholesterol levels, and a reduced risk of diabetes (86, 87). For cancer, mixed effects have been observed. N-6 fatty acids intake has been associated with increased incidence and progression of colorectal cancers and prostate cancer in men, as well as breast cancer in women, while links between AA intake and skin and pancreatic cancer incidence and progression have been observed (88). However, decreases in lung cancer and ER+ breast cancer growth have been observed as well, showing inconsistency between and within types of cancer (88). Typically LA and AA are the predominant fatty acids investigated. Although there is less literature compared to LA and AA, the effects of the intermediates in the n-6 pathway have been found to differ from LA and AA. GLA has been found to possibly have anti-inflammatory and cardio-protective effects, enhance breast cancer chemotherapy sensitivity, reduce glucose-insulin indices, improve glucose transport in muscle, and slow down the development of diet-induced atherosclerosis in various animal and cell culture models (89-92). Many of these effects are predicted to be due to GLA's conversion to DGLA, but DGLA's effects has not been well documented. DGLA has been found, however, to have anti-cancer effects on cervical and breast cancer *in vitro* (93, 94). Overall, intake of n-6 fatty acids has been reported

to have a mix of positive and negative effects on the human health, especially cancer.

Despite the beneficial effects of GLA and there being dietary sources of GLA, the sources are not readily available to Canadians resulting in a low dietary intake of GLA. In attempt to increase GLA content in the diet, novel plants with a high GLA content are being produced for commercial purposes. Novel soybean, fungus *Mortierella alpina*, and safflower plants have been reported to have average GLA contents of 2.16-4.21%, 10.7% (up to 50%), and 58.6% (up to 77.4%), respectively (95, 96). Similarly, there have been attempts at creating a high DGLA oil from *Mortierella alpine*, through which a 43.9% DGLA oil has been produced (97). Like AA, DGLA is also used as a precursor to produce eicosanoids, however, these eicosanoids have been associated with more anti-inflammatory effects. Other effects of DGLA have not been tested directly.

C. Interactions Between N-3 & N-6 Fatty Acids

Considerable overlap exists in the metabolism of n-3 and n-6 fatty acids. Not only do they overlap in their content in some plant sources, but they also share the same elongase and low flux desaturase enzymes in their metabolic pathways. These enzymes are also used in other pathways, so, when multiple types of fatty acids are ingested, these substrates compete for the same enzymes. Enzymes, however, also have substrate specificity, so they will catalyze some reactions more than others. Δ6-desaturase has been found to prefer ALA > LA > C18:1n-9 (OA) as substrates (98). Δ5-desaturase substrate preferences, however, are still unknown, as most of the studies have focused on the enzyme's activity in the n-6 pathway and not its competition with the n-3 pathway. Elongases have been shown to prefer GLA > C16:0 > AA > ALA > LA > DGLA (99). Other than substrate preferences, enzyme activity can also be affected by substrate or product inhibition, as studies have shown ALA, EPA, DHA, LA and AA to inhibit LA conversion to AA (100-104). Due to these differences, ratios in which n-3 and n-6 fatty acids in the diet should be considered to regulate the amounts of AA, EPA and DHA in tissues. High ratios of n-6:n-3 will then inhibit n-3 fatty

acid conversion, while low ratios will inhibit n-6 fatty acid conversion. But what is a good balance?

N-3 fatty acid intake has been associated with decreased oxidative damage in the brain, and a low n-6:n-3 ratio has been suggested in the prevention of Alzheimer's disease (105, 106). A ratio of 4:1 was associated with partially alleviate learning and motor problems rats with simulated multiple sclerosis (107). On top of brain-related functions, cardiovascular functions have also been assessed in regards to balance between n-6 and n-3 fatty acids. Heart cells treated with a 0.9:1 n-6:n-3 ratio was able to respond normally to a high Ca^{2+} stimulus which usually makes normal cells experience an arrhythmia (108). In humans, a study examining n-3 fatty acid intake and coronary heart disease risk found that a low n-6:n-3 significantly reduced the risk of sudden cardiac death by 35.1% and an almost significant 16.6% reduction of total fatal coronary events, but non-fatal myocardial infarction risk was not reduced (109). Another study found that decreased n-6:n-3 ratios were associated with a 22% reduction of non-fatal myocardial infarction (110). Inflammatory diseases like rheumatoid arthritis have also shown improvements with increased n-3 intake and a n-6:n-3 ratio of 2.7:1, due to the anti-inflammatory properties of n-3 fatty acids (111, 112). In prostate cancer, a high n-6:n-3 ratio over 9.5:1 has been associated with a 355% increased risk versus ratios under 7:1 (113). Statistical difference was even observed when comparing the n-6:n-3 ratios in healthy men (1.1:1) and men with prostate cancer (2:1) (114). When comparing various levels of n-6:n-3 ratios, the 0.68:1 n-6:n-3 level was the most efficient at decreasing the number of colon adenocarinoma tumours per rat compared to an n-6:n-3 of 100:1, although no differences were observed with adenomas (115). In breast cancer, low n-6:n-3 ratios in tumour cell lipids decreased prostaglandin E_2 production and proliferation and increased cell apoptosis, while low ratios in adipose tissue were significantly associated with a decreased risk of breast cancer (odds ratio (OR) 0.33, 95% CI 0.17-0.66) (116, 117). Overall, low n-6:n-3 ratios have been found to be beneficial for the prevention of various chronic diseases.

Despite the observed benefits, the dietary ratio is still fairly high and varies significantly throughout the world. Dietary ratios of Indians living in urban populations and Western diets have been found to range from 38:1-50:1 and 15:1-20:1, respectively, and have been linked to a higher risk of chronic disease (118). Even in Israel, where diets are typically low in total energy, fat, and high in cholesterol-lowering PUFA, it has been hypothesized that the dietary n-6:n-3 ratio of 22-26:1 contributes to the country's similar rates of coronary artery disease, diabetes and cancer to the West (118). In Japan, however, the ratio is about 4:1, and is hypothesized to contribute to the lower risk of cardiovascular disease and breast cancer in this population (118, 119). The ratio, however, is slowly increasing due to the Westernization of the Japanese diet (119). Japanese who have immigrated to Hawaii also showed significant increases in the occurrence of various cancers including prostate, lung and breast cancers, to levels higher than those of Japanese in Japan, and this observation has been suggested to be due to the Westernization of their diets (120). Compared to the 2.4:1 n-6:n-3 ratio of the traditional hunter-gatherer diet and lifestyle and the estimated Palaeolithic 0.79:1 ratio from which our genes have been selected from, our modern n-6:n-3 ratios are much higher (118). Unlike the significant changes to our diets over the years, our genes have not changed, as mutations of DNA are estimated to be 0.05% per million years (118). As genes are linked to disease susceptibility, changes in diet may be a reason for our increased chronic disease incidence. Due to these differences, more effort needs to be put into decreasing these high ratios, by either increasing n-3 intake or lowering n-6 intake in order to reduce our risk for chronic diseases.

III. Dietary Fat & Breast Cancer

About 35% of a North American's diet's calories come from fat, of which a third comes from saturated fat, and a third from monounsaturated fat (121, 122). Due to this large proportion, it is important to determine how fat could influence diseases when studying at the effects of diet on breast cancer. A meta-analysis of 100 animal studies showed that high caloric intakes as well as high fat intake, in

the form of corn oil, independently increased mammary tumour incidence (26). In a mice study examining different concentrations of corn oil on breast tumour growth, a 20% w/w corn oil diet group had much higher tumour volumes than those fed a 5% corn oil diet (27). Rats fed a 20% lard diet, high in saturated fatty acids (SFA), also showed tumour-enhancing effects versus a 0.5% lard diet (28, 29).

Not all components of the diet have the same effects on all types of breast cancer cells, however. An American study investigated diet quality in postmenopausal women and their risk of breast cancer, and found that women following the Alternate Healthy Eating Index, a Mediterranean diet, or had a high Recommended Food Score had a lower risk of ER- breast cancer (123). These differences may be due to the regulation of different genes by different fatty acids, as observed by Alquobail et al. (2010) (124). The importance of the quality of the diet is further supported when mice fed a 20% w/w corn oil diet had the greatest tumour volume, followed by a 20% w/w butter diet, a 19%w/w beef tallow/1%w/w corn oil diet, and then a 19%w/w fish oil/1%w/w corn oil diet in both ER+PR+ and ER-PR- breast tumours (27). Another group studying PUFAs (mainly LA) and SFA found that the incidence of tumours was significantly higher in rats fed the PUFA diet versus the SFA diet regardless of what they were fed before tumour induction (125).

Despite the negative associations between fat and breast cancer, not all fats have been negatively associated with breast cancer. An animal study using a 10% flax seed diet showed a significant reduction in growth rate and a 45% reduction in total breast cancer cell metastasis, including metastasis to lungs and lymph nodes (44). The reasons for these decreases may be partially due to reductions in insulin-like growth factor I and epidermal growth factor receptor expression and increased lipid peroxidation, as well as changes in ER pathways and prolactin in the hypothalamic-pituitary system (27, 28, 44, 126). Although flax seeds are composed of more than just the oil, Saggar et al. found that although the lignans themselves could decrease breast cell proliferation and increase apoptosis significantly by 29% each, the oil was more effective,

decreasing cell proliferation by 35% and increasing apoptosis by 76% (126). Together, they did not show an additive effect, showing proliferation and apoptotic changes of 25% and 28% respectively (126). Oils high in n-3 fatty acids have also been found to have positive effects on breast cancer outcomes. Reports indicate that the tumour growth rate was lower in mice fed either menhaden oil or fish oil concentrate diets in comparison to a corn oil diet, as well as decreased bone metastasis and increased efficacy to chemotherapy drug doxorubicin (127-129). More specifically, DHA in the fish oil has also been shown to have inhibitory effects on breast cancer. A significant 30% reduction in mammary tumour incidence was observed when rats were fed a 7% DHA diet compared to a 7% rapeseed/peanut oil diet, and rats fed a 16% n-3 diet had a lower incidence of induced breast tumours than a 16% n-6 diet (130, 131).

Despite the supporting animal research, the negative effects of a high saturated fat diet on breast cancer are highly debatable in human studies. Many studies show no or weak correlations between fat and breast cancer risk in humans, possibly due to the high variability of human diets (132-137). Even examining a specific fatty acid, namely LA, the major component in corn oil, the results from epidemiological studies are highly variable in humans (35). A meta-analysis of epidemiological studies concluded that the relative risk for breast cancer was 0.84 (95% CI 0.71-1.00) in case-control studies and 1.05 (95% CI 0.83-1.34) in prospective cohort studies when comparing high to low intakes of LA (138). Despite this weak evidence, it has generally been considered that diets high in saturated and trans fat are considered to increase the risk of breast cancer, and instead, people should opt for diets high in fruits and vegetables, whole grains and n-3 fatty acids.

IV. Cell Membranes, Dietary Fat & Breast Cancer

Within cells, membranes play an important role in the functioning of a cell. Membranes not only protect the cell from its external environment, but control the flow of certain substances into or out of the cell, both passively and actively, with or without the aid of anchored proteins, or through endocytosis or

exocytosis. Proteins in the membrane can be used to receive or transmit signals from or to other cells. Cellular signaling activity is also concentrated in membrane microdomains in the plasma cell membrane called lipid rafts, which are enriched in SFA (139). Membranes are essential to creating microenvironments within the cell for cell homeostasis (140).

Membranes are composed of a variety of lipids, including PLs and cholesterol, of which the proportions vary depending on the organism, tissue, and membrane type. Of the various membranes in cells, the largest membrane is that of the plasma membrane. The most abundant lipids in cells are the amphipathic PL, which can make up 40-80% of the dried total lipid weight depending on the tissue (excluding adipose tissue) (141). These lipids interact with each other and hold the membrane together, as well as anchor in proteins through various interactions like hydrophobic interactions and Van der Waals forces (140). It has been found that mammalian cells will readily incorporate fatty acids and cholesterol from media into their membranes, while suppressing fatty acid synthesis in the cell (142). These observations lead to the possibility of changing cell membrane fatty acid composition through changes in the diet, which could be important due to the large amount of fat ingested daily. As there are a wide variety of lipids, the composition of cell membranes can also change depending on the diet. PL types, fatty acid chain length, degree of desaturation, and amount of cholesterol all change the membrane fluidity, and in turn, will influence the functions of the membrane (140, 141). Of these differences, membrane fatty acid desaturation has been found to play a major role in regulating cell function. Hydrolysation of sn-2 fatty acyl groups in glycerophospholipids by phospholipase A_2 has been found to alter glucose uptake and a sodium/potassium adenosine triphosphatase activity much more than the removal of PL head groups or the hydrolysation of phosphatidylcholine in erythrocyte cells (143). Amino acid transport, adenylatecyclase, AA metabolism, and insulin binding have also been found to be moderated by SFA and PUFAs (83, 142, 144-146).

Due to the observed effects of membrane composition on cell signaling, various health concerns have also implicated differences in membrane

composition as a mechanism. Cognitive decline was associated with high stearic acid (C18:0) (OR 1.91, 95% CI 1.16-3.15) and n-6 PUFA (OR 1.59, 95% CI 1.04-2.44) in erythrocyte membranes, while high n-3 PUFA content was associated with decreased cognitive decline (OR 0.59, 95% CI 0.38-0.93) (147). Changes in the composition of saturated fat palmitic acid (C16:0) and AA in muscle and adipocyte PLs have been found to alter insulin resistance (148, 149). Vaccenic acid (C18:1 trans-11) and elaidic acid (C18:1 trans-9) fed obese rats were found to have improved T-cell-stimulated cytokine production, although LPS-stimulated cytokine responses were enhanced (150). Many of the aforementioned fatty acids that change cell function are dietary fatty acids, suggesting a connection between dietary fat and disease progression.

Breast cancer is no exception in regards to observed changes in disease progression and changes in membrane composition. It has been reported that the fatty acid composition of malignant and normal fatty acid composition differ, in that cultured malignant cells have been reported to have lower PUFA content and higher SFA:PUFA ratios in whole cell lipids (151). Of the fatty acids implicated, PUFA have been consistently found to influence growth and progression of human breast cancer cells and mammary tumours in animals. Multiple PUFAs have been shown to play important roles in breast cancer progression, including ALA which has anti-carcinogenic properties, and LA which can promote or inhibit growth depending on the environment (152, 153). However, of all the PUFAs, long chain PUFAs EPA and DHA have been found to be among the most potent in their anti-carcinogenic activity (152, 153). There is evidence that the effects of these fatty acids on growth and death of breast cancer cells are due, at least in part, to changes in membrane composition.

One of the main mechanisms PUFAs are thought to exert their effects on breast cancer is through their incorporation into PL membranes. Compared to normal breast cells *in vitro*, anti-cancer PUFAs have been found to be incorporated at much higher rates into tumour PLs than other lipid classes (152). The majority of PUFAs are incorporated into phosphatidylethanolamine, phosphatidylinositol (PI), phosphatidylserine (PS) and phosphatidylcholine (PC)

fractions in the whole cell, and are also incorporated into the PI, PS and PC of lipid rafts, despite the rafts having higher levels of SFA than the rest of the membrane (154). Other fatty acids like conjugated linoleic acid (CLA, C18:2), which has been shown to reduce tumour growth when fed to animals or tumour cells, have also been reported to be incorporated into the PLs and caveolae membranes (a type of lipid raft) of breast cancer cells, suggesting a common mechanism for the anti-cancer effects of both CLA isomers and ω-fatty acids (155-157).

V. N-3 and N-6 Fatty Acids, Membranes & Breast Cancer

Some of the most compelling evidence of the biological effects of n-3 and n-6 fatty acids has been reported against breast cancer. This evidence comes from studies in variety of systems, including cell culture, animals, and humans, that have found anti- and pro-carcinogenic properties of these fatty acids in breast cancers of different receptor statuses. However, evidence is weak concerning the effects of the intermediates SDA, GLA and DGLA on breast cancer cell growth, and generally only speculative, while literature regarding the specificities of the action of ETA on breast cancer has not been studied. On the whole, these fatty acids intermediates have not been well studied in breast cancer.

A. Cell Culture Studies

Cell culture studies are a way to determine the mechanisms through which the dietary lipids exert their effects on cancer progression/treatment. For breast cancer, some of these mechanisms include changes in inflammatory molecule and angiogenic factor production, cell cycle signaling and metastasis, as well as changes in membrane composition, to which is the topic of this thesis (158, 159).

Many studies have examined the effects of n-3 and n-6 fatty acids on breast cell viability and lipids, some of which are summarized in Table 2.2. From this table, it can be summarized that ALA has inhibitory effects on tumorigenic MDA-MB-231 and MCF-7 cell lines and does not affect the growth of non-malignant cells MCF-10A. These effects were associated with the conversion of

ALA to DHA in both these tumorigenic cell lines' lipids. SDA, however, has not been as well studied, and no significant effects have been observed on MDA-MB-231 cell viability, although significant conversion of SDA to DPA has been observed. The effects of SDA on breast cells need to be further investigated on other breast cell lines including non-tumorigenic cells, in order to evaluate its safety as a supplement. Effects of ETA have not been studied at all on breast cells, to our knowledge. EPA and DHA's anti-tumorigenic properties, on the other hand, are very well known. They are consistently found to decrease breast cell growth, with DHA being more potent, and do not affect normal cell growth even at high concentrations. EPA and DPA have also been found to increase the contents of EPA, DPA and DHA in the tumour lipids of MCF-7 cells. Conversely, the effects of LA are more contested: both inhibitory and promoting effects have been associated with LA, as well as the degree of conversion down the pathway is disputed. However, LA may have an inverted U-shaped curve on cell viability, with inhibitory effects at both low and high concentrations, and promoting effects in mid-range concentrations. Like SDA, research on GLA and DGLA's effects on breast cells is limited. Existing literature, does suggest that these two PUFAs possess anti-tumorigenic properties on breast tumour cells, however, little is known about its fate after treatment. AA has also been found to have no or inhibitory effects on breast cells, and can be retroconverted to DGLA or elongated to C22:4n6 in tumorigenic MCF-7 and non-tumorigenic MCF-10A cells.

Dietary supplementation of fatty acids seem to change tumour membrane composition, and it is possible that it is through incorporation into membranes, factors like membrane fluidity and protein delocalization from rafts, may activate anti- and/or pro-carcinogenic mechanisms. Many of the proteins implicated in the progression of breast cancer are directly or indirectly involved in pathways involving membrane-bound proteins; therefore, changes in the membrane may affect their activity. Fatty acid enzyme activity may be involved as well, as *de novo* fatty acid synthesis and PL remodeling pathways have been implicated in breast cancer tumour growth (160). A study has reported that cell membrane

composition differs between normal and malignant lines (151). Low levels of Δ6-desaturase enzyme protein have also been associated with impaired breast cancer prognosis, which would affect n-3 and n-6 fatty acid conversion (161). Whether n-3 and n-6 fatty acids exert their effects by modulating the activity of these enzymes has not been investigated in breast cancer, specifically. Links between desaturase activity and risk of diseases like cardiovascular disease and cystic fibrosis have been found previously (162, 163). Overall, many n-3 and n-6 fatty acids have been found to have anti-cancer effects *in vitro*, however, the effects of the intermediates have not been well studied. Whether their effects are due to their conversion down their respective metabolic pathways or are an intrinsic property of the fatty acid are unknown.

Table 2.2 Cell Culture Studies of N-3 and N-6 Fatty Acids on Breast Cancer Growth and Lipids

Ref.	Cell Line	Treatments	Control	Viability Results	Lipid Changes
(164)	MCF-7	ALA at 50μM for 5 days	No fatty acid	ALA ↓ proliferation 33%	N/A
(165)	MCF-7	30μg/mL LA, ALA methyl ester	No fatty acid	Proliferation: control> LA > ALA	Membrane PL: LA: ↓MUFA, DHA; ↑n-6, LA, C20 :2n6, DGLA, AA ALA: ↓MUFA, ↑n-3, ALA, C20 :3n3, EPA, DPA, DHA, DGLA, AA
(166)	MDA-MB-231	ALA, AA, SDA methyl esters with BSA at 25-200μM for 24h	No fatty acid BSA	↓ with ALA at 25-100μM No change with SDA, though ↓ vs. AA at 100μM No change with AA from 25-100μM	Total Lipids: SDA ↑ETA, EPA, DPA, ΣPUFA, Σn-3, ↓AA, and no changes in ALA, SDA or DHA at 50μM (n-6:n-3 1.1-1.3)
(167)	MCF-7	Borage & echium extracts for 48hr	N/A	Borage, echium ↓ proliferation by 15% and 4%, respectively	N/A
(168)	ER+ : MCF-7, ZR-75, T47D ER- : MDA-MB-231, HBL-100	20μg/mL OA, GLA, ALA, EPA, DHA ethyl esters for 10 days	No fatty acid	Proliferation: MDA-MB-231 : OA ↑; ALA, EPA, DHA↓ HBL-100: OA↑; ALA, EPA, DHA, GLA ↓ MCF-7: no differences ZR-75: ALA, EPA, DHA, GLA ↓ T47D: ALA, EPA, DHA ↓	N/A

Table 2.2 Cell Culture Studies of N-3 and N-6 Fatty Acids on Breast Cancer Growth and Lipids Continued

Ref.	Cell Line	Treatments	Control	Viability Results	Lipid Changes
(169)	MDA-MB-435	^{14}C Labeled LA, EPA	N/A	N/A	1μg/mL EPA uptake > 1.28μg/mL LA up to 16h EPA and LA more in PL vs. neutral lipids at 1 & 4μg/mL Opposite at 16μg/mL In PL with1μg/mL EPA or 1.28μg/mL LA: LA content: PC > PE, PI, PS EPA content : PE > PC, PI, PS 1.28μg/mL LA uptake not affected by addition of EPA (0-16μg/mL) Increasing EPA concentrations with 1.3μM LA ↓LA and ↑EPA in PL and vice versa for increasing LA concentrations with 1.0μg/mL EPA (no changes in AA)
(170)	MDA-MB-231	^{14}C and non-labelled AA and EPA at 50 or 100μM FFA	OA	No cytotoxicity after 24h	7-16% treatment fatty acid uptake at 3h (plateau afterwards) AA > OA > EPA uptake Uptake was higher with 100μM than 50μM treatment Incorporation into cells was in PL > TAG >> CE, DAG, FFA after 3h
(171)	MDA-MB-231	I - 75μM LA; II - 60μM EPA, 40μM DHA; III - 45μM EPA, 30μM DHA, 75μM LA; IV - 150μM LA; V - 100μM DHA; VI - 75μM DHA, 75μM LA; VII - 100μM EPA; VIII - 75μM EPA, 75μM LA; IX - 75μM LA FFA for 72h	No fatty acid or IX	Viability: II, III ↓ vs. I, IV, control V <VII VI < VIII < IV, IX	N/A

Table 2.2 Cell Culture Studies of N-3 and N-6 Fatty Acids on Breast Cancer Growth and Lipids Continued

Ref.	Cell Line	Treatments	Control	Viability Results	Lipid Changes
(172)	MCF-7 MDA-MB-231 HBL-100	0-20μM ALA, EPA, DHA, LA, GLA, AA-BSA-bound for 3 days	No fatty acid	ALA, EPA, DHA, LA, GLA, AA, inhibited HBL-100 EPA no effect on MCF-7 or MDA-MB-231 (other fatty acids not tested)	N/A
(173)	MDA-MB-231	LA, DHA methyl esters at 100μM for 48 h	Ethanol	LA ↑ viability DHA ↓ viability	N/A
(174)	MCF-7	EPA, DHA, LA, AA methyl esters	No fatty acid	EPA, DHA ↓colony growth from 10-100μM (dose response) LA no effect from 1-100μM AA ↓growth @ 100μM	N/A

Table 2.2 Cell Culture Studies of N-3 and N-6 Fatty Acids on Breast Cancer Growth and Lipids Continued

Ref.	Cell Line	Treatments	Control	Viability Results	Lipid Changes
(152)	MCF-7 MCF-10A	6-30μM ALA, EPA, DHA, LA or AA BSA-bound or in liposomes for 7 days	OA	Cell growth: MCF-7 (BSA): ALA, EPA, DHA showed dose response from 6-30μM (EPA, DHA > ALA) AA=ALA LA no effect MCF-10A (BSA): No inhibition by any treatments < 24 μM Inhibition by AA and EPA at 30μM greater than MCF-7 BSA vs. Liposomes in MCF-7: AA: ↑inhibition with BSA-AA at 18-30μM vs. liposome LA: ↑ inhibition with BSA-LA at 30μM vs. liposome DHA: ↑ inhibition with liposome DHA from 12-30μM vs. BSA-DHA ALA: ↑inhibition with liposome EPA from 12-24μM vs. BSA-ALA; equal inhibition at 30μM	Tumour lipids: Up to 40% of tumour lipid was from treatments MCF-7 : LA: ↑ LA, C20:2n6 AA: ↑ DGLA, AA, C22:4n6 ALA: ↑ALA, SDA, ETA, C22:3n3, C22:4n3 EPA : ↑ETA, EPA, DPA DHA : ↑EPA, DPA, DHA MCF-10A: LA: ↑LA, GLA, C20 :2n6, DGLA, AA, C22 :4n6 AA : ↑DGLA, AA, C22 :4n6 ALA : ↑ALA, SDA, C20 :3n3 ETA, EPA, DPA EPA : ↑EPA, DPA, DHA DHA : ↑EPA, DPA, DHA MCF-7 & MCF-10A: Most treatment fatty acids go into PC, PE > PI, PS, PA, TAG, FFA EPA and DHA preferably go to PE ALA, LA, AA preferably go into PC Greater incorporation and conversion of treatment with BSA vs. liposomes
(175)	MDA-MB-435	LA, GLA FFA for 6 days	No fatty acid	Cell counts: 0.5-2μg/mL LA ↑ 10μg/mL LA ↓ 0.5μg/mL GLA ↑ 10μg/mL GLA↓	N/A

Table 2.2 Cell Culture Studies of N-3 and N-6 Fatty Acids on Breast Cancer Growth and Lipids Continued

Ref.	Cell Line	Treatments	Control	Viability Results	Lipid Changes
(176)	ZR-75-1	70μM GLA FFA	No fatty acid	Drop to 39%, 11% and 7% of control by days 5, 6, and 7	N/A
(177)	MCF-7	GLA methyl esters	No fatty acid	Inhibitory concentration at 50% ↓ with GLA from 6-72h Cell growth ↓ at 20-50μg/mL GLA	N/A
(178)	MCF-7	50μM GLA or EPA FFA	No fatty acid	Non-significant decrease vs. no fatty acid by GLA and EPA	N/A
(179)	MDA-MB-231	GLA, OA methyl esters	N/A	GLA dose- and time-dependent response on MDA-MB-231 OA stimulated cells at high concentrations (125-500μg/mL), while inhibited at low (50μg/mL) Other cell lines not tested	N/A
(94)	SKBR3 MDA-MB-231 HMEC	DGLA ethyl ester at 10 or 20 μM for 48 h	No fatty acid	DGLA ↓cell viability at 20μM for SKBR3 & MDA-MB-231 No effect on HMEC	N/A

Abbreviations: AA arachidonic acid; ALA α-linolenic acid; BSA bovine serum albumin; CE cholesterol ester; DAG diacylglycerol; DGLA dihomo-γ linolenic acid; DHA docosahexaenoic acid; DPA docosahexaenoic acid; EPA eicosapentaenoic acid; ER estrogen receptor; ETA eicosatetraenoic acid; FFA free fatty acid; GLA γ-linolenic acid; LA linoleic acid; MUFA monounsaturated fatty acid; N/A non-applicable; OA oleic acid; PC phosphatidylcholine; PE phosphatidylethanolamine; PI phosphatidylinositol; PL phospholipid; PS phosphatidylserine; SDA stearidonic acid; TAG triacyglycerols

B. Animal Studies

Unlike cell culture studies, animal studies focus more on the effects of these fatty acids within a living system rather than mechanisms, as other factors like the immune system and circulating hormones in the body may affect the anti-cancer effects of fatty acids observed in cell culture studies. When investigating the effects of feeding n-3 and n-6 fatty acids in animal models with breast cancer, animal studies correlate fairly well with *in vitro* studies, in terms of effects on growth, as shown in Table 2.3. However, consistency is not as strong as that in cell culture, possibly due to other complicated variables that must be considered in animal models, like animal species and the other components of the diet, including the mixture of fatty acids that would be part of a diet. People typically do not consume a single fatty acid, but rather a mixture of fat sources. These sources provide fat primarily in the form of triglycerides. Therefore, animal studies usually use oils to make up the diet's fat composition. Differences compared to *in vitro* studies may be due to this difference, as usually free fatty acids, albumin-conjugated fatty acids or fatty acid esters are studied *in vitro*. By using a mixture of fatty acids, the observed activity of bioactive fatty acids may be reduced in comparison to *in vitro* studies, as positive effects may be countered by other fatty acids. Fatty acid proportions also vary between the same types of oils as well, and may also account for different activities on tumour progression. Due to this difference, some studies have also taken into account the n-6:n-3 ratios of diets.

In summary of Table 2.3, it was observed that feeding animals diets containing oils high in ALA generally showed decreased tumour progression, as well as some conversion to EPA and DHA and incorporation in tumour lipids to replace LA and AA. Few studies have examined giving diets containing significant levels of SDA to animals, most likely due to lack of natural source to include in diets. Diets using blackcurrant oil, which contains significant levels of SDA, still contain less than 10% SDA. The inability to feed a higher dose (similar to EPA/DHA studies) may explain the lack of anti-tumorigenic activity reported for these diets on tumours. Some conversion of SDA to EPA, DPA and

DHA has been observed. No studies have examined the effects of ETA on tumour development in animals. Extensive research has been done on fish oils containing both EPA and DHA. The use of fish oil in the diet have consistently been found to decrease tumour growth in tumour-bearing animals, typically compared to corn oil control diets. Incorporation of these fatty acids and conversion to DPA has also been observed in tumour cells and adipose tissue. Diets containing high levels of LA in the form of corn and safflower oils, have generally been used as control diets in these studies, so its anti-tumorigenic activities have not been well assessed. However, low fat diets containing mainly LA have been found show decreased tumour progression compared to high fat diets, and has been shown to have anti-tumorigenic activity compared to a commercial diet. Diets containing GLA have showed minimal benefits on tumour progression. Like with studies examining SDA effects, these differences may be due to the low dosages of GLA. Despite the minimal benefits, increases in GLA elongation-product DGLA have been observed with GLA treatment.

In summary, it seems that many oils reduce the growth of breast cancer tumours in animal models; however, some of the dosages that were used in these experiments were unrealistically high for human consumption (even in a therapeutic scenario), so interpretation of the results need to be done with caution. These oils increased the content on their component fatty acids and some conversion down their metabolic pathways was observed in the tumour PLs. As in the *in vitro* studies, anti-tumorigenic effects may be initiated by their changes in the membrane; however, a thorough investigation has not been done on changes in the membranes in animal models.

Table 2.3 Animal Studies of N-3 and N-6 Fatty Acids on Breast Cancer Growth and Lipids

Ref.	Animal Model	Tumour	Diet (w/w unless otherwise noted)	Survival/Tumour Growth/Metastasis	Lipid Changes
(180)	Athymic nude mice	M2, MM3	I – 5% mistol oil (13% LA, 22% ALA) II – control (10% LA, 3% ALA)	Metastasis : II > I Latency, survival : I > II (M2) Tumour size, weight: II > I (MM3)	N/A
(181)	Sprague Dawley rats	NMU induced	I – 15% LO (59% ALA, 15% LA) II – 15% palm oil/sunflower oil (4% LA, 40% OA)	Multiplicity: I > II Latency, incidence: no difference	N/A
(182)	Athymic nude mice	MDA-MB-231	I – 8% CO (n-6:n-3 57:1) II – 8% canola oil (n-6:n-3 2:1) (CO 57% LA; canola oil 21% LA, 10% ALA)	After 25d of diet, tumour volume ↑ with I	II ↑EPA & DHA in tumour ↓LA & AA
(164)	Athymic mice	MCF-7	I – 20% CO II – 4% FSO, 16% CO	II ↓tumour size 33% ↓ proliferation 38% ↑apoptosis 110%	Serum ALA, EPA, DHA ↑ in II
(183)	TG.NK mice	N/A	Oral gavage: I – 0.05ml FSO, 0.15ml CO II – 0.10mL FSO, 0.10 mL CO III – 0.20mL FSO IV – 0.20mL CO	III ↓ tumour weight No difference in tumour incidence	N/A
(184)	Athymic nude mice	MDA-MB-435	I – 20% CO II – 10% FSO, 10% CO	Growth rate ↓ in II No differences in tumour volume weight, metastasis or apoptosis	N/A

Table 2.3 Animal Studies of N-3 and N-6 Fatty Acids on Breast Cancer Growth and Lipids Continued

Ref.	Animal Model	Tumour	Diet (w/w unless otherwise noted)	Survival/Tumour Growth/Metastasis	Lipid Changes
(185)	Sprague-Dawley rats	NMU induced	10% rapeseed oil (21% LA, 9% ALA) n-6:n-3 2.4 :1	NMU caused invasive mammary carcinoma	Fatty acid profile of adipose tissue no different between control (no tumours) and NMU-treated rats
(186)	Sprague Dawley rats	DMBA induced	I – 0.5% CO + 0.5mL coconut oil II – 20% CO + 0.5mL coconut oil III – 20% CO + 0.5mL EPA ethyl ester IV – 20% CO + 0.5mL DHA ethyl ester For 20wk	Incidence and tumours per rat: II > I, III, IV Tumour weight, doubling time: no differences	Tumour PL: LA: I < II, III, IV EPA : I, II, IV < III DPA: I, II, IV < III DHA: I, II, III < IV I < III
(187)	Sprague Dawley rats	MNU induced	I – 9.5% EPA, 0.5% LA II – 4.75% EPA,4.75%DHA,0.5% LA III – 9.5% DHA, 0.5% LA (EPA, DHA, LA were ethyl esters)	Incidence : III << II , I Latency : No differences	Mammary fat: AA: I, II > III EPA: I, II > III DPA: I, II > III DHA: III > II > I
(188)	Athymic nude mice	MDA-MB-231	20% fat using SFO, coconut oil, RBD-DHASCO I – 8% LA II – 4% LA III – 4% LA, 2% DHA IV – 4% LA, 4% DHA	Growth Rate : IV < II < I Incidence: no difference Tumour weight: III < II; IV < I Proliferation: IV < I, II Apoptosis: IV > II > I	N/A
(189)	Athymic nude mice	Mouse 4526	I – 10% SFO (0.2g/100g n-3) II- 5% SFO, 5% FO (1.9g/100g n-3) III – 10% FO (3.5g/100g n-3) IV – 20% SFO (0.3g/100g n-3) V – 10% SFO, 10% FO (3.7g/100g n-3) VI – 20% FO (7g/100g n-3) n-6:n-3: I, IV - 20:1; II, V - 1.9:1; III, VI - 0.3:1	VI ↑ tumour latency + ↓ lung metastatic load vs. I, II, IV, V VI ↓ growth rate vs. IV, II III, VI ↓ lung metastasis vs. I, II, IV, V II, III, V, VI ↓ pulmonary nodules	N/A

Table 2.3 Animal Studies of N-3 and N-6 Fatty Acids on Breast Cancer Growth and Lipids Continued

Ref.	Animal Model	Tumour	Diet (w/w unless otherwise noted)	Survival/Tumour Growth/Metastasis	Lipid Changes
(131)	Sprague Dawley rats	DMBA induced	Low fat (16% energy) I – 65g CO, 5g FO/kg II – 35g CO, 35g FO/kg High fat (32% energy) III – 175g CO, 15g FO/kg IV – 120g CO, 70g FO/kg (CO – 54% n-6; FO – 27% n-3) I, III – n-6:n-3 ~16-17:1; II, IV – 2-3:1	Incidence: II < I, III< IV Latency: No differences Apoptosis: II > I, IV Proliferation: II < I, III < IV	N/A
(128)	Sprague Dawley rats	MDA-MB-231	I – 5% CO II – 2% CO, 3% FO concentrate (CO - ~50% LA, < 0.6% n-3; FO concentrate 34% EPA, 24% DHA, ~10% ALA)	Growth : II < I	Tumour : EPA : II > I DHA: no difference
(190)	Sprague Dawley rats	DMBA induced	I – 5% CO II – 5% MO III – 20% CO IV – 20% MO (CO 59% LA; MO 17% EPA, 13% DHA) For 14 wk	Incidence, tumour number, tumour weight, latency: no differences at 14wk Incidence with II, III > I, IV at 12wk	N/A
(191)	Athymic nude mice	MDA-MB-435	23% fat I – 23% CO II – 18% MO, 5% CO	Tumour weight, surface area, macroscopic metastasis: I > II	N/A
(192)	Athymic nude mice	MX-1	I – 20% MO, 5% CO II – 5% CO	Tumour weight: II > I	N/A

Table 2.3 Animal Studies of N-3 and N-6 Fatty Acids on Breast Cancer Growth and Lipids Continued

Ref.	Animal Model	Tumour	Diet (w/w unless otherwise noted)	Survival/Tumour Growth/Metastasis	Lipid Changes
(27)	Athymic nude mice	MCF-7 MDA-MB-231	I – 5% CO II – 20% CO III – 19% MO, 1% CO (CO: 56% LA MO : 13% DHA, 10% EPA, 1% LA, 1% ALA) for 8wk	Tumour volume : II > I > III	N/A
(127, 193)	Athymic nude mice	MDA-MB-435	I – 18% CO, 5% FO II – 11.5% CO, 11.5% FO III – 5% CO, 18% FO (CO 56% LA; MO 17% EPA, 7% DHA, 1.5% LA) for 13 wk	Tumour incidence and weight : no differences Tumour surface area: I > II, III Metastasis: III < I	Tumour PL: LA: I > II > III ALA : I < II, III AA: I > II, III EPA: I < II < III DHA: I < II, III n-6:n-3: I > II > III
(194)	Fisher 344 rats	NMU induced	I – 18% MO, 5% CO II – 11% MO, 11.8% CO III – 5% MO, 18% CO IV – 23% CO V – 5% CO (CO 56% LA; MO 13% EPA, 11% DHA)	Incidence : I, II, V < III, IV	N/A
(195)	Athymic nude mice, beige-XID-athymic mice, SCID mice	MCF-7, MDA-MB-231	I – 19% FO, 1% CO II – 20% CO (CO 56% LA; FO 10% EPA, 13% DHA)	Growth : I > II in XID and SCID mice with MDA-MB-231 or MCF-7 tumours	N/A

Table 2.3 Animal Studies of N-3 and N-6 Fatty Acids on Breast Cancer Growth and Lipids Continued

Ref.	Animal Model	Tumour	Diet (w/w unless otherwise noted)	Survival/Tumour Growth/Metastasis	Lipid Changes
(196)	Athymic nude mice	MDA-MB-435	23% high fat from SFO and coconut oil to make: I – 2% LA II – 8% LA III – 12% LA IV – 5% low fat CO diet	Growth: I, IV < II, III Macroscopic lung metastasis: I < II, III No differences in microscopic or total metastasis Total volume of metastasis: I < II, III; IV < II	N/A
(197)	Wistar rats	Walker 256	6% fat diets : I – 43% LA (control) II – 48% LA, 3% GLA III – 46% LA, 6% GLA	Tumour weight : I > II, III	Tumour total lipid: II, III ↑ C20:3n6
(198)	MF1 nude mice	MCF-7 B1M	I - 5% CO II - 2% GLA free fatty acid, 3% CO	Growth rate/ survival: II↓ vs. I	In tumours: ↓LA in II vs. I ↑GLA, DGLA in II vs. I
(175)	Athymic nude mice	MDA-MB-435	20% fat (coconut oil + I: 8% LA from SFO II:8% GLA for 10wk	No differences in primary tumour weight or lung metastasis	II ↓LA, ↑DGLA + AA in tumour PL; no changes in EPA/DHA, GLA could not be detected
(199)	Athymic nude mice	M2 and MM3	I: Commercial diet, (10%LA, 3% ALA) II: 5% EPO (68%LA, 13% GLA) III: 5% CO (47%LA) IV: 5% olein (3% LA, 1% ALA)	M2: survival time ↑ with II, III, IV vs. I tumour volume↓ with II vs I metastasis ↓with III vs. I, II, O MM3:tumour volume ↓ with II, III, IV vs. I metastasis ↓with IV vs. I	N/A
(200)	Sprague-Dawley rats	DMBA-induced	I – 10% EPO (53% LA, 5% GLA, 3%ALA) n-6:n-3 ~23 II – 10% FO (12% LA, 3% ALA, 10% EPA, 5% DHA) n-6 :n-3 ~0.6 III – 10% LO (19% LA, 34% ALA) n-6 :n-3 ~0.6 For 20wk	Incidence : III > II > I Tumour weight : I > II > III	In tumour PC: LA ↓ in I vs. III; AA ↓ in I vs. II DHA ↓ in I and II vs. III In tumour PE: LA, AA, DHA ↓ in I and II vs. III

Table 2.3 Animal Studies of N-3 and N-6 Fatty Acids on Breast Cancer Growth and Lipids Continued

Ref.	Animal Model	Tumour	Diet (w/w unless otherwise noted)	Survival/Tumour Growth/Metastasis	Lipid Changes
(201)	Athymic nude mice	MDA-MB-231	20% fat I: CO II: olive oil III: LO IV: EPO V: canola oil VI: MO	V ↓ growth; other diets had no effect	N/A
(153)	Athymic nude mice	M3	I: 5% mistol oil (14%LA, 22% ALA) II: 5% EPO (68%LA, 13% GLA) III: 5% CO (37%LA) IV: 5% olein (3% LA, 1% ALA) V: Commercial diet (10%LA, 3%ALA)	Survival time ↑ with I, II, IV vs. V Tumour size ↓ with I, II, III, IV vs. V Metastasis ↓ with II, III vs. V	N/A
(202)	Fischer 344 rats	13762MAT:B (derived from DMBA-induced tumours)	I-23.52% BCO (43% LA, 18% GLA, 16% ALA, 5% SDA) II-23.52%CO (61% LA, 1% GLA) III-15.52% BCO + 8% FO (32% LA, 12% GLA, 11% ALA, 5% SDA, 6% EPA, 1% DPA, 3% DHA) IV-20.52% FO + 3% CO (12% LA, 4% ALA, 6% SDA, 2% AA, 14% EPA, 1% DPA, 8% DHA) V-5% CO	V ↓ survival II ↑metastatic foci and tumour volume III ↓metastatic foci and tumour volume II, V ↑ metastatic foci vs. III, IV	In RBC PL: III, IV, V ↓LA I ↑ GLA I, III ↑DGLA IV ↓RBC AA vs. I III, IV ↑EPA I, III, IV ↑DPA III, IV ↑DHA

Abbreviations: AA arachidonic acid; ALA α-linolenic acid; CO corn oil; DGLA dihomo- γ linolenic acid; DHA docosahexaenoic acid; DMBA 7,12-dimethylbenz(a)anthracene; DPA docosahexaenoic acid; EPA eicosapentaenoic acid; EPO evening primrose oil; ETA eicosatetraenoic acid; FO fish oil; FSO flaxseed oil; GLA γ-linolenic acid; LA linoleic acid; LO linseed oil; MO menhaden oil; N/A not applicable; NMU N-methyl-N-nitrosourea; OA oleic acid; PL phospholipid; RBC red blood cell; SDA stearidonic acid; SFO safflower oil

C. Human Studies

From Table 2.4, it can be seen that many observational studies examining cohorts of women have found no consistent association between fish intake, dietary or serum n-3 or n-6 fatty acids, or even fat intake with breast cancer risk in various populations. A problem with many of these observational studies' populations is that the subjects do not have a wide variety of fish intake in the first place. It is also difficult to accurately assess dietary content over a long time when only a 24 hour recall is used. Dietary records are also reliant on a subject, and may be biased or incomplete. The large variety of food available also poses challenges in the recording of accurate nutrient intakes. It is also possible that gene polymorphisms that have been associated with positive or negative effects on breast cancer risk are not corrected for. For example, LA intake was associated with a certain polymorphism in 5-lipoxygenase protein gene, which increases eicosanoid production from AA. This gene has then been associated with a higher invasive breast cancer risk (OR 1.8, 95% CI 1.2-2.9) (203). Certain low activity polymorphisms in the glutathione S-transferases, that eliminate peroxidation products, have also been associated with lower breast cancer risk in postmenopausal women (OR 0.71, 95% CI 0.52-0.96) (204). In intervention studies, study populations are typically very small, and due to drop outs, can sometimes lack statistical power. Dosages may also not be high enough to show significant effects, either. There is also inconsistency between the effects of n-3 and n-6 intake in the form of food or supplements on the prevention/risk of breast cancer. In addition, any statistical significant results may not be significant physiologically, as many ratios are still close to 1. The wide variation in the genetic makeup, environment, and diet of humans, as well as the researcher's time constraints and methods to collect the data are just a few of the variables that make it difficult for researchers to find consistent relationships between fat and breast cancer. Other difficulties with these studies lay in how many studies only examine total fat in the diets of patient, and possibly saturated, monounsaturated and polyunsaturated fat in general, but do not stratify the types of polyunsaturated fat. As previously discussed in other biological systems, different types of

PUFAs have been shown to have different effects on cancer outcomes, and should be a point of examination in epidemiological studies. Many intervention studies also investigate improving the whole diet quality, so the effects of any changes in fat intake are difficult to differentiate. In the event that interventions supplement fatty acids, these supplements are usually in the form of fish oil. The use of other oil supplements higher in levels of n-3 and n-6 intermediates like evening primrose oil has been recorded in patients, but its effect as well as the effects of novel plant oils high in n-3 and n-6 intermediates on breast cancer incidence or mortality has not been assessed in the human population. Despite these inconsistencies, evidence suggests a possible link between increased total n-3, EPA and DHA, decreased total n-6 intake and n-6:n-3 ratios, and decreased breast cancer risk, but have yet to consistently support cell culture and animal studies. Although these changes may be due to changes in n-3 and n-6 lipids in the body, a thorough investigation has not been conducted in humans (231).

Studies examining the treatment of cancer with n-3 fatty acids, however, have been more successful in a variety of cancer populations, and are more consistent with animal studies. For example, the progression and survival of breast cancer patients have been shown to be improved with n-3 and GLA intake (90, 205-207). These effects are not limited to breast cancer patients, either, as fish oil has also been found to improve chemotherapy response in nonsmall lung cancer patients, as well as improve side effects like cachexia in oesophageal, and head and neck cancers (208, 209).

Table 2.4 Human Studies of N-3 and N-6 Fatty Acids on Breast Cancer Risk

Ref.	Type of Study	Results
(210)	Cohort (China)	No associations between breast cancer risk and LA, AA, ALA and marine n-3 PUFA. An interaction, however, was found between n-6 intake and marine n-3 PUFA and breast cancer, where high n-6 and low marine n-3 intake increased breast cancer risk (RR 2.06; 95% CI 1.27-3.34)
(211)	Cohort (France)	Hazard ratios for breast cancer risk and total n-6, total n-3, LA, AA, ALA, long chain n-3, and n-6:n-3 were non-significant. When subdivided by food source, significant trends in ALA and LA intake and breast cancer risk were observed when ALA and LA were from vegetable oils, fruit and vegetables, and nut mixes.
(212)	Cohort (USA)	Although there was a significant trend for fish intake and cancer mortality, no significant trend was observed for fish intake or n-3 intake and breast cancer incidence
(213)	Cohort (USA)	Women with < 20% energy from fat had a slight increase in their risk for breast cancer compared to women with 30% energy from fat. 5% increments of total, animal and vegetable fat, and PUFA slightly decreased risk of breast cancer, while 0.1% increases in n-3 from fish slightly increased breast cancer risk.
(214)	Cohort (USA)	Higher EPA and DHA intake from food were associated with 25% decrease in breast cancer. No association was observed with EPA and DHA from supplements and breast cancer risk.
(215)	Case-control (France)	No significant differences between cases and controls for n-3 PUFA intake (total, ALA, EPA, DPA, DHA, from plants, or from animals), fatty fish intake, or n-6 PUFA intake
(216)	Case-control (France)	Significant relative risk of breast cancer with decreased levels of ALA, DHA, total n-3, ALA:LA and long chain n-3:n-6 in breast adipose tissue
(217)	Case-control (France)	Significant relative risk of breast cancer with decreased levels of ALA in breast adipose tissue
(218)	Case-control (France)	Significant relative risk of breast cancer metastasis with decreases levels of ALA in breast adipose tissue. A trend towards low DHA levels in breast adipose tissue and the increased risk of metastasis was also observed, but was not significant.
(219)	Case-control (France)	Inverse association between breast cancer metastasis and risk and ALA levels in adipose tissue

Table 2.4 Human Studies of N-3 and N-6 Fatty Acids on Breast Cancer Growth and Lipids Continued

Ref.	Type of Study	Results
(220)	Case-control (Greece)	Cases had significantly lower total n-3, EPA, DPA and DHA levels and higher LA, C20:2n6 and DGLA levels in adipose tissue. The ratio of n-6:n-3 fatty acids in adipose tissue was also higher in cases.
(221)	Case-control (Uruguay)	Increase odds ratio for breast cancer with decreased levels of PUFA, LA, ALA
(222)	Case-control (USA)	No associations between breast cancer risk and n-3 or n-6 status, SFA, MUFA or trans fats in serum fatty acids.
(223)	Case-Control (USA)	Fish oil supplement users were found to have decreased risks of breast cancer. Dietary AA nor grapeseed supplements (high in LA) were not associated with breast cancer risk.
(224)	Case-control (USA)	No associations between fatty acid intake and breast cancer risk was observed. However, in premenopausal women, a trend towards decreased breast cancer risk was observed with low n-6:n-3 intake.
(225)	Case-control (USA)	High fat intake was associated with breast cancer risk, but LA intake was not. The use of LA-rich corn oil in cooking was associated with breast cancer risk.
(226)	Case-control (USA)	↑ Age-adjusted n-6 PUFA levels in breast adipose tissue correlated with cases Trend for n-6 PUFA to correlate with protective n-3 EPA +DHA effects at given level Trend for increased n-6:n-3 ratio and breast cancer cases
(227)	Meta-analysis	Cohort studies: protective effect by n-3 PUFA on breast cancer Case control studies: ALA had a close, but non-significant protective effect on breast cancer No other significant findings were observed (total n-6, LA, AA, EPA, DHA, total n-3)
(228)	Meta-analysis	Cohort studies: significant RR between PUFA and breast cancer (1.091, 95% CI 1.001-1.184) Case-control studies: no significant relationship between fat and breast cancer in general. In postmenopausal women, however, relationships between breast cancer and total fat (1.042, 95% CI 1.013-1.073) and PUFA intake (1.22; 95% CI 1.08-1.381) were observed
(229)	Meta-analysis (Italy)	Breast cancer risk was not associated with fish intake

Table 2.4 Human Studies of N-3 and N-6 Fatty Acids on Breast Cancer Growth and Lipids Continued

Ref.	Type of Study	Results
(230)	Intervention: 6g corn oil/day or 6g DHA-rich algae oil /day (2.14g DHA/day)	Algae oil decreased cholesterol but did not affect breast cancer risk (estimated by urinary 2-hydroxyestrone:16 α-hydroxyestrone) in postmenopausal vegetarian women

Abbreviations: AA arachidonic acid; ALA α-linolenic acid; CI confidence interval; DGLA dihomo-γ-linolenic acid; DHA docosahexaenoic acid; EPA eicosapentaenoic acid; LA linoleic acid; MUFA monounsaturated fatty acids; PUFA polyunsaturated fatty acids; RR relative risk; SFA saturated fatty acids; Tam tamoxifen

VI. Conjugated Linoleic Acid

Like LA, conjugated linoleic acid (CLA, C18:2) is a PUFA 18 carbons long with 2 carbon-carbon double bonds. It is different, however, in that the two double bonds are conjugated, only differing in position by 2 carbons anywhere along the chain, unlike LA whose double bond location differs by 3 carbons and has a specific conformation. With possibly over thirty-two positional (e.g. 9-11, 10-12) and geometric (e.g. cis-cis, cis-trans) isomers identified, c9, t11-CLA, also known as rumenic acid, is the most common isomer, representing up to 80% of total CLA in food, and will be the focus of the proceeding discussion (232).

CLA is mainly produced from the anaerobic *Butyrivibrio* bacteria in ruminants, as an intermediate in the conversion of ALA, LA and GLA to stearic acid (233-235). However, the c9, t11-CLA isomer is made when *Butyrivibrio fibrisolvens* isomerizes LA to c9, t11-CLA, before hydrogenation to vaccenic acid (C18:1t11) and then stearic acid (C18:0) (233). Rats fed LA in the free fatty acid form have been reported to have increased CLA content in tissues (supporting conversion in the gut), but this was not observed with LA in triglyceride form (236). Humans fed LA in triglyceride form did not show increases in CLA content in tissues, either, however, *Bifidobacterium* species that are capable of converting LA in free fatty acid form to c9, t11-CLA, have been found in the human microflora, indicating potential for endogenous conversion (237-239).

Another pathway to create CLA in humans is through the desaturation of vaccenic acid to c9, t11-CLA catalyzed by the Δ9-desaturase (240, 241). Although humans can produce CLA from dietary vaccenic acid, it is predicted that the endogenous synthesis of CLA is a minor source of CLA, and that CLA mainly comes from the diet (242).

In the human diet, the main isomer of CLA consumed is c9, t11-CLA. It is mainly found in beef and beef products, where about 69% of CLA is from dairy and 24% of CLA is from beef in the American diet (243). Other sources of CLA include pork and poultry, but, the CLA content may be due to the intake of beef products by the animals (243).

In Americans, total CLA intake is estimated to be 212 mg/day in men and 151 mg/day in women, where c9, t11-CLA intake is about 193 mg/day in men and 140 mg/day in women. Estimations, however, are difficult as CLA content in food depends largely on animal breed and upbringing, and can even be different between animals of the same breed (243). In addition, different processing methods and raw ingredient quality can alter the CLA content of dairy products like milk and cheese, further complicating the determination of CLA in the diet (244-246). Despite the potential benefits of CLA, as is not required in the body, Canada has no recommendation regarding its intake. It is, however, recognized as not being detrimental and is excluded from the estimations of total trans fatty acids in a food.

Concerning health, c9, t11-CLA has the potential to have many positive physiological effects on chronic diseases, including, but not limited to anti-atherogenic, anti-diabetogenic and anti-carcinogenic effects and improvements in inflammation. CLA diet supplementation was associated with improved lipid profiles and improved atherosclerosis (247, 248). Higher levels of c9, t11-CLA in adipose tissue was also associated with decreased risk of diabetes, possibly due to lower plasma glucose and insulin, improved lipid profiles, decreased inflammation, increased adiponectin, and/or decreased endoplasmic reticulum stress (249, 250). In cancer, c9, t11-CLA has shown significant decreases in breast, lung and colon tumour cell proliferation, but not prostate, melanoma or

glioblastoma cells (251, 252). Improvements in the immune function due to CLA have also been found, and include decreased macrophage adhesion, as well as decreased levels of prostaglandin E_2 and inflammatory cytokines like tumour necrosis factor-α, interleukin-1 and -6 (253-255). Overall, c9, t11-CLA has been associated with many beneficial effects on health.

VII. Conjugated Linoleic Acid, Membranes & Breast Cancer

CLA isomers c9, t11-CLA and t10, c12-CLA alone and in mixtures have been consistently shown to inhibit breast tumour growth in both cell culture and animal experiments, where t10, c12-CLA is generally more potent then c9, t11-CLA (Tables 2.5-6). Some studies have found negative effects of CLA on breast cancer development in the mouse Erb B2 models, however, survival was not affected. Despite the extensive research on breast tumour cells, few studies have examined the effects on non-tumorigenic breast cells. A single study suggests that CLA isomers may inhibit normal cell growth at concentrations above 30μM. Comparing isomers, it seems that t10, c12-CLA consistently inhibits MCF-7 growth more than the c9, t11-isomer. Similar effects on MDA-MB-231 cells comparing the two isomers have also been observed, however, both no and an inhibitory effect have been found with c9, t11-CLA incubations. From these experiments, many mechanisms for the anti-carcinogenic activities of CLA have been suggested. One of these mechanisms includes changes to the membrane composition, which can change the membrane's physical properties and protein organization (256). Although changes in tumour membranes are not well documented in breast cells, the few studies examining lipids find significant incorporation into tumour lipids, more specifically PLs. C9, t11-CLA and t10, c12-CLA have also been shown to be readily incorporated into caveolae, and the trans-linkages have the ability to decrease membrane fluidity and potentially change protein function (257, 258). When CLA is incorporated into membranes and caveolae, decreases in membrane AA content have also been observed, and may account for decreased pro-inflammatory eicosanoid synthesis, which has been implicated in tumorigenesis (258, 259). Therefore, the incorporation of

CLA may be compensated by decreases in n-6 fatty acid content. C9, t11-CLA has also been found to be isomerized into t10, c12-CLA, which may contribute to its anti-tumorigenic effects.

Table 2.5 Cell Culture Studies of CLA on Breast Cancer Growth and Lipids

Ref.	Cell Line	Treatments	Control	Viability Results	Lipid Changes
(260)	MCF-10A	0, 10, 20, 30, 40μM LA, c9, t11-CLA, t10, c12-CLA, t10, t12-CLA methyl esters	No fatty acid	Between treatments: No differences at 10, 20μM At 30μM all CLA < LA At 40μM all CLA < LA Within treatments: ↑ with 10μM for all treatments and 20μM for LA Dose-dependent inhibition from 10-40μM	N/A
(261)	MCF-7	25, 50, 100μM c9, t11-CLA or t10, c12-CLA	Ethanol	C9, t11-CLA no effect on proliferation T10, c12-CLA ↓ proliferation at all concentrations (50 and 100μM > 25μM)	N/A
(262)	T47D, MDA-MB-231	CLA mix, c9, t11-CLA and t10,c12-CLA BSA conjugated	No fatty acid	T47D Inhibition from 8-128μM CLA mix. Dose response was observed with c9, t11-CLA. CLA mix had maximal inhibition at 16μM. T10,c12 had maximal inhibition at 8-16μM MDA-MB-231 C9, t11-CLA increased growth at 8-16μM and inhibited at 64-128μM T10, c12-CLA inhibited growth at 8-128μM, maximal at 128μM	N/A

Table 2.5 Cell Culture Studies of CLA on Breast Cancer Growth and Lipids Continued

Ref.	Cell Line	Treatments	Control	Viability Results	Lipid Changes
(263)	MCF-7	T, t-CLA-BSA cfrom 5-60μM And 40 μM LA, c9, t11-CLA, t10, c12-CLA, t, t-CLA	No fatty acid	All t,t-CLA dosages ↓ growth after 4 days; 40-60 μ M > 10-20 μM > 5 μM Other treatments not tested	Plasma membrane : LA content: LA > control > c9, t11-CLA > t10, c12-CLA, t,t- CLA ALA content :Control > t, t-CLA > t10, c12-CLA; LA, c9, t11-CLA no different from t, t-CLA and t10, c12-CLA AA content : control > t10, c12-CLA > t, t-CLA; LA and c9, t11-CLA no different from t,t-CLA and t10, c12-CLA C9, t11-CLA content : c9, t11-CLA > t10, c12-CLA, t, t-CLA (not detectable in control/LA) T10, c12-CLA content : t10, c12-CLA > c9, t11-CLA (not detectable in other treatments) T, t-CLA content: Only detectable in t, t-CLA treatments
(264)	MDA-MB-231	100μM LA, c9, t11-CLA, t10, c12-CLA free fatty acids	Ethanol	C9, t11-CLA ↓ viability at 10 and 100μM and not 50 μM T10, c12-CLA ↓ viability in dose-dependent manner from 10-100 μM LA did not affect viability from 10-100 μM	N/A
(265)	MDA-MB-231 MCF-7	CLA (isomers not specified), LA for 6 days	Ethanol	Dose-dependent response from 17-71 μM in MCF-7 Proliferation inhibited by CLA at 35 μM vs. LA and control in MCF-7 LA, CLA has no effects on MDA-MB-231 cell proliferation	N/A

Table 2.5 Cell Culture Studies of CLA on Breast Cancer Growth and Lipids Continued

Ref.	Cell Line	Treatments	Control	Viability Results	Lipid Changes
(266)	MCF-7	C9, t11-CLA, t10, c12-CLA, CLA mixture (21% c11, t13-CLA, 29% t10, c12-CLA, 30% c9, t11-CLA, c8, t10-CLA) or LA free fatty acids	Ethanol	No inhibition at 24hr at 5 and 16μg/mL Similar inhibition by all CLA treatments after 4 days at 5 and 16μg/mL	AA preferentially incorporated into tumour phospholipids > triglycerides > monoglycerides CLA mixture and c9, t11-CLA ↑ AA incorporation into monoglycerides C9, t11-CLA ↓phosphatidylcholine AA and ↑phosphatidylethanolamine AA (no other differences with CLA or LA treatment on AA incorporation)
(267)	MDA-MB-231	LA, C9, t11-CLA, t10, c12-CLA and 1 :1 c9, t11-CLA and t10, c12-CLA mix with LA cotreatment	No fatty acid	N/A	Phosphatidylcholine : LA content : 120μM LA, CLA mix, t10, c12-CLA > 60 μMLA, c9, t11-CLA> control AA content : 60 μM, 120 μM LA > c9, t11-CLA, t10, c12-CLA > control, CLA mix C9, t11-CLA content : CLA mix > other treatments T10, C12-CLA content :CLA mix > t10, c12-CLA> other treatments Phosphatidylethanolamine : LA and c9, t11-CLA content as above AA content : control > 60 μM, 120 μM LA, c9, t11-CLA > other treatments
(268)	MDA-MB-231, MCF-7	C9, t11-CLA; t10, c12-CLA; CLA mixture complexed to BSA	No fatty acid	N/A	C9, t11-CLA ↑ c9, t11-CLA in total lipids T10, c12-CLA ↑t10, c12-CLA in total lipids CLA mixture ↑ c9, t11-CLA and t10, c12-CLA

Table 2.5 Cell Culture Studies of CLA on Breast Cancer Growth and Lipids Continued

Ref.	Cell Line	Treatments	Control	Viability Results	Lipid Changes
(269)	MCF-7	CLA mixture (50% c9, t11-CLA; 40% t10, c12-CLA; 10% c10, c12-CLA)	No fatty acid	Dose dependent CLA inhibition from 40-160μM (trypan blue) and 10-160μM (MTT assay)	N/A
(270)	MCF-7	Milk fat or c9, t11-CLA, LA for 4 days	No fatty acid	Milk fat and CLA treatments ↓ growth from 60-80μM CLA. Dose-dependent response was observed with milk fat LA↑growth at 45μM, no effect at 60μM, ↓at 153μM	N/A
(271)	MCF-7	10μM c9, t11-CLA; t10, c12-CLA or CLA mix (49% c9, t11-CLA, 41%	No fatty acid	Growth : control > t10, c12-CLA > CLA mix > c9, t11-CLA	N/A
(272)	MDA-MB-231, MCF-7	CLA mix or individual isomers c9, c11; t10, c12; t9, t11; c11, t13; c9, t11	No fatty acid	Dose dependent inhibition of MCF-7 by CLA mix CLA mix no effect on MDA-MB-231 growth All isomers inhibited MCF-7 growth All isomers by c9, c11 and c9, t11 inhibited MDA-MB-231 growth	N/A
(273)	Mouse mammary 4526	T10, c12-CLA; c9, t11-CLA; LA at 0-50μM	Ethanol	Dose-dependent response with t10, c12-CLA Inhibition by c9, t11-CLA only at 10μM Time-dependent effects seen by both CLA treatments	N/A

Table 2.5 Cell Culture Studies of CLA on Breast Cancer Growth and Lipids Continued

Ref.	Cell Line	Treatments	Control	Viability Results	Lipid Changes
(274)	MCF-7	CLA (not specified)	No fatty acid	CLA at 5-100μM ↓ growth by 72h CLA at 100μM ↓ growth by 48 h	N/A
(275)	MDA-MB-231	CLA (not specified)	No fatty acid	Slight inhibitory effect; no time or concentration dependency	N/A
(276)	MCF-7	Mixtures of CLA or total fat based off beef samples; 9, 11-CLA mix	No fatty acid	All mixtures ↓ cell proliferation	N/A
(251)	MCF-7 T47D	100μM C18:2 isomers, ALA for 48h	No fatty acid	Proliferation with LA > ALA in both cell lines; T47D > MCF-7 CLA isomers generally have stronger inhibitory effects vs. non-conjugated homologs	N/A
(225)	MCF-7	40μM c9, t11-CLA or t10, c12-CLA	No fatty acid	CLA isomers inhibited MCF-7 growth equally	N/A
(277)	MCF-10A MCF-7	50μM CLA (mixed isomers)	No fatty acid	N/A	50μM CLA preferentially incorporated into PL (2-3 times)

Abbreviations: AA arachidonic acid; ALA α-linoelic acid; BSA bovine serum albumin; CLA conjugated linoleic acid; LA linoleic acid; N/A non-applicable; PL phospholipid

Table 2.6 Animal Studies of CLA on Breast Cancer Growth and Lipids

Ref.	Animal Model	Tumour	Diet (w/w unless otherwise noted)	Survival/Tumour Growth/Metastasis	Lipid Changes
(278)	Sprague Dawley	MNU induced	I – control AIN93G II – 1% trans, trans-CLA III – 1% c9, t11-CLA IV – 1% t10, c12-CLA V – 1% LA	Incidence : I > V>III > IV > II Multiplicity, size: I, V > III, IV > II Apoptosis: I, V> III>IV>II	Tumour PL: LA: V > I, III > II, IV; ALA : I, III, IV, V > II; AA: II> IV, I > III, V; C9, t11-CLA: III > IV > I, II, V; T10,c12-CLA: IV> III> I, II, V Trans,trans-CLA : II > I, III, IV, V
(279)	PyMT mice	N/A	I - 7% energy soybean oil II – 6% energy soybean oil, 1% CLA mix (39% c9, t11-CLA, 41% t10,c12-CLA, 20% other CLA isomers)	Tumour burden: II > I No difference in latency	N/A
(280)	Sprague Dawley	MNU or DMBA	I – control AIN-76A II – 0.05% CLA; III – 0.1% CLA; IV – 0.25% CLA; V – 0.5% CLA; (CLA 43% t10, c12-CLA, 45% c9, t11-CLA, 3% LA)	Incidence : I, II, III> IV, V	N/A
(281)	Erb B2 mice	N/A	I – control AIN-76A II – 0.5% c9, t11-CLA III – 0.5% t10, c12-CLA	Latency : III < II, I Metastasis : III > I , II Survival : no differences	N/A
(282)	ErbB2 mice	N/A	I – control AIN-76A II – 0.5% t10, c12-CLA	II fed continuously ↓ latency and survival vs.II fed for a few weeks or I. Tumour diameter > II vs. I	N/A

Abbreviations: CLA conjugated linoleic acid; LA linoleic acid; N/A non-applicable; PL phospholipid

Despite extensive research of CLA in cell culture and in animal models, the evidence concerning the effects of CLA on breast cancer in humans have not been well studied. CLA intake and content in breast adipose tissue was not significantly associated with breast cancer women in Sweden, France or the Netherlands, although some weak positive trends were observed in the Netherlands for breast cancer risk (283-285). In Finland, however, CLA intake was associated with a 0.4 hazard ratio (95% CI 0.2-0.9) for the risk of breast cancer, and in America, c9, t11-CLA intake in pre-menopausal women showed significant correlation with ER- tumours (286, 287). Overall, human evidence for risk of cancer is very weak and conflicting, concerning the relationship between CLA and breast cancer, possibly due to low intake. An accurate assessment of CLA content in the diet is also a concern, as content can change significantly between similar products depending on the production method, as previously mentioned. As with n-3 fatty acid, human studies aimed at treatment with CLA need to be done to clarify the relationship between CLA and breast tumour survival.

VIII. Summary & Directions for Future Research

Major n-3 and n-6 fatty acids have been studied extensively in their relationship with breast cancer and their changes to tumour membranes and tissues, however, there is a lack of literature on the specific activities of the pathway intermediates. Current research suggests significant bioactivity of some of these intermediates, namely SDA, GLA and DGLA on breast cancer. The bioactivity of another intermediate, ETA, is not known, but could possess anti-cancer properties. It has been suggested that these fatty acids may exert their anti-carcinogenic properties through their conversion to their longer chained counterparts; however, confirmation has not been done. It would be important to study these fatty acids to determine their bioactivity at physiological dosages as potential therapies in breast cancer, as well as determine whether their modes of action are due to the fatty acid itself or due to their conversion to their longer chained bioactive counterparts in the improvement of breast cancer outcomes, as

well as their safety. Changes in the membrane have been implicated in the progression of breast cancer, however, have not been thoroughly investigated.

Most *in vitro* studies have focused on supplying single fatty acids to cells, while most fat we consume are in the form of oil mixtures, so it is important to assess the effects of fatty acid mixtures *in vitro* as well. With the growing popularity of novel plant oils high in n-3 and n-6 pathway intermediates, their bioactivity needs to be studied further. To our knowledge, none of these oils have been tested on breast cancer despite potential anti-carcinogenic effects, and therefore, mechanisms of action, if any, have not been suggested, either. Fatty acid mixtures could possibly do anything from being biologically inactive to work synergistically compared to pure fatty acids. As a potential novel commercial source of n-3 and n-6 fatty acid intermediates, the bioactivity of these oils in comparison to their individual components in breast cancer, as well as their modes of action and safety need to be resolved.

While extensive research has been done on the anti-carcinogenic properties of c9, t11-CLA on breast cancer cells and animal models, its effects on non-tumorigenic breast cells have yet to be well established. Additionally, the effect of CLA on membrane PL composition and the potential similarities to the effects of anti-cancer n-3 and n-6 fatty acids needs further study.

IX. References

1. Canadian Cancer Society's Steering Committee on Cancer Statistics. Canadian cancer statistics 2011. Toronto, ON: Canadian Cancer Society; 2011.

2. Hirshaut Y, Pressman PI. Breast cancer: The complete guide. Fifth Edition, Fully Revised ed. New York, New York: Bantam Dell; 2008.

3. Dian D, Herold H, Mylonas I, Scholz C, Janni W, Sommer H, et al. Survival analysis between patients with invasive ductal and invasive lobular breast cancer. Arch Gynecol Obstet. 2009 Jan;279(1):23-8.

4. Roder DM, de Silva P, Zorbas HM, Kollias J, Malycha PL, Pyke CM, et al. Age effects on survival from early breast cancer in clinical settings in australia. ANZ J Surg. 2012 Jul 8.

5. Truin W, Voogd AC, Vreugdenhil G, van der Heiden-van der Loo,M., Siesling S, Roumen RM. Effect of adjuvant chemotherapy in postmenopausal patients with invasive ductal versus lobular breast cancer. Ann Oncol. 2012 Jun 27.

6. Fortunato L, Mascaro A, Poccia I, Andrich R, Amini M, Costarelli L, et al. Lobular breast cancer: Same survival and local control compared with ductal cancer, but should both be treated the same way? analysis of an institutional database over a 10-year period. Ann Surg Oncol. 2012 Apr;19(4):1107-14.

7. Dunnwald LK, Rossing MA, Li CI. Hormone receptor status, tumor characteristics, and prognosis: A prospective cohort of breast cancer patients. Breast Cancer Res. 2007;9(1):R6.

8. Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, McGuire WL. Human breast cancer: Correlation of relapse and survival with amplification of the HER-2/neu oncogene. Science. 1987 Jan 9;235(4785):177-82.

9. Slamon DJ, Godolphin W, Jones LA, Holt JA, Wong SG, Keith DE, et al. Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. Science. 1989 May 12;244(4905):707-12.

10. Tan M, Yao J, Yu D. Overexpression of the c-erbB-2 gene enhanced intrinsic metastasis potential in human breast cancer cells without increasing their transformation abilities. Cancer Res. 1997 Mar 15;57(6):1199-205.

11. Xu YL, Sun Q, Shan GL, Zhang J, Liao HB, Li SY, et al. A case-control study on risk factors of breast cancer in china. Arch Med Sci. 2012 May 9;8(2):303-9.

12. Trichopoulos D, MacMahon B, Cole P. Menopause and breast cancer risk. J Natl Cancer Inst. 1972 Mar;48(3):605-13.

13. Marchant DJ. Epidemiology of breast cancer. Clin Obstet Gynecol. 1982 Jun;25(2):387-92.

14. Kelsey JL. A review of the epidemiology of human breast cancer. Epidemiol Rev. 1979;1:74-109.

15. Connolly BS, Barnett C, Vogt KN, Li T, Stone J, Boyd NF. A meta-analysis of published literature on waist-to-hip ratio and risk of breast cancer. Nutr Cancer. 2002;44(2):127-38.

16. Yang XR, Chang-Claude J, Goode EL, Couch FJ, Nevanlinna H, Milne RL, et al. Associations of breast cancer risk factors with tumor subtypes: A pooled analysis from the breast cancer association consortium studies. J Natl Cancer Inst. 2011 Feb 2;103(3):250-63.

17. Albanes D, Blair A, Taylor PR. Physical activity and risk of cancer in the NHANES I population. Am J Public Health. 1989 Jun;79(6):744-50.

18. Thompson HJ, Ronan AM, Ritacco KA, Tagliaferro AR, Meeker LD. Effect of exercise on the induction of mammary carcinogenesis. Cancer Res. 1988 May 15;48(10):2720-3.

19. Ellison RC, Zhang Y, McLennan CE, Rothman KJ. Exploring the relation of alcohol consumption to risk of breast cancer. Am J Epidemiol. 2001 Oct 15;154(8):740-7.

20. Cox DG, Dostal L, Hunter DJ, Le Marchand L, Hoover R, Ziegler RG, et al. N-acetyltransferase 2 polymorphisms, tobacco smoking, and breast cancer risk in the breast and prostate cancer cohort consortium. Am J Epidemiol. 2011 Dec 1;174(11):1316-22.

21. Xue F, Willett WC, Rosner BA, Hankinson SE, Michels KB. Cigarette smoking and the incidence of breast cancer. Arch Intern Med. 2011 Jan 24;171(2):125-33.

22. Preston DL, Mattsson A, Holmberg E, Shore R, Hildreth NG, Boice JD,Jr. Radiation effects on breast cancer risk: A pooled analysis of eight cohorts. Radiat Res. 2002 Aug;158(2):220-35.

23. Jansen-van der Weide MC, Greuter MJ, Jansen L, Oosterwijk JC, Pijnappel RM, de Bock GH. Exposure to low-dose radiation and the risk of breast cancer among women with a familial or genetic predisposition: A meta-analysis. Eur Radiol. 2010 Nov;20(11):2547-56.

24. Reeves GK, Beral V, Green J, Gathani T, Bull D, Million Women Study Collaborators. Hormonal therapy for menopause and breast-cancer risk by histological type: A cohort study and meta-analysis. Lancet Oncol. 2006 Nov;7(11):910-8.

25. Shah NR, Borenstein J, Dubois RW. Postmenopausal hormone therapy and breast cancer: A systematic review and meta-analysis. Menopause. 2005 Nov-Dec;12(6):668-78.

26. Freedman LS, Clifford C, Messina M. Analysis of dietary fat, calories, body weight, and the development of mammary tumors in rats and mice: A review. Cancer Res. 1990 Sep 15;50(18):5710-9.

27. Gonzalez MJ, Schemmel RA, Gray JI, Dugan L,Jr, Sheffield LG, Welsch CW. Effect of dietary fat on growth of MCF-7 and MDA-MB231 human breast carcinomas in athymic nude mice: Relationship between carcinoma growth and lipid peroxidation product levels. Carcinogenesis. 1991 Jul;12(7):1231-5.

28. Chan PC, Didato F, Cohen LA. High dietary fat, elevation of rat serum prolactin and mammary cancer. Proc Soc Exp Biol Med. 1975 May;149(1):133-5.

29. Chan PC, Head JF, Cohen LA, Wynder EL. Influence of dietary fat on the induction of mammary tumors by N-nitrosomethylurea: Associated hormone changes and differences between sprague-dawley and F344 rats. J Natl Cancer Inst. 1977 Oct;59(4):1279-83.

30. Tvrzicka E, Kremmyda LS, Stankova B, Zak A. Fatty acids as biocompounds: Their role in human metabolism, health and disease--a review. part 1: Classification, dietary sources and biological functions. Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub. 2011 Jun;155(2):117-30.

31. Sprecher H. Metabolism of highly unsaturated n-3 and n-6 fatty acids. Biochim Biophys Acta. 2000 Jul 19;1486(2-3):219-31.

32. Pawlosky RJ, Hibbeln JR, Novotny JA, Salem N,Jr. Physiological compartmental analysis of alpha-linolenic acid metabolism in adult humans. J Lipid Res. 2001 Aug;42(8):1257-65.

33. Burdge GC, Wootton SA. Conversion of alpha-linolenic acid to eicosapentaenoic, docosapentaenoic and docosahexaenoic acids in young women. Br J Nutr. 2002 Oct;88(4):411-20.

34. Yamazaki K, Fujikawa M, Hamazaki T, Yano S, Shono T. Comparison of the conversion rates of alpha-linolenic acid (18:3(n - 3)) and stearidonic acid (18:4(n - 3)) to longer polyunsaturated fatty acids in rats. Biochim Biophys Acta. 1992 Jan 3;1123(1):18-26.

35. USDA national nutrient database for standard reference, release 24. nutrient data laboratory home page [Internet].; 2012. Available from: http://www.ars.usda.gov/ba/bhnrc/ndl.

36. Passi S, Cataudella S, Di Marco P, De Simone F, Rastrelli L. Fatty acid composition and antioxidant levels in muscle tissue of different mediterranean marine species of fish and shellfish. J Agric Food Chem. 2002 Dec 4;50(25):7314-22.

37. Khan MN, Cho JY, Lee MC, Kang JY, Park NG, Fujii H, et al. Isolation of two anti-inflammatory and one pro-inflammatory polyunsaturated fatty acids from the brown seaweed undaria pinnatifida. J Agric Food Chem. 2007 Aug 22;55(17):6984-8.

38. Surette ME, Edens M, Chilton FH, Tramposch KM. Dietary echium oil increases plasma and neutrophil long-chain (n-3) fatty acids and lowers serum

triacylglycerols in hypertriglyceridemic humans. J Nutr. 2004 Jun;134(6):1406-11.

39. Del Castillo ML, Dobson G, Brennan R, Gordon S. Fatty acid content and juice characteristics in black currant (ribes nigrum L.) genotypes. J Agric Food Chem. 2004 Feb 25;52(4):948-52.

40. Sewon P, Tyystjarvi E. Stearidonic and gamma-linolenic acid contents of common borage leaves. Phytochemistry. 1993 Jul;33(5):1029-32.

41. Lands W. Biochemistry and physiology of N-3 fatty-acids. FASEB J. 1992 May;6(8):2530-6.

42. Harper CR, Edwards MJ, DeFilippis AP, Jacobson TA. Flaxseed oil increases the plasma concentrations of cardioprotective (n-3) fatty acids in humans. J Nutr. 2006 Jan;136(1):83-7.

43. Lucas M, Mirzaei F, O'Reilly EJ, Pan A, Willett WC, Kawachi I, et al. Dietary intake of n-3 and n-6 fatty acids and the risk of clinical depression in women: A 10-y prospective follow-up study. Am J Clin Nutr. 2011 Jun;93(6):1337-43.

44. Chen J, Stavro PM, Thompson LU. Dietary flaxseed inhibits human breast cancer growth and metastasis and downregulates expression of insulin-like growth factor and epidermal growth factor receptor. Nutr Cancer. 2002;43(2):187-92.

45. Lu J, Borthwick F, Hassanali Z, Wang Y, Mangat R, Ruth M, et al. Chronic dietary n-3 PUFA intervention improves dyslipidaemia and subsequent cardiovascular complications in the JCR:LA- cp rat model of the metabolic syndrome. Br J Nutr. 2011 Jun;105(11):1572-82.

46. Balvers MG, Verhoeckx KC, Plastina P, Wortelboer HM, Meijerink J, Witkamp RF. Docosahexaenoic acid and eicosapentaenoic acid are converted by 3T3-L1 adipocytes to N-acyl ethanolamines with anti-inflammatory properties. Biochim Biophys Acta. 2010 Oct;1801(10):1107-14.

47. Song KS, Jing K, Kim JS, Yun EJ, Shin S, Seo KS, et al. Omega-3-polyunsaturated fatty acids suppress pancreatic cancer cell growth in vitro and in vivo via downregulation of Wnt/Beta-catenin signaling. Pancreatology. 2011;11(6):574-84.

48. Song C, Zhao S. Omega-3 fatty acid eicosapentaenoic acid. A new treatment for psychiatric and neurodegenerative diseases: A review of clinical investigations. Expert Opin Investig Drugs. 2007 Oct;16(10):1627-38.

49. Kalupahana NS, Claycombe K, Newman SJ, Stewart T, Siriwardhana N, Matthan N, et al. Eicosapentaenoic acid prevents and reverses insulin resistance in high-fat diet-induced obese mice via modulation of adipose tissue inflammation. J Nutr. 2010 Nov;140(11):1915-22.

50. Fearon KC, Von Meyenfeldt MF, Moses AG, Van Geenen R, Roy A, Gouma DJ, et al. Effect of a protein and energy dense N-3 fatty acid enriched oral supplement on loss of weight and lean tissue in cancer cachexia: A randomised double blind trial. Gut. 2003 Oct;52(10):1479-86.

51. Petrik MB, McEntee MF, Johnson BT, Obukowicz MG, Whelan J. Highly unsaturated (n-3) fatty acids, but not alpha-linolenic, conjugated linoleic or gamma-linolenic acids, reduce tumorigenesis in apc(min/+) mice. J Nutr. 2000 Oct;130(10):2434-43.

52. Hsueh HW, Zhou Z, Whelan J, Allen KG, Moustaid-Moussa N, Kim H, et al. Stearidonic and eicosapentaenoic acids inhibit interleukin-6 expression in ob/ob mouse adipose stem cells via toll-like receptor-2-mediated pathways. J Nutr. 2011 Jul;141(7):1260-6.

53. Oie E, Ueland T, Dahl CP, Bohov P, Berge C, Yndestad A, et al. Fatty acid composition in chronic heart failure: Low circulating levels of eicosatetraenoic acid and high levels of vaccenic acid are associated with disease severity and mortality. J Intern Med. 2011 Sep;270(3):263-72.

54. Eating well with canada's food guide [Internet].; 2011. Available from: http://www.hc-sc.gc.ca/fn-an/food-guide-aliment/index-eng.php/.

55. Committee,Canada.Health and Welfare Canada.Scientific Review. Nutrition recommendations: The report of the scientific review committee. Health and Welfare Canada; 1990.

56. Macronutrients,A Report of the Panel on, Intakes,Subcommittees on Upper Reference Levels of Nutrients and Interpretation and Uses of Dietary Reference, Intakes,Standing Committee on the Scientific Evaluation of Dietary Reference. Dietary reference intakes for energy, carbohydrate, fiber, fat, fatty acids, cholesterol, protein, and amino acids (macronutrients). The National Academies Press; 2005.

57. Reference values for macronutrients [Internet].; 2006. Available from: http://www.hc-sc.gc.ca/fn-an/nutrition/reference/table/ref_macronutr_tbl-eng.php.

58. Bjerve K. N-3 fatty-acid deficiency in man. J Intern Med. 1989;225:171-5.

59. Perusse-Lachance E, Tremblay A, Drapeau V. Lifestyle factors and other health measures in a canadian university community. Appl Physiol Nutr Metab. 2010 Aug;35(4):498-506.

60. Lucas M, Asselin G, Plourde M, Cunnane SC, Dewailly E, Dodin S. n-3 fatty acid intake from marine food products among quebecers: Comparison to worldwide recommendations. Public Health Nutr. 2010 Jan;13(1):63-70.

61. Fratesi JA, Hogg RC, Young-Newton GS, Patterson AC, Charkhzarin P, Thomas KB, et al. Direct quantitation of omega-3 fatty acid intake of canadian residents of a long-term care facility. Appl Physiol Nutr Metab. 2009 Feb;34(1):1-9.

62. Hu J, La Vecchia C, DesMeules M, Negri E, Mery L, Canadian Canc Registries Epidemiol. Meat and fish consumption and cancer in canada. Nutr Cancer. 2008 May-Jun;60(3):313-24.

63. Denomme J, Stark K, Holub B. Directly quantitated dietary (n-3) fatty acid intakes of pregnant canadian women are lower than current dietary recommendations. J Nutr. 2005 Feb;135(2):206-11.

64. Wang C, Chung M, Lichtenstein A, Balk E, Kupelnick B, DeVine D, et al. Effects of omega-3 fatty acids on cardiovascular disease. Evid Rep Technol Assess (Summ). 2004 Mar;(94)(94):1-8.

65. Multiple ingredient oil products [Internet].; 2012. Available from: http://webprod.hc-sc.gc.ca/nhpid-bdipsn/atReq.do?atid=multiple.oil&lang=eng.

66. Mortensen JZ, Schmidt EB, Nielsen AH, Dyerberg J. The effect of N-6 and N-3 polyunsaturated fatty acids on hemostasis, blood lipids and blood pressure. Thromb Haemost. 1983 Aug 30;50(2):543-6.

67. Schirmer MA, Phinney SD. Gamma-linolenate reduces weight regain in formerly obese humans. J Nutr. 2007 Jun;137(6):1430-5.

68. Schwab US, Callaway JC, Erkkila AT, Gynther J, Uusitupa MI, Jarvinen T. Effects of hempseed and flaxseed oils on the profile of serum lipids, serum total and lipoprotein lipid concentrations and haemostatic factors. Eur J Nutr. 2006 Dec;45(8):470-7.

69. Mortensen J, Schmidt E, Nielsen A, Dyerberg J. The effect of N-6 and N-3 poly-unsaturated fatty-acids on hemostasis, blood-lipids and blood-pressure. Thromb Haemost. 1983;50(2):543-6.

70. Kelley D, Taylor P, Nelson G, Mackey B. Dietary docosahexaenoic acid and immunocompetence in young healthy men. Lipids. 1998 Jun;33(6):559-66.

71. Umegaki K, Hashimoto M, Yamasaki H, Fujii Y, Sugisawa A, Shinozuka K. Docosahexaenoic acid supplementation-increased oxidative damage in bone marrow DNA in aged rats and its relation to antioxidant vitamins. Free Radic Res. 2001;34(4):427-35.

72. Song J, Miyazawa T. Enhanced level of n-3 fatty acid in membrane phospholipids induces lipid peroxidation in rats fed dietary docosahexaenoic acid oil. Atherosclerosis. 2001 Mar;155(1):9-18.

73. Ruiz-Lopez N, Haslam RP, Venegas-Caleron M, Larson TR, Graham IA, Napier JA, et al. The synthesis and accumulation of stearidonic acid in transgenic plants: A novel source of 'heart-healthy' omega-3 fatty acids. Plant Biotechnology Journal. 2009 Sep;7(7):704-16.

74. Ursin V. Modification of plant lipids for human health: Development of functional land-based omega-3 fatty acids. J Nutr. 2003 Dec;133(12):4271-4.

75. Sato S, Xing A, Ye X, Schweiger B, Kinney A, Graef G, et al. Production of gamma-linolenic acid and stearidonic acid in seeds of marker-free transgenic soybean. Crop Sci. 2004 Mar-Apr;44(2):646-52.

76. Lemke SL, Vicini JL, Su H, Goldstein DA, Nemeth MA, Krul ES, et al. Dietary intake of stearidonic acid-enriched soybean oil increases the omega-3 index: Randomized, double-blind clinical study of efficacy and safety. Am J Clin Nutr. 2010 Oct;92(4):766-75.

77. Rodriguez A, Sarda P, Nessmann C, Boulot P, Leger CL, Descomps B. Delta6- and delta5-desaturase activities in the human fetal liver: Kinetic aspects. J Lipid Res. 1998 Sep;39(9):1825-32.

78. Carnielli V, Wattimena D, Luijendijk I, Boerlage A, Degenhart H, Sauer P. The very low birth weight premature infant is capable of synthesizing arachidonic and docosahexaenoic acids from linoleic and linolenic acids. Pediatr Res. 1996 Jul;40(1):169-74.

79. Anel A, Naval J, Gonzalez B, Uriel J, Pineiro A. Fatty acid metabolism in human lymphocytes. II. activation of fatty acid desaturase-elongase systems during blastic transformation. Biochim Biophys Acta. 1990 Jun 14;1044(3):332-9.

80. Dietary reference intakes: The essential guide to nutrient requirements. Otten JJ, Hellwig JP, Meyers LD, editors. The National Academies Press; 2006.

81. Ghasemnezhad A, Honermeier B. Yield, oil constituents, and protein content of evening primrose (oenothera biennis L.) seeds depending on harvest time, harvest method and nitrogen application. Industrial Crops and Products. 2008 Jul;28(1):17-23.

82. Jeppesen P, Hoy C, Mortensen P. Essential fatty acid deficiency in patients receiving home parenteral nutrition. Am J Clin Nutr. 1998 Jul;68(1):126-33.

83. Field CJ, Toyomizu M, Clandinin MT. Relationship between dietary fat, adipocyte membrane composition and insulin binding in the rat. J Nutr. 1989 Oct;119(10):1483-9.

84. Bergstrom S, Klenberg D, Danielsson H, Samuelsson B. Enzymatic conversion of essential fatty acids into prostaglandins - prostaglandins + related factors 34. J Biol Chem. 1964;239(11):4006,&.

85. Lewis GP. Immunoregulatory activity of metabolites of arachidonic acid and their role in inflammation. Br Med Bull. 1983 Jul;39(3):243-8.

86. Willett WC. The role of dietary n-6 fatty acids in the prevention of cardiovascular disease. J Cardiovasc Med. 2007 Sep;8:S42-5.

87. Salmeron J, Hu F, Manson J, Stampfer M, Colditz G, Rimm E, et al. Dietary fat intake and risk of type 2 diabetes in women. Am J Clin Nutr. 2001 Jun;73(6):1019-26.

88. Lof M, Olivo-Marston S, Hilakivi-Clarke L. n-6 polyunsaturated fatty acids and cancer. Milner JR,DF, editor. Totowa; 999 Riverview Dr, STE 208, Totowa, NJ 07512-1165 USA: Humana Press INC; 2010.

89. Charnock J. Gamma-linolenic acid provides additional protection against ventricular fibrillation in aged rats fed linoleic acid rich diets. Prostaglandins Leukotrienes Essential Fatty Acids. 2000 Feb;62(2):129-34.

90. Kenny F, Pinder S, Ellis I, Gee J, Nicholson R, Bryce R, et al. Gamma linolenic acid with tamoxifen as primary therapy tn breast cancer. International Journal of Cancer. 2000 Mar 1;85(5):643-8.

91. Peth J, Kinnick T, Youngblood E, Tritschler H, Henriksen E. Effects of a unique conjugate of alpha-lipoic acid and gamma-linolenic acid on insulin action in obese zucker rats. American Journal of Physiology-Regulatory Integrative and Comparative Physiology. 2000 Feb;278(2):R453-9.

92. Fan Y, Ramos K, Chapkin R. Dietary gamma-linolenic acid suppresses aortic smooth muscle cell proliferation and modifies atherosclerotic lesions in apolipoprotein E knockout mice. J Nutr. 2001 Jun;131(6):1675-81.

93. Das UN, Madhavi N. Effect of polyunsaturated fatty acids on drug-sensitive and resistant tumor cells in vitro. Lipids in Health and Disease. 2011 Sep 14;10:159.

94. Zhang W, Chakravarty B, Zheng F, Gu Z, Wu H, Mao J, et al. Crystal structure of FAS thioesterase domain with polyunsaturated fatty acyl adduct and inhibition by dihomo-gamma-linolenic acid. Proc Natl Acad Sci U S A. 2011 Sep 20;108(38):15757-62.

95. Qin F, Kang L, Guo L, Lin J, Song J, Zhao Y. Composition of transgenic soybean seeds with higher gamma-linolenic acid content is equivalent to that of conventional control. J Agric Food Chem. 2012 Mar 7;60(9):2200-4.

96. Nykiforuk CL, Shewmaker C, Harry I, Yurchenko OP, Zhang M, Reed C, et al. High level accumulation of gamma linolenic acid (C18:3 delta 6.9,12 cis) in transgenic safflower (carthamus tinctorius) seeds. Transgenic Res. 2012 Apr;21(2):367-81.

97. Kawashima H, Akimoto K, Higashiyama K, Fujikawa S, Shimizu S. Industrial production of dihomo-gamma-linolenic acid by a delta 5 desaturase-defective mutant of mortierella alpina 1S-4 fungus. J Am Oil Chem Soc. 2000 Nov;77(11):1135-8.

98. Brenner R, Peluffo R. Effect of sturated and unsaturated fatty acids on desaturation in vitro of palmitic stearic oleic linoleic linolenic acids. J Biol Chem. 1966;241(22):5213,&.

99. Cook H. Chain elongation in the formation of poly-unsaturated fatty-acids by brain - some properties of the microsomal system. Arch Biochem Biophys. 1982;214(2):695-704.

100. Sauerwald T, Hachey D, Jensen C, Chen H, Anderson R, Heird W. Effect of dietary alpha-linolenic acid intake on incorporation of docosahexaenoic and arachidonic acids into plasma phospholipids of term infants. Lipids. 1996 Mar;31:S131-5.

101. Chen Q, Nilsson A. Desaturation and chain elongation of N-3 and N-6 polyunsaturated fatty-acids in the human caco-2 cell-line. Biochim Biophys Acta. 1993 Feb 24;1166(2-3):193-201.

102. Emken E, Adlof R, Duval S, Nelson G. Effect of dietary docosahexaenoic acid on desaturation and uptake in vivo of isotope-labeled oleic, linoleic, and linolenic acids by male subjects. Lipids. 1999 Aug;34(8):785-91.

103. Emken E, Adlof R, Gulley R. Dietary linoleic-acid influences desaturation and acylation of deuterium-labeled linoleic and linolenic acids in young-adult males. Biochimica Et Biophysica Acta-Lipids and Lipid Metabolism. 1994 Aug 4;1213(3):277-88.

104. Emken E, Adlof R, Duval S, Nelson G. Effect of dietary arachidonic acid on metabolism of deuterated linoleic acid by adult male subjects. Lipids. 1998 May;33(5):471-80.

105. Corsinovi L, Biasi F, Poli G, Leonarduzzi G, Isaia G. Dietary lipids and their oxidized products in alzheimer's disease. Mol Nutr Food Res. 2011 Sep;55 Suppl 2:S161-72.

106. Hashimoto M, Tozawa R, Katakura M, Shahdat H, Haque AM, Tanabe Y, et al. Protective effects of prescription n-3 fatty acids against impairment of spatial cognitive learning ability in amyloid beta-infused rats. Food Funct. 2011 Jul;2(7):386-94.

107. Yehuda S, Rabinovitz S, Mostofsky DI, Huberman M, Sredni B. Essential fatty acid preparation improves biochemical and cognitive functions in experimental allergic encephalomyelitis rats. Eur J Pharmacol. 1997 Jun 5;328(1):23-9.

108. Kang J, Leaf A. Antiarrhythmic effects of polyunsaturated fatty acids - recent studies. Circulation. 1996 Oct 1;94(7):1774-80.

109. Musa-Veloso K, Binns MA, Kocenas A, Chung C, Rice H, Oppedal-Olsen H, et al. Impact of low v. moderate intakes of long-chain n-3 fatty acids on risk of coronary heart disease. Br J Nutr. 2011 Oct;106(8):1129-41.

110. Ramsden CE, Hibbeln JR, Majchrzak SF, Davis JM. n-6 fatty acid-specific and mixed polyunsaturate dietary interventions have different effects on CHD risk: A meta-analysis of randomised controlled trials. Br J Nutr. 2010 Dec;104(11):1586-600.

111. Miles EA, Calder PC. Influence of marine n-3 polyunsaturated fatty acids on immune function and a systematic review of their effects on clinical outcomes in rheumatoid arthritis. Br J Nutr. 2012 Jun;107 Suppl 2:S171-84.

112. Hagfors L, Nilsson I, Skoldstam L, Johansson G. Fat intake and composition of fatty acids in serum phospholipids in a randomized, controlled, mediterranean dietary intervention study on patients with rheumatoid arthritis. Nutr Metab (Lond). 2005 Oct 10;2:26.

113. Williams CD, Whitley BM, Hoyo C, Grant DJ, Iraggi JD, Newman KA, et al. A high ratio of dietary n-6/n-3 polyunsaturated fatty acids is associated with increased risk of prostate cancer. Nutr Res. 2011 Jan;31(1):1-8.

114. Yang YJ, Lee SH, Hong SJ, Chung BC. Comparison of fatty acid profiles in the serum of patients with prostate cancer and benign prostatic hyperplasia. Clin Biochem. 1999 8;32(6):405-9.

115. Reddy BS, Sugie S. Effect of different levels of omega-3 and omega-6 fatty acids on azoxymethane-induced colon carcinogenesis in F344 rats. Cancer Res. 1988 Dec 1;48(23):6642-7.

116. Ge Y, Chen Z, Kang ZB, Cluette-Brown J, Laposata M, Kang JX. Effects of adenoviral gene transfer of C. elegans n-3 fatty acid desaturase on the lipid profile and growth of human breast cancer cells. Anticancer Res. 2002 Mar-Apr;22(2A):537-43.

117. Maillard V, Bougnoux P, Ferrari P, Jourdan ML, Pinault M, Lavillonniere F, et al. N-3 and N-6 fatty acids in breast adipose tissue and relative risk of breast cancer in a case-control study in tours, france. Int J Cancer. 2002 Mar 1;98(1):78-83.

118. Omega-6/Omega-3 essential fatty acid ratio: The scientific evidence. Simopoulos AP and Cleland LG, editors. Basel; New York: Karger; 2003.

119. Lands W, Hamazaki T, Yamazaki K, Okuyama H, Sakai K, Goto Y, et al. Changing dietary patterns. Am J Clin Nutr. 1990 Jun;51(6):991-3.

120. Kolonel L, Hankin J, Lee J, Chu S, Nomura A, Hinds M. Nutrient intakes in relation to cancer incidence in hawaii. Br J Cancer. 1981;44(3):332-9.

121. Freedman MR, King J, Kennedy E. Popular diets: A scientific review. Obes Res. 2001 Mar;9 Suppl 1:1S-40S.

122. A Report of the Panel,on Macronutrients, Subcommittees on Upper Reference Levels of Nutrients and Interpretation and Uses of Dietary,Reference Intakes, Standing Committee on the Scientific Evaluation of Dietary,Reference Intakes. Dietary reference intakes for energy, carbohydrate, fiber, fat, fatty acids, cholesterol, protein, and amino acids (macronutrients). The National Academies Press; 2005.

123. Fung TT, Hu FB, McCullough ML, Newby PK, Willett WC, Holmes MD. Diet quality is associated with the risk of estrogen receptor-negative breast cancer in postmenopausal women. J Nutr. 2006 Feb;136(2):466-72.

124. Alquobaili F, Miller SA, Muhie S, Day A, Jett M, Hammamieh R. Estrogen receptor-dependent genomic expression profiles in breast cancer cells in response to fatty acids. J Carcinog. 2010 Feb 4;8:17.

125. Hopkins GJ, West CE, Hard GC. Effect of dietary fats on the incidence of 7,12-dimethylbenz(a)-anthracene-induced tumors in rats. Lipids. 1976 Apr;11(4):328-33.

126. Saggar JK, Chen J, Corey P, Thompson LU. Dietary flaxseed lignan or oil combined with tamoxifen treatment affects MCF-7 tumor growth through estrogen receptor- and growth factor-signaling pathways. Mol Nutr Food Res. 2010 Mar;54(3):415-25.

127. Rose DP, Rayburn J, Hatala MA, Connolly JM. Effects of dietary fish oil on fatty acids and eicosanoids in metastasizing human breast cancer cells. Nutr Cancer. 1994;22(2):131-41.

128. Hardman WE, Avula CP, Fernandes G, Cameron IL. Three percent dietary fish oil concentrate increased efficacy of doxorubicin against MDA-MB 231 breast cancer xenografts. Clin Cancer Res. 2001 Jul;7(7):2041-9.

129. Mandal CC, Ghosh-Choudhury T, Yoneda T, Choudhury GG, Ghosh-Choudhury N. Fish oil prevents breast cancer cell metastasis to bone. Biochem Biophys Res Commun. 2010 Nov 26;402(4):602-7.

130. Jourdan ML, Maheo K, Barascu A, Goupille C, De Latour MP, Bougnoux P, et al. Increased BRCA1 protein in mammary tumours of rats fed marine omega-3 fatty acids. Oncol Rep. 2007 Apr;17(4):713-9.

131. Olivo SE, Hilakivi-Clarke L. Opposing effects of prepubertal low- and high-fat n-3 polyunsaturated fatty acid diets on rat mammary tumorigenesis. Carcinogenesis. 2005 Sep;26(9):1563-72.

132. Kushi LH, Byers T, Doyle C, Bandera EV, McCullough M, McTiernan A, et al. American cancer society guidelines on nutrition and physical activity for cancer prevention: Reducing the risk of cancer with healthy food choices and physical activity. CA Cancer J Clin. 2006 Sep-Oct;56(5):254,81; quiz 313-4.

133. Cho E, Spiegelman D, Hunter DJ, Chen WY, Stampfer MJ, Colditz GA, et al. Premenopausal fat intake and risk of breast cancer. Journal of the National Cancer Institute. 2003 July 16;95(14):1079-85.

134. Howe G, Hirohata T, Hislop T, Iscovich J, Yuan J, Katsouyanni K, et al. Dietary factors and risk of breast-cancer - combined analysis of 12 case control studies. J Natl Cancer Inst. 1990 APR 4;82(7):561-9.

135. Turner LB. A meta-analysis of fat intake, reproduction, and breast cancer risk: An evolutionary perspective. Am J Hum Biol. 2011 Sep-Oct;23(5):601-8.

136. Sieri S, Krogh V, Ferrari P, Berrino F, Pala V, Thiebaut AC, et al. Dietary fat and breast cancer risk in the european prospective investigation into cancer and nutrition. Am J Clin Nutr. 2008 Nov;88(5):1304-12.

137. Boyd NF, Stone J, Vogt KN, Connelly BS, Martin LJ, Minkin S. Dietary fat and breast cancer risk revisited: A meta-analysis of the published literature. Br J Cancer. 2003 print;89(9):1672-85.

138. Zock PL, Katan MB. Linoleic acid intake and cancer risk: A review and meta-analysis. Am J Clin Nutr. 1998 Jul;68(1):142-53.

139. Yaqoob P, Shaikh SR. The nutritional and clinical significance of lipid rafts. Curr Opin Clin Nutr Metab Care. 2010 Mar;13(2):156-66.

140. Yeagle PL. Lipid regulation of cell membrane structure and function. FASEB J. 1989 May;3(7):1833-42.

141. Finean J. Phospholipids in biological membranes and the study of phospholipid-protein interactions. In: Ansell GB, Hawthorne JN, Dawson RMC, editors. Form and Function of Phospholipids. Second ed. Amsterdam, The Netherlands: Elsevier Scientific Publishing Company; 1973. p. 171.

142. Spector AA, Mathur SN, Kaduce TL. Lipid nutrition and metabolism of cultured mammalian cells. Prog Lipid Res. 1980;19(3–4):155-86.

143. Kahlenberg A, Banjo B. Involvement of phospholipids in the D-glucose uptake activity of isolated human erythrocyte membranes. J Biol Chem. 1972 Feb 25;247(4):1156-60.

144. Garg ML, Thomson AB, Clandinin MT. Interactions of saturated, n-6 and n-3 polyunsaturated fatty acids to modulate arachidonic acid metabolism. J Lipid Res. 1990 Feb;31(2):271-7.

145. Innis SM, Clandinin MT. Dynamic modulation of mitochondrial membrane physical properties and ATPase activity by diet lipid. Biochem J. 1981 Jul 15;198(1):167-75.

146. Field CJ, Ryan EA, Thomson AB, Clandinin MT. Diet fat composition alters membrane phospholipid composition, insulin binding, and glucose metabolism in adipocytes from control and diabetic animals. J Biol Chem. 1990 Jul 5;265(19):11143-50.

147. Heude B, Ducimetiere P, Berr C, EVA Study. Cognitive decline and fatty acid composition of erythrocyte membranes--the EVA study. Am J Clin Nutr. 2003 Apr;77(4):803-8.

148. Aldamiz-Echevarria L, Prieto JA, Andrade F, Elorz J, Sanjurjo P, Rodriguez Soriano J. Arachidonic acid content in adipose tissue is associated with insulin resistance in healthy children. J Pediatr Gastroenterol Nutr. 2007 Jan;44(1):77-83.

149. Yang C, Aye CC, Li X, Diaz Ramos A, Zorzano A, Mora S. Mitochondrial dysfunction in insulin resistance: Differential contributions of chronic insulin and saturated fatty acid exposure in muscle cells. Biosci Rep. 2012 Jun 29.

150. Ruth MR, Wang Y, Yu HM, Goruk S, Reaney MJ, Proctor SD, et al. Vaccenic and elaidic acid modify plasma and splenocyte membrane phospholipids and mitogen-stimulated cytokine production in obese insulin resistant JCR: LA-cp rats. Nutrients. 2010 Feb;2(2):181-97.

151. Meng X, Riordan NH, Riordan HD, Mikirova N, Jackson J, Gonzalez MJ, et al. Cell membrane fatty acid composition differs between normal and malignant cell lines. P R Health Sci J. 2004 Jun;23(2):103-6.

152. Grammatikos S, Subbaiah P, Victor T, Miller W. N-3 and N-6 fatty-acid processing and growth effects in neoplastic and non-cancerous human mammary epithelial-cell lines. Br J Cancer. 1994 Aug;70(2):219-27.

153. Munoz S, Piegari M, Guzman C, Eynard A. Differential effects of dietary oenothera, zizyphus mistol and corn oils, and essential fatty acid deficiency on the progression of a murine mammary gland adenocarcinoma. Nutrition. 1999 Mar;15(3):208-212.

154. Corsetto PA, Cremona A, Montorfano G, Jovenitti IE, Orsini F, Arosio P, et al. Chemical-physical changes in cell membrane microdomains of breast cancer cells after omega-3 PUFA incorporation. Cell Biochem Biophys. 2012 May 24.

155. Huot PS, Sarkar B, Ma DW. Conjugated linoleic acid alters caveolae phospholipid fatty acid composition and decreases caveolin-1 expression in MCF-7 breast cancer cells. Nutr Res. 2010 Mar;30(3):179-85.

156. Lokesh BR, Wrann M. Incorporation of palmitic acid or oleic acid into macrophage membrane lipids exerts differential effects on the function of normal mouse peritoneal macrophages. Biochim Biophys Acta. 1984 Feb 9;792(2):141-8.

157. Field CJ, Schley PD. Evidence for potential mechanisms for the effect of conjugated linoleic acid on tumor metabolism and immune function: Lessons from n-3 fatty acids. Am J Clin Nutr. 2004 Jun;79(6 Suppl):1190S-8S.

158. Kobayashi N, Barnard RJ, Henning SM, Elashoff D, Reddy ST, Cohen P, et al. Effect of altering dietary omega-6/omega-3 fatty acid ratios on prostate cancer membrane composition, cyclooxygenase-2, and prostaglandin E2. Clin Cancer Res. 2006 Aug 1;12(15):4662-70.

159. Sawyer MB, Field CJ. Possible mechanisms of omega-3 PUFA anti-tumour action. Dietary Omega-3 Polyunsaturated Fatty Acids and Cancer. 2010;1:3-38.

160. Hilvo M, Denkert C, Lehtinen L, Muller B, Brockmoller S, Seppanen-Laakso T, et al. Novel theranostic opportunities offered by characterization of altered membrane lipid metabolism in breast cancer progression. Cancer Res. 2011 May 1;71(9):3236-45.

161. Lane J, Mansel RE, Jiang WG. Expression of human delta-6-desaturase is associated with aggressiveness of human breast cancer. Int J Mol Med. 2003 Aug;12(2):253-7.

162. Do HJ, Chung HK, Moon J, Shin M. Relationship between the estimates of desaturase activities and cardiometabolic phenotypes in koreans. Journal of Clinical Biochemistry and Nutrition. 2011 Sep 1;49(2):131-5.

163. Njoroge SW, Seegmiller AC, Katrangi W, Laposata M. Increased delta 5-and delta 6-desaturase, cyclooxygenase-2, and lipoxygenase-5 expression and activity are associated with fatty acid and eicosanoid changes in cystic fibrosis. Biochimica Et Biophysica Acta-Molecular and Cell Biology of Lipids. 2011 Jul-Aug;1811(7-8):431-40.

164. Truan JS, Chen JM, Thompson LU. Flaxseed oil reduces the growth of human breast tumors (MCF-7) at high levels of circulating estrogen. Mol Nutr Food Res. 2010 Oct;54(10):1414-21.

165. Gore J, Besson P, Hoinard C, Bougnoux P. Na(+)-H+ antiporter activity in relation to membrane fatty acid composition and cell proliferation. Am J Physiol. 1994 Jan;266(1 Pt 1):C110-20.

166. Horia E, Watkins B. Comparison of stearidonic acid and alpha-linolenic acid on PGE(2) production and COX-2 protein levels in MDA-MB-231 breast cancer cell cultures. J Nutr Biochem. 2005 Mar;16(3):184-92.

167. Conforti F, Ioele G, Statti GA, Marrelli M, Ragno G, Menichini F. Antiproliferative activity against human tumor cell lines and toxicity test on mediterranean dietary plants. Food and Chemical Toxicology. 2008 Oct;46(10):3325-32.

168. Chajes V, Sattler W, Stranzl A, Kostner GM. Influence of n-3 fatty acids on the growth of human breast cancer cells in vitro: Relationship to peroxides and vitamin-E. Breast Cancer Res Treat. 1995 Jun;34(3):199-212.

169. Hatala MA, Rayburn J, Rose DP. Comparison of linoleic acid and eicosapentaenoic acid incorporation into human breast cancer cells. Lipids. 1994 Dec;29(12):831-7.

170. Kaur B, Jorgensen A, Duttaroy AK. Fatty acid uptake by breast cancer cells (MDA-MB-231): Effects of insulin, leptin, adiponectin, and TNFalpha. Prostaglandins Leukot Essent Fatty Acids. 2009 Feb-Mar;80(2-3):93-9.

171. Schley P, Jijon H, Robinson L, Field C. Mechanisms of omega-3 fatty acid-induced growth inhibition in MDA-MB-231 human breast cancer cells. Breast Cancer Res Treat. 2005 Jul;92(2):187-95.

172. Hawkins R, Sangster K, Arends M. The apoptosis-inducing effects of polyunsaturated fatty acids (PUFAs) on benign and malignant breast cells in vitro. Breast. 1999 Feb;8(1):16-20.

173. Rogers KR, Kikawa KD, Mouradian M, Hernandez K, McKinnon KM, Ahwah SM, et al. Docosahexaenoic acid alters epidermal growth factor receptor-related signaling by disrupting its lipid raft association. Carcinogenesis. 2010 Sep;31(9):1523-30.

174. Chamras H, Ardashian A, Heber D, Glaspy J. Fatty acid modulation of MCF-7 human breast cancer cell proliferation, apoptosis and differentiation. J Nutr Biochem. 2002 Dec;13(12):711-6.

175. Rose D, Connolly, Liu X. Effects of linoleic-acid and gamma-linolenic acid on the growth and metastasis of a human breast-cancer cell-line in nude mice and on its growth and invasive capacity in-vitro. Nutrition and Cancer - An International Journal. 1995;24(1):33-45.

176. Ells G, Chisholm K, Simmons V, Horrobin D. Vitamin E blocks the cytotoxic effect of gamma-linolenic acid when administered as late as the time of onset of cell death - insight into the mechanism of fatty acid induced cytotoxicity. Cancer Lett. 1996 Jan 2;98(2):207-11.

177. Menendez J, Ropero S, del Mar Barbacid M, Montero S, Solanas M, Escrich E, et al. Synergistic interaction between vinorelbine and gamma-linolenic acid in breast cancer cells. Breast Cancer Res Treat. 2002 Apr;72(3):203-19.

178. Pasqualini ME, Heyd VL, Manzo P, Eynard AR. Association between E-cadherin expression by human colon, bladder and breast cancer cells and the 13-HODE:15-HETE ratio. A possible role of their metastatic potential. Prostaglandins Leukot Essent Fatty Acids. 2003 Jan;68(1):9-16.

179. Menendez J, Barbacid M, Montero S, Sevilla E, Escrich E, Solanas M, et al. Effects of gamma-linolenic acid and oleic acid on paclitaxel cytotoxicity in human breast cancer cells. Eur J Cancer. 2001 Feb;37(3):402-13.

180. Munoz SF, Silva RA, Lamarque A, Guzman CA, Eynard AR. Protective capability of dietary zizyphus mistol seed oil, rich in 18:3, n-3, on the

development of two murine mammary gland adenocarcinomas with high or low metastatic potential. Prostaglandins Leukot Essent Fatty Acids. 1995 Aug;53(2):135-8.

181. Cognault S, Jourdan ML, Germain E, Pitavy R, Morel E, Durand G, et al. Effect of an alpha-linolenic acid-rich diet on rat mammary tumor growth depends on the dietary oxidative status. Nutr Cancer. 2000;36(1):33-41.

182. Hardman WE. Dietary canola oil suppressed growth of implanted MDA-MB 231 human breast tumors in nude mice. Nutr Cancer. 2007;57(2):177-83.

183. Rao GN, Ney E, Herbert RA. Effect of melatonin and linolenic acid on mammary cancer in transgenic mice with c-neu breast cancer oncogene. Breast Cancer Res Treat. 2000 Dec;64(3):287-96.

184. Wang L, Chen J, Thompson LU. The inhibitory effect of flaxseed on the growth and metastasis of estrogen receptor negative human breast cancer xenograftsis attributed to both its lignan and oil components. Int J Cancer. 2005 Sep 20;116(5):793-8.

185. Lhuillery C, Bougnoux P, Groscolas R, Durand G. Time-course study of adipose tissue fatty acid composition during mammary tumor growth in rats with controlled fat intake. Nutr Cancer. 1995;24(3):299-309.

186. Noguchi M, Minami M, Yagasaki R, Kinoshita K, Earashi M, Kitagawa H, et al. Chemoprevention of DMBA-induced mammary carcinogenesis in rats by low-dose EPA and DHA. Br J Cancer. 1997;75(3):348-53.

187. Yuri T, Danbara N, Tsujita-Kyutoku M, Fukunaga K, Takada H, Inoue Y, et al. Dietary docosahexaenoic acid suppresses N-methyl-N-nitrosourea-induced mammary carcinogenesis in rats more effectively than eicosapentaenoic acid. Nutrition and Cancer-an International Journal. 2003;45(2):211-7.

188. Connolly JM, Gilhooly EM, Rose DP. Effects of reduced dietary linoleic acid intake, alone or combined with an algal source of docosahexaenoic acid, on MDA-MB-231 breast cancer cell growth and apoptosis in nude mice. Nutr Cancer. 1999;35(1):44-9.

189. Hubbard NE, Lim D, Erickson KL. Alteration of murine mammary tumorigenesis by dietary enrichment with n-3 fatty acids in fish oil. Cancer Lett. 1998 Feb 13;124(1):1-7.

190. Imrhan V, Hsueh A. Effects of type and level of dietary fat during the pre-initiation phase of mammary carcinogenesis in rats. Nutr Res. 1998 MAR;18(3):543-55.

191. Connolly JM, Liu XH, Rose DP. Effects of dietary menhaden oil, soy, and a cyclooxygenase inhibitor on human breast cancer cell growth and metastasis in nude mice. Nutr Cancer. 1997;29(1):48-54.

192. Shao Y, Pardini L, Pardini R. Intervention of transplantable human mammary carcinoma MX-1 chemotherapy with dietary menhaden oil in athymic mice: Increased therapeutic effects and decreased toxicity of cyclophosphamide. Nutr Cancer. 1997;28(1):63-73.

193. Rose D, Connolly J. Effects of dietary omega-3-fatty-acids on human breast-cancer growth and metastases in nude-mice. J Natl Cancer Inst. 1993 Nov 3;85(21):1743-7.

194. Cohen LA, Chen-Backlund JY, Sepkovic DW, Sugie S. Effect of varying proportions of dietary menhaden and corn oil on experimental rat mammary tumor promotion. Lipids. 1993 May;28(5):449-56.

195. Welsch CW, Oakley CS, Chang CC, Welsch MA. Suppression of growth by dietary fish oil of human breast carcinomas maintained in three different strains of immune-deficient mice. Nutr Cancer. 1993;20(2):119-27.

196. Rose DP, Hatala MA, Connolly JM, Rayburn J. Effect of diets containing different levels of linoleic acid on human breast cancer growth and lung metastasis in nude mice. Cancer Res. 1993 Oct 1;53(19):4686-90.

197. Colquhoun A, Schumacher RI. Modifications in mitochondrial metabolism and ultrastructure and their relationship to tumour growth inhibition by gamma-linolenic acid. Mol Cell Biochem. 2001 Feb;218(1-2):13-20.

198. Kenny F, Gee J, Nicholson R, Ellis I, Morris T, Watson S, et al. Effect of dietary GLA plus /-tamoxifen on the growth, ER expression and fatty acid profile of ER positive human breast cancer xenografts. International Journal of Cancer. 2001 May 1;92(3):342-347.

199. Munoz S, Lopez C, Valentich M, Eynard A. Differential modulation by dietary n-6 or n-9 unsaturated fatty acids on the development of two murine mammary gland tumors having different metastatic capabilities. Cancer Letters. 1998 Apr 24;126(2):149-155.

200. Jelinska M, Tokarz A, Oledzka R, Czorniuk-Sliwa A. Effects of dietary linseed, evening primrose or fish oils on fatty acid and prostaglandin E-2 contents in the rat livers and 7,12-dimethylbenz[a]anthracene-induced tumours. Biochim Biophys Acta-Mol Basis Dis. 2003 Apr 17;1637(3):193-9.

201. Welsch C, Welsch M, Huelskamp L, Gonzalez M, Vanderploeg L. Influence of dietary-fat on growth of MDA-MB231 human breast carcinomas maintained in

female athymic nude-mice. International Journal of Oncology. 1995 Jan;6(1):55-64.

202. Karmali R, Adams L, Trout J. Plant and marine N-3 fatty-acids inhibit experimental metastasis of rat mammary adenocarcinoma cells. Prostaglandins Leukotrienes Essential Fatty Acids. 1993 Apr;48(4):309-14.

203. Wang J, John EM, Ingles SA. 5-lipoxygenase and 5-lipoxygenase-activating protein gene polymorphisms, dietary linoleic acid, and risk for breast cancer. Cancer Epidemiol Biomarkers Prev. 2008 Oct;17(10):2748-54.

204. Gago-Dominguez M, Castelao J, Sun C, Van den Berg D, Koh W, Lee H, et al. Marine n-3 fatty acid intake, glutathione S-transferase polymorphisms and breast cancer risk in post-menopausal chinese women in singapore. Carcinogenesis. 2004 Nov;25(11):2143-2147.

205. Bougnoux P, Hajjaji N, Ferrasson MN, Giraudeau B, Couet C, Le Floch O. Improving outcome of chemotherapy of metastatic breast cancer by docosahexaenoic acid: A phase II trial. British Journal of Cancer. 2009 Dec 8;101(12):1978-1985.

206. Patterson RE, Flatt SW, Newman VA, Natarajan L, Rock CL, Thomson CA, et al. Marine fatty acid intake is associated with breast cancer prognosis. J Nutr. 2011 Feb;141(2):201-6.

207. Gogos C, Ginopoulos P, Salsa B, Apostolidou E, Zoumbos N, Kalfarentzos F. Dietary omega-3 polyunsaturated fatty acids plus vitamin E restore immunodeficiency and prolong survival for severely ill patients with generalized malignancy - A randomized control trial. Cancer. 1998 Jan 15;82(2):395-402.

208. Murphy RA, Mourtzakis M, Chu QSC, Baracos VE, Reiman T, Mazurak VC. Supplementation with fish oil increases first-line chemotherapy efficacy in patients with advanced nonsmall cell lung cancer. Cancer. 2011 Aug 15;117(16):3774-80.

209. Murphy RA, Yeung E, Mazurak VC, Mourtzakis M. Influence of eicosapentaenoic acid supplementation on lean body mass in cancer cachexia. Br J Cancer. 2011 Nov 8;105(10):1469-73.

210. Murff HJ, Shu X, Li H, Yang G, Wu X, Cai H, et al. Dietary polyunsaturated fatty acids and breast cancer risk in chinese women: A prospective cohort study. Int J Cancer. 2011 Mar 15;128(6):1434-41.

211. Thiebaut AC, Chajes V, Gerber M, Boutron-Ruault MC, Joulin V, Lenoir G, et al. Dietary intakes of omega-6 and omega-3 polyunsaturated fatty acids and the risk of breast cancer. Int J Cancer. 2009 Feb 15;124(4):924-31.

212. Folsom AR, Demissie Z. Fish intake, marine omega-3 fatty acids, and mortality in a cohort of postmenopausal women. Am J Epidemiol. 2004 Nov 15;160(10):1005-10.

213. Holmes M, Hunter D, Colditz G, Stampfer M, Hankinson S. Association of dietary intake of fat and fatty acids with risk of breast cancer. JAMA. 1999 Mar 10;281(10):914-920.

214. Patterson RE, Flatt SW, Newman VA, Natarajan L, Rock CL, Thomson CA, et al. Marine fatty acid intake is associated with breast cancer prognosis. Journal of Nutrition. 2011 Feb;141(2):201-206.

215. Touvier M, Kesse-Guyot E, Andreeva VA, Fezeu L, Charnaux N, Sutton A, et al. Modulation of the association between plasma intercellular adhesion molecule-1 and cancer risk by n-3 PUFA intake: A nested case-control study. Am J Clin Nutr. 2012 Apr;95(4):944-50.

216. Maillard V, Bougnoux P, Ferrari P, Jourdan ML, Pinault M, Lavillonniere F, et al. N-3 and N-6 fatty acids in breast adipose tissue and relative risk of breast cancer in a case-control study in tours, france. Int J Cancer. 2002 Mar 1;98(1):78-83.

217. Klein V, Chajes V, Germain E, Schulgen G, Pinault M, Malvy D, et al. Low alpha-linolenic acid content of adipose breast tissue is associated with an increased risk of breast cancer. Eur J Cancer. 2000 Feb;36(3):335-40.

218. Bougnoux P, Koscielny S, Chajes V, Descamps P, Couet C, Calais G. Alpha-linolenic acid content of adipose breast tissue: A host determinant of the risk of early metastasis in breast cancer. Br J Cancer. 1994 Aug;70(2):330-4.

219. Chajes V, Cognault S, Maillard V, Pinault M, Jourdan M, Lhuillery C, et al. Alpha-linolenic acid, anti-oxidants and the growth of breast tumours. OCL-Ol Corps Gras Lipides. 2000 Jan-Feb;7(1):64-7.

220. Mamalakis G, Hatzis C, de Bree E, Sanidas E, Tsiftsis DD, Askoxylakis J, et al. Adipose tissue fatty acids in breast cancer patients versus healthy control women from crete. Ann Nutr Metab. 2009;54(4):275-82.

221. De Stefani E, Deneo-Pellegrini H, Mendilaharsu M, Ronco A. Essential fatty acids and breast cancer: A case-control study in uruguay. Int J Cancer. 1998 May 18;76(4):491-4.

222. Takata Y, King IB, Neuhouser ML, Schaffer S, Barnett M, Thornquist M, et al. Association of serum phospholipid fatty acids with breast cancer risk among postmenopausal cigarette smokers. Cancer Causes Control. 2009 May;20(4):497-504.

223. Brasky TM, Lampe JW, Potter JD, Patterson RE, White E. Specialty supplements and breast cancer risk in the VITamins and lifestyle (VITAL) cohort. Cancer Epidemiology Biomarkers & Prevention. 2010 JUL;19(7):1696-1708.

224. Goodstine S, Zheng T, Holford T, Ward B, Carter D, Owens P, et al. Dietary (n-3)/(n-6) fatty acid ratio: Possible relationship to premenopausal but not postmenopausal breast cancer risk in US women. Journal of Nutrition. 2003 May;133(5):1409-1414.

225. Wang J, John EM, Horn-Ross PL, Ingles SA. Dietary fat, cooking fat, and breast cancer risk in a multiethnic population. Nutr Cancer. 2008 Jul-Aug;60(4):492-504.

226. Bagga D, Anders KH, Wang HJ, Glaspy JA. Long-chain n-3-to-n-6 polyunsaturated fatty acid ratios in breast adipose tissue from women with and without breast cancer. Nutr Cancer. 2002;42(2):180-5.

227. Saadatian-Elahi M, Norat T, Goudable J, Riboli E. Biomarkers of dietary fatty acid intake and the risk of breast cancer: A meta-analysis. Int J Cancer. 2004 Sep 10;111(4):584-91.

228. Turner LB. A meta-analysis of fat intake, reproduction, and breast cancer risk: An evolutionary perspective. Am J Hum Biol. 2011 Sep-Oct 2011;23(5):601-8.

229. Vecchia Cl. Mediterranean diet and cancer. Public Health Nutr. 2004 2004;7(7):965-8.

230. Wu WH, Lu SC, Wang TF, Jou HJ, Wang TA. Effects of docosahexaenoic acid supplementation on blood lipids, estrogen metabolism, and in vivo oxidative stress in postmenopausal vegetarian women. Eur J Clin Nutr. 2006 Mar;60(3):386-92.

231. Witt PM, Christensen JH, Ewertz M, Aardestrup IV, Schmidt EB. The incorporation of marine n-3 PUFA into platelets and adipose tissue in pre- and postmenopausal women: A randomised, double-blind, placebo-controlled trial. Br J Nutr. 2010 Aug;104(3):318-25.

232. Schmid A, Collomb M, Sieber R, Bee G. Conjugated linoleic acid in meat and meat products: A review. Meat Sci. 2006 May;73(1):29-41.

233. Palmquist DL, Lock AL, Shingfield KJ, Bauman DE. Biosynthesis of conjugated linoleic acid in ruminants and humans. Adv Food Nutr Res. 2005;50:179-217.

234. Kepler CR, Hirons KP, Mcneill JJ, Tove SB. Intermediates and products of biohydrogenation of linoleic acid by butyrivibrio fibrisolvens. J Biol Chem. 1966 1966;241(6):1350,&.

235. Kemp P, Lander DJ. The hydrogenation of gamma-linolenic acid by pure cultures of 2 rumen bacteria. Biochem J. 1983 1983;216(2):519-22.

236. Chin SF, Storkson JM, Albright KJ, Pariza MW. Conjugated linoleic-acid (9,11-octadecadienoic and 10,12-octadecadienoic acid) is produced in conventional but not germ-free rats fed linoleic-acid. J Nutr. 1994 May 1994;124(5):694-701.

237. Herbel BK, McGuire MK, McGuire MA, Shultz TD. Safflower oil consumption does not increase plasma conjugated linoleic acid concentrations in humans. Am J Clin Nutr. 1998 Feb;67(2):332-7.

238. Rosberg-Cody E, Ross RP, Hussey S, Ryan CA, Murphy BP, Fitzgerald GF, et al. Mining the microbiota of the neonatal gastrointestinal tract for conjugated linoleic acid-producing bifidobacteria. Appl Environ Microbiol. 2004 Aug 2004;70(8):4635-41.

239. Coakley M, Ross RP, Nordgren M, Fitzgerald G, Devery R, Stanton C. Conjugated linoleic acid biosynthesis by human-derived bifidobacterium species. J Appl Microbiol. 2003 2003;94(1):138-45.

240. Mahfouz MM, Valicenti AJ, Holman RT. Desaturation of isomeric trans-octadecenoic acids by rat liver microsomes. Biochim Biophys Acta. 1980 Apr 18;618(1):1-12.

241. Turpeinen AM, Mutanen M, Aro A, Salminen I, Basu S, Palmquist DL, et al. Bioconversion of vaccenic acid to conjugated linoleic acid in humans. Am J Clin Nutr. 2002 Sep 2002;76(3):504-10.

242. Salminen I, Mutanen M, Jauhiainen M, Aro A. Dietary trans fatty acids increase conjugated linoleic acid levels in human serum. J Nutr Biochem. 1998 Feb 1998;9(2):93-8.

243. Ritzenthaler KL, McGuire MK, Falen R, Shultz TD, Dasgupta N, McGuire MA. Estimation of conjugated linoleic acid intake by written dietary assessment methodologies underestimates actual intake evaluated by food duplicate methodology. J Nutr. 2001 May;131(5):1548-54.

244. Ha YL, Grimm NK, Pariza MW. Newly recognized anticarcinogenic fatty acids identification and quantification in natural and processed cheeses. J Agric Food Chem. 1989 1989;37(1):75-81.

245. Garcialopez S, Echeverria E, Tsui I, Balch B. Changes in the content of conjugated linoleic-acid (cla) in processed cheese during processing. Food Res Int. 1994 1994;27(1):61-4.

246. Shantha NC, Decker EA. Conjugated linoleic-acid concentrations in processed cheese containing hydrogen donors, iron and dairy-based additives. Food Chem. 1993 1993;47(3):257-61.

247. Lee KN, Kritchevsky D, Pariza MW. Conjugated linoleic-acid and atherosclerosis in rabbits. Atherosclerosis. 1994 Jul 1994;108(1):19-25.

248. Yi Dan, Lin XueZheng, Shen JiHong, Liu FaYi, Liang XiaoFang, Yi D, et al. Effect of conjugated linoleic acid in lowering blood lipids and anti-atherosclerosis. Progress in Modern Biomedicine. 2011 2011;11(7):1228-30.

249. Castro-Webb N, Ruiz-Narvaez EA, Campos H. Cross-sectional study of conjugated linoleic acid in adipose tissue and risk of diabetes. Am J Clin Nutr. 2012 Jul 2012;96(1):175-81.

250. Rungapamestry V, McMonagle J, Reynolds C, Rucklidge G, Reid M, Duncan G, et al. Inter-organ proteomic analysis reveals insights into the molecular mechanisms underlying the anti-diabetic effects of cis-9, trans-11-conjugated linoleic acid in ob/ob mice. Proteomics. 2012 Feb 2012;12(3):461-76.

251. De La Torre A, Debiton E, Durand D, Chardigny JM, Berdeaux O, Loreau O, et al. Conjugated linoleic acid isomers and their conjugated derivatives inhibit growth of human cancer cell lines. Anticancer Res. 2005 Nov-Dec 2005;25(6B):3943-9.

252. Schonberg S, Krokan HE. The inhibitory effect of conjugated dienoic derivatives (cla) of linoleic-acid on the growth of human tumor-cell lines is in part due to increased lipid-peroxidation. Anticancer Res. 1995 Jul-Aug 1995;15(4):1241-6.

253. Stachowska E, Siennicka A, Baskiewcz-Halasa M, Bober J, Machalinski B, Chlubek D. Conjugated linoleic acid isomers may diminish human macrophages adhesion to endothelial surface. Int J Food Sci Nutr. 2012 Feb 2012;63(1):30-5.

254. Park Y, Yang M, Storkson JM, Albright KJ, Liu W, Cook ME, et al. Effects of conjugated linoleic acid isomers on serum tumor necrosis factor-a concentration in mice. J Food Biochem. 2007 Apr 2007;31(2):252-65.

255. Changhua L, Jindong Y, Defa L, Lidan Z, Shiyan Q, Jianjun X. Conjugated linoleic acid attenuates the production and gene expression of proinflammatory cytokines in weaned pigs challenged with lipopolysaccharide. J Nutr. 2005 Feb 2005;135(2):239-44.

256. Amaru DL, Biondo PD, Field CJ. The role of conjugated linoleic acid in breast cancer growth and development. The Open Nutraceuticals Journal. 2010;3:30-46.

257. Amaru DL, Field CJ. Conjugated linoleic acid decreases mcf-7 human breast cancer cell growth and insulin-like growth factor-1 receptor levels. Lipids. 2009 May;44(5):449-58.

258. Huot PSP, Sarkar B, Ma DWL. Conjugated linoleic acid alters caveolae phospholipid fatty acid composition and decreases caveolin-1 expression in MCF-7 breast cancer cells. Nutr Res. 2010 Mar;30(3):179-85.

259. Banni S, Angioni E, Casu V, Melis M, Carta G, Corongiu F, et al. Decrease in linoleic acid metabolites as a potential mechanism in cancer risk reduction by conjugated linoleic acid. Carcinogenesis. 1999 Jun;20(6):1019-24.

260. Rakib MA, Kim YS, Jang WJ, Jang JS, Kang SJ, Ha YL. Preventive effect of t,t-conjugated linoleic acid on 12-O-tetradecanoylphorbol-13-acetate-induced inhibition of gap junctional intercellular communication in human mammary epithelial MCF-10A cells. J Agric Food Chem. 2011 Apr 27;59(8):4164-70.

261. Lau DS, Archer MC. The 10t,12c isomer of conjugated linoleic acid inhibits fatty acid synthase expression and enzyme activity in human breast, colon, and prostate cancer cells. Nutr Cancer. 2010;62(1):116-21.

262. Donnelly C, Olsen AM, Lewis LD, Eisenberg BL, Eastman A, Kinlaw WB. Conjugated linoleic acid (CLA) inhibits expression of the spot 14 (THRSP) and fatty acid synthase genes and impairs the growth of human breast cancer and liposarcoma cells. Nutr Cancer. 2009;61(1):114-22.

263. Islam MA, Kim YS, Jang WJ, Lee SM, Kim HG, Kim SY, et al. A mixture of trans, trans conjugated linoleic acid induces apoptosis in MCF-7 human breast cancer cells with reciprocal expression of bax and bcl-2. J Agric Food Chem. 2008 Jul 23;56(14):5970-6.

264. Kim J, Hubbard N, Ziboh V, Erickson K. Attenuation of breast tumor cell growth by conjugated linoleic acid via inhibition of 5-lipoxygenase activating protein. Biochim Biophys Acta Mol Cell Biol Lipids. 2005 Oct 1;1736(3):244-50.

265. Durgam VR, Fernandes G. The growth inhibitory effect of conjugated linoleic acid on MCF-7 cells is related to estrogen response system. Cancer Lett. 1997 Jun 24;116(2):121-30.

266. Miller A, Stanton C, Devery R. Modulation of arachidonic acid distribution by conjugated linoleic acid isomers and linoleic acid in MCF-7 and SW480 cancer cells. Lipids. 2001 Oct;36(10):1161-8.

267. Ma D, Field C, Clandinin M. An enriched mixture of trans-10,cis-12-CLA inhibits linoleic acid metabolism and PGE(2) synthesis in NWA-MB-231 cells. Nutrition and Cancer-an International Journal. 2002;44(2):202-12.

268. Choi Y, Park Y, Storkson JM, Pariza MW, Ntambi JM. Inhibition of stearoyl-CoA desaturase activity by the cis-9, trans-11 isomer and the trans-10,cis-12 isomer of conjugated linoleic acid in MDA-MB-231 and MCF-7 human breast cancer cells. Biochem Biophys Res Commun. 2002 Jun 21;294(4):785-90.

269. Kemp MQ, Jeffy BD, Romagnolo DF. Conjugated linoleic acid inhibits cell proliferation through a p53-dependent mechanism: Effects on the expression of G1-restriction points in breast and colon cancer cells. J Nutr. 2003 Nov;133(11):3670-7.

270. Miller A, Stanton C, Murphy J, Devery R. Conjugated linoleic acid (CLA)-enriched milk fat inhibits growth and modulates CLA-responsive biomarkers in MCF-7 and SW480 human cancer cell lines. Br J Nutr. 2003 Nov;90(5):877-85.

271. Chujo H, Yamasaki M, Nou S, Koyanagi N, Tachibana H, Yamada K. Effect of conjugated linoleic acid isomers on growth factor-induced proliferation of human breast cancer cells. Cancer Lett. 2003 Dec 8;202(1):81-7.

272. Tanmahasamut P, Liu J, Hendry LB, Sidell N. Conjugated linoleic acid blocks estrogen signaling in human breast cancer cells. J Nutr. 2004 Mar;134(3):674-80.

273. Kim JH, Hubbard NE, Ziboh V, Erickson KL. Conjugated linoleic acid reduction of murine mammary tumor cell growth through 5-hydroxyeicosatetraenoic acid. Biochim Biophys Acta. 2005 Feb 21;1687(1-3):103-9.

274. Miglietta A, Bozzo F, Gabriel L, Bocca C, Canuto RA. Extracellular signal-regulated kinase 1/2 and protein phosphatase 2A are involved in the antiproliferative activity of conjugated linoleic acid in MCF-7 cells. Br J Nutr. 2006 Jul;96(1):22-7.

275. Miglietta A, Bozzo F, Bocca C, Gabriel L, Trombetta A, Belotti S, et al. Conjugated linoleic acid induces apoptosis in MDA-MB-231 breast cancer cells through ERK/MAPK signalling and mitochondrial pathway. Cancer Lett. 2006 Mar 28;234(2):149-57.

276. De La Torre A, Debiton E, Juaneda P, Durand D, Chardigny JM, Barthomeuf C, et al. Beef conjugated linoleic acid isomers reduce human cancer cell growth even when associated with other beef fatty acids. Br J Nutr. 2006 Feb;95(2):346-52.

277. Albright CD, Klem E, Shah AA, Gallagher P. Breast cancer cell-targeted oxidative stress: Enhancement of cancer cell uptake of conjugated linoleic acid, activation of p53, and inhibition of proliferation. Exp Mol Pathol. 2005 Oct;79(2):118-25.

278. Islam MA, Kim YS, Oh TW, Kim GS, Won CK, Kim HG, et al. Superior anticarcinogenic activity of trans,trans-conjugated linoleic acid in N-methyl-N-nitrosourea-induced rat mammary tumorigenesis. J Agric Food Chem. 2010 May 12;58(9):5670-8.

279. Flowers M, Schroeder JA, Borowsky AD, Besselsen DG, Thomson CA, Pandey R, et al. Pilot study on the effects of dietary conjugated linoleic acid on tumorigenesis and gene expression in PyMT transgenic mice. Carcinogenesis. 2010 Sep;31(9):1642-9.

280. Ip C, Singh M, Thompson HJ, Scimeca JA. Conjugated linoleic acid suppresses mammary carcinogenesis and proliferative activity of the mammary gland in the rat. Cancer Res. 1994 Mar 1;54(5):1212-5.

281. Ip MM, McGee SO, Masso-Welch PA, Ip C, Meng X, Ou L, et al. The t10,c12 isomer of conjugated linoleic acid stimulates mammary tumorigenesis in transgenic mice over-expressing erbB2 in the mammary epithelium. Carcinogenesis. 2007 Jun;28(6):1269-76.

282. Meng X, Shoemaker SF, McGee SO, Ip MM. t10,c12-conjugated linoleic acid stimulates mammary tumor progression in Her2/ErbB2 mice through activation of both proliferative and survival pathways. Carcinogenesis. 2008 May;29(5):1013-21.

283. Larsson SC, Bergkvist L, Wolk A. Conjugated linoleic acid intake and breast cancer risk in a prospective cohort of swedish women. Am J Clin Nutr. 2009 SEP 1;90(3):556-60.

284. Chajes V, Lavillonniere F, Maillard V, Giraudeau B, Jourdan M, Sebedio J, et al. Conjugated linoleic acid content in breast adipose tissue of breast cancer patients and the risk of metastasis. Nutr Cancer. 2003;45(1):17-23.

285. Voorrips L, Brants H, Kardinaal A, Hiddink G, van den Brandt P, Goldbohm R. Intake of conjugated linoleic acid, fat, and other fatty acids in relation to postmenopausal breast cancer: The netherlands cohort study on diet and cancer. Am J Clin Nutr. 2002 Oct;76(4):873-82.

286. Aro A, Mannisto S, Salminen I, Ovaskainen M, Kataja V, Uusitupa M. Inverse association between dietary and serum conjugated linoleic acid and risk of breast cancer in postmenopausal women. Nutr Cancer. 2000;38(2):151-7.

287. McCann SE, Ip C, Ip MM, McGuire MK, Muti P, Edge SB, et al. Dietary intake of conjugated linoleic acids and risk of premenopausal and postmenopausal breast cancer, western new york exposures and breast cancer study (WEB study). Cancer Epidemiology Biomarkers & Prevention. 2004 Sep 2004;13(9):1480-4.

Chapter Three – Materials and Methods

I. Introduction

As the methods are referred to in each study are repeated, all of the methods and the rationale for their use were combined into a single chapter. In this chapter, the techniques used for cell line maintenance, fatty acid preparations, and cell viability and PL composition determination, as well as the more complicated calculations used and statistical analysis are outlined.

II. Cell Line Maintenance

To study both ER+PR+ and ER-PR- breast cancer cells, two tumorigenic human cell lines, the MDA-MB-231 and MCF-7, were chosen as the models for our cell culture studies. MDA-MB-231 is an ER-/PR- ductal breast cell line, representing about 20% of all breast cancer cases, while the MCF-7 cell line is a ductal ER+PR+ cell line representing about 63% of all breast cancer cases (1). Together, they represent over 80% of all breast cancer cases and provide relevance to a large population. A non-tumorigenic breast cell line, the MCF-12A cell line, was chosen as a control to the tumorigenic cell lines, to determine whether treatments had effects on normal cells.

MDA-MB-231 breast cancer cells obtained from the American Type Culture Centre (Manassas, VA, USA) were cultured in Iscove's Modified Dulbecco's Medium with L-glutamine and 25mM HEPES buffer (Invitrogen Corporation, Grand Island, NY) containing 5% v/v fetal bovine serum (Thermo Fisher Scientific Inc., Logan, UT, USA) and 1% v/v antibiotic-antimycotic solution (Mediatech Inc., Manassas, VA, USA). MCF-7 breast cancer cells obtained from the American Type Culture Centre (Manassas, VA, USA) were cultured in minimal essential media with Earle's salts, L-glutamine, and non-essential amino acids (Invitrogen Corporation, Grand Island, NY) containing 5% v/v fetal bovine serum and 1% v/v antibiotic-antimycotic solution (as indicated above). MCF-12A breast cells kindly provided by Dr. Michael Weinfeld's lab of the Department of Oncology at the University of Alberta, were cultured with Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (Ham) 1:1

(Invitrogen Corporation, Grand Island, NY) containing 0.1µg/mL cholera toxin (Calbiochem, Darmstadt, Germany), 20ng/mL human epidermal growth factor (EGF), 0.5µg/mL hydrocortisone, 10µg/mL bovine insulin (EGF, hydrocortisone and insulin purchased from Sigma-Aldrich Co. LLC., Oakville, ON), 10% v/v fetal bovine serum and 1% v/v antibiotic-antimycotic solution (as indicated above).

Cells were maintained in 75cm^2 tissue culture flasks (Fisher Scientific, Ottawa, Ont., Canada) at 37°C and 5% v/v CO_2. Media was changed 3 times per week and passaged regularly at 80-90% confluence. Cells were washed twice with phosphate buffered saline before being detached from the flasks using 5mL HyClone Trypsin 0.25% (Thermo Scientific, Logan, UT, USA), stopped with their respective medias and centrifuged at 377x for 5 minutes in a 15mL sterile centrifuge tube (Fisher Scientific, Ottawa, ON). Cells were resuspended in media and a 20µL aliquot was mixed with 20µL trypan blue 0.4% in normal saline. Viable cells were then counted using a Hausser Scientific Bright Line hemacytometer (Fisher Scientific, Ottawa, ON) and light microscope. Cells were then used for experiments and/or cell maintenance in a flask.

III. Fatty Acid Preparation and Complexing to Bovine Serum Albumin

Treatment fatty acids were supplied to cells through conjugation to BSA. Fats obtained from the diet are usually in triglyceride form, and are broken down for absorption into the lymphatic system in the intestine, where they are reassembled and stored as triglycerides or PLs in the body. When signals for the mobilization of lipids occur, triglycerides are catabolized by the liver and sent into circulation to be used by other tissues. As free fatty acids are not water soluble, they are not very stable in the blood, and therefore not commonly found in the blood. Instead, esterfied fatty acids travel as lipoproteins, while non-esterfied fatty acids are bound to plasma albumin (2). Although it is possible to incorporate lipids from liposomes into membranes, it has been shown that fatty acid incorporation is significantly higher when fatty acids are bound to albumin versus liposomes (3). Therefore, we used fatty acids conjugated to BSA as not

only is it is physiological method by which fatty acids are moved around the body for use by tissues, but it also ensures a greater amount of fatty acid incorporation into the membranes.

SDA, ETA, EPA, GLA, DGLA, stearic acid, 11(Z),14(Z)-eicosatrienoic acid (Cayman Chemical Company, Ann Arbor, MI, USA), OA, ALA, DHA, AA, LA, c9, t11-CLA, and palmitic acid (Matreya, Pleasant Gap, PA, USA) were used in the following experiments. Stock fatty acid solutions were prepared by dissolving the fatty acid in ethanol to a concentration of 10mg/mL and stored at –20°C. Fatty acids were then conjugated to fatty acid-free bovine serum albumin (BSA, Fisher Scientific, Ottawa, ON) at a 4:1 ratio as follows (4): 1.2 mL (12mg) of the stock solution was then dried down in a 15mL acid washed glass tube under nitrogen before being saponified with 1mL of 0.1M KOH in a shaking 50°C water bath for 10 minutes, mixing intermittently. Fatty acids were then conjugated to BSA by adding 9mL of 7.5% BSA in doubly distilled water mixture and incubated at room temperature for 3 hours. Mixtures were then left overnight at 4°C before aliquoting into sterile 1.5mL eppendorf microcentrifuge tubes (Fisher Scientific, Ottawa, ON) and stored at –20°C until further use. A fatty acid mixture was made using the above fatty acids to represent the fatty acid composition of a stearidonic acid-enriched flax oil (SO) created by Dr. Randall Weselake's Bioactive Oils Program at the University of Alberta (Table 3.1), and stored at -20°C.

Table 3.1 Fatty Acid Composition of a Stearidonic Acid-Enriched Flax Oil (SO)

Fatty Acid	**% Composition**
C16:0	6.02
C18:0	3.08
C18:1-n-9	13.4
C18:2 n-6	3.40
C18:3 n-6	15.0
C18:3 n-3	31.2
C18:4 n-3	26.1
C20:2	0.72
Total	**98.9**

IV. Determination of Cell Viability in the Presence of Fatty Acid

Cells were detached from 75cm^2 flasks as described previously, and plated in triplicate at a density of 2.6x10^4 cells per well for MDA-MB-231 and MCF-12A cells, and 5.2x10^4 cells per well for MCF-7 cells in a 24 well flat bottomed cell culture plate (Corning Inc., Corning, NY, USA) with 2mL of their respective media for 72 hours. Media was replaced with fresh media containing 40μM OA and 40μM LA (OA/LA) to mimic a physiological membrane composition, as previously determined, as a control.

Research has shown that the growth and development of breast tumour cells *in vitro* have been show to require OA and LA in low doses, as higher doses are associated with inhibitory effects (5, 6). LA deficiency has been associated with increased tumour growth in animal models, further supporting the requirement of this essential fatty acid in the diet (6). Therefore, our control treatment had both of these fatty acids to better represent what might occur *in vivo* and to ensure optimal growth. Optimal concentrations of OA and LA for the growth of MDA-MB-231 cells have been suggested to be 0.25μg/mL and 0.75μg/mL, respectively, translating to about concentrations of about 2μM and 3μM, respectively by Rose et al. (5). However, the culture conditions that these cells were grown in, including media, BSA-conjugation, fetal bovine serum, and ethanol, were different from those of our study, and therefore, these concentrations may not translate to the same effects in our culture experiments. We added these fatty acids, based on previous experiments, to ensure that the fatty acid concentration of n-6 essential fatty acids was sufficient to ensure both cell growth and presence of AA in the cell, as well as mimic physiological membrane composition (7, 8).

In order to determine a working concentration for the fatty acid treatments, experiments were done examining cell viability over physiologically possible plasma concentrations, between 30μM-300μM (data not shown). From this data, the concentration of 150μM was chosen as it exhibited significant inhibition of tumour growth with well known anti-carcinogenic fatty acids EPA and DHA. Using this concentration, we further supplemented 150μM of either ALA, SDA,

ETA, EPA, DHA, LA, GLA, DGLA, AA, CLA, or SO to cells in triplicate. Wells with media free of additional fatty acids were also used. Every 24 hours for 48 hours, the media was replaced with treatment media. After 48 hours, media was removed with an aspirator and cells were washed twice with phosphate buffered saline (PBS). Cells were then detached with 200μL of Trypsin 0.25% as described above and moved into 1.5mL eppendorf microcentrifuge tubes. Cell viability was assessed using the trypan blue exclusion method. Cells were resuspended in media and a 20μL aliquot was taken and mixed with trypan blue 0.4% w/v. Live cells were then counted using a hemacytometer, as previously described. MDA-MB-231 and MCF-7 cancer cell lines treatments were done five times while MCF-12A treatments were done three times.

We had previously attempted to use the WST-1 assay to determine cell proliferation by measuring the metabolic activities of our cells, however, we found a large discrepancy between the results from this assay and the number of cells observed under a light microscope, and opted for the trypan blue assay in order to determine cell viability.

V. Determination of Phospholipid Fatty Acid Composition

Dietary fatty acids have been previously reported to be significantly incorporated in the cell plasma membrane of tumour cells (9). Thus, we investigated the effect of our fatty acid treatments on the cell plasma membrane PL, to estimate conversion and incorporation of the treatment fatty acids into the structural and functional lipid in cell membranes. Although intracellular membranes are also composed of PL, the majority of PLs are found in the plasma membrane; therefore, we decided to isolate whole cell PLs for our analyses and draw conclusions for the plasma membrane.

Cells were detached from 75cm^2 flasks as described above and aliquoted into flasks. At about 50% confluence, cells were treated with fatty acid as described above for 48 hours. Cells were then washed twice with PBS and detached with 5mL of Trypsin 0.25% as described above.

Lipids were then extracted from whole cell pellets using a modified Folch procedure as previously described (10, 11). Total PLs were then separated on silica gel 'G' thin layer chromatography plates (Analtech Inc., Newark, DE, USA) using an 80:20:1 v/v/v ratio of petroleum ether: diethyl ether: glacial acetic acid for 40 minutes (8). The PL band was visualized using 8-anilino-1-naphthalene-sulfonic acid under ultraviolet light.

PLs were methylated by heating samples in boron trifluoride and hexane at 110°C for 1hr. 1mL of double distilled water was then added per sample and stood overnight at 4°C. CLA samples were methylated by incubating samples in 1mL sodium methoxide and 1mL hexane at room temperature for 30 minutes. Double distilled water (300μL) was then added to each sample and stood overnight at 4°C. Hexane layers from the methylation steps were then removed and dried down, before being reconstituted in 75μL hexane.

Fatty acids were separated and quantified with an Agilent 7890A Gas Chromatograph System using a 100m CP-Sil 88 fused capillary column, (Agilent Technologies, Mississauga, ON, Can) following the method described by Sørensen et al. (12). The capillary column is coated in a highly polar CP-Sil stationary phase (poly(bicyanopropyl siloxane)). The sample adsorbs onto this phase, with some fatty acids being more strongly attracted to this phase than others, depending on their polarity. The stronger the attractions, the less likely the compound will be carried down the column by the mobile hydrogen phase, and vice versa, resulting in the polar molecules taking longer to elute from the column. Also, as the oven temperature increases, more compounds reach their boiling point, go into their gaseous form and elute from the column, therefore, fatty acids with low boiling points will elute out the fastest. Therefore, small non-polar compounds with low boiling points will elute from the column first, while large polar compounds with high boiling points will elute last. When compounds elute from the column, the flame ionizer ionizes the compounds, creating a current proportional to the amount of compound, which is then sensed by the detector and recorded as peaks based on the time of elution, or retention time.

Retention times can be compared to those from a standard to identify the peaks on the chromatogram, and peak areas can be used to quantify samples.

VI. Calculations

Typical estimations of desaturase enzymes' catalytic activity compare the ratios of only a few fatty acids: n-6's LA and AA and n-3's ALA, EPA, DHA and possibly DPA, neglecting the intermediates, as they are not commonly found in the diet. As we supplied n-3 and n-6 pathway intermediates to cells, we had to come up with a different method to estimate desaturase activities that would include the intermediates and account for any conversion that occured. In the end, we decided to estimate desaturase enzyme activities by dividing all of the enzyme's downstream products by all the reactants within a pathway as seen in Figures 3.1-4.

$$\Delta 6\text{-desaturase activity}_{n-3} = \frac{\sum(\text{SDA} + \text{ETA} + \text{EPA} + \text{DPA} + \text{DHA})}{\text{ALA}}$$

Figure 3.1 Estimated Δ6-Desaturase Activity Formula in the N-3 Pathway

$$\Delta 6\text{-desaturase activity}_{n-6} = \frac{\sum(\text{GLA} + \text{DGLA} + \text{AA})}{\text{LA}}$$

Figure 3.2 Estimated Δ6-Desaturase Activity Formula in the N-6 Pathway

$$\Delta 5\text{-desaturase activity}_{n-3} = \frac{\sum(\text{EPA} + \text{DPA} + \text{DHA})}{\sum(\text{ALA} + \text{SDA} + \text{ETA})}$$

Figure 3.3 Estimated Δ5-Desaturase Activity Formula in the N-3 Pathway

$$\Delta 5 - \text{desaturase activity}_{n-6} = \frac{\text{AA}}{\sum(\text{LA+GLA+DGLA})}$$

Figure 3.4 Estimated Δ5-Desaturase Activity Formula in the N-6 Pathway

VII. Statistical Analysis

Statistical analyses were conducted using SPSS, version 18.0.0 to compare the effects of fatty acid treatment on the viability of different cell lines. A normal

distribution was confirmed using the Kolmogorov-Smirnov test statistic. A one-way analysis of variance was used to determine significant treatment effects. When a significant difference was determined ($p<0.05$), a Dunnett test was used for post-hoc analysis against a control to determine treatment effect within a cell line. When equal variances could not be assumed through Levene's test, Dunnett's T3 test was used. OA/LA treatments were used as a control in all experiments cell lines except in the viability assays where untreated cells were used as a control. Tukey's test and independent t-tests were performed for cell line comparisons when 3 and 2 lines were compared, respectively, in PL analyses. Data is presented as means ± SEM.

VIII. References

1. Dunnwald LK, Rossing MA, Li CI. Hormone receptor status, tumor characteristics, and prognosis: A prospective cohort of breast cancer patients. Breast Cancer Res. 2007;9(1):R6.

2. Tvrzicka E, Kremmyda LS, Stankova B, Zak A. Fatty acids as biocompounds: Their role in human metabolism, health and disease--a review. part 1: Classification, dietary sources and biological functions. Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub. 2011 Jun;155(2):117-30.

3. Grammatikos S, Subbaiah P, Victor T, Miller W. N-3 and N-6 fatty-acid processing and growth effects in neoplastic and non-cancerous human mammary epithelial-cell lines. Br J Cancer. 1994 AUG;70(2):219-27.

4. Keating AF, Kennelly JJ, Zhao FQ. Characterization and regulation of the bovine stearoyl-CoA desaturase gene promoter. Biochem Biophys Res Commun. 2006 May 26;344(1):233-40.

5. Rose D, Connolly J. Effects of fatty-acids and inhibitors of eicosanoid synthesis on the growth of a human breast-cancer cell-line in culture. Cancer Res. 1990 NOV 15;50(22):7139-44.

6. Munoz S, Piegari M, Guzman C, Eynard A. Differential effects of dietary oenothera, zizyphus mistol and corn oils, and essential fatty acid deficiency on the progression of a murine mammary gland adenocarcinoma. Nutrition. 1999 Mar;15(3):208-212.

7. Schley P, Jijon H, Robinson L, Field C. Mechanisms of omega-3 fatty acid-induced growth inhibition in MDA-MB-231 human breast cancer cells. Breast Cancer Res Treat. 2005 Jul;92(2):187-95.

8. Amaru DL, Field CJ. Conjugated linoleic acid decreases mcf-7 human breast cancer cell growth and insulin-like growth factor-1 receptor levels. Lipids. 2009 May;44(5):449-58.

9. Spector AA, Mathur SN, Kaduce TL. Lipid nutrition and metabolism of cultured mammalian cells. Prog Lipid Res. 1980;19(3–4):155-86.

10. Field CJ, Ryan EA, Thomson AB, Clandinin MT. Dietary fat and the diabetic state alter insulin binding and the fatty acyl composition of the adipocyte plasma membrane. Biochem J. 1988 Jul 15;253(2):417-24.

11. Folch J, Lees M, Sloane Stanley GH. A simple method for the isolation and purification of total lipides from animal tissues. J Biol Chem. 1957 May;226(1):497-509.

12. Sorensen BM, Chris Kazala E, Murdoch GK, Keating AF, Cruz-Hernandez C, Wegner J, et al. Effect of CLA and other C18 unsaturated fatty acids on DGAT in bovine milk fat biosynthetic systems. Lipids. 2008 Oct;43(10):903-12.

Chapter Four – N-3 and N-6 Fatty Acids on Cell Viability and Phospholipid Composition in Breast Cells

I. Introduction

The anti-carcinogenic effects of long chain n-3 (PUFAs EPA and DHA) as well as an n-6 PUFA (AA) on breast cancer have been well documented in cell culture (1-3). However, due to low intakes in the North American diet, their beneficial effects are not realized in order to reduce the progression of breast cancer (4).

Humans production of these long chain PUFAs from dietary precursors ALA and LA are very low due to the low catalytic activity of the Δ6-desaturase enzyme that catalyses these fatty acids' conversion to SDA and GLA, respectively (5-10). To increase the endogenous synthesis of long chain PUFA, it may be possible to supply intermediates in the n-3 and n-6 pathways to bypass this low-activity enzyme. A number of metabolic intermediates exist in these pathways: SDA and ETA in the n-3 metabolic pathway for their conversions to EPA and DHA, as well as GLA and DGLA to be converted to AA in the n-6 metabolic pathway. SDA and GLA have been shown to exhibit anti-carcinogenic properties in breast cancer, however their mechanisms have yet to be elucidated (11, 12). Whether these intermediates have anti-carcinogenic effects that do not harm normal cells have yet to be confirmed.

Since dietary fatty acids have been shown to be incorporated into tumour membranes, and changes in membrane composition have also been found to play important roles in cancer progression (13). Changes regarding the cell membrane composition with fatty acid intermediates are important to determine as a possible mechanism for the effects these fatty acids may exhibit. Literature has predicted that SDA and GLA exhibit their anti-tumorigenic effects from their conversions to at least EPA and DGLA, respectively, if not further, rather than through direct anti-tumorigenic properties, however, this hypothesis has yet to be confirmed. Therefore, the objective of this study is to determine the anti-tumorigenic properties of fatty acid intermediates SDA, ETA, GLA and DGLA, and to determine whether their effects are due to their own properties or to their

conversions to other fatty acids. We also want to determine the safety of their use in regards to effects on normal cells.

By supplying tumorigenic and non-tumorigenic breast cells with intermediates SDA, ETA, GLA and DGLA, their bioactivities can be assessed *in vitro*. Then, from PL analysis, it can be determined whether these intermediates are incorporated directly into membranes or are converted down their respective pathways to long chain PUFAs.

II. Materials and Methods

Please refer to chapter three.

III. Results

A. N-3 Fatty Acid Treatments

i. Cell Viability

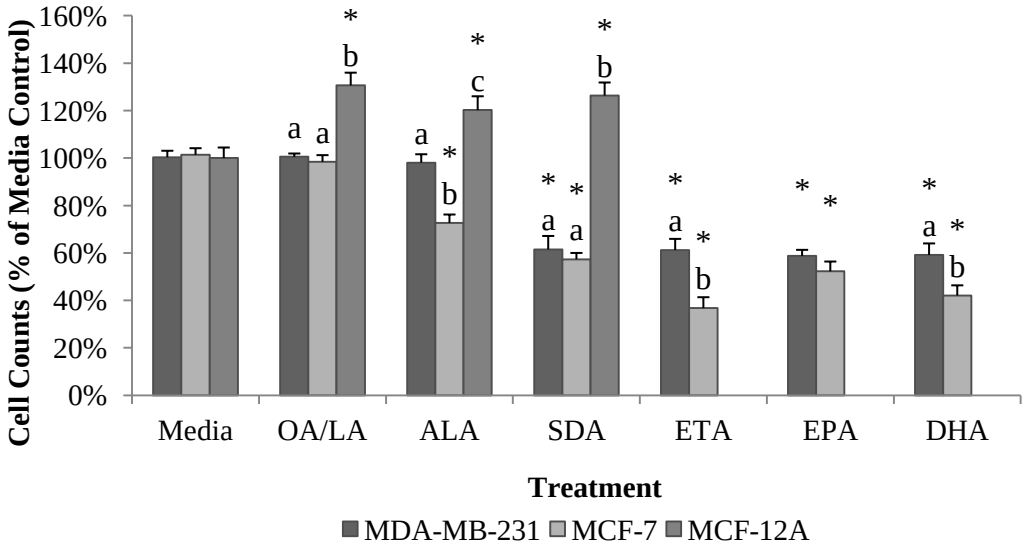

Figure 4.1 Cell Viability of MDA-MB-231, MCF-7 and MCF-12A Cells Incubated with 150μM of N-3 Fatty Acid. Values are mean ± SEM, expressed as percentage of the media only treatment (n=3 per cell line per treatment). ETA, EPA, and DHA treatments were not studied with MCF-12A cells. * indicates a significant difference compared to the cell line's respective media treatment ($p < 0.05$). Different letters within a treatment indicates a significant difference between cell lines ($p < 0.05$).

No significant differences were observed between the media and the fatty acid control treatments in the two tumorigenic lines (Figure 4.1). In our non-tumorigenic MCF-12A cell line, providing additional fatty acid (control OA/LA fatty acids, ALA or SDA) significantly increased cell viability in comparison to our media treatment that did not contain added fatty acids. In the MDA-MB-231 cell line, significant decreases in cell viability (to about 60%) were observed with all n-3 treatments, except ALA. All n-3 treatments decreased MCF-7 cell viability.

When comparing between cell lines within a treatment, the non-tumorigenic MCF-12A cells' viability was significantly higher than either of the tumorigenic cell lines after all of the fatty acid treatments studied, including the

OA/LA treatment. ALA and ETA treatment resulted in a significantly greater decrease in viability in MCF-7 cells as compared to MDA-MB-231 cells.

ii. Phospholipid Composition

Complete whole cell PL profiles are shown in Tables 4.1-3 for MDA-MB-231, MCF-7 and MCF-12A cells, respectively, treated with n-3 treatments ALA, SDA, ETA, EPA and DHA, as well as comparisons within and between cell lines. This data will be illustrated in the subsequent sections to compare the effect of fatty acid treatments on fatty acid changes in PLs and to compare the effects on the different cell lines.

Table 4.1 Whole Cell Phospholipid Composition of MDA-MB-231 Cells Incubated with 150μM ALA, SDA, ETA, EPA or DHA

Fatty Acid	Fatty Acid Treatment						
	Media	OA/LA	ALA	SDA	ETA	EPA	DHA
C14:0	1.79±0.08^{A}	1.22±0.07	1.50±0.14	1.41±0.26	1.69±0.22	1.28±0.12	1.16±0.10
C16:0	17.3±1.1^{AC}	15.1±0.6	16.2±0.8	16.6±1.5	19.0±0.8	17.8±1.2	16.8±1.0
C16:1n9	**4.15±0.37**^{A}	1.02±0.16^{A}	0.87±0.12	0.86±0.07^{AC}	1.27±0.21	0.87±0.12^{A}	0.90±0.10^{A}
C17:0	1.03±0.10^{AC}	0.64±0.09	1.14±0.35	1.05±0.17	0.78±0.11	1.10±0.26	1.06±0.29
C18:0	25.1±1.2^{AC}	18.53±0.40^{AC}	**24.3±0.5**^{C}	**23.8±0.8**^{AC}	18.5±2.7	22.4±0.8^{A}	22.9±0.9^{A}
C18:1t11	3.86±0.83^{C}	3.42±0.44^{AC}	2.94±0.15^{AC}	3.73±0.81^{C}	1.34±0.78^{A}	3.57±0.67	3.56±0.54
C18:1n9	26.6±0.6^{AC}	29.9±1.2^{A}	**17.9±1.1**^{A}	**19.8±1.0**^{A}	**19.4±0.8**^{A}	**19.9±0.8**^{A}	**21.8±0.9**^{A}
C18:1c11	ND^{C}	0.19±0.11	0.25±0.11	0.22±0.09	0.07±0.07	0.14±0.07	0.18±0.09
C18:2n6	**1.60±0.30**	13.4±1.3^{AC}	15.7±1.7	14.1±1.3	12.2±1.8	14.6±1.4^{A}	15.9±1.3
C20:0	0.22±0.14	0.12±0.07	0.05±0.05	0.05±0.05	0.09±0.07	0.11±0.07	0.03±0.03
C18:3n6	0.34±0.06^{C}	0.60±0.33	0.15±0.15	0.03±0.03	0.41±0.31	0.08±0.02	0.15±0.04
C18:3n3	1.03±0.29	0.46±0.16	**8.67±0.80**^{C}	0.29±0.20	0.26±0.15	0.13±0.06^{A}	0.34±0.07
C18:4n3	0.25±0.11	0.11±0.05	0.05±0.05	1.08±0.18^{AC}	0.20±0.13	0.23±0.10	0.02±0.02
C20:2n6	**0.58±0.16**^{A}	1.50±0.18^{AC}	0.98±0.21^{A}	**0.30±0.12**	**0.66±0.31**	0.83±0.16	**0.66±0.23**
C20:3n6	1.95±0.06^{A}	1.47±0.14^{AC}	0.85±0.25	1.18±0.22	1.42±0.26^{A}	0.93±0.15	1.40±0.23
C20:3n3	0.26±0.19	ND	**1.15±0.44**	0.24±0.24	0.20±0.20	ND	0.03±0.03
C20:4n6	5.07±0.98^{C}	4.26±0.19^{AC}	4.17±0.44^{AC}	**2.30±0.26**^{AC}	**1.73±0.11**^{A}	**1.77±0.16**	2.96±0.24^{A}
C20:4n3	0.12±0.08	0.10±0.05	**0.54±0.07**^{AC}	**6.74±0.33**^{AC}	**12.9±1.7**	0.14±0.06	0.07±0.04
C20:5n3	0.63±0.19	0.06±0.03	0.98±0.17^{A}	**1.92±0.14**^{AC}	**1.81±0.19**^{A}	**5.30±0.47**^{A}	0.42±0.10
C22:3n3	2.44±0.57^{AC}	0.99±0.41	1.08±0.35	0.54±0.30	0.70±0.19^{A}	0.51±0.26	1.10±0.22^{A}
C22:4n6	1.01±0.32	1.65±0.49	1.02±0.22^{C}	1.04±0.38	1.14±0.25^{A}	1.08±0.19^{A}	0.78±0.26
C22:1n9	**0.29±0.13**^{C}	1.48±0.15^{AC}	0.53±0.15^{C}	0.59±0.06^{C}	0.97±0.16^{A}	**0.23±0.11**	0.30±0.17
C22:5n6	0.04±0.04^{C}	0.34±0.12	0.28±0.18	0.50±0.10^{A}	0.43±0.26	0.33±0.09	0.27±0.07
C22:5n3	0.95±0.20^{C}	0.46±0.01^{C}	1.46±0.19^{A}	1.66±0.20^{A}	1.76±0.33^{A}	**5.38±0.22**^{A}	0.44±0.06^{A}
C22:6n3	1.35±0.18$^{A\,C}$	0.40±0.10$^{A\,C}$	0.14±0.09^{C}	0.20±0.09^{C}	0.11±0.07	0.23±0.06	**6.46±0.32**^{A}
ΣSFA	45.2±1.6^{C}	35.9±0.9^{C}	**43.2±0.9**^{C}	44.1±1.7^{C}	39.8±3.6	43.9±1.8^{A}	42.0±1.7
ΣMUFA	34.6±1.8^{A}	37.0±1.1	22.4±1.1^{A}	26.1±0.3^{A}	23.0±1.5^{A}	24.6±1.1^{A}	26.7±0.7^{A}
ΣPUFA	**18.9±0.8**^{A}	26.2±0.7^{C}	**34.5±1.0**^{AC}	29.8±1.9^{C}	**36.0±2.5**^{A}	29.6±1.1	31.0±1.6^{A}
Σn-3	**7.33±0.78**^{A}	2.78±0.57^{A}	**13.5±0.5**^{AC}	**12.0±1.3**^{AC}	**18.0±1.4**^{A}	**11.9±0.6**^{A}	**8.88±0.68**^{A}
Σn-6	**11.6±1.3**	23.4±0.8^{C}	21.0±1.4^{AC}	17.8±1.0^{C}	18.0±2.3	18.1±1.1^{A}	22.1±1.9

Values are mean percent composition ± SEM (n=5 per treatment). Bold values indicate significant difference compared to OA/LA ($p < 0.05$). ^{A} and ^{C} indicate significant differences compared to MCF-7 and MCF-12A values, respectively ($p < 0.05$). ND indicates not detectable.

Table 4.2 Whole Cell Phospholipid Composition of MCF-7 Cells Incubated with 150μM ALA, SDA, ETA, EPA or DHA

Fatty Acid	Fatty Acid Treatment Media	OA/LA	ALA	SDA	ETA	EPA	DHA
C14:0	**3.53±0.31** ^{AB}	1.46±0.19	1.22±0.36	1.22±0.20	1.78±0.29	1.51±0.11	1.78±0.40
C16:0	**23.3±0.4** ^{A}	17.1±0.5	17.6±0.4 ^{B}	16.5±0.5	19.2±0.1	16.2±0.7	17.8±0.3
C16:1n9	**8.69±0.35** $^{A B}$	1.80±0.12 ^{A}	1.12±0.15	1.53±0.12 ^{A}	1.16±0.25	1.45±0.12 ^{A}	1.82±0.18 ^{A}
C17:0	0.52±0.03 ^{A}	0.51±0.02	0.67±0.03	0.64±0.01	0.56±0.14	0.65±0.01	0.48±0.12
C18:0	14.3±0.4 ^{A}	16.7±0.5 $^{A B}$	**22.4±1.2** ^{B}	**21.2±0.3** $^{A B}$	**22.0±0.8**	19.6±0.8 ^{A}	19.0±0.5 ^{A}
C18:1t11	3.98±0.38 ^{B}	5.14±0.46 $^{A B}$	4.50±0.39 $^{A B}$	3.64±0.81 ^{B}	4.26±0.41 ^{A}	2.10±1.05	4.73±0.29
C18:1n9	34.1±0.6 ^{A}	33.8±0.4 $^{A B}$	**23.7±0.9** $^{A B}$	**28.6±0.5** $^{A B}$	27.4±1.0 ^{A}	30.8±0.8 ^{A}	**30.0±0.5** ^{A}
C18:1c11	ND ^{B}	0.22±0.14	0.27±0.17	0.01±0.01	ND	0.09±0.06	ND
C18:2n6	**1.92±0.33**	18.9±0.2 ^{A}	**14.3±0.4**	17.5±0.4	**11.3±0.4**	19.6±1.0 ^{A}	18.0±0.5
C20:0	ND	ND	ND	ND	ND	ND	ND
C18:3n6	0.27±0.08	0.08±0.03	0.27±0.09	0.19±0.09	0.35±0.09	0.06±0.04	0.14±0.04
C18:3n3	0.53±0.03	0.44±0.13	**9.59±0.32** ^{B}	0.29±0.07	0.32±0.26	0.32±0.06 ^{A}	0.30±0.10
C18:4n3	ND	0.02±0.02	0.06±0.05	**3.09±0.25** ^{A}	0.13±0.08	0.04±0.02	0.04±0.03
C20:2n6	0.05±0.04 ^{A}	0.80±0.16 $^{A B}$	0.32±0.09 ^{A}	0.06±0.04	0.01±0.01	0.50±0.09	0.52±0.22
C20:3n6	0.86±0.22 ^{A}	0.41±0.11 ^{A}	0.51±0.14	0.99±0.15	0.68±0.11 ^{A}	0.78±0.06	0.84±0.13
C20:3n3	ND	ND	0.02±0.02	ND	ND	**0.04±0.02**	ND
C20:4n6	**4.93±0.34** ^{B}	1.52±0.11 $^{A B}$	1.04±0.24 $^{A B}$	1.19±0.13 $^{A B}$	**0.41±0.09** ^{A}	1.25±0.18	1.39±0.19 ^{A}
C20:4n3	0.02±0.02	0.01±0.01	ND ^{AB}	1.07±0.18 ^{AB}	**9.46±0.45**	0.07±0.02	0.05±0.04
C20:5n3	0.50±0.12	0.26±0.12	ND $^{A B}$	0.47±0.13 $^{A B}$	0.58±0.04 ^{A}	**1.97±0.19** ^{A}	0.21±0.09
C22:3n3	0.45±0.09 ^{A}	0.29±0.14	0.32±0.10	0.28±0.04	0.16±0.09 ^{A}	0.25±0.02	0.29±0.08 ^{A}
C22:4n6	0.73±0.17	0.66±0.14	0.55±0.14	0.49±0.18	0.39±0.11 ^{A}	0.26±0.12 ^{A}	0.51±0.13
C22:1n9	ND ^{B}	ND $^{A B}$	0.17±0.16 ^{B}	0.17±0.15 ^{B}	0.41±0.17 ^{A}	0.52±0.21	0.01±0.01
C22:5n6	ND ^{B}	0.18±0.09 ^{B}	0.06±0.03	0.13±0.08 ^{A}	ND	0.31±0.14	0.12±0.09
C22:5n3	0.77±0.07 ^{B}	0.31±0.10 ^{B}	0.19±0.03 $^{A B}$	0.51±0.11 $^{A B}$	0.25±0.05 ^{A}	**1.53±0.17** ^{A}	0.14±0.05 ^{A}
C22:6n3	**0.61±0.04** $^{A B}$	0.03±0.02 $^{A B}$	0.02±0.02 ^{B}	0.06±0.05 ^{B}	ND	0.09±0.04	**1.69±0.20** ^{A}
ΣSFA	**41.6±0.5**	35.8±0.4	41.8±1.5 ^{B}	39.5±0.7	**43.6±0.9**	38.0±1.1 ^{A}	39.1±0.6
ΣMUFA	46.8±0.9 ^{AB}	40.8±0.8	**29.7 ±1.2** $^{A B}$	**34.0±0.8** $^{A B}$	**33.2±0.5** ^{A}	**34.94±1.36** ^{A}	36.6±0.6 ^{A}
ΣPUFA	**11.6±1.0** $^{A B}$	23.8±0.6 ^{B}	27.2±0.9 $^{A B}$	26.3±1.0 ^{B}	24.0±1.0 ^{A}	27.1±1.8	24.3±1.0 ^{A}
Σn-3	2.88±0.09 $^{A B}$	1.35±0.34 ^{AB}	**10.2±0.4** $^{A B}$	**5.76±0.52** $^{A B}$	**10.9±0.6** ^{A}	4.31±0.49 ^{A}	2.72±0.16 ^{A}
Σn-6	**8.76±1.01**	22.5±0.3 ^{B}	**17.0±0.6** ^{AB}	20.6±0.5	**13.1±0.5**	22.8±1.3 ^{A}	21.5±1.0

Values are mean percent composition ± SEM (n=5 per treatment). Bold values indicate significant difference compared to OA/LA ($p < 0.05$). ^{A} and ^{B} indicate significant differences compared to MDA-MB-231 and MCF-12A values, respectively ($p < 0.05$). ND indicates not detectable.

Table 4.3 Whole Cell Phospholipid Composition of MCF-12A Cells Incubated with 150μM ALA or SDA

Fatty Acid	Fatty Acid Treatment			
	Media	OA/LA	ALA	SDA
C14:0	**2.19±0.03**^{B}	1.47±0.05	**1.04±0.05**	**1.16±0.02**
C16:0	**23.3±0.5**^{C}	16.9±0.6	**14.3±0.4** ^{B}	15.2±0.3
C16:1n9	**4.59±0.28**^{B}	1.48±0.06	1.13±0.05	1.33±0.04^{C}
C17:0	0.52±0.03^{C}	0.51±0.04	**0.67±0.04**	**0.70±0.02** ^{B}
C18:0	13.6±0.6^{C}	13.4±0.1 ^{BC}	**17.6±0.3** ^{BC}	**18.1±0.3** ^{BC}
C18:1t11	0.31±0.12^{BC}	0.11±0.04 ^{BC}	0.22±0.05 ^{BC}	0.13±0.02
C18:1n9	**33.5±0.4**^{C}	28.7±0.2 ^{B}	**19.1±0.4** ^{B}	**22.0±0.5** ^{B}
C18:1c11	**0.08±0.00**^{BC}	0.05±0.00	0.08±0.01	0.07±0.01
C18:2n6	**2.58±0.09**	20.5±0.8^{C}	**15.1±0.2**	**15.7±0.1**
C20:0	0.15±0.01	0.07±0.00	0.09±0.00	**0.11±0.01**
C18:3n6	0.03±0.03^{C}	0.04±0.00	0.05±0.05	0.05±0.03
C18:3n3	0.89±0.05	0.79±0.04	**13.5±0.8** ^{BC}	**0.21±0.04**
C18:4n3	0.17±0.04	0.05±0.01	0.02±0.02	**2.48±0.22**^{C}
C20:2n6	**0.14±0.01**	2.25±0.02 ^{BC}	**0.83±0.03**	**0.23±0.01**
C20:3n6	1.34±0.10	0.83±0.02 ^{C}	0.88±0.00	1.09±0.04
C20:3n3	0.03±0.03	0.02±0.02	ND	ND
C20:4n6	**8.01±0.09**^{BC}	6.29±0.04 ^{BC}	**9.61±0.25** ^{BC}	**4.63±0.13**
C20:4n3	0.07±0.02	0.05±0.00	0.30±0.08^{BC}	**9.51±0.41**
C20:5n3	**1.05±0.03**	0.32±0.01	0.67±0.10 ^{B}	**3.50±0.14**
C22:3n3	0.87±0.02^{C}	0.17±0.14	0.50±0.01	0.03±0.01
C22:4n6	0.05±0.01	0.28±0.13	0.20±0.01^{C}	0.48±0.06
C22:1n9	**1.60±0.06**^{BC}	2.78±0.09 ^{BC}	**1.89±0.07** ^{BC}	**1.20±0.04**
C22:5n6	**0.23±0.00**^{BC}	0.63±0.02 ^{B}	0.49±0.06	**0.16±0.01**
C22:5n3	**2.00±0.07**$^{B\ C}$	1.10±0.04 $^{B\ C}$	1.06±0.10 ^{B}	**1.52±0.11** ^{B}
C22:6n3	**2.71±0.17**$^{B\ C}$	1.20±0.09 $^{B\ C}$	**0.74±0.07** $^{B\ C}$	**0.52±0.06** ^{B}
ΣSFA	**39.8±0.3**^{C}	32.3±0.5 ^{C}	33.7±0.4 ^{BC}	**35.2±0.3** ^{C}
ΣMUFA	**40.1±0.5**^{B}	33.1±0.2	**22.4±0.5** ^{B}	**24.7±0.5** ^{B}
ΣPUFA	**20.2±0.4**^{B}	34.6±0.7 ^{BC}	**43.9±0.8** ^{BC}	**40.1±0.6** ^{BC}
Σn-3	**7.79±0.15** ^{B}	3.70±0.05 ^{B}	**16.8±0.6** ^{BC}	**17.8±0.6** ^{BC}
Σn-6	**12.4±0.2**	30.9±0.7^{BC}	27.2±0.2 ^{BC}	22.3±0.1^{C}

Values are mean percent composition ± SEM (n=3 per treatment). Bold values indicate significant difference compared to OA/LA ($p<0.05$). ^{B} and ^{C} indicate significant differences compared to MDA-MB-231 and MCF-7 values, respectively ($p < 0.05$). ND indicates not detectable.

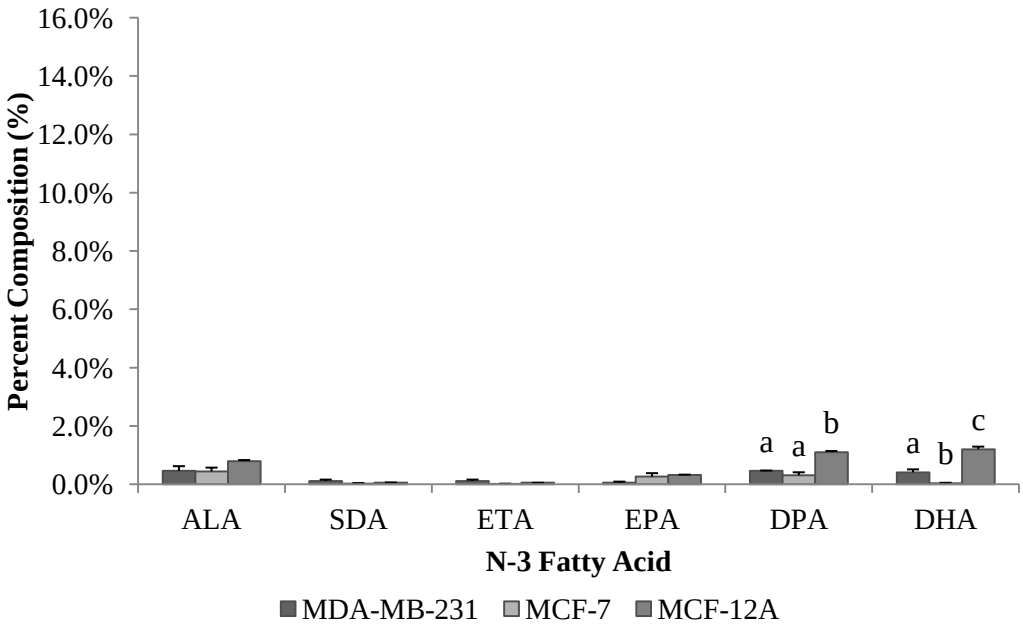

Figure 4.2 Phospholipid N-3 Fatty Acid Profile of MDA-MB-231, MCF-7 and MCF-12A Cells After Incubation with OA/LA Control Media. Values are mean percent composition ± SEM (n=3-5 per cell line). Different letters within a fatty acid indicate a significant difference between cell lines ($p < 0.05$).

In Figure 4.2, it can be seen that n-3 fatty acid concentration in PLs was low in all the cell lines incubated with the OA/LA treatment. MCF-12A cells, however, have significantly higher levels of DPA and DHA in PLs than either MDA-MB-231 or MCF-7 cells. This n-3 fatty acid profile will serve as the control to which n-3 treatments' n-3 fatty acid profiles are compared to.

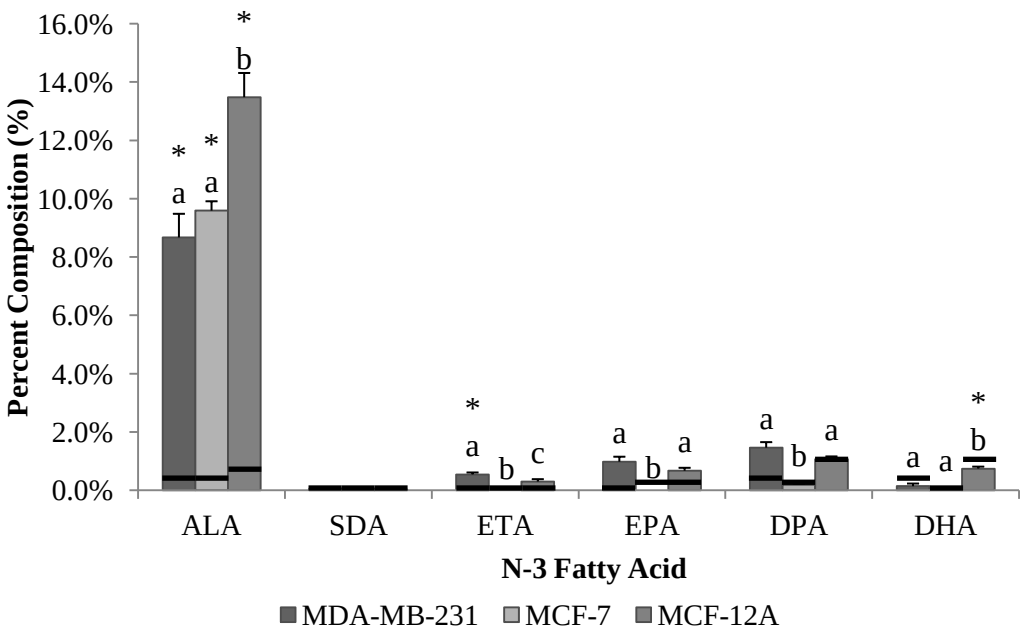

Figure 4.3 Phospholipid N-3 Fatty Acid Profile of MDA-MB-231, MCF-7 and MCF-12A Cells After Incubation with 150μM ALA. Values are mean percent composition ± SEM (n=3-5 per cell line). Black horizontal bars indicate the concentration of the fatty acid after treatment with OA/LA (Figure 4.2). * indicate a significant difference ($p<0.05$) in the incorporation into the PLs in comparison to the OA/LA treatment. Different letters within a fatty acid indicate a significant difference between cell lines ($p < 0.05$).

ALA was significantly incorporated into the cell PLs in all three cell lines in Figure 4.3. Between cell lines, ALA incorporation was the greatest in MCF-12A cells. ETA, EPA and DPA content was significantly lower in MCF-7 cells compared to either MDA-MB-231 and MCF-12A cells. The only significant increase in downstream fatty acid levels was observed in MDA-MB-231 cells, where there was an significant increase in the ETA content, compared to the OA/LA treatment. It should be noted that ALA treatment also increased C20:3n3 content in the cell membranes of MDA-MB-231 cells (Table 4.1). C20:3n3 is an intermediate in an alternate pathway, where ALA is elongated to C20:3n3 before it is desaturated by the catalytic activity of Δ8-desaturase to ETA, bypassing conversion to SDA. This activity was not observed in the other cell lines (Table 4.2 and Table 4.3).

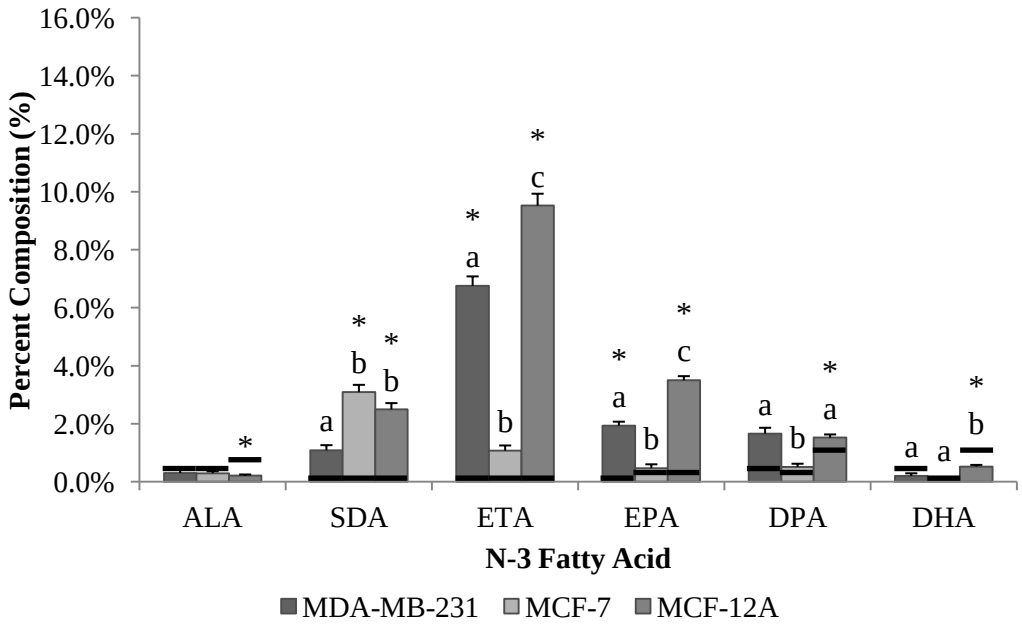

Figure 4.4 Phospholipid N-3 Fatty Acid Profile of MDA-MB-231, MCF-7 and MCF-12A Cells After Incubation with 150μM SDA. Values are mean percent composition ± SEM (n=3-5 per cell line). Black horizontal bars indicate the concentration of the fatty acid after treatment with OA/LA (Figure 4.2). * indicate a significant difference ($p<0.05$) in the incorporation into the PLs in comparison to the OA/LA treatment. Different letters within a fatty acid indicate a significant difference between cell lines ($p < 0.05$).

SDA treatment increased SDA PL content in MCF-7 and MCF-12A cell lines, but not the MDA-MB-231 cells (Figure 4.4). Instead, MDA-MB-231 cells showed significantly increased contents of ETA and EPA in PLs. MCF-12A cells also showed significantly higher concentrations of ETA, EPA and DPA in cell PLs after ALA treatment. No significant incorporation of downstream fatty acids were observed in MCF-7 cells. Between cell lines, SDA, ETA, EPA and DHA content in PLs was higher in MCF-12A cells compared to MDA-MB-231 cells.

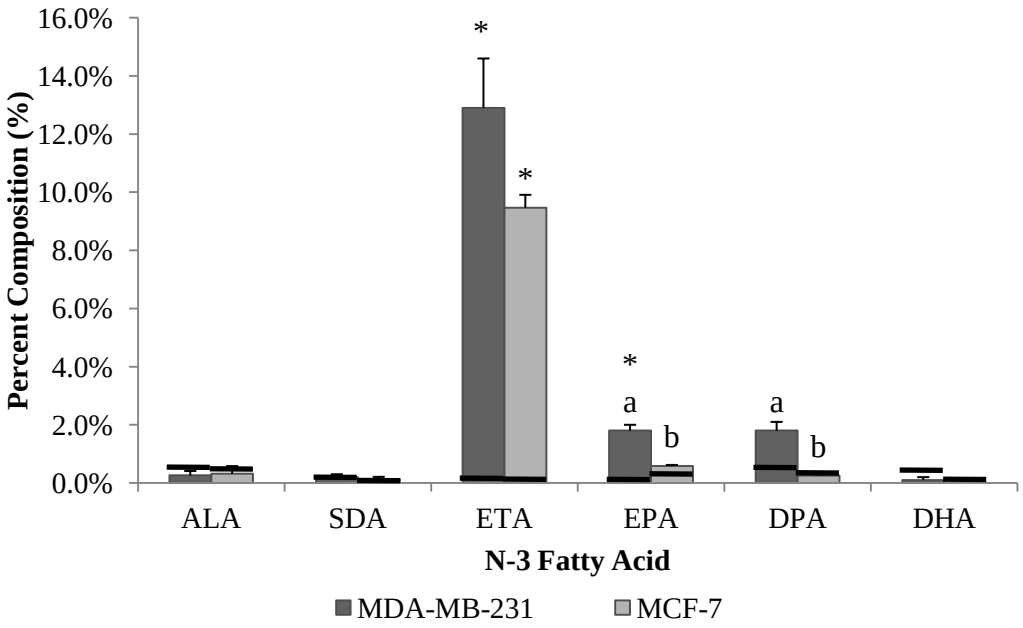

Figure 4.5 Phospholipid N-3 Fatty Acid Profile of MDA-MB-231 and MCF-7 Cells After Incubation with 150μM ETA. Values are mean percent composition ± SEM (n=3-5 per cell line). Black horizontal bars indicate the concentration of the fatty acid after treatment with OA/LA (Figure 4.2). * indicate a significant difference ($p<0.05$) in the incorporation into the PLs in comparison to the OA/LA treatment. Different letters within a fatty acid indicate a significant difference between cell lines ($p < 0.05$).

ETA treatment showed significant incorporation of ETA into the PLs of both tumorigenic cell lines (Figure 4.5). Increased levels of EPA and DPA was observed in MDA-MB-231 cells, however, only changes in the EPA content was significant. MCF-7 cells did not show significant changes downstream to EPA, DPA or DHA. ETA treatment was not tested on MCF-12A cells.

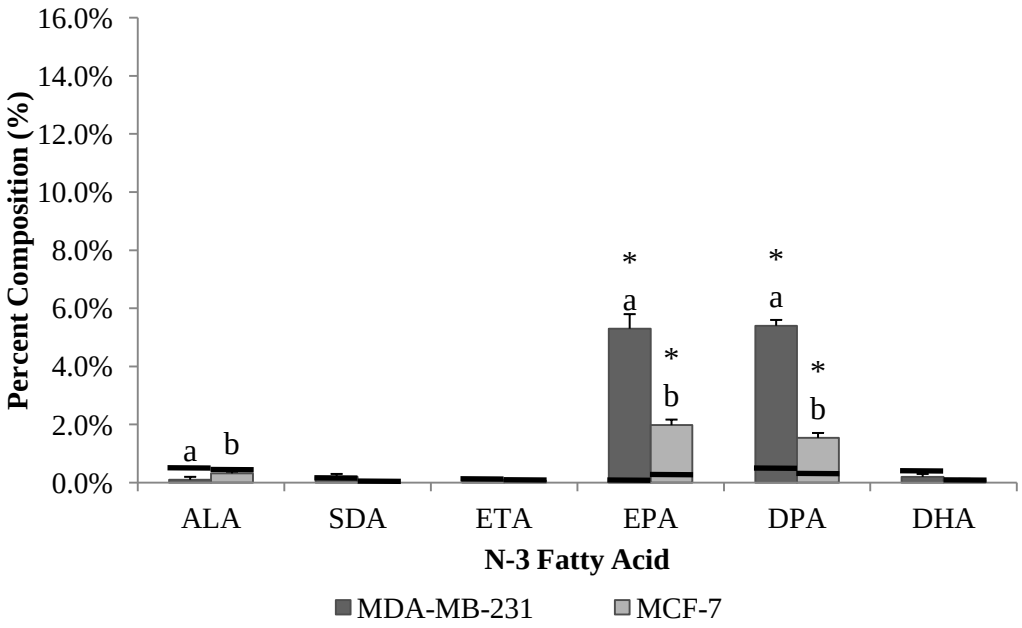

Figure 4.6 Phospholipid N-3 Fatty Acid Profile of MDA-MB-231 and MCF-7 Cells Incubated with 150μM EPA. Values are mean percent composition ± SEM (n=3-5 per cell line). Black horizontal bars indicate the concentration of the fatty acid after treatment with OA/LA (Figure 4.2). * indicate a significant difference ($p<0.05$) in the incorporation into the PLs in comparison to the OA/LA treatment. Different letters within a fatty acid indicate a significant difference between cell lines ($p < 0.05$).

From Figure 4.6, EPA was significantly incorporated into both tumorigenic cell lines and significant increases in DPA content, but not DHA content was observed in both tumorigenic cell lines. EPA and DPA levels were significantly higher in MDA-MB-231 cells than MCF-7 cells. No significant changes were observed in other n-3 fatty acid levels. EPA treatment was not tested on MCF-12A cells.

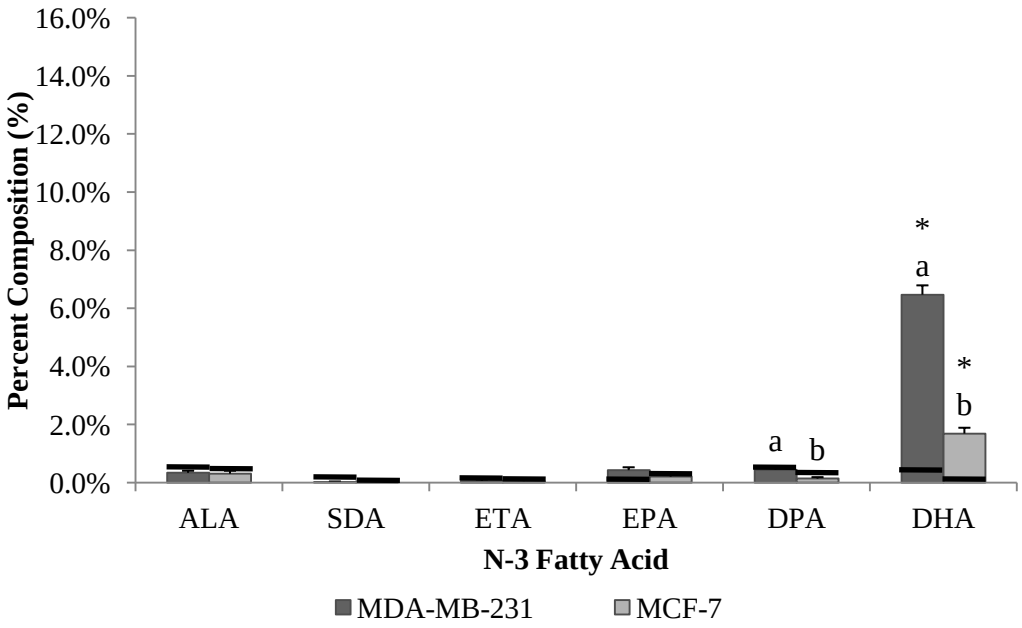

Figure 4.7 Phospholipid N-3 Fatty Acid Profile of MDA-MB-231 and MCF-7 Cells After Incubation with 150μM DHA. Values are mean percent composition ± SEM (n=3-5 per cell line). Black horizontal bars indicate the concentration of the fatty acid after treatment with OA/LA (Figure 4.2). * indicate a significant difference ($p<0.05$) in the incorporation into the PLs in comparison to the OA/LA treatment. Different letters within a fatty acid indicate a significant difference between cell lines ($p < 0.05$).

DHA was incorporated into MDA-MB-231 and MCF-7 cells significantly, as seen in Figure 4.7, but incoporation was much higher in MDA-MB-231 cells than MCF-7 cells. No significant changes in the content of other n-3 fatty acids were observed in PLs. DHA treatment was not tested on MCF-12A cells.

iii. N-6:N-3 Ratios

Treatment	Cell Line		
	MDA-MB-231	MCF-7	MCF-12A
Media	1.55±0.30[a]	2.73±0.32[b]	**1.59±0.03 [ab]**
OA/LA	7.14±1.02	17.5±4.1	8.34±0.38
ALA	1.77±0.14	1.67±0.06	**1.63±0.07**
SDA	1.56±0.13 [a]	3.64±0.20[b]	**1.26±0.05** [a]
ETA	1.15±0.09	1.21±0.05	
EPA	1.65±0.13[a]	5.43±0.33 [b]	
DHA	2.63±0.25 [a]	8.39±0.57 [b]	

Table 4.4 N-6:N-3 Ratios of Whole Cell Phospholipids of MDA-MB-231, MCF-7 and MCF-12A Cells Incubated with 150μM ALA, SDA, ETA, EPA or DHA. Values are means ± SEM (n=3-5 per cell line per treatment). Bold values indicate a significant difference compared to the cell line's respective OA/LA control ($p < 0.05$). Different letters within a treatment indicate a significant difference between cell lines ($p < 0.05$). ETA, EPA and DHA treatments were not studied with MCF-12A cells.

N-6:n-3 ratios in whole cell PLs were under 10, varying between 1.15-2.63 in the MDA-MB-231 cell line and 1.67-8.39 in the MCF-7 cell line, and did not vary significantly within the tumorigenic cell lines (Table 4.4). However, a significant decrease in the ratio was observed in MCF-12A cell lines, dropping down from 8.34 to 1.63 and 1.26 in ALA and SDA treatments, respectively, compared to the OA/LA control. Between cell lines, MCF-7 cells showed significant higher ratios in SDA, EPA and DHA treatments. A significant increase in n-6:n-3 ratio was also observed in MCF-7 cells compared to MCF-12A cells with SDA treatment.

iv. Estimated Desaturase Activity

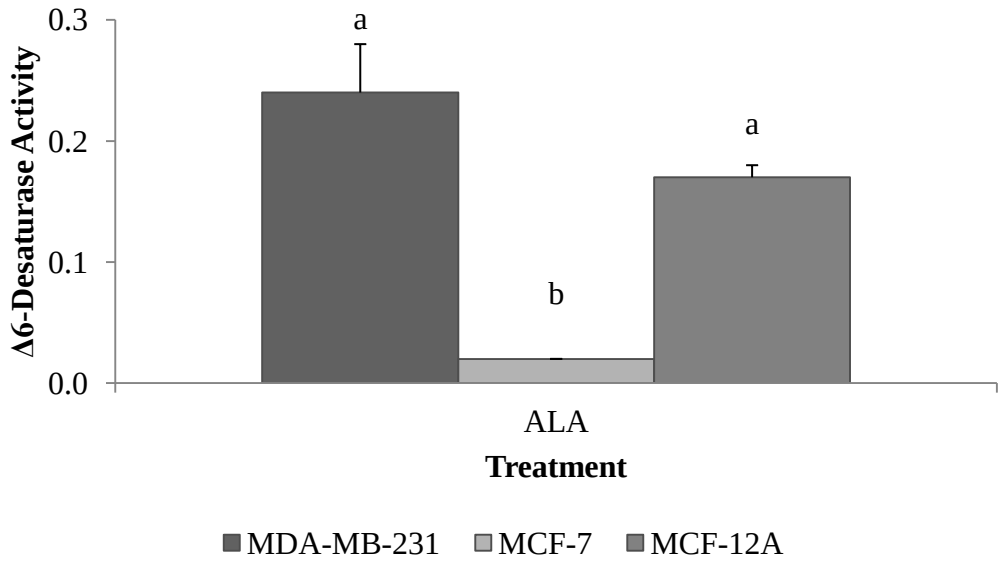

Figure 4.8 Estimated Δ6-Desaturase Activity in MDA-MB-231, MCF-7 and MCF-12A Cells in the N-3 Pathway. Values are means ± SEM (n=3-5 per cell line). Different letters within a treatment indicates a significant difference between cell lines ($p < 0.05$).

Fatty acid ratios used to estimate Δ6-desaturase's catalytic activities are below 1.0 in all cell lines in Figure 4.8. Estimation of desaturase activity using the ALA treatment's PL percent composition values was significantly lower in MCF-7 cells compared to both MDA-MB-231 and MCF-12A cells. Using this ratio, the Δ6-desaturase's catalytic activity to convert ALA to SDA could not be distinguished from the use of the Δ8-desaturase pathway.

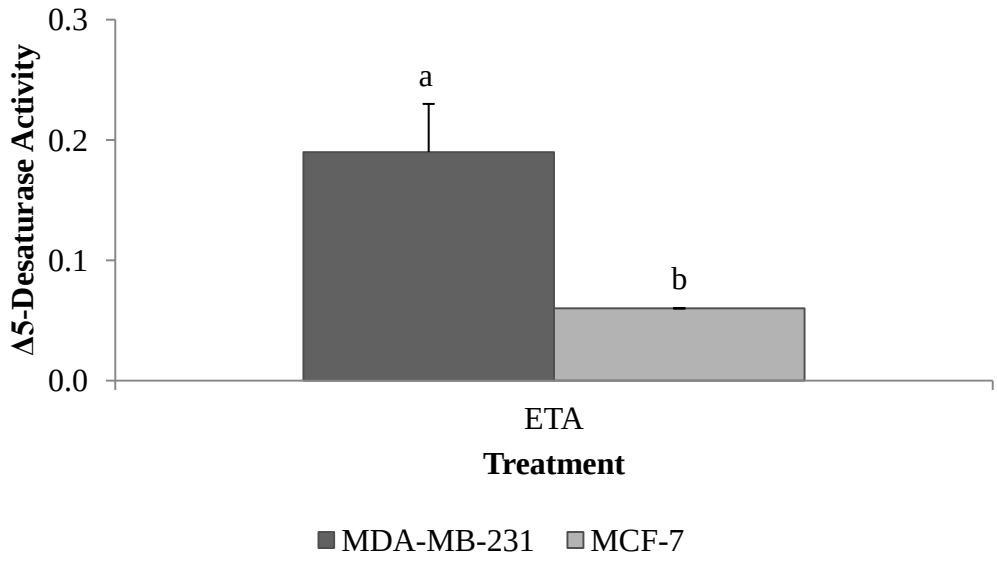

Figure 4.9 Estimated Δ5-Desaturase Activity in MDA-MB-231and MCF-7 Cells in the N-3 Pathway. Values are means ± SEM (n=3-5 per cell line). Different letters within a treatment indicates a significant difference between cell lines ($p < 0.05$).

Estimated Δ5-desaturase catalytic activity in both tumorigenic cell lines are well below 1.0 (Figure 4.9). Activity in MCF-7 cells is significantly lower than that of MDA-MB-231 cells. ETA treatment was not studied with MCF-12A cells.

B. N-6 Fatty Acid Treatments

i. *Cell Viability*

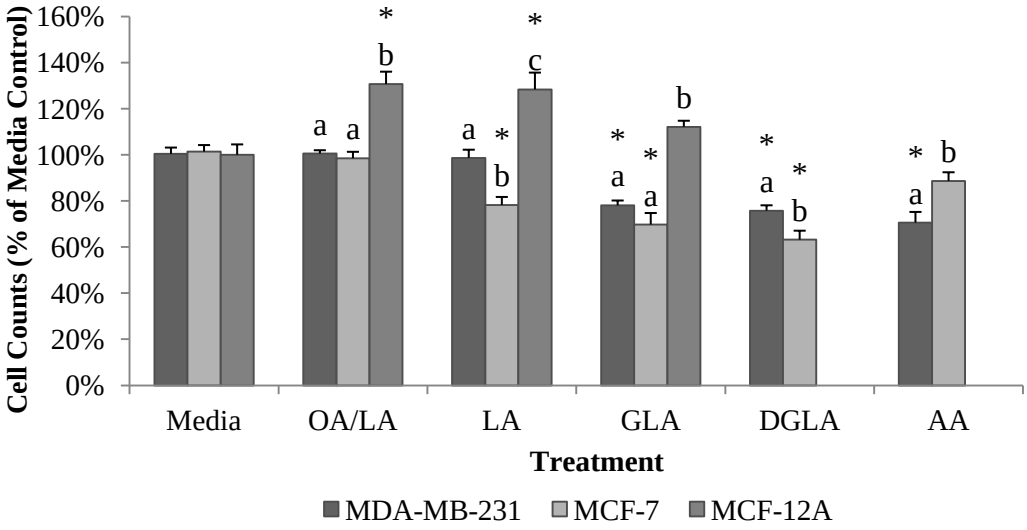

Figure 4.10 Cell Viability of MDA-MB-231, MCF-7 and MCF-12A Cells Incubated with 150μM of N-6 Fatty Acid. Values are mean ± SEM, expressed as percentage of the media only treatment (n=3-5 per cell line per treatment). DGLA and AA treatments were not studied with MCF-12A cells. * indicates a significant difference compared to the cell line's respective media treatment ($p < 0.05$). Different letters within a treatment indicates a significant difference between cell lines ($p < 0.05$).

When cells were incubated with n-6 fatty acids, the non-tumorigenic MCF-12A cells also showed an increase in cell viability in comparison to the media control with LA and GLA treatment, however, only the LA treatment's growth reached significance (Figure 4.10). In the tumorigenic MDA-MB-231 cell line, decreases in cell viability were observed with all n-6 fatty acid treatments except LA. On the other hand, MCF-7 cells showed decreases in cell viability with all n-6 fatty acids except AA.

Between cell lines, once again, MCF-12A cells' viability was significantly different from the tumorigenic cell lines for all treatments. MCF-7 cells had significantly lower viability than that of MDA-MB-231 cells with LA and DGLA treatments, while MDA-MB-231 cells have a lower viability than MCF-7 cells only with AA treatment.

ii. Phospholipid Composition

Complete whole cell PL profiles are shown in Tables 4.4-6 for MDA-MB-231, MCF-7 and MCF-12A cells, respectively, treated with n-6 treatments LA, GLA, DGLA and AA. Comparisons within and between cell lines are also shown. Again, this data will be illustrated in the subsequent sections to compare the effect of fatty acid treatments on fatty acid changes in PLs and to compare the effects on the different cell lines.

Table 4.5 Whole Cell Phospholipid Composition of MDA-MB-231 Cells Incubated with 150μM LA, GLA, DGLA or AA

Fatty Acid	Fatty Acid Treatment					
	Media	OA/LA	LA	GLA	DGLA	AA
C14:0	1.79±0.08 [A]	1.22±0.07	1.33±0.20	1.86±0.23	1.60±0.13	1.73±0.16
C16:0	17.3±1.1 [AC]	15.1±0.6	17.0±1.7	17.7±0.7	19.1±0.7	**19.3±0.8**
C16:1n9	**4.15±0.37** [A]	1.02±0.16 [A]	0.75±0.11 [A]	0.91±0.11	0.87±0.15	1.05±0.13
C17:0	1.03±0.10 [AC]	0.64±0.09	0.68±0.05	0.76±0.06	0.73±0.08	1.83±0.12
C18:0	25.1±1.2 [AC]	18.53±0.40 [AC]	23.0±1.6 [C]	22.0±1.1 [C]	20.3±0.6	18.3±1.9
C18:1t11	3.86±0.83 [C]	3.42±0.44 [AC]	3.07±0.81	1.99±0.53 [A]	2.77±0.26	2.43±0.44 [A]
C18:1n9	26.6±0.6 [AC]	29.9±1.2 [A]	**18.0±1.2**	**20.9±1.2**	**19.9±0.7**	**19.9±1.6** [A]
C18:1c11	ND [C]	0.19±0.11	0.11±0.11	0.04±0.04	0.05±0.05	ND
C18:2n6	**1.60±0.30**	13.4±1.3 [AC]	**26.2±1.2** [AC]	7.99±0.39 [AC]	6.85±0.57 [A]	8.34±0.50 [A]
C20:0	0.22±0.14	0.12±0.07	0.10±0.06	1.03±0.85	0.06±0.06	0.23±0.13
C18:3n6	0.34±0.06 [C]	0.60±0.33	0.12±0.04	**2.57±0.18** [AC]	0.48±0.14	0.22±0.09
C18:3n3	1.03±0.29	0.46±0.16	0.28±0.10	0.26±0.18	0.32±0.13	0.46±0.12
C18:4n3	0.25±0.11	0.11±0.05	0.05±0.05	0.13±0.13	0.37±0.17	0.39±0.32
C20:2n6	**0.58±0.16** [A]	1.50±0.18 [AC]	**3.43±0.16** [A]	**0.29±0.09**	**0.43±0.15**	**0.57±0.24**
C20:3n6	1.95±0.06 [A]	1.47±0.14 [AC]	1.55±0.24 [AC]	**12.6±0.3** [A]	**17.8±1.8**	1.15±0.26
C20:3n3	0.26±0.19	ND	ND	ND	ND	0.33±0.33
C20:4n6	5.07±0.98 [C]	4.26±0.19 [AC]	3.14±0.22 [AC]	4.76±0.29 [AC]	3.35±0.32 [A]	**13.8±0.5** [A]
C20:4n3	0.12±0.08	0.10±0.05	0.01±0.01	0.04±0.02	ND	0.20±0.13
C20:5n3	0.63±0.19	0.06±0.03	0.20±0.07	0.52±0.52	2.41±0.41 [A]	0.84±0.42
C22:3n3	2.44±0.57 [AC]	0.99±0.41	0.90±0.32	1.80±0.44 [A]	0.48±0.20	0.87±0.35
C22:4n6	1.01±0.32	1.65±0.49	0.57±0.25	0.38±0.25	1.21±0.35	**7.58±0.60** [A]
C22:1n9	**0.29±0.13** [C]	1.48±0.15 [AC]	1.57±0.22 [A]	1.73±0.29 [A]	0.79±0.41	**0.17±0.08**
C22:5n6	0.04±0.04 [C]	0.34±0.12	0.70±0.21	0.46±0.38	ND	0.12±0.08
C22:5n3	0.95±0.20 [C]	0.46±0.01 [C]	0.28±0.09 [C]	0.50±0.06 [A C]	0.40±0.17	0.43±0.21
C22:6n3	1.35±0.18 [A C]	0.40±0.10 [A C]	0.30±0.08 [C]	0.26±0.04 [C]	0.16±0.04 [A]	0.22±0.12
ΣSFA	45.2±1.6 [C]	35.9±0.9 [C]	38.7±1.2 [C]	41.8±1.2 [C]	41.8±0.9	41.7±1.8
ΣMUFA	34.6±1.8 [A]	37.0±1.1	24.1±1.4	25.4±0.7	24.4±0.9	24.4±0.9 [A]
ΣPUFA	**18.9±0.8** [A]	26.2±0.7 [C]	**37.1±1.5** [C]	32.0±1.3 [AC]	**34.2±0.7**	**34.0±1.3** [A]
Σn-3	**7.33±0.78** [A]	2.78±0.57 [A]	2.52±0.46	3.51±0.88 [A]	4.13±0.43	2.17±0.70
Σn-6	**11.6±1.3**	23.4±0.8 [C]	**35.2±1.1** [C]	28.5±0.9 [AC]	30.1±0.7	31.8±1.5 [A]

Values are mean percent composition ± SEM (n=5 per treatment). Bold values indicate significant difference compared to OA/LA control ($p < 0.05$). [A] and [C] indicate significant differences compared to MCF-7 and MCF-12A, respectively ($p < 0.05$). ND indicates not detectable.

Table 4.6 Whole Cell Phospholipid Composition of MCF-7 Cells Incubated with 150μM LA, GLA, DGLA or AA

Fatty Acid	Fattv Acid Treatment Media	OA/LA	LA	GLA	DGLA	AA
C14:0	**3.53±0.31** ^{AB}	1.46±0.19	1.53±0.33	1.51±0.21	1.36±0.06	1.95±0.34
C16:0	**23.3±0.4** ^{A}	17.1±0.5	17.7±0.8	18.5±0.6^{B}	19.1±0.5	18.7±0.6
C16:1n9	**8.69±0.35** $^{A\ B}$	1.80±0.12 ^{A}	1.24±0.11 ^{A}	1.04±0.29	1.09±0.29	1.36±0.16
C17:0	0.52±0.03 ^{A}	0.51±0.02	0.40±0.14	0.57±0.14	0.59±0.15	0.70±0.04
C18:0	14.3±0.4 ^{A}	16.7±0.5 $^{A\ B}$	**21.3±0.8** ^{B}	**22.2±1.2** ^{B}	**21.0±0.8**	**21.8±0.7**
C18:1t11	3.98±0.38 ^{B}	5.14±0.46 $^{A\ B}$	3.38±0.91	4.50±0.63 $^{A\ B}$	2.91±0.69	4.48±0.51 ^{A}
C18:1n9	34.1±0.6 ^{A}	33.8±0.4 $^{A\ B}$	**17.5±0.9**	**23.8±0.5** ^{B}	**19.8±0.4**	**26.4±0.5** ^{A}
C18:1c11	ND ^{B}	0.22±0.14	0.13±0.09	0.04±0.04	0.04±0.04	0.08±0.08
C18:2n6	**1.92±0.33**	18.9±0.2 ^{A}	**33.3±0.8** ^{A}	**12.3±0.2** ^{A}	**10.2±0.8** ^{A}	15.6±0.7^{A}
C20:0	ND	ND	ND	ND	ND	ND
C18:3n6	0.27±0.08	0.08±0.03	0.14±0.07	**7.09±0.22** $^{A\ B}$	0.91±0.23	0.24±0.10
C18:3n3	0.53±0.03	0.44±0.13	0.35±0.15	0.23±0.13	1.10±0.57	0.24±0.13
C18:4n3	ND	0.02±0.02	0.01±0.01	0.04±0.02	0.81±0.29	0.17±0.11
C20:2n6	0.05±0.04^{A}	0.80±0.16 $^{A\ B}$	1.40±0.11 $^{A\ B}$	0.05±0.03^{B}	0.32±0.04	0.32±0.06
C20:3n6	0.86±0.22 ^{A}	0.41±0.11 ^{A}	0.19±0.05 ^{A}	**3.31±0.06** $^{A\ B}$	**16.4±0.9**	0.96±0.34
C20:3n3	ND	ND	ND	ND	ND	ND
C20:4n6	**4.93±0.34** ^{B}	1.52±0.11 $^{A\ B}$	1.21±0.29 $^{A\ B}$	2.20±0.16 $^{A\ B}$	1.40±0.18 ^{A}	**5.44±0.25** ^{A}
C20:4n3	0.02±0.02	0.01±0.01	0.10±0.08	0.03±0.03	0.01±0.01	0.04±0.03
C20:5n3	0.50±0.12	0.26±0.12	0.06±0.04	0.22±0.16	0.07±0.04 ^{A}	0.03±0.03
C22:3n3	0.45±0.09 ^{A}	0.29±0.14	0.08±0.08	0.42±0.06 ^{A}	0.66±0.14	0.24±0.04
C22:4n6	0.73±0.17	0.66±0.14	0.27±0.13	0.61±0.11	0.94±0.13	1.50±0.22 ^{A}
C22:1n9	ND ^{B}	ND $^{A\ B}$	0.09±0.09 $^{A\ B}$	ND $^{A\ B}$	ND	0.02±0.02
C22:5n6	ND ^{B}	0.18±0.09 ^{B}	0.30±0.15 ^{B}	0.02±0.02	0.12±0.10	0.08±0.05
C22:5n3	0.77±0.07^{B}	0.31±0.10 ^{B}	0.29±0.07 ^{B}	0.24±0.06 $^{A\ B}$	0.11±0.07	0.27±0.06
C22:6n3	**0.61±0.04** $^{A\ B}$	0.03±0.02 $^{A\ B}$	0.07±0.05 ^{B}	0.23±0.14^{B}	0.01±0.01 ^{A}	0.01±0.01
ΣSFA	**41.6±0.5**	35.8±0.4	40.9±1.7	44.0±1.4^{B}	**42.1±0.9**	43.1±1.6
ΣMUFA	46.8±0.9^{AB}	40.8±0.8	**22.1±1.4**	**29.4±1.3** ^{B}	**24.4±0.4**	**32.4±0.5** ^{A}
ΣPUFA	**11.6±1.0** $^{A\ B}$	23.8±0.6 ^{B}	**36.3±0.6** ^{B}	27.0±0.53^{AB}	**33.0±0.7**	25.2±0.9 ^{A}
Σn-3	2.88±0.09$^{A\ B}$	1.35±0.34 ^{AB}	0.96±0.24	1.12±0.14^{A}	3.27±0.48	1.01±0.27
Σn-6	**8.76±1.01**	22.5±0.3 ^{B}	**36.6±0.6** ^{B}	**25.6±0.4** ^{AB}	**30.2±1.2**	24.1±1.0^{A}

Values are mean percent composition ± SEM (n=5 per treatment). Bold values indicate significant difference compared to OA/LA control ($p < 0.05$). ^{A} and ^{C} indicate significant differences compared to MCF-7 and MCF-12A, respectively ($p < 0.05$). ND indicates not detectable.

Table 4.7 Whole Cell Phospholipid Composition of MCF-12A Cells Incubated with 150μM LA or GLA

Fatty Acid	Fatty Acid Treatment			
	Media	OA/LA	LA	GLA
C14:0	**2.19±0.03**^{B}	1.47±0.05	**1.02±0.02**	**1.27±0.04**
C16:0	**23.3±0.5**^{C}	16.9±0.6	**14.2±0.4**	15.5±0.4 ^{B}
C16:1n9	**4.59±0.28**^{B}	1.48±0.06	**0.96±0.05**	1.19±0.02
C17:0	0.52±0.03^{C}	0.51±0.04	0.64±0.03	**0.68±0.02**
C18:0	13.6±0.6^{C}	13.4±0.1 ^{BC}	**16.1±0.3** ^{BC}	**17.0±0.4** ^{BC}
C18:1t11	0.31±0.12^{BC}	0.11±0.04 ^{BC}	0.17±0.06	0.19±0.04 ^{B}
C18:1n9	**33.5±0.4**^{C}	28.7±0.2 ^{B}	**15.5±0.1**	**20.4±0.2** ^{B}
C18:1c11	**0.08±0.00**^{BC}	0.05±0.00	0.07±0.00	0.07±0.01
C18:2n6	**2.58±0.09**	20.5±0.8^{C}	**35.9±1.0**^{C}	**12.9±0.6**^{C}
C20:0	0.15±0.01	0.07±0.00	0.09±0.01	0.04±0.02
C18:3n6	0.03±0.03^{C}	0.04±0.00	ND	**4.70±0.18** ^{BC}
C18:3n3	0.89±0.05	0.79±0.04	**0.36±0.03**	**0.13±0.03**
C18:4n3	0.17±0.04	0.05±0.01	0.03±0.02	0.04±0.02
C20:2n6	**0.14±0.01**	2.25±0.02 ^{BC}	4.03±0.20^{B}	**0.45±0.02** ^{B}
C20:3n6	1.34±0.10	0.83±0.02 ^{C}	0.68±0.02^{C}	**12.4±0.2** ^{B}
C20:3n3	0.03±0.03	0.02±0.02	ND	ND
C20:4n6	**8.01±0.09**^{BC}	6.29±0.04 ^{BC}	**4.65±0.17** ^{BC}	8.03±0.20^{BC}
C20:4n3	0.07±0.02	0.05±0.00	0.21±0.11	0.06±0.02
C20:5n3	**1.05±0.03**	0.32±0.01	0.28±0.13	**0.09±0.01**
C22:3n3	0.87±0.02^{C}	0.17±0.14	0.02±0.00	0.81±0.01
C22:4n6	0.05±0.01	0.28±0.13	0.42±0.03	0.80±0.16
C22:1n9	**1.60±0.06**^{BC}	2.78±0.09 ^{BC}	**1.84±0.13** ^{B}	**1.24±0.07** ^{B}
C22:5n6	**0.23±0.00**^{BC}	0.63±0.02 ^{B}	**1.30±0.04** ^{B}	**0.21±0.01**
C22:5n3	**2.00±0.07**$^{B\,C}$	1.10±0.04 $^{B\,C}$	**0.73±0.04** $^{B\,C}$	**0.76±0.04** $^{B\,C}$
C22:6n3	**2.71±0.17**$^{B\,C}$	1.20±0.09 $^{B\,C}$	**0.75±0.06** $^{B\,C}$	**0.72±0.09** ^{BC}
ΣSFA	**39.8±0.3**^{C}	32.3±0.5 ^{C}	32.0±0.5 ^{C}	34.5±0.5^{BC}
ΣMUFA	**40.1±0.5**^{B}	33.1±0.2	**18.6±0.2**	**22.7±0.2** ^{B}
ΣPUFA	**20.2±0.4**^{B}	34.6±0.7 ^{BC}	**49.4±0.7** ^{BC}	**42.1±0.8** ^{BC}
Σn-3	**7.79±0.15** ^{B}	3.70±0.05 ^{B}	2.37±0.11	2.62±0.08
Σn-6	**12.4±0.2**	30.9±0.7^{BC}	**47.0±0.6** ^{BC}	**39.5±0.7** ^{BC}

Values are mean percent composition ± SEM (n=3 per treatment). Bold values indicate significant difference compared to OA/LA ($p < 0.05$). ^{B} and ^{C} indicate significant differences compared to MDA-MB-231 and MCF-7 values, respectively ($p < 0.05$). ND indicates not detectable.

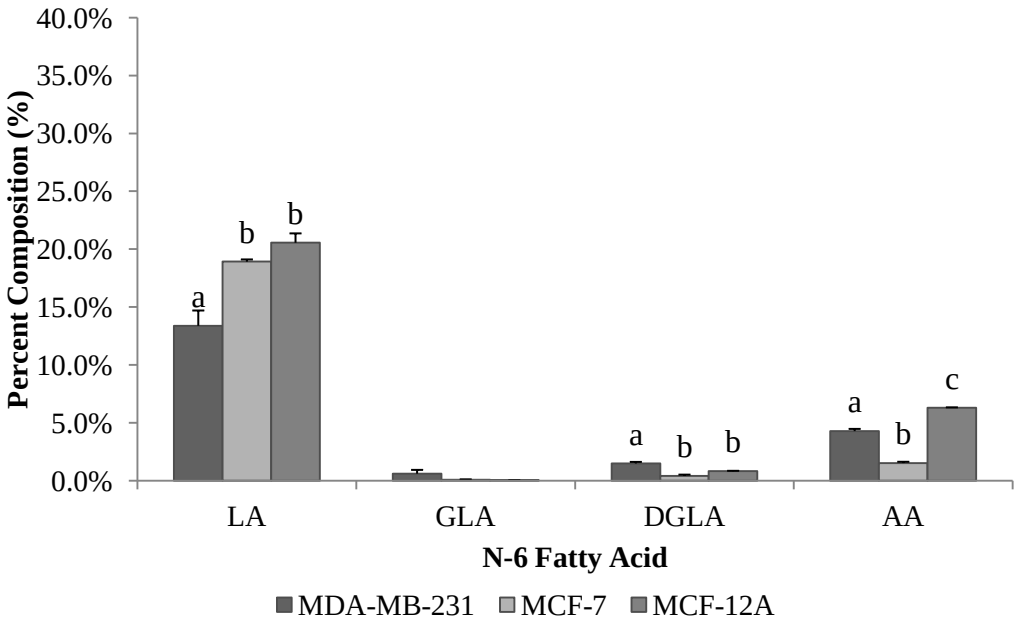

Figure 4.11 Phospholipid N-6 Fatty Acid Profile of MDA-MB-231, MCF-7 and MCF-12A Cells After Incubation with OA/LA Control Media. Values are mean percent composition ± SEM (n=3-5 per cell line). Different letters within a fatty acid indicate a significant difference between cell lines ($p < 0.05$).

LA from the OA/LA control media was incorporated to a significant degree into the PLs by all cell lines in comparison to cells grown in plain media (Table 4.1-3, Figure 4.11). Low levels of the intermediates GLA and DGLA were found in all cell lines, although there was significantly more DGLA in MDA-MB-231 cells than MCF-7 or MCF-12A cells. AA levels were significantly higher in MCF-12A cells than MDA-MB-231 cells which had higher levels than MCF-7 cells. This n-6 fatty acid profile will be used as the control for n-6 fatty acid treatments' n-6 fatty acid profiles.

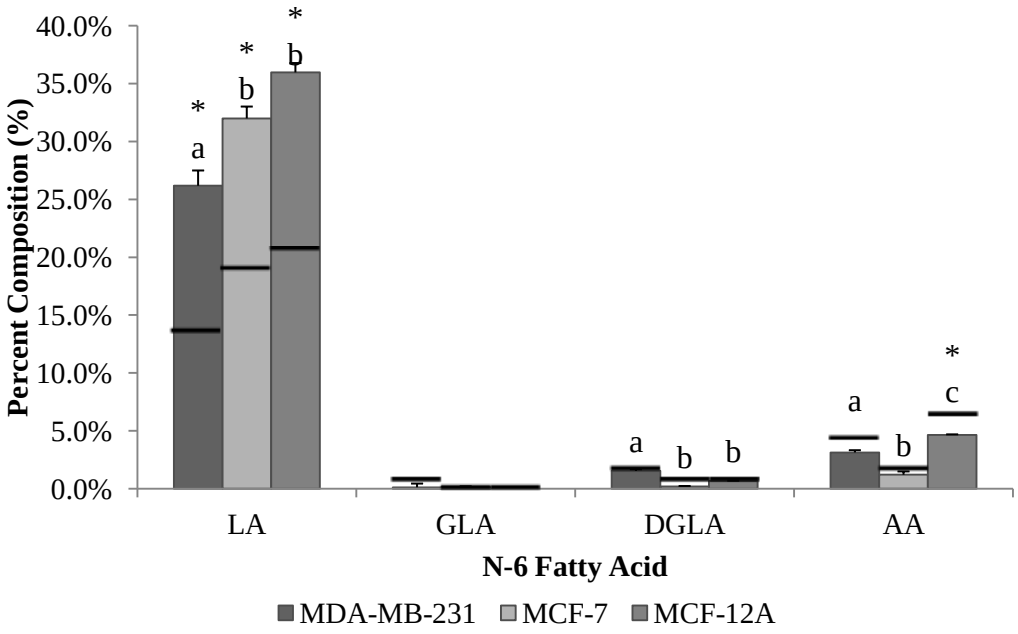

Figure 4.12 Phospholipid N-6 Fatty Acid Profile of MDA-MB-231, MCF-7 and MCF-12A Cells After Incubation with 150μM LA. Values are mean percent composition ± SEM (n=3-5 per cell line). Black horizontal bars indicate the concentration of the fatty acid after treatment with OA/LA (Figure 4.11). * indicate a significant difference ($p<0.05$) in the incorporation into the PLs in comparison to the OA/LA treatment. Different letters within a fatty acid indicate a significant difference between cell lines ($p < 0.05$).

From Figure 4.12, LA treatment resulted in significant increases in LA PL content in all cell lines. No significant changes to levels of downstream counterparts was observed, except for a significant decrease in AA content in MCF-12A cells. Incorporation was the greatest in MCF-7 and MCF-12A cells.

LA treatment also increased C20:2n6 content in the cell membranes of all cell lines, however only MDA-MB-231's C20:2n6 increase was signficant (Tables 4.5-7). C20:2n6 is an intermediate in an alternate pathway where LA is elongated to C20:2n6 before it is desaturated by the catalytic activity of Δ8-desaturase to DGLA, bypassing conversion to GLA.

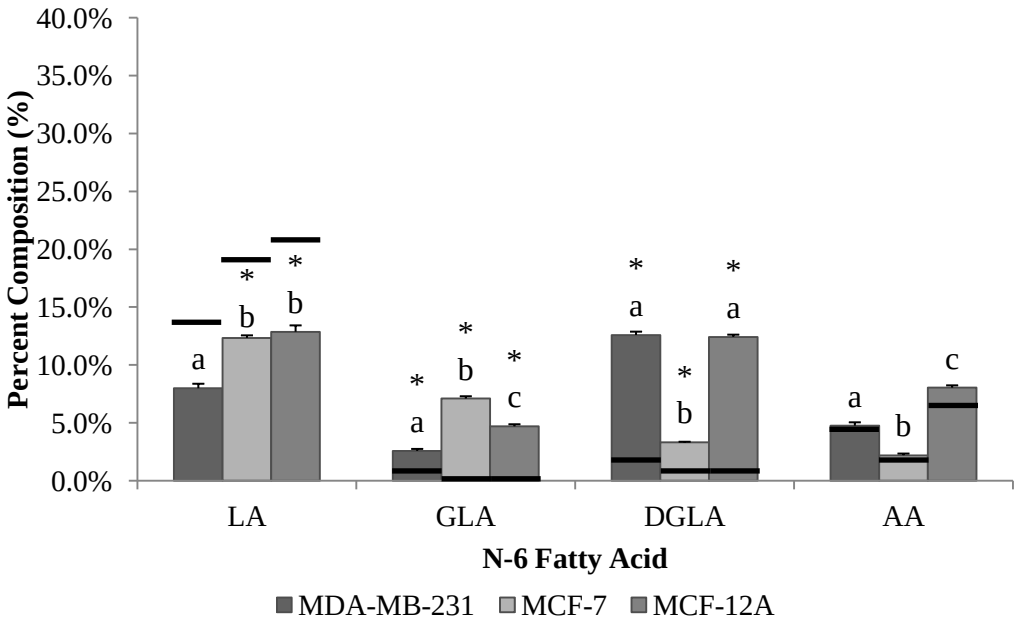

Figure 4.13 Phospholipid N-6 Fatty Acid Profile of MDA-MB-231, MCF-7 and MCF-12A Cells After Incubation with 150µM GLA. Values are mean percent composition ± SEM (n=3-5 per cell line). Black horizontal bars indicate the concentration of the fatty acid after treatment with OA/LA (Figure 4.11). * indicate a significant difference ($p<0.05$) in the incorporation into the PLs in comparison to the OA/LA treatment. Different letters within a fatty acid indicate a significant difference between cell lines ($p < 0.05$).

GLA treatment resulted in significant increases in GLA content in the PLs of all cell lines, as seen in Figure 4.13. All cell lines also showed significant increases in levels of DGLA. Incorporation of GLA was greatest by MCF-7 cells, however, increases in DGLA content was the greatest in MDA-MB-231 and MCF-12A cells.

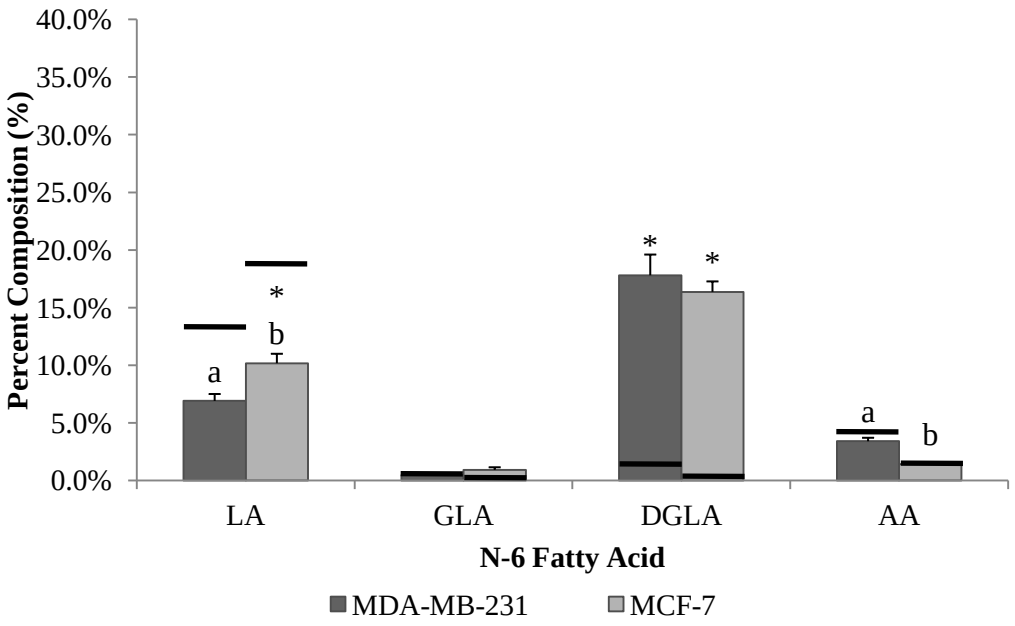

Figure 4.14 Phospholipid N-6 Fatty Acid Profile of MDA-MB-231 and MCF-7 Cells After Incubation with 150μM DGLA. Values are mean percent composition ± SEM (n=3-5 per cell line). Black horizontal bars indicate the concentration of the fatty acid after treatment with OA/LA (Figure 4.11). * indicate a significant difference ($p<0.05$) in the incorporation into the PLs in comparison to the OA/LA treatment. Different letters within a fatty acid indicate a significant difference between cell lines ($p < 0.05$).

DGLA was significantly incorporated into the PLs of both tumorigenic cell lines to a similar degree, however, no significant changes downsteam to AA was observed (Figure 4.14). DGLA treatment was not tested on MCF-12A cells.

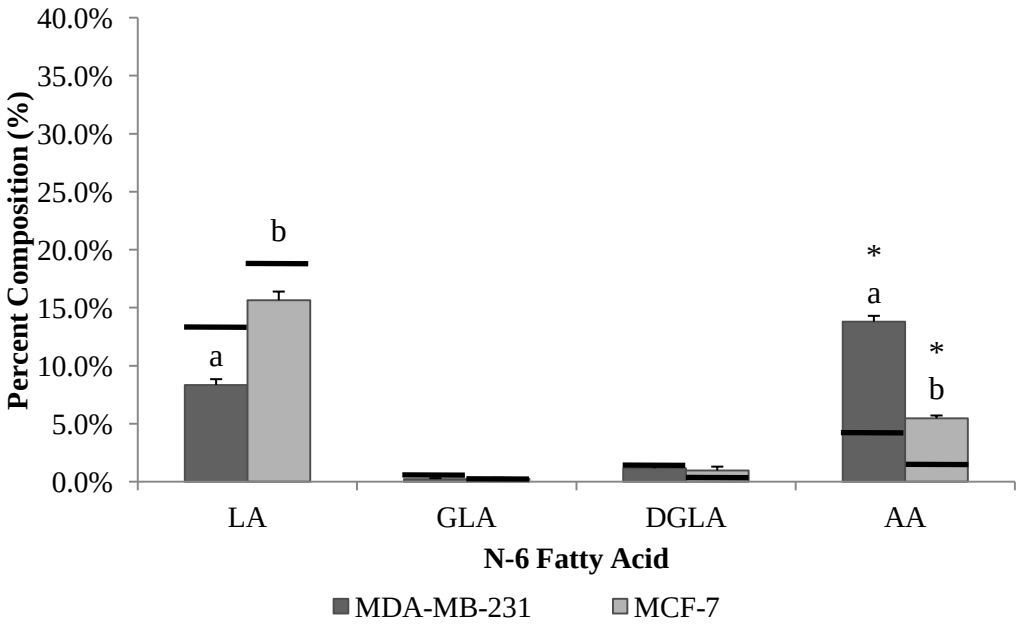

Figure 4.15 Phospholipid N-6 Fatty Acid Profile of MDA-MB-231 and MCF-7 Cells After Incubation with 150μM AA. Values are mean percent composition ± SEM (n=3-5 per cell line). Black horizontal bars indicate the concentration of the fatty acid after treatment with OA/LA (Figure 4.11). * indicate a significant difference ($p<0.05$) in the incorporation into the PLs in comparison to the OA/LA treatment. Different letters within a fatty acid indicate a significant difference between cell lines ($p < 0.05$).

AA from the AA treatment was significantly incorporated into the PLs of MDA-MB-231 and MCF-7 cell lines, where the incorporation into MDA-MB-231 cells was significantly higher than that of MCF-7 cells (Figure 4.15). No significant changes were observed in other n-6 fatty acid levels. AA treatment was not tested on MCF-12A cells.

iii. N-6:N-3 Ratios

Table 4.8 N-6:N-3 Ratios of Whole Cell Phospholipids of MDA-MB-231, MCF-7 and MCF-12A Cells Incubated with 150μM LA, GLA, DGLA or AA

Treatment	Cell Line		
	MDA-MB-	MCF-7	MCF-12A
Media	1.55±0.30[a]	2.73±0.32[b]	**1.59±0.03 [ab]**
OA/LA	7.14±1.02	17.5±4.1	8.34±0.38
LA	10.7±0.9[a]	26.9±5.0[b]	**20.0±1.0**[ab]
GLA	6.27±0.93[a]	20.9±2.7[b]	**15.1±0.5[b]**
DGLA	7.65±0.90	7.76±1.04	
AA	13.3±3.3 [a]	34.1±3.8 [b]	

Values are means ± SEM (n=3-5 per cell line per treatment). Bold values indicate a significant difference compared to the cell line's respective OA/LA control ($p < 0.05$). Different letters within a treatment indicate a significant difference between cell lines ($p < 0.05$). DGLA and AA treatments were not studied with MCF-12A cells.

In Table 4.8, it can be seen that n-6:n-3 ratios vary greatly with n-6 treatments. In MDA-MB-231 cells, the ratios vary between 6.27-13.25, while MCF-7 ratios vary between 7.76 and 34.07. Despite the variation, these ratios were not significantly different from the OA/LA control's ratio. The n-6:n-3 ratio significantly increased with LA and GLA treatments in MCF-12A cells. MCF-7 cells' n-6:n-3 ratio was generally higher than those of MDA-MB-231 or MCF-12A cells, however, only LA, GLA and AA treatments resulted in significantly higher than MDA-MB-231 ratios.

iv. Estimated Desaturase Activity

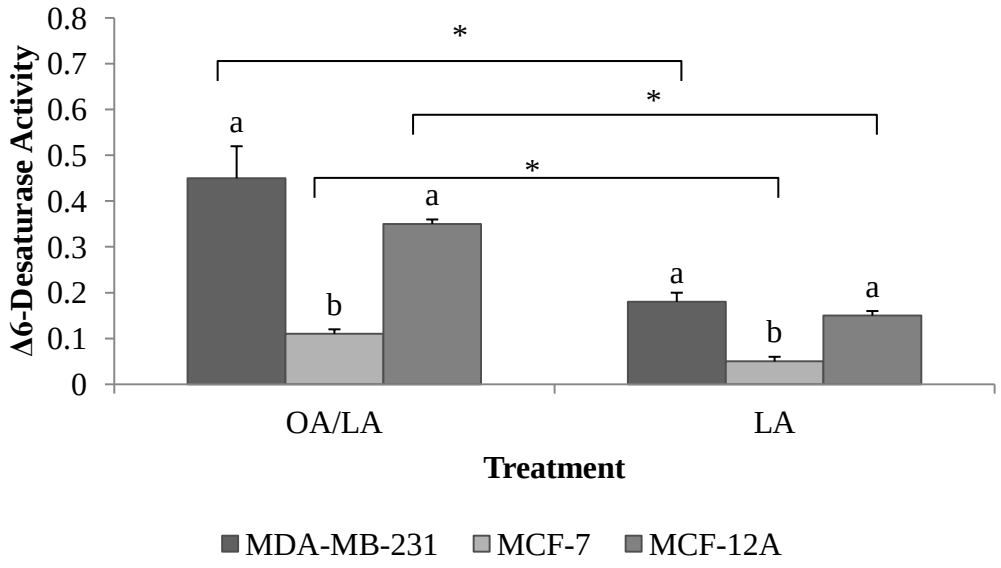

Figure 4.16 Estimated Δ6-Desaturase Activity in MDA-MB-231, MCF-7 and MCF-12A Cells in the N-6 Pathway. Values are means ± SEM (n=3-5 per cell line per treatment). * indicates a significant difference between treatments ($p < 0.05$). Different letters within a treatment indicates a significant difference between cell lines ($p < 0.05$).

From Figure 4.16, estimated Δ6-desaturase's catalytic activity is significantly lower with a higher treatment concentration of LA in all cell lines. MCF-7 cells' estimated Δ6-desaturase activity is also significantly lower than those of MDA-MB-231 and MCF-12A cells. Use of the Δ6-desaturase pathway could not be distinguished from the use of the Δ8-desaturase pathway in cells.

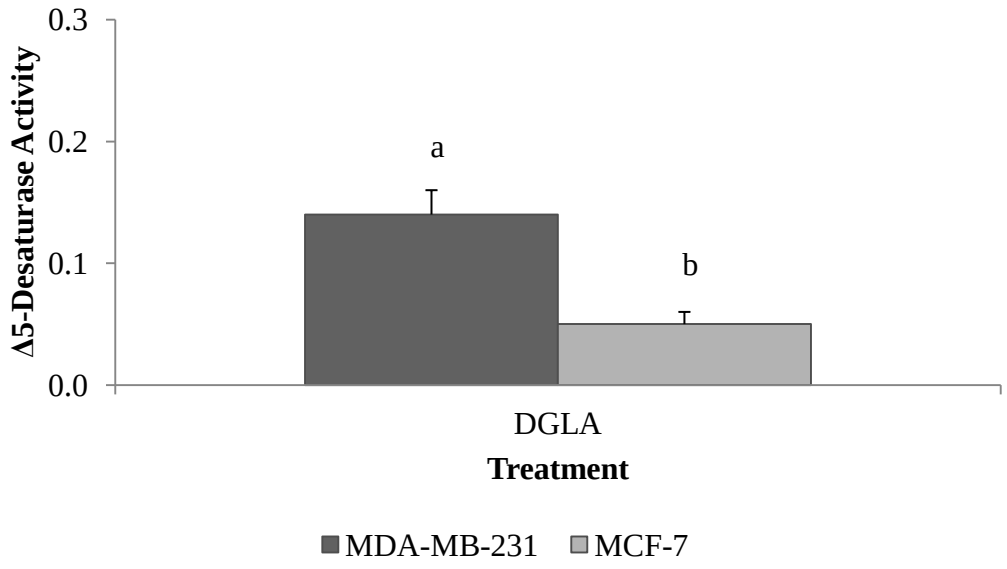

Figure 4.17 Estimated Δ5-Desaturase Activity in MDA-MB-231 and MCF-7 Cells in the N-6 Pathway. n=3/5 Values are means ± SEM (n=3-5 per cell line). Different letters within a treatment indicates a significant difference between cell lines ($p < 0.05$).

Estimated Δ5-desaturase activity is below 1.0 in both tumorigenic cell lines in Figure 4.17. Estimated Δ5-desaturase activity of MCF-7 cells is significantly lower than that of MDA-MB-231 cells. DGLA treatment was not studied with MCF-12A cells.

IV. Discussion

A. Cell Viability

Using an untreated media control as a guide for optimal tumour growth, we compared the viability of tumour cells incubated with OA/LA to untreated media cells and found no significant differences in viability (Figure 4.1). As we observed no significant differences in viability in the tumorigenic cell lines, we determined that these dosages of each OA and LA were not high enough to exhibit inhibitory effects. The non-tumorigenic MCF-12A cells, however, exhibited significantly higher cell growth with the OA/LA fatty acid control media in comparison to normal media, showing that OA/LA is required for enhanced growth. Incorporation of LA into the membrane would also ensure that growth changes are not due to changes in essential fatty acid content in the PLs.

In Figure 4.1, ALA was shown to have inhibitory effects on the viability of MCF-7 cells but not MDA-MB-231 cells. Previous findings have shown that ALA concentrations less than 150μM significantly inhibit MDA-MB-231 cell growth (11, 14). Some of the differences between our results and these studies may be due to differences in culture conditions, as not only were the type of media used different, but no OA or LA were provided, and ALA was in the form of methyl esters dissolved in ethanol instead of BSA conjugates. In MCF-7 cells *in vitro*, similar inhibitory effects were observed at ALA concentrations up to 100μM (15). Flaxseed, which has a high ALA content, has also been found to inhibit MCF-7 cell growth in mice, however, whether effects are due to the ALA or other components of the oil were not distinguished (16, 17). Our observation that ALA was not observed to inhibit MCF-12A cell growth, and appeared to promote growth, to our knowledge, has not been previously studied, and illustrates the tumour-specific action of ALA.

In this study, SDA was shown to significantly inhibit the growth of both breast cancer cell lines to similar degrees, while MCF-12A showed significant increases in growth. The effects of SDA on breast cancer have not been studied extensively, but a previous study has showed treatment with SDA up to 100μM showed a trend towards inhibiting MDA-MB-231 cell growth within 24 hours

(11). The longer incubation period and higher concentration we used may have contributed to the greater inhibitory effect on viability, as inhibitory effects of DHA have been found to increase with time (18).

ETA was found to significantly inhibit the viability of both tumorigenic cell lines; however, the effect was significantly greater in MCF-7 cells than MDA-MB-231 cells. To our knowledge, this is the first time that ETA has been shown to have anti-tumorigenic activity. This property of ETA further supports the importance of long chain PUFAs in the progression of breast cancer and increase the interest in ETA as a bioactive fatty acid. ETA may not have been previously studied as there are few dietary and supplemental sources. Additionally, few biological functions have been attributed to ETA, compared to SDA and GLA which can make up a significant amount of certain plant oils, or DGLA which can be used to synthesize eicosanoids.

EPA significantly inhibited the viability of both MDA-MB-231 and MCF-7 cells. These observations confirm the inhibitory effect of EPA in MCF-7 and MDA-MB-231 cells (13, 14, 19).

DHA also inhibited MDA-MB-231 and MCF-7 viability, however, at the concentration used in this study, the effect was significantly lower in MCF-7 cells. The inhibitory effects agree with existing literature in both cell lines (13, 19-22). Comparing the growth effects between cell lines, however, have not been determined in the same study. A study has shown that DHA has similar effects on the two lines in terms of increasing oxidative stress, which contributes to toxicity (23). However, DHA was delivered at 30μM in the absence of other fatty acids, the dosage was much lower than the one we used. Since DHA has been shown to inhibit cell growth in breast tumour cells in a dose-dependent manner, the conclusions from that study are less relevant to our study or the *in vivo* situation. The different inhibitions between cell lines indicate a possible connection between fatty acid inhibition and breast cancer receptor status, as ER+ and ER- cells have been found to activate different pathways in response to incubation with EPA and AA (24).

Like ALA, we found that LA treatment significantly lowered the viability of MCF-7 cells but not MDA-MB-231 cells. A previous study of LA methyl esters at 100μM was not found to inhibit the growth of MCF-7 cells (25). However, another study using albumin bound LA found a slight but significant inhibition of MCF-7 cells at 30μM, though not at 6-24μM, suggesting that method of fatty acid delivery may impact anti-tumorigenic activity (13). In comparison to this latter study, we used a much higher concentration which may account for the changes in MCF-7 viability. The effect of LA free fatty acid treatments on MDA-MB-435 and MDA-MB-231 cells have been suggested to have an inverted U-shaped curve with no LA and high concentrations of LA inhibiting tumour growth (26-28). As we only used one concentration, it is difficult to compare results. In non-tumorigenic cells, the increase in growth observed in MCF-12A cells has not been previously reported, however, LA treatment in MCF-10A cells, another non-tumorigenic cell line, has been examined. It was found that at low concentrations (6-12μM) of LA methyl esters increased the growth of MCF-10A cells, however, high concentrations (30μM) inhibited growth in comparison to their control. Differences in results may be accountable to the different cell line and media conditions.

GLA significantly inhibited the growth of both tumorigenic cell lines similarly. Literature is in conflict over the anti-tumorigenic properties of GLA. Chajes et al. (1995) found variable effects of GLA at 20μg/mL (about 70μM) on breast tumour growth depending on the cell line, however, MDA-MB-231 and MCF-7 were not found to be affected by GLA supplementation. Menendez et al. (2002), however, found inhibitory effects on MCF-7 cells at over 200μM, and strongly suggested synergistic effects of GLA with chemotherapy drug vinorelbine, used to treat metastatic breast cancer, in MCF-7 and MDA-MB-231 cells at 40μg/mL (about 143μM) (29). Although cell viability was not assessed directly in MDA-MB-231 cells by Menendez et al., GLA given with anti-mitotic vinorelbine and pre-treatment before vinorelbine at the same dosages suggest some anti-tumorigenic activity by GLA on MDA-MB-231 cells (29). The non-significant increase in cell viability when MCF-12A cells were treated with GLA

has not been previously studied in literature, and suggests a tumour-specific activity by GLA.

DGLA also significantly inhibited the growth of both tumorigenic cell lines, with a more pronounced in MCF-7 than MDA-MB-231 cells. These results are in agreement with previous findings in MDA-MB-231 cells, however, to our knowledge, this is the first time DGLA has been shown to have anti-tumorigenic activity in MCF-7 cells (30, 31). It has been predicted that GLA exerts its anti-tumorigenic effects through its conversion to DGLA, and now we have confirmed anti-tumorigenic activity of DGLA in two breast cell lines (30, 31).

AA only inhibited MDA-MB-231 cell growth and not MCF-7 cells. Bocca et al. found that AA provided as a free fatty acid inhibited both MDA-MB-231 and MCF-7 cells at concentrations below 50μM (3). AA methyl esters (100μM) were also shown to have inhibitory effects on MCF-7 cells (25). Some of these differences may be due to culture conditions and AA delivery method.

To our knowledge, this is the first time that the bioactivities of ETA and DGLA on multiple tumorigenic breast cell line and a non-tumorigenic breast cell line have been examined. From these findings, it is possible that SDA and GLA exhibit their anti-carcinogenic effects through their conversion to downstream counterparts ETA and DGLA, respectively, however, whether the conversion stops at ETA and DGLA or continues further down the pathway has yet to be thoroughly investigated. SDA supplementation has been shown to increases levels of EPA in breast cancer cell culture, so it is possible that SDA, as well as ETA, exhibit anti-tumorigenic effects through conversion to EPA rather than ETA (11).

Our results clearly demonstrate the inhibitory effects of the n-3 and n-6 pathway fatty acids on the growth of breast cancer cells. Inhibitory effects of common fatty acids ALA, EPA, DHA, LA and AA were confirmed by our studies. Pathway intermediates SDA and GLA had similar effects on both tumorigenic cell lines, while longer chained fatty acids ETA and DGLA showed more inhibition in MCF-7 than MDA-MB-231 cells. It has been proposed that we might have produced fatty acid toxicity on the cells, as total fatty acid treatment is

230μM (32). However, growth inhibition was not significant for MDA-MB-231 cells with ALA or LA treatment, or with MCF-7 cells with LA treatment, and no inhibitory effects on MCF-12A growth were observed at this total fatty acid concentration. As Jiang et al. did not use a mixture of fatty acids, like our OA/LA treatment, and single fatty acids may be more toxic to cells, our results are not comparable. By adding LA to the media, inhibitory effects from LA deficiency and anti-tumorigenic effects of treatment fatty acids can be isolated (19, 33). Inhibitory effects on cell viability observed in tumorigenic cells were not observed in the non-tumorigenic MCF-12A cells, suggesting tumour-specific activities of ALA, SDA, LA and GLA. Therefore, fatty acid toxicity is unlikely to have played a role in decreasing cell growth of tumour cells with our treatments.

B. Phospholipid Composition

We compared all treatments to the OA/LA control, as both OA and LA were added to all treatments, and OA/LA would change the lipid composition of the cells in comparison to media that does not contain fatty acids. Comparisons to media without any fatty acids would not represent an *in vivo* condition.

Using the main n-3 and n-6 pathways, we assumed that any increases in pathway fatty acid levels from control levels that were not supplied directly through the media were due to the conversion of the treatment fatty acid through the main pathway. First examining the n-3 fatty acid treatments, we found that ALA treatment did not significantly change the content of most downstream n-3 fatty acids in the main pathway, suggesting impairment in the catalytic activity of the Δ6-desaturase enzyme (Figure 4.3). The only significant conversion with ALA was observed in MDA-MB-231 cells to ETA, however, no changes in SDA levels were observed. Although it is possible that any SDA created from the desaturation of ALA was immediately converted to ETA, significant increases in C20:3n3 content suggests that the ETA may have been created through an alternate pathway involving the elongation of ALA to C20:3n3, before Δ8-desaturase catalyzes the reaction converting C20:3n3 to ETA in MDA-MB-231

cells. Therefore, caution has to be taken when analyzing fatty acid conversion data in the n-3 pathway, as it is possible that an alternate pathway may be used. More research needs to be done to differentiate the activity of these two pathways. Previous studies have noted impaired Δ6-desaturase enzyme activity in humans and breast tumours; however, investigation into the alternate pathway using the Δ8-desaturase enzyme is lacking (6, 7, 10, 34).

SDA treatment showed fatty acid conversion downstream, however, although there was a trend towards conversion to DPA, only the conversion of SDA to EPA was significant in MDA-MB-231 cells. Therefore, the increased levels of longer-chained fatty acids may contribute to the anti-tumorigenic effects of SDA on MDA-MB-231 cells. Due to the lack of conversion in MCF-7 cells, anti-tumorigenic effects of SDA on cell viability may be due to effects by SDA itself, rather than to its conversion products. Similar observations have been found in MDA-MB-231 cells, but, SDA treatment on MCF-7 cells have not been previously studied (11). This lack of fatty acid conversion may be due to differences in metabolic rates compared to the MDA-MB-231 cell line. More conversion may have been observed with longer incubations.

When tumour cells were treated with ETA, similar to SDA treatment, conversion to EPA was only observed in MDA-MB-231 cells although there was a trend towards conversion to DPA. Therefore, the anti-tumorigenic activity observed in Figure 4.1 could be due to properties of ETA rather than its conversion. The observed lack of significant conversion of ETA to EPA suggests some impairment in the function of Δ5-desaturase, that catalyses this reaction. To our knowledge, these results are the first to study the effects of ETA on PUFA conversion, and gives insight to possible anti-tumorigenic mechanisms exhibited by ETA through changes in breast tumour membrane PL composition.

EPA was found to be readily elongated to DPA in both tumorigenic cell lines. The lack of conversion to DHA by Δ6-desaturase further supports the low catalytic activity of this enzyme. DHA treatment only resulted in the accumulation of DHA into the PL membranes, and no further changes in n-3 fatty acid levels were observed, confirming that the biological end product of this

pathway to be DHA. The conversion from EPA to DHA and the accumulation of DHA with no retroconversion has been previously shown in literature with EPA and DHA supplementation, respectively (35).

In the n-6 treatments, LA treatment did not show significant changes in levels of downstream n-6 pathway fatty acids, indicating no significant conversion (Figure 4.12). An increase was observed in the levels of C20:2n6, the n-6 intermediate in an alternate pathway in all cell lines, however, only the increase in MDA-MB-231 cell lines was significant, showing that this cell line uses this pathway. This pathway would bypass the creation of GLA in the n-6 pathway. Therefore, the catalytic conversion of LA may be through the use of another pathway using a Δ8-desaturase enzyme rather than the pathway using the Δ6-desaturase enzyme. Grammatikos et al. (1994) has previously shown the inability for LA to be converted to AA in MCF-7 cells and increases in C20:2n-6 levels in tumour PLs.

GLA treatment showed significant conversion to DGLA, however, not to AA in all cell lines (Figure 4.13). Therefore, anti-tumorigenic properties of GLA may be through their conversion to DGLA. However, GLA may have its own anti-tumorigenic properties as conversion was less in the MCF-7 cells (as seen by lower DGLA content) and similar anti-tumorigenic effects were observed between MCF-7 and MDA-MB-231 cell lines. The conversion of GLA has not been previously examined on breast tumour cells. To our knowledge, our experiments are the first ones that indicate the conversion of GLA to DGLA.

DGLA was not further converted to AA in the DGLA treatment in the tumorigenic cell lines (Figure 4.14). Therefore, anti-tumorigenic properties observed in the cell viability experiment are most likely due to DGLA itself. These results are the first of their kind to our knowledge, and suggest anti-tumorigenic properties of DGLA treatment itself on breast tumour cells.

AA treatment only resulted in AA accumulation in the PL, although some conversion was observed downstream to C22:4n6 in both tumorigenic cell lines but was only significant in MDA-MB-231 cell lines (Figure 4.15, Table 4.5-6). Other treatments did not show this change. This result suggests dysfunction of

the catalytic activity of the Δ5-desaturase enzyme in the conversion of DGLA to AA. It also suggests that part of the inhibitory effect of AA on MDA-MB-231 could be possibly due to this extra conversion. Through this conversion, there is less AA available to produce pro-inflammatory eicosanoids that have been associated with aggravating tumour cell growth. It is also possible that the observed elongation of AA creates a product which is not a precursor for eicosanoids.

Overall, fatty acids not impeded by the requirement of the catalytic activity of a desaturase enzyme, especially the Δ6-desaturase enzyme, were readily converted down their metabolic pathways in MDA-MB-231 cells. Conversion rates tended to be much greater in MDA-MB-231 and MCF-12A cells than MCF-7 cells, which may be due to the higher metabolic rates of these cell lines, as their doubling time was about twice that of MCF-7 cells.

Treatment of MCF-12A cells with the fatty acids of the n-3 and n-6 pathways show the ability for normal cells to convert PUFAs. ALA was not able to be converted to downstream products, emphasizing the low catalytic activity of Δ6-desaturases as found in literature. However, once past the requirement of the Δ6-desaturase, SDA treatment fatty acids were readily elongated and desaturated to DPA, where it encountered the Δ6-desaturase enzyme again, which may have limited conversion to DHA. Similar observations were found in the n-6 pathway, where the desaturation of LA to GLA by the Δ6-desaturase enzyme was not observed. However, past it, GLA was readily elongated to DGLA. Unlike in the n-3 pathway, the Δ5-desaturase did not show significant catalytic activity converting DGLA to AA, suggesting a fatty acid substrate preference of ETA over DGLA. Overall, in normal breast cells, the desaturase enzymes significantly hindered the conversion of PUFAs, which is similar to the conversion abilities observed in tumorigenic cells. Grammatikos et al. (1994), however, observed significant conversion of fatty acids in both n-3 and n-6 pathways in non-tumorigenic MCF-10A cells, however, cells were incubated with for four days and estrogen was provided in the media, which would stimulate fatty acid conversion in the cells. In MCF-12A cells, significant decreases in LA and long

chain PUFAs were observed with the short-chain treatments ALA, SDA and LA, as well. These results suggest that these treatment fatty acids may replace the content of other PUFAs or are preferentially taken into the cell over LA.

C. N-6:N-3 Ratios

N-6:n-3 ratios of the treatments were compared to those of the OA/LA control as all treatments contained these fatty acids. The addition of OA/LA to the media would increase LA content in PL, thus, increasing the n-6:n-3 ratio. As expected, n-6:n-3 ratios were lower with n-3 fatty acid treatment than n-6 fatty acid treatment. Despite lower and higher ratios with n-3 and n-6 fatty acid treatments, respectively, they were not significantly different from the OA/LA control, possibly due to large variability between replicates or impaired cell regulation with high supplementation of fatty acids. It would have been predicted that lower ratios would be associated with higher reduced viability, however, in the current study, changes in the ratio did not predict changes in viability in either tumorigenic cell line. Significant increases in cell viability with MCF-12A cells were not associated with changes in the n-6:n-3 ratios with n-3 and n-6 fatty acid treatments, as well. This lack of any significant associations in membrane PLs does not support the predicted importance of n-6:n-3 ratios on breast cancer progression, as supported by other groups (36-38). Many other human studies did not find significant effects regarding n-6:n-3 ratios and breast cancer risk (39, 40). A problem with the n-6:n-3 ratio is that it does not take into account types of n-3 and n-6 fatty acids. Instead, it generally groups all n-3 fatty acids as having equal positive effects on health, while all n-6 fatty acids as having negative effects on health. From our viability results, all of the tested fatty acids showed some anti-tumorigenic activity. Therefore, the n-6:n-3 ratio may not be the best predictor of disease progression or health status, and it may be more important to examine individual fats, rather than ratios of total n-6 and total n-3 levels.

D. Estimated Desaturase Activity

It has been noted that the desaturation of fatty acids in the n-3 and n-6 pathway is very low in humans (41). Low Δ6-desaturase protein levels have also been previously associated with breast cancer compared to normal breast tissue, where women with the poorest prognosis had the lowest levels of Δ6-desaturase (42). However, desaturase activity has not been previously studied in breast cells *in vitro*. We estimated desaturase activity in the current study by calculating the ratio of the percent composition of fatty acids downstream of the desaturase in question to those upstream of the desaturase within a pathway. Using this estimate, we observed low activity under 1.0 in all the cell lines studied. Despite the inverse association between Δ6-desaturase protein levels and breast cancer prognosis, we observed similar estimated desaturase activity ratios between tumorigenic MDA-MB-231 cells and non-tumorigenic MCF-12A cells with both n-3 and n-6 fatty acid stimulation. However, as we did not perform a radio-labeled experiment, Δ6- and Δ8-desaturase activity could not be distinguished. Δ8-desaturase is the enzyme used in an alternate pathway that elongates ALA to C20:3n3 before it catalyzes the desaturation of C20:3n3 to ETA, thereby bypassing SDA production. Similarly LA is elongated to C20:2n6 before desaturation to DGLA, bypassing GLA production. The use of this pathway could have artificially increased our apparent Δ6-desaturase activity due to our assumption that all conversion would be through the catalytic activity of the Δ6-desaturase. MCF-7 cells, however, have considerably lower activity, which concurs with previous studies that showed MCF-7 cells lack Δ6-desaturase activity (13). We have also examined different concentrations of LA supplementation on the Δ6-desaturase ratio, and found that higher concentrations of LA significantly decreased the estimated Δ6-desaturase activity in both tumorigenic and non-tumorigenic cell lines. Although this suppression has not been previously studied in breast cells, this observation has been observed in humans (10).

In the estimated Δ5-desaturase activity, we found a significantly higher ratio in MDA-MB-231 cells than MCF-7 cells in both n-3 and n-6 pathways. It

has previously been found that MCF-7 cells lack Δ5-desaturase activity, agreeing with our findings (13). To our knowledge, Δ5-desaturase activity or protein levels has not been previously assessed in MDA-MB-231 cells.

Despite the apparent changes in estimated activity with both Δ5- and Δ6-desaturases, these estimates are not accurate, especially with the differentiation between Δ6- and Δ8-desaturase activities. In order to distinguish these activities, an investigation using with radio-labeled fatty acids and examining enzyme proteins levels would be required, as these have not been previously investigated in breast cells.

Estrogen has also been found to enhance desaturase activity, and may be of interest in the use of these fatty acids in the treatment of breast cancer (6, 7, 34). As estrogen would also have effects on ER+ breast tumours, it would be interesting to determine whether the enhancements of tumour growth would be offset by desaturase activity and higher levels of long chain PUFAs with fatty acid intermediate treatments.

V. Summary

N-3 and n-6 fatty acids SDA, ETA, EPA, DHA, GLA and DGLA inhibited the growth of both tumorigenic cell lines tested, while ALA, LA and AA showed different degrees of inhibition between cell lines. Although inhibitory effects of ALA, SDA, EPA, DHA, LA, GLA and AA have been previously studied in breast tumours, our research shows the results concerning the inhibitory effects of ETA and DGLA. Treatments with fatty acids that did not require a desaturase step, especially the Δ6-desaturase in the next step of the metabolic pathway, were readily converted downstream in MDA-MB-231 cells, however, conversion was limited in MCF-7 cells. Although discrepancies between cell lines may be due to the higher metabolic rate of MDA-MB-231 cells, similar inabilities to convert fatty acids through the catalytic activity of the Δ6-desaturase were found within cell lines, as there was limited conversion of ALA, EPA and LA to SDA, EPA and GLA.

In non-tumorigenic cells, we found that none of the treatments studied negatively affected the viability. Grammatikos et al. (1994) has also show similar results with MCF-10A cells. In cell PL, conversion of SDA to DPA and GLA to DGLA, but no other conversions in the treatments tested was observed. Whether supplementation of ETA and DGLA will result in increased levels of downstream fatty acids down to DHA and AA, respectively is not know and may be of interest in normal cells. A previous study has found that fatty acid conversion in non-tumorigenic MCF-10A cells is much greater than in tumorigenic MCF-7 cells, where the cells converted fatty acids down to DHA and AA (13).

Investigation of n-6:n-3 ratios of whole cell PLs showed that low ratios do not correlate with changes in cell viability. Therefore, it should not be used as a method to predict tumour progression. Fatty acid composition (considering both chain length and degrees of desaturation) is of importance, as some n-6 fatty acids have been found to significantly inhibit breast tumour growth like GLA and DGLA, and should be taken into account when predicting tumour progression (13).

Using ratios of fatty acids in the PLs along the pathway our data suggests there were low catalytic activities of Δ6- and Δ5-desaturases in all cell lines tested. This has been previously reported *in vivo* (6, 7, 34). The Δ6-desaturase enzyme activity estimate was also higher with lower concentrations of LA than higher levels. This difference in activity also matches that found in literature, where high levels of LA can inhibit Δ6-desaturase activity (13). We also found evidence of the use of an alternate pathway that involves a Δ8-desaturase. We could not distinguish the activities of Δ6- and Δ8-desaturases in our ratio calculations. More investigation towards the use of this alternate pathway needs to be done.

VI. References

1. Schley PD, Brindley DN, Field CJ. (n-3) PUFA alter raft lipid composition and decrease epidermal growth factor receptor levels in lipid rafts of human breast cancer cells. J Nutr. 2007 Mar;137(3):548-53.

2. Schonberg S, Krokan HE. The inhibitory effect of conjugated dienoic derivatives (cla) of linoleic-acid on the growth of human tumor-cell lines is in part due to increased lipid-peroxidation. Anticancer Res. 1995 Jul-Aug 1995;15(4):1241-6.

3. Bocca C, Bozzo F, Martinasso G, Canuto RA, Miglietta A. Involvement of PPAR alpha in the growth inhibitory effect of arachidonic acid on breast cancer cells. Br J Nutr. 2008 Oct;100(4):739-50.

4. Macronutrients,A Report of the Panel on, Intakes,Subcommittees on Upper Reference Levels of Nutrients and Interpretation and Uses of Dietary Reference, Intakes,Standing Committee on the Scientific Evaluation of Dietary Reference. Dietary reference intakes for energy, carbohydrate, fiber, fat, fatty acids, cholesterol, protein, and amino acids (macronutrients). The National Academies Press; 2005.

5. Sprecher H. Metabolism of highly unsaturated n-3 and n-6 fatty acids. Biochim Biophys Acta. 2000 Jul 19;1486(2-3):219-31.

6. Burdge GC, Wootton SA. Conversion of alpha-linolenic acid to eicosapentaenoic, docosapentaenoic and docosahexaenoic acids in young women. Br J Nutr. 2002 Oct;88(4):411-20.

7. Pawlosky RJ, Hibbeln JR, Novotny JA, Salem N,Jr. Physiological compartmental analysis of alpha-linolenic acid metabolism in adult humans. J Lipid Res. 2001 Aug;42(8):1257-65.

8. Sauerwald T, Hachey D, Jensen C, Chen H, Anderson R, Heird W. Effect of dietary alpha-linolenic acid intake on incorporation of docosahexaenoic and arachidonic acids into plasma phospholipids of term infants. Lipids. 1996 Mar;31:S131-5.

9. Chen Q, Nilsson A. Desaturation and chain elongation of N-3 and N-6 polyunsaturated fatty-acids in the human caco-2 cell-line. Biochim Biophys Acta. 1993 Feb 24;1166(2-3):193-201.

10. Emken E, Adlof R, Gulley R. Dietary linoleic-acid influences desaturation and acylation of deuterium-labeled linoleic and linolenic acids in young-adult males. Biochimica Et Biophysica Acta-Lipids and Lipid Metabolism. 1994 Aug 4;1213(3):277-88.

11. Horia E, Watkins B. Comparison of stearidonic acid and alpha-linolenic acid on PGE(2) production and COX-2 protein levels in MDA-MB-231 breast cancer cell cultures. J Nutr Biochem. 2005 Mar;16(3):184-92.

12. Jiang W, Hiscox S, Horrobin D, Bryce R, Mansel R. Gamma linolenic acid regulates expression of maspin and the motility of cancer cells. Biochem Biophys Res Commun. 1997 Aug 28;237(3):639-44.

13. Grammatikos S, Subbaiah P, Victor T, Miller W. N-3 and N-6 fatty-acid processing and growth effects in neoplastic and non-cancerous human mammary epithelial-cell lines. Br J Cancer. 1994 Aug;70(2):219-27.

14. Chajes V, Sattler W, Stranzl A, Kostner GM. Influence of n-3 fatty acids on the growth of human breast cancer cells in vitro: Relationship to peroxides and vitamin-E. Breast Cancer Res Treat. 1995 Jun;34(3):199-212.

15. Kim JY, Park HD, Park E, Chon JW, Park YK. Growth-inhibitory and proapoptotic effects of alpha-linolenic acid on estrogen-positive breast cancer cells. Ann N Y Acad Sci. 2009 Aug;1171:190-5.

16. Truan JS, Chen JM, Thompson LU. Comparative effects of sesame seed lignan and flaxseed lignan in reducing the growth of human breast tumors (MCF-7) at high levels of circulating estrogen in athymic mice. Nutr Cancer. 2012;64(1):65-71.

17. Chen J, Saggar JK, Corey P, Thompson LU. Flaxseed cotyledon fraction reduces tumour growth and sensitises tamoxifen treatment of human breast cancer xenograft (MCF-7) in athymic mice. Br J Nutr. 2011 Feb;105(3):339-47.

18. Blanckaert V, Ulmann L, Mimouni V, Antol J, Brancquart L, Chenais B. Docosahexaenoic acid intake decreases proliferation, increases apoptosis and decreases the invasive potential of the human breast carcinoma cell line MDA-MB-231. Int J Oncol. 2010 Mar;36(3):737-42.

19. Schley P, Jijon H, Robinson L, Field C. Mechanisms of omega-3 fatty acid-induced growth inhibition in MDA-MB-231 human breast cancer cells. Breast Cancer Res Treat. 2005 Jul;92(2):187-95.

20. Byon CH, Hardy RW, Ren C, Ponnazhagan S, Welch DR, McDonald JM, et al. Free fatty acids enhance breast cancer cell migration through plasminogen activator inhibitor-1 and SMAD4. Lab Invest. 2009 Nov;89(11):1221-8.

21. Rogers KR, Kikawa KD, Mouradian M, Hernandez K, McKinnon KM, Ahwah SM, et al. Docosahexaenoic acid alters epidermal growth factor receptor-related signaling by disrupting its lipid raft association. Carcinogenesis. 2010 Sep;31(9):1523-30.

22. Sun H, Berquin IM, Owens RT, O'Flaherty JT, Edwards IJ. Peroxisome proliferator-activated receptor gamma-mediated up-regulation of syndecan-1 by

n-3 fatty acids promotes apoptosis of human breast cancer cells. Cancer Res. 2008 Apr 15;68(8):2912-9.

23. Vibet S, Goupille C, Bougnoux P, Steghens J, Gore J, Maheo K. Sensitization by docosahexaenoic acid (DHA) of breast cancer cells to anthracyclines through loss of glutathione peroxidase (GPx1) response. Free Radical Biology and Medicine. 2008 Apr 1;44(7):1483-91.

24. Alquobaili F, Miller SA, Muhie S, Day A, Jett M, Hammamieh R. Estrogen receptor-dependent genomic expression profiles in breast cancer cells in response to fatty acids. J Carcinog. 2010 Feb 4;8:17.

25. Chamras H, Ardashian A, Heber D, Glaspy J. Fatty acid modulation of MCF-7 human breast cancer cell proliferation, apoptosis and differentiation. J Nutr Biochem. 2002 Dec;13(12):711-6.

26. Earashi M, Noguchi M, Tanaka M. In vitro effects of eicosanoid synthesis inhibitors in the presence of linoleic acid on MDA-MB-231 human breast cancer. Breast Cancer Res Treat. 1996;37(1):29-37.

27. Rose D, Connolly J, Liu X. Effects of linoleic-acid and gamma-linolenic acid on the growth and metastasis of a human breast-cancer cell-line in nude-mice and on its growth and invasive capacity in-vitro. Nutrition and Cancer- An International Journal. 1995;24(1):33-45.

28. Rose D, Connolly J. Effects of fatty-acids and inhibitors of eicosanoid synthesis on the growth of a human breast-cancer cell-line in culture. Cancer Res. 1990 Nov 15;50(22):7139-44.

29. Menendez J, Ropero S, del Mar Barbacid M, Montero S, Solanas M, Escrich E, et al. Synergistic interaction between vinorelbine and gamma-linolenic acid in breast cancer cells. Breast Cancer Res Treat. 2002 Apr;72(3):203-19.

30. Wang X, Lin H, Gu Y. Multiple roles of dihomo-gamma-linolenic acid against proliferation diseases. Lipids in Health and Disease. 2012 Feb 14;11:25.

31. Zhang W, Chakravarty B, Zheng F, Gu Z, Wu H, Mao J, et al. Crystal structure of FAS thioesterase domain with polyunsaturated fatty acyl adduct and inhibition by dihomo-gamma-linolenic acid. Proc Natl Acad Sci U S A. 2011 Sep 20;108(38):15757-62.

32. Jiang W, Bryce R, Horrobin D. Essential fatty acids: Molecular and cellular basis of their anti-cancer action and clinical implications. Critical Reviews in Oncology Hematology. 1998 Apr;27(3):179-209.

33. Amaru DL, Field CJ. Conjugated linoleic acid decreases mcf-7 human breast cancer cell growth and insulin-like growth factor-1 receptor levels. Lipids. 2009 May;44(5):449-58.

34. Yamazaki K, Fujikawa M, Hamazaki T, Yano S, Shono T. Comparison of the conversion rates of alpha-linolenic acid (18:3(n - 3)) and stearidonic acid (18:4(n - 3)) to longer polyunsaturated fatty acids in rats. Biochim Biophys Acta. 1992 Jan 3;1123(1):18-26.

35. Yuri T, Danbara N, Tsujita-Kyutoku M, Fukunaga K, Takada H, Inoue Y, et al. Dietary docosahexaenoic acid suppresses N-methyl-N-nitrosourea-induced mammary carcinogenesis in rats more effectively than eicosapentaenoic acid. Nutrition and Cancer-an International Journal. 2003;45(2):211-7.

36. Ge Y, Chen Z, Kang ZB, Cluette-Brown J, Laposata M, Kang JX. Effects of adenoviral gene transfer of C. elegans n-3 fatty acid desaturase on the lipid profile and growth of human breast cancer cells. Anticancer Res. 2002 Mar-Apr;22(2A):537-43.

37. Maillard V, Bougnoux P, Ferrari P, Jourdan ML, Pinault M, Lavillonniere F, et al. N-3 and N-6 fatty acids in breast adipose tissue and relative risk of breast cancer in a case-control study in tours, france. Int J Cancer. 2002 Mar 1;98(1):78-83.

38. Zhu Z, Jiang W, McGinley JN, Prokopczyk B, Richie JP, Jr., El Bayoumy K, et al. Mammary gland density predicts the cancer inhibitory activity of the N-3 to N-6 ratio of dietary fat. Cancer Prevention Research. 2011 Oct;4(10):1675-85.

39. Murff HJ, Shu X, Li H, Yang G, Wu X, Cai H, et al. Dietary polyunsaturated fatty acids and breast cancer risk in chinese women: A prospective cohort study. Int J Cancer. 2011 Mar 15;128(6):1434-41.

40. Takata Y, King IB, Neuhouser ML, Schaffer S, Barnett M, Thornquist M, et al. Association of serum phospholipid fatty acids with breast cancer risk among postmenopausal cigarette smokers. Cancer Causes Control. 2009 May;20(4):497-504.

41. Brenner R, Peluffo R. Effect of sturated and unsaturated fatty acids on desaturation in vitro of palmitic stearic oleic linoleic linolenic acids. J Biol Chem. 1966;241(22):5213,&.

42. Lane J, Mansel RE, Jiang WG. Expression of human delta-6-desaturase is associated with aggressiveness of human breast cancer. Int J Mol Med. 2003 Aug;12(2):253-7.

Chapter Five – SDA-Enriched Flax Seed Oil Mixture on Cell Viability and Phospholipid Composition in Breast Cells

I. Introduction

N-3 and n-6 pathways intermediates SDA and GLA have been found to have anti-carcinogenic properties, but, they are usually only present in trace amounts in the diet and are found in foods that are not typically part of the North American diet (1-6). Novel sources of these intermediates, however, are being created in the form of commercial plants enriched with fatty acid intermediates, and could become a possible source of these fatty acids. Plants with enriched SDA content include linseed, canola and soybeans which have been found to have SDA contents of 13.4%, 16-23%, and 4.2-28.2%, respectively (7-10). Soybean, fungus and safflower plants have been enriched to have GLA contents of 2.16-4.21%, 10.7%, and 58.6%, respectively, while a fungus has been enriched to have a DGLA content of 43.9% (11-13).

With these novel sources of n-3 and n-6 pathway intermediates, it would be possible to supplement the diet with these plants or their extracts in order to gain their anti-carcinogenic properties. However, it is first important to explore whether these oils can exhibit their bioactive properties in breast cancer cells. Since fish oils containing EPA and DHA have been shown to have similar properties to EPA and DHA individually, it may be possible for these novel oils to exhibit their anti-carcinogenic properties on breast cancer cells. As oils come as mixtures of fatty acids, it is possible that fatty acids could have opposing effects and negate any positive effects on tumour cells. Therefore, it would be important to compare these oils to their components to see whether their anti-carcinogenic properties and effects on the cell membranes are similar. The safety of these plants is also of consumer concern, so it is also important to assess their safety on normal cells.

In partnership with Dr. Randall Weselake's Bioactive Oils Program at the University of Alberta, we obtained oil from an SDA-enriched flax seed plant with an SDA content of 26.1%. A mixture of fatty acids representing the composition

of this oil was incubated with tumorigenic and non-tumorigenic cells to determine its anti-carcinogenic properties and effect on membrane PL composition. The main components of this mixture are ALA, SDA and GLA.

II. Materials and Methods

Please refer to chapter three.

III. Results

A. Cell Viability

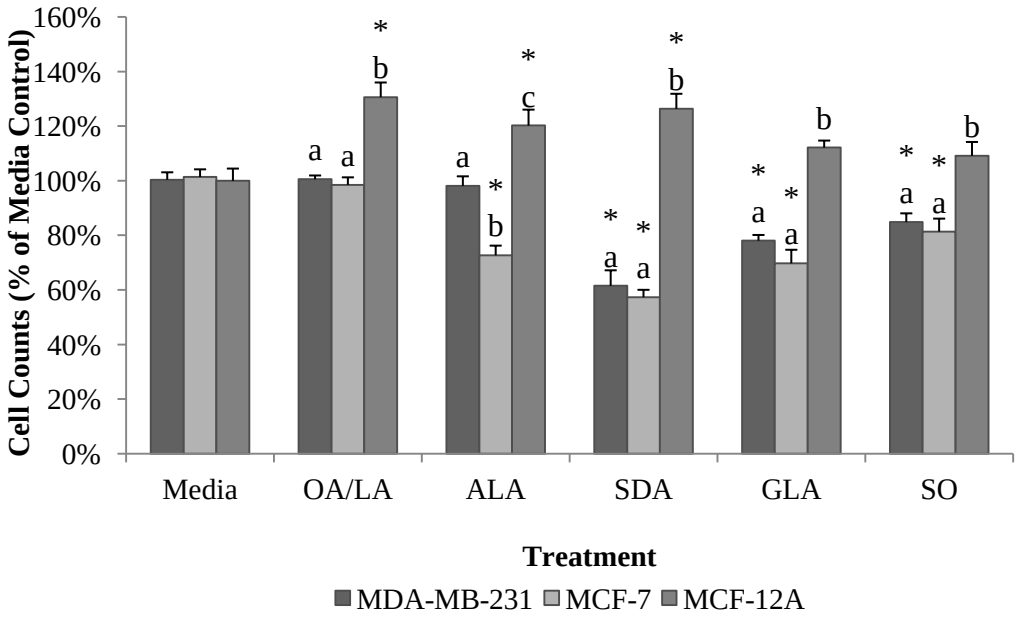

Figure 5.1 Cell Viability of MDA-MB-231, MCF-7 and MCF-12A Cells Incubated with 150μM of ALA, SDA, GLA or SO. Values are mean ± SEM (n=3 per cell line per treatment), expressed as percentage of the media only treatment. * indicates a significant difference compared to the cell line's respective media treatment ($p < 0.05$). Different letters within a treatment indicates a significant difference between cell lines ($p < 0.05$).

From Figure 5.1, it can be seen that each of the main fatty acids (with the exception of ALA treatment on MDA-MB-231 cells) contained in SO and the SO mixture significantly decreased cell viability as determined by live cell counts in both the MDA-MB-231 and MCF-7 tumorigenic cell lines. SDA, GLA and SO

decreased cell viability similarly between the tumorigenic cell lines. None of the treatments decreased the cell viability of MCF-12A cells, but, n-3 treatments ALA and SDA significantly increased cell viability.

B. Phospholipid Composition

Tables 5.1-5.3 show the complete PL composition of the cell lines treated with ALA, SDA, GLA and SO, as well as comparisons to OA/LA control and between cell lines. This data will be illustrated in the subsequent sections to compare the effect of fatty acid treatments on fatty acid changes in PLs and to compare the effects on the different cell lines.

Table 5.1 Whole Cell Phospholipid Composition of MDA-MB-231 Cells Incubated with 150μM of ALA, SDA, GLA or SO.

Fatty Acid	Fatty Acid Treatment				
	OA/LA	ALA	SDA	GLA	SO
C14:0	1.22±0.07	1.50±0.14	1.41±0.26	1.86±0.23	**0.78±0.06**
C16:0	15.1±0.6	16.2±0.8	16.6±1.5	17.7±0.7	14.4±0.3 ^{C}
C16:1n9	1.02±0.16 ^{A}	0.87±0.12	0.86±0.07	0.91±0.11	1.04±0.07 ^{A}
C17:0	0.64±0.09	1.14±0.35	1.05±0.17	0.76±0.06	0.45±0.07
C18:0	18.5±0.4 ^{AC}	**24.3±0.5** ^{C}	**23.8±0.8** ^{AC}	22.0±1.1 ^{C}	20.3±0.4 ^{A}
C18:1t11	3.42±0.44	2.94±0.15	3.73±0.81 ^{C}	1.99±0.53 ^{A}	**0.29±0.16**
C18:1n9	29.9±1.2 ^{A}	**17.9±1.1** ^{A}	**19.8±1.0** ^{A}	**20.9±1.2**	**23.1±0.** ^{A}
C18:1c11	0.19±0.11	0.25±0.11	0.22±0.09	0.04±0.04	0.01±0.01
C18:2n6	13.4±1.3 ^{AC}	15.7±1.7	14.1±1.3	7.99±0.39	13.2±0.3 ^{AC}
C20:0	0.12±0.07	0.05±0.05	0.05±0.05	1.03±0.85	ND ^{C}
C18:3n6	0.60±0.33	0.15±0.15	0.03±0.03	**2.57±0.18**	0.78±0.03
C18:3n3	0.46±0.16	**8.67±0.80** ^{C}	0.29±0.20	0.26±0.18	**2.53±0.14**
C18:4n3	0.11±0.05	0.05±0.05	1.08±0.18 ^{A}	0.13±0.13	0.28±0.02
C20:2n6	1.50±0.18	0.98±0.21 ^{A}	**0.30±0.12**	**0.29±0.09**	0.85±0.03
C20:3n6	1.47±0.14	0.85±0.25	1.18±0.22	**12.6±0.3** ^{A}	**4.77±0.15**
C20:3n3	ND	**1.2±0.4**	0.24±0.24	ND	0.34±0.08 ^{C}
C20:4n6	4.26±0.19	4.17±0.44	**2.30±0.26**	4.76±0.29	5.16±0.08 ^{A}
C20:4n3	0.10±0.05	**0.54±0.07**	**6.74±0.33**	0.04±0.02	**3.00±0.13**
C20:5n3	0.06±0.03	0.98±0.17 ^{A}	**1.92±0.14**	0.52±0.52	**1.73±0.03**
C22:3n3	0.99±0.41	1.08±0.35	0.54±0.30	1.80±0.44 ^{A}	0.25±0.00
C22:4n6	1.65±0.49	1.02±0.22 ^{C}	1.04±0.38	0.38±0.25	1.03±0.13 ^{A}
C22:1n9	1.48±0.15	0.53±0.15 ^{C}	0.59±0.06 ^{C}	1.73±0.29 ^{A}	1.69±0.09
C22:5n6	0.34±0.12	0.28±0.18	0.50±0.10 ^{A}	0.46±0.38	0.36±0.02
C22:5n3	0.46±0.01 ^{C}	1.46±0.19 ^{A}	1.66±0.20 ^{A}	0.50±0.06 ^{A}	**3.17±0.07**
C22:6n3	0.40±0.10 ^{A}	0.14±0.09 ^{C}	0.20±0.09 ^{C}	0.26±0.04 ^{C}	0.44±0.02 ^{A}
ΣSFA	35.5±0.8 ^{C}	**43.2±0.9** ^{C}	41.4±1.5 ^{C}	41.8±1.2 ^{C}	35.9±0.2 ^{AC}
ΣMUFA	35.1±2.2	22.4±1.1 ^{A}	26.1±0.3 ^{A}	25.4±0.7	26.1±0.7 ^{A}
ΣPUFA	26.2±0.7 ^{C}	**34.5±1.0** ^{AC}	29.8±1.9 ^{C}	32.0±1.3 ^{AC}	**37.9±0.7** ^{AC}
Σn-3	3.32±0.38 ^{A}	**14.1±0.7** ^{AC}	**12.7±1.3** ^{AC}	4.14±0.71 ^{A}	**11.8±0.3** ^{AC}
Σn-6	23.4±0.8 ^{C}	22.3±0.8 ^{AC}	17.8±1.0 ^{C}	28.5±0.9 ^{AC}	26.2±0.4

Values are mean percent composition ± SEM (n=5 per treatment). Bold values indicate a significant difference in comparison to the OA/LA control ($p < 0.05$). ^{A} and ^{C} indicate significant differences compared to MCF-7 and MCF-12A cell lines, respectively ($p < 0.05$). ND indicates not detectable.

Table 5.2 Whole Cell Phospholipid Composition of MCF-7 Cells Incubated with 150μM of ALA, SDA, GLA or SO.

Fatty Acid	Fatty Acid Treatment				
	OA/LA	ALA	SDA	GLA	SO
C14:0	1.46±0.19	1.22±0.36	1.22±0.20	1.51±0.21	0.85±0.04
C16:0	17.1±0.5	17.6±0.4 ^{B}	16.5±0.5	18.5±0.6 ^{B}	15.9±0.3 ^{B}
C16:1n9	1.80±0.12	1.12±0.15	1.53±0.12	1.04±0.29	2.58±0.04
C17:0	0.51±0.02	0.67±0.03	0.64±0.01	0.57±0.14	0.23±0.10
C18:0	16.7±0.5 ^{A}	**22.4±1.2** ^{B}	**21.2±0.3** ^{A}	**22.2±1.2** ^{B}	16.8±0.2
C18:1t11	5.14±0.46	4.50±0.39	3.64±0.81	4.50±0.63	**0.02±0.01**
C18:1n9	33.8±0.4 ^{A}	**23.7±0.9** ^{A}	**28.6±0.5** ^{A}	**23.8±0.5** ^{B}	**29.7±0.1**
C18:1c11	0.22±0.14	0.27±0.17	0.01±0.01	0.04±0.04	0.04±0.1
C18:2n6	18.9±0.2 ^{A}	**14.3±0.4**	17.5±0.4	**12.3±0.2** ^{A}	18.3±0.2
C20:0	ND	ND	ND	ND	ND ^{B}
C18:3n6	0.08±0.03	0.27±0.09	0.19±0.09	**7.09±0.22**	**1.34±0.03**
C18:3n3	0.44±0.13	**9.59±0.32**	0.29±0.07	0.23±0.13	**3.71±0.06**
C18:4n3	0.02±0.02	0.06±0.05	**3.09±0.25**	0.04±0.02	**0.42±0.01**
C20:2n6	0.80±0.16	0.32±0.09	0.06±0.04	0.05±0.03^{B}	0.40±0.00
C20:3n6	0.41±0.11	0.51±0.14	0.99±0.15	**3.31±0.06**	**3.10±0.06**
C20:3n3	ND	0.02±0.02	ND	ND	**0.18±0.01**
C20:4n6	1.52±0.11	1.04±0.24	1.19±0.13	2.20±0.16	**2.93±0.07**
C20:4n3	0.01±0.01	ND ^{AB}	1.07±0.18^{A}	0.03±0.03	**0.68±0.02**
C20:5n3	0.26±0.12	ND $^{A B}$	0.47±0.13	0.22±0.16	**0.72±0.03**
C22:3n3	0.29±0.14	0.32±0.10	0.28±0.04	0.42±0.06	0.07±0.01
C22:4n6	0.66±0.14	0.55±0.14	0.49±0.18	0.61±0.11	0.43±0.03
C22:1n9	ND $^{A B}$	0.17±0.16	0.17±0.15	ND $^{A B}$	**0.80±0.04**
C22:5n6	0.18±0.09	0.06±0.03	0.13±0.08	0.02±0.02	0.12±0.02
C22:5n3	0.31±0.10	0.19±0.03	0.51±0.11	0.24±0.06	**1.04±0.05**
C22:6n3	0.03±0.02	0.02±0.02	0.06±0.05	0.23±0.14^{B}	**0.19±0.01**
ΣSFA	35.8±0.4	40.6±0.8 ^{B}	39.5±0.7	44.0±1.4^{B}	33.8±0.1^{AB}
ΣMUFA	40.8±0.8	**29.7 ±1.2** ^{A}	**34.0±0.8** ^{A}	**29.4±1.3** ^{B}	**32.6±0.1** ^{A}
ΣPUFA	23.8±0.6 ^{B}	27.2±0.9 ^{A}	25.4±0.4 ^{B}	27.0±0.5 ^{A}	**33.6±0.2** ^{A}
Σn-3	1.55±0.32	**10.2±0.4** ^{A}	**5.76±0.52**	1.12±0.14^{A}	**7.02±0.08**
Σn-6	22.5±0.3 ^{B}	**17.0±0.6**	20.6±0.5	**25.6±0.4**	**26.6±0.1**

Values are mean percent composition ± SEM (n=5 per treatment). Bold values indicate a significant difference in comparison to the OA/LA control ($p < 0.05$). ^{A} and ^{B} indicate significant differences compared to MDA-MB-231 and MCF-12A cell lines, respectively ($p < 0.05$). ND indicates not detectable.

Table 5.3 Whole Cell Phospholipid Composition of MCF-12A Cells Incubated with 150μM of ALA, SDA, GLA or SO.

Fatty Acid	Fatty Acid OA/LA	ALA	SDA	GLA	SO
C14:0	1.47±0.05	**1.04±0.05**	**1.16±0.02**	**1.27±0.04**	**0.64±0.03**
C16:0	16.9±0.6	**14.3±0.4** ^{B}	15.2±0.3	15.5±0.4 ^{B}	17.8±0.3 ^{BC}
C16:1n9	1.48±0.06	1.13±0.05	1.33±0.04^{C}	1.19±0.02	1.07±0.12
C17:0	0.51±0.04	**0.67±0.04**	**0.70±0.02**	**0.68±0.02**	0.60±0.03
C18:0	13.4±0.1 ^{BC}	**17.6±0.3** ^{BC}	**18.1±0.3** ^{BC}	**17.0±0.4** ^{BC}	**20.3±1.1** ^{B}
C18:1t11	0.11±0.04	0.22±0.05	0.13±0.02	0.19±0.04	**0.56±0.11**
C18:1n9	28.7±0.2 ^{B}	**19.1±0.4** ^{B}	**22.0±0.5** ^{B}	**20.4±0.2** ^{B}	**23.3±0.2** ^{B}
C18:1c11	0.05±0.00	0.08±0.01	0.07±0.01	0.07±0.01	0.07±0.01
C18:2n6	20.5±0.8^{C}	**15.1±0.2**	**15.7±0.1**	**12.9±0.6**^{C}	**14.4±0.3** ^{BC}
C20:0	0.07±0.00	0.09±0.00	**0.11±0.01**	0.04±0.02	**0.67±0.01**
C18:3n6	0.04±0.00	0.05±0.05	0.05±0.03	**4.70±0.18**	**ND** ^{BC}
C18:3n3	0.79±0.04	**13.5±0.8** ^{BC}	**0.21±0.04**	**0.13±0.03**	**3.57±0.06**^{C}
C18:4n3	0.05±0.01	0.02±0.02	**2.48±0.22**^{C}	0.04±0.02	**0.39±0.02**^{C}
C20:2n6	2.25±0.02	**0.83±0.03**	**0.23±0.01**	**0.45±0.02**	**0.65±0.07**^{B}
C20:3n6	0.83±0.02	0.88±0.00	1.09±0.04	**12.4±0.2** ^{B}	**3.77±0.26**
C20:3n3	0.02±0.02	ND	ND	ND	ND ^{C}
C20:4n6	6.29±0.04	**9.61±0.25**	**4.63±0.13**	8.03±0.20^{B}	6.09±0.09
C20:4n3	0.05±0.00	0.30±0.08^{B}	**9.51±0.41**	0.06±0.02	2.31±0.24^{B}
C20:5n3	0.32±0.01	0.67±0.10	**3.50±0.14**	**0.09±0.01**	1.04±0.06^{B}
C22:3n3	0.17±0.14	0.50±0.01	0.03±0.01	0.81±0.01	0.14±0.06^{C}
C22:4n6	0.28±0.13	0.20±0.01^{C}	0.48±0.06	0.80±0.16	0.89±0.02
C22:1n9	2.78±0.09	**1.89±0.07**	**1.20±0.04**	**1.24±0.07**	**0.25±0.12**
C22:5n6	0.63±0.02	0.49±0.06	**0.16±0.01**	**0.21±0.01**	0.08±0.08^{C}
C22:5n3	1.10±0.04	1.06±0.10	**1.52±0.11**	**0.76±0.04**	1.19±0.02
C22:6n3	1.20±0.09	**0.74±0.07**	**0.52±0.06**	**0.72±0.09**	**0.35±0.05**
ΣSFA	32.3±0.5 ^{C}	33.7±0.4 ^{BC}	**35.2±0.3** ^{C}	34.5±0.5^{BC}	**38.7±0.9**^{BC}
ΣMUFA	33.1±0.2	**22.4±0.5** ^{B}	**24.7±0.5** ^{B}	**22.7±0.2** ^{B}	**25.1±0.3** ^{B}
ΣPUFA	34.6±0.7 ^{BC}	**43.9±0.8** ^{BC}	**40.1±0.6** ^{BC}	**42.1±0.8** ^{BC}	35.4±0.2 ^{C}
Σn-3	3.70±0.05	**16.8±0.6** ^{BC}	**17.8±0.6** ^{BC}	2.62±0.08	**9.18±0.14**^{B}
Σn-6	30.9±0.7^{BC}	27.2±0.2 ^{BC}	22.3±0.1^{C}	**39.5±0.7** ^{BC}	26.3±0.1

Values are mean percent composition ± SEM (n=3 per treatment). Bold values indicate a significant difference in comparison to the OA/LA control ($p < 0.05$). ^{B} and ^{C} indicate significant differences compared to MCF-7 and MDA-MB-231 cell lines, respectively ($p < 0.05$). ND indicates not detectable.

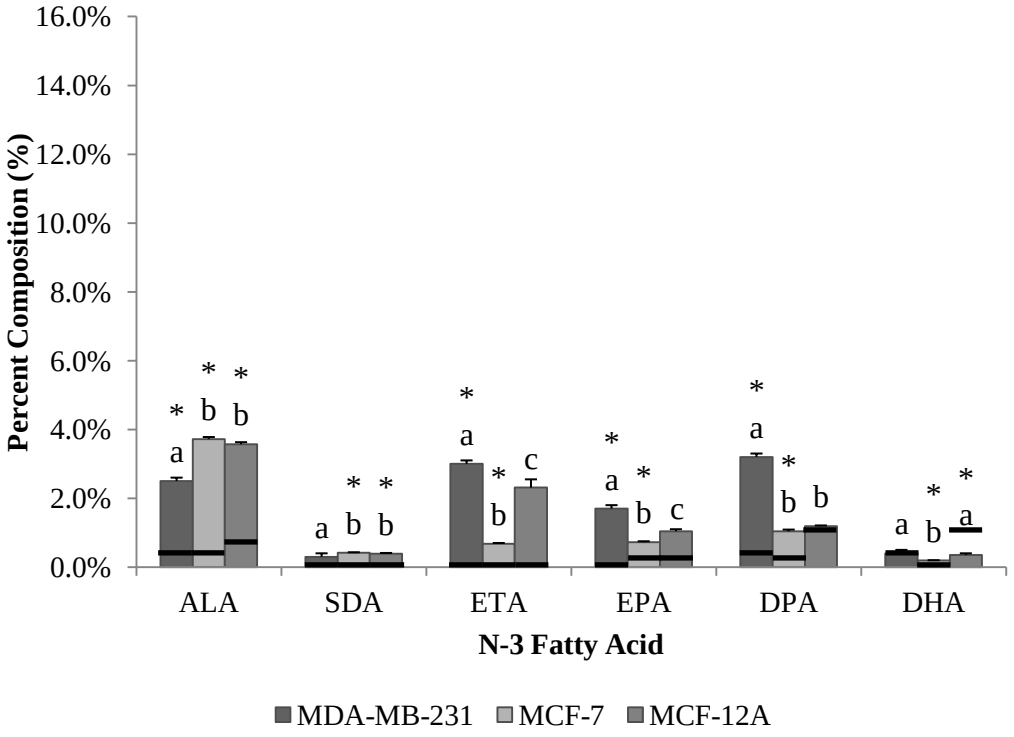

Figure 5.2 Phospholipid N-3 Fatty Acid Profile of MDA-MB-231, MCF-7 and MCF-12A Cells After Incubation with 150μM SO. Values are mean percent composition ± SEM (n=3-5 per cell line). Black horizontal bars indicate the concentration of the fatty acid after treatment with OA/LA(Figure 4.2. * indicate a significant difference ($p<0.05$) in the incorporation into the PLs in comparison to the OA/LA treatment). Different letters within a fatty acid indicate a significant difference between cell lines ($p < 0.05$).

ALA and SDA from the SO treatment were significantly incorporated into the MCF-7 and MCF-12A cell membranes as ALA and SDA, respectively (Figure 5.2). In MDA-MB-231 cells, only ALA was significantly incorporated into the cell membrane. Significant increases in levels of downstream fatty acids down to DPA and DHA were observed in MDA-MB-231 and MCF-7 cells, respectively. ETA, EPA and DPA levels were significantly higher in MDA-MB-231 cells than the other cell lines, while ALA and SDA levels were significantly lower. An increase in ETA content in membrane lipids was observed in MCF-12A cells, but was not significant ($p = 0.06$).

A significant increase in C20:3n3 was also observed in MCF-7 cells only (Table 5.2).

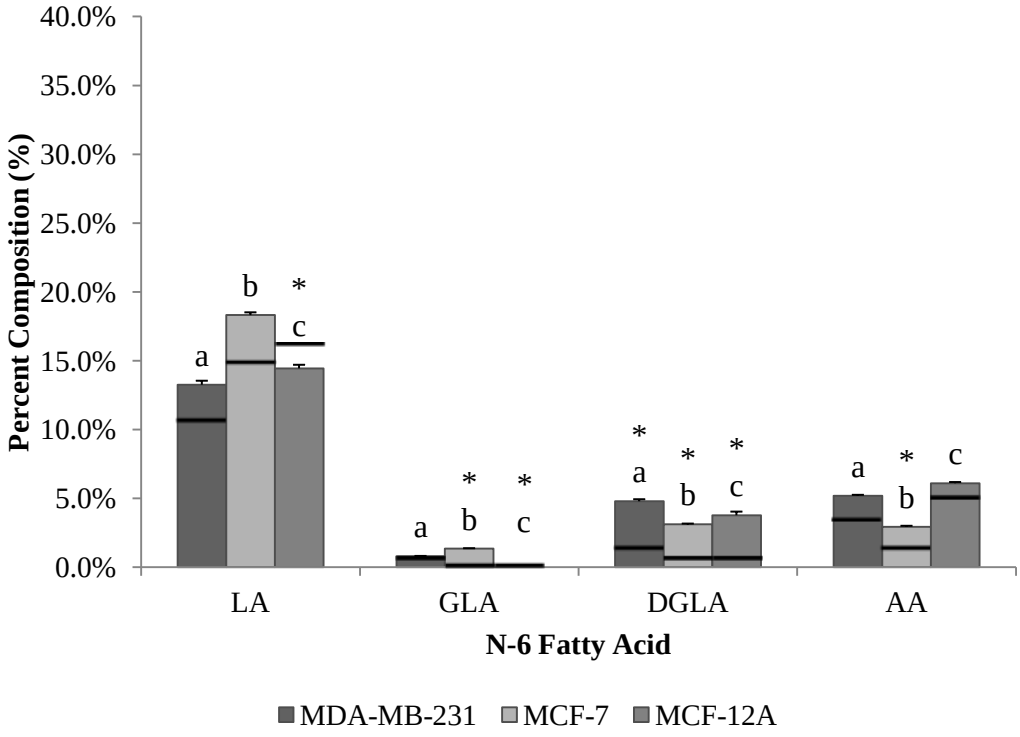

Figure 5.3 Phospholipid N-6 Fatty Acid Profile of MDA-MB-231, MCF-7 and MCF-12A Cells Incubated with 150μM SO. Values are mean percent composition ± SEM (n=3-5 per cell line). Black horizontal bars indicate the concentration of the fatty acid after treatment with OA/LA(Figure 4.2. * indicate a significant difference ($p<0.05$) in the incorporation into the PLs in comparison to the OA/LA treatment). Different letters within a fatty acid indicate a significant difference between cell lines ($p < 0.05$).

N-6 fatty acids analysis is shown in Figure 5.3. GLA was only significantly incorporated in the PLs of MCF-7 cells as GLA, but a significant increase in the amount of DGLA was observed in the PLs of all cell lines. Significant increases in AA content was also observed in MCF-7 cells. LA and GLA levels were significantly higher in MCF-7 cells than either MDA-MB-231 or MCF-12A cells, but DGLA and AA levels were significantly lower.

C. N-6:N-3 Ratios

Table 5.4 N-6:N-3 Ratios of Whole Cell Phospholipids of MDA-MB-231, MCF-7 and MCF-12A Cells Incubated with 150μM Fatty Acid Treatment.

Treatment	Cell Line		
	MDA-MB-231	MCF-7	MCF-12A
Media	1.55±0.30[a]	2.73±0.32[b]	**1.59±0.03**[ab]
OA/LA	7.14±1.02	17.5±4.1	8.34±0.38
ALA	1.77±0.14	1.67±0.06	**1.63±0.07**
SDA	1.56±0.13[a]	3.64±0.20[b]	**1.26±0.05**[a]
GLA	6.27±0.93[a]	20.9±2.7[b]	**15.1±0.5**[b]
SO	2.23±0.04[a]	3.79±0.04[b]	**2.86±0.05**[c]

Values are means ± SEM (n=3-5 per cell line per treatment). Bold values indicate a significant difference compared to the cell line's respective OA/LA control ($p < 0.05$). Different letters within a treatment indicate a significant difference between cell lines ($p < 0.05$).

N-6:n-3 ratios were low with SO and n-3 ALA and SDA treatments in Table 5.4, varying between 1.26-3.79 in all cell lines. The ratio was higher with GLA treatments, ranging from 6.27-20.90. Despite this difference, none of the treatment's n-6:n-3 ratios were significantly different from the OA/LA control within the tumorigenic cell lines. Ratios were significantly lower with ALA, SDA and SO treatments compared to the OA/LA control in the MCF-12A cell line, while it was significantly higher with GLA treatment. MCF-7 n-6:n-3 ratios were higher than MDA-MB-231 ratios with media, SDA, GLA and SO treatment, and were higher than MCF-12A ratios in SDA and SO treatments.

IV. Discussion

A. Cell Viability

Similar inhibition of cell viability by the SO mixture was observed on our tumorigenic cell lines compared to each of its main individual components, ALA, SDA and GLA, showing that the anti-tumorigenic effects observed by these fatty acids individually also work when presented in a fatty acid mixture. The lack of an effect of the SO treatment on the MCF-12A cells indicates a tumour-specific action, similar to individual ALA, SDA and GLA treatments. Although this particular fatty acid mixture or any other oils with a similar composition have not

been tested on breast cells before, previous studies have shown that fatty acid mixtures composed of anti-tumorigenic fatty acids also have anti-tumorigenic properties in culture and animal studies. Other mixtures of EPA and DHA were found to significantly inhibit MDA-MB-231 cell growth, as well as mixtures with either EPA or DHA with LA, and mixtures with all three fatty acids (14). A mixture of 5% w/w EPA and 5% w/w DHA diet also resulted in reduced tumor incidence in N-methyl-N-nitrosourea-induced tumours in rats similar to that of a 10% w/w EPA diet, but was less suppressive than 10% w/w DHA diet alone, suggesting that the effects of mixtures of n-3 fatty acid have anti-cancer properties but their effects may not be additive when fed in diets (15).

The use of dietary oils composed of various n-3 and n-6 fatty acids that make up a significant amount of their oils have also been found to have inhibitory effects on breast cancer. Mistol oil (22% ALA, 14% LA of fat), evening primrose oil (68% LA, 13% GLA of fat), fish oil (12% LA, 10% EPA, 5% DHA of fat) and linseed oil (34% ALA, 19% LA of fat) all have been found to inhibit breast tumour growth (16, 17). From these results, it is possible that the SO oil used in the current study also possesses anti-tumorigenic activity, as over 70% of this mixture is composed of fatty acids we have shown to have anti-tumorigenic activity on both ER+PR+ and ER-PR- human breast cancer cell lines.

B. Phospholipid Composition

As with the PLs in chapter four, we assumed that increases in fatty acid levels past the supplied fatty acids in SO was due to the conversion of the supplied fatty acids to their longer chained counterparts. Using this assumption, we found that the ALA and SDA in the SO treatment were significantly converted down the n-3 metabolic pathway to DPA in both the MDA-MB-231 and MCF-7 cell lines (Figure 5.2). These effects seem to be a combination of the effects of ALA and SDA alone (Figure 4.3-4), where, in brief, ALA from the ALA treatment was incorporated into the membrane and was only significantly converted to ETA in the MDA-MB-231 cell line. SDA from the SDA treatment was significantly incorporated into the MCF-7 and MCF-12A cell lines and

significant conversion to EPA was observed in the MDA-MB-231 cell line, and to DPA in the MCF-12A cell line but no significant conversion was observed in the MCF-7 cell line. Previous studies have found that a mixture of EPA and DHA resulted in increased DPA levels, supporting that fatty acids in mixtures can be converted through the pathway (15). However, mixtures in literature have focused on fish oil mixtures with EPA and DHA. Conversion is limited at the end of the pathway, and there is little literature that has examined fatty acid mixtures with n-3 or n-6 pathway intermediates regarding PL composition.

Comparing the SO treatment to the ALA and SDA treatments, significantly more fatty acid conversion down the n-3 metabolic pathway was observed with SO treatment than ALA and SDA treatments alone. Although the percent composition of each individual fatty acid was lower with SO treatment, this effect is most likely due to the lower concentrations of each of these fatty acids in this treatment, which was 47μM ALA and 39μM SDA in the 150μM SO treatment. This difference between conversion ability between ALA and SDA treatments and the SO treatment may be due to inhibition of the enzymes in the pathway by the very high levels of substrate, as our results in Figure 4.16 and previous literature have indicated (18-22).

A significant increase was also observed in C20:3n3 levels in MCF-7 cells, indicating the use of an alternate metabolic pathway in MCF-7 cells, and may have enhanced conversion of ALA to longer-chained n-3 PUFAs. The use of this pathway was not observed in MCF-7 cells in with ALA treatment in chapter four, and may be due to ALA's high concentration of fatty acid that can inhibit Δ6-desaturase catalytic activity. The use of this pathway in MDA-MB-231 cells with ALA treatment, however, was not significant with SO treatment, although, a trend was observed. This difference may be due to lack of substrate, as the high levels of ALA did not seem to inhibit Δ8-desaturase's catalytic activity, and various fatty acids seem to have inhibitory effects on Δ6-desaturase (Figure 4.16) (18-22). To our knowledge, this is the first time fatty acid conversion has been investigated with the use of enriched plant oils, or in the Δ8-desaturase pathway

in breast tumour cells. As in chapter 4, our findings suggest the use of Δ8-desaturase enzymes in breast tumour cells.

In the n-6 fatty acid profile shown in Figure 5.3, the increases in DGLA content were most likely due to the conversion of GLA in the SO treatment to DGLA. This result matches that of the GLA treatment (Figure 4.13), where GLA was significantly converted to DGLA in all cell lines. However, further conversion in the MCF-7 cell line of GLA in SO to AA was also observed, which may be due to less inhibition of the pathway enzymes due to lower concentrations of inhibiting fatty acids, as previously discussed.

Between tumorigenic cell lines, MDA-MB-231 cells showed significantly higher levels of downstream conversion products ETA, EPA, DHA and DGLA than MCF-7 cells suggesting higher levels of conversion in these cells. This difference may be due to the higher metabolic rate of MDA-MB-231 cells, as the doubling rate is about twice that of MCF-7 cells. A non-significant trend towards conversion to EPA was observed in the MCF-12A cell line. As ALA from the ALA treatment was not converted downstream in MCF-12A cells (Figure 4.3), SDA is most likely the main substrate in the conversions. Compared to the SDA-only treatment (Figure 4.4), where significant conversion up to DPA was observed, there seems to be an inhibition of the converting ability of MCF-12A cells with SO treatment. This observation suggests that the PUFA converting ability in this cell line may not be as high as the tumorigenic cell lines, as MCF-12A's growth rate is similar to that of the MDA-MB-231 cells. However, there may also be regulatory mechanisms in normal cells that would normally control fatty acid conversion, and warrants further study. It has previously been shown that non-tumorigenic MCF-10A cells have a great ability to convert fatty acids from LA to AA (28%), ALA to EPA (21%), and EPA to DHA(14%) (23). However, culture conditions were different, including the supplementation of estrogen, which could stimulate fatty acid conversion.

Significant displacement of DHA observed in the MCF-12A cell line with the ALA, SDA and SO treatments further supporting the suggestion that these treatments preferentially replace some of the DHA in the membrane upon

incorporation of other fatty acids in the membrane. As no negative effects on cell viability were observed, low levels of DHA in the PLs may not have a negative effect on cells, and suggest that there may be a balance between fatty acids in the membrane of normal cells that still allow it to function properly. This regulation of fatty acid balance, namely DHA levels, was not observed in tumorigenic cells in the present study, and may contribute to the tumour-specific effects of the essential fatty acids on cell growth in the current study. Changes in non-tumour cell membrane PLs have not been studied previously in oils or fatty acid mixtures and are a novel contribution of this thesis.

Overall, the SO treatment showed fatty acid conversion similar to that of a combination of each of its main components, ALA, SDA and GLA in all cell lines, where conversion to EPA and DGLA was observed in MDA-MB-231 cells, to DGLA in MCF-7 cells, and to DPA and DGLA in MCF-12A cells. Although the individual content of each fatty acid was lower with SO treatment than with ALA, SDA or GLA treatment, fatty acids were converted further down the pathway with the SO treatment than with any of the individual fatty acids. In MDA-MB-231 cells, conversion to DPA and DGLA was observed, while in MCF-7 cells conversion to DHA and AA was observed. These results may possibly be due to less inhibition of the catalytic activities of the Δ5- and Δ6-desaturases from fatty acids in tumorigenic cell lines. MCF-12A cells, however, only showed conversion to DGLA, suggesting possible PUFA metabolism dysfunction or regulation not observed in tumorigenic cells, and requires further study.

C. N-6:N-3 Ratios

Many experiments have examined the effects of dietary n-6:n-3 ratios on breast tumour progression and found that the diets with lower n-6:n-3 ratios were associated with a significantly lower development of mammary cancer in rats (24, 25). Despite the support for the use of the n-6:n-3 ratios in predicting tumour progression, many of these studies are limited by their comparisons of only LA to ALA, EPA and DHA. Other pathway intermediates are not taken into account in

these calculations, most likely due to their low abundance in the diet. An experiment studying evening primrose oil, fish oil and linseed oil supplemented diets with n-6:n-3 of 22.73, 0.65 and 0.57, respectively, for rats showed the highest mammary tumour incidence in the linseed oil diet and the lowest in the evening primrose diet which has high levels of GLA (17). These results show the potential of other pathway intermediates to have anti-carcinogenic properties, as well as suggest the n-6:n-3 ratio may not be accurate (17).

The oil used in our experiment had an n-6:n-3 ratio of 0.32, which would predict anti-tumorigenic activity. The n-6:n-3 ratios in the PLs with SO treatment, however, do not reflect the prediction that lower ratios are related to decreased cell viability in breast cancer cells, as the ratios were not significantly different from the OA/LA control. These observations are possibly due to the wide variability of the replicates. Although viability between the MDA-MB-231 cells and MCF-7 cells were similar, the n-6:n-3 ratio was significantly higher in MCF-7 cells than MDA-MB-231 cells. A significantly lower ratio than the control in MCF-12A cells also did not reflect an increase in cell growth, either, which supports the suggestion that whole cell PL composition may not be a good predictor of cell viability, and that there may be a finer balance of membrane of fatty acids that is required for normal cell function. These observations further support data presented in chapter four, that n-6:n-3 ratios in whole cell PLs may not play a significant role in determining the anti-tumorigenic effects of SO, and anti-tumorigenic effects are dependent on specific fatty acid properties. Plant oils enriched with n-3 or n-6 pathway intermediates have not been tested on breast cancer previously. Therefore, to our knowledge, this is the first time that an oil enriched with an n-3 pathway intermediate, SDA, has been found to significantly decrease the n-6:n-3 ratio in normal cell PL, but not tumorigenic PLs.

V. Summary

The inhibitory activity of an SDA-enriched flax oil was found to be similar to those of its main individual components ALA, SDA and GLA. The fatty acids in the oil appeared to be taken up by the cells and converted down their

respective pathways to DPA and DGLA in MDA-MB-231 and DHA and DGLA in MCF-7 cells. This was not observed with individual fatty acids, where conversion only went as far as ETA with SDA treatment and DGLA with GLA treatment in MDA-MB-231 cells, and conversion to DGLA from GLA treatments in MCF-7 cells. This increased conversion may be due to the effects of mixtures of fatty acids in different concentrations on enzymes in the n-3 and n-6 metabolic pathways as previously reported (18-22). Increases in C20:3n3 levels in MCF-7 PLs were also observed, suggesting metabolism through an alternate fatty acid metabolic pathway. The presence of these longer chain fatty acids in cellular PLs may explain some of the anti-tumorigenic effects observed with this oil mixture on tumorigenic breast cells.

In non-tumorigenic MCF-12A cells, conversion was also observed, however, not to the same extent as tumorigenic cells or treatments of the individual components, suggesting either lower conversion ability or possibly controlling mechanisms that suppress fatty acid conversion, and needs to be elucidated through further analysis of enzyme activity and protein levels.

Changes in the n-6:n-3 ratio in membrane PLs with SO treatment did not predict tumour viability. This observations further supports our observations in chapter 4, where n-6:n-3 ratios in PLs did not predict tumour growth, possibly due to the inhibitory effects of n-6 pathway intermediates GLA and DGLA and inconsistent inhibitory effects of ALA on tumour growth as observed in chapter 4, Figure 4.1. The work in this chapter suggests that the composition of the individual long chain n-6 and n-3 fatty acids is an important characteristic to take into account when predicting tumour progression.

VI. References

1. Horia E, Watkins B. Comparison of stearidonic acid and alpha-linolenic acid on PGE(2) production and COX-2 protein levels in MDA-MB-231 breast cancer cell cultures. J Nutr Biochem. 2005 Mar;16(3):184-92.

2. Jiang W, Hiscox S, Horrobin D, Bryce R, Mansel R. Gamma linolenic acid regulates expression of maspin and the motility of cancer cells. Biochem Biophys Res Commun. 1997 Aug 28;237(3):639-44.

3. Passi S, Cataudella S, Di Marco P, De Simone F, Rastrelli L. Fatty acid composition and antioxidant levels in muscle tissue of different mediterranean marine species of fish and shellfish. J Agric Food Chem. 2002 Dec 4;50(25):7314-22.

4. Ghasemnezhad A, Honermeier B. Yield, oil constituents, and protein content of evening primrose (oenothera biennis L.) seeds depending on harvest time, harvest method and nitrogen application. Industrial Crops and Products. 2008 Jul;28(1):17-23.

5. Sewon P, Tyystjarvi E. Stearidonic and gamma-linolenic acid contents of common borage leaves. Phytochemistry. 1993 Jul;33(5):1029-32.

6. Surette ME, Edens M, Chilton FH, Tramposch KM. Dietary echium oil increases plasma and neutrophil long-chain (n-3) fatty acids and lowers serum triacylglycerols in hypertriglyceridemic humans. J Nutr. 2004 Jun;134(6):1406-11.

7. Ruiz-Lopez N, Haslam RP, Venegas-Caleron M, Larson TR, Graham IA, Napier JA, et al. The synthesis and accumulation of stearidonic acid in transgenic plants: A novel source of 'heart-healthy' omega-3 fatty acids. Plant Biotechnology Journal. 2009 Sep;7(7):704-16.

8. Ursin V. Modification of plant lipids for human health: Development of functional land-based omega-3 fatty acids. J Nutr. 2003 Dec;133(12):4271-4.

9. Sato S, Xing A, Ye X, Schweiger B, Kinney A, Graef G, et al. Production of gamma-linolenic acid and stearidonic acid in seeds of marker-free transgenic soybean. Crop Sci. 2004 Mar-Apr;44(2):646-52.

10. Lemke SL, Vicini JL, Su H, Goldstein DA, Nemeth MA, Krul ES, et al. Dietary intake of stearidonic acid-enriched soybean oil increases the omega-3 index: Randomized, double-blind clinical study of efficacy and safety. Am J Clin Nutr. 2010 Oct;92(4):766-75.

11. Qin F, Kang L, Guo L, Lin J, Song J, Zhao Y. Composition of transgenic soybean seeds with higher gamma-linolenic acid content is equivalent to that of conventional control. J Agric Food Chem. 2012 Mar 7;60(9):2200-4.

12. Nykiforuk CL, Shewmaker C, Harry I, Yurchenko OP, Zhang M, Reed C, et al. High level accumulation of gamma linolenic acid (C18:3 delta 6.9,12 cis) in transgenic safflower (carthamus tinctorius) seeds. Transgenic Res. 2012 Apr;21(2):367-81.

13. Kawashima H, Akimoto K, Higashiyama K, Fujikawa S, Shimizu S. Industrial production of dihomo-gamma-linolenic acid by a delta 5 desaturase-defective

mutant of mortierella alpina 1S-4 fungus. J Am Oil Chem Soc. 2000 Nov;77(11):1135-8.

14. Schley P, Jijon H, Robinson L, Field C. Mechanisms of omega-3 fatty acid-induced growth inhibition in MDA-MB-231 human breast cancer cells. Breast Cancer Res Treat. 2005 Jul;92(2):187-95.

15. Yuri T, Danbara N, Tsujita-Kyutoku M, Fukunaga K, Takada H, Inoue Y, et al. Dietary docosahexaenoic acid suppresses N-methyl-N-nitrosourea-induced mammary carcinogenesis in rats more effectively than eicosapentaenoic acid. Nutrition and Cancer-an International Journal. 2003;45(2):211-7.

16. Munoz S, Piegari M, Guzman C, Eynard A. Differential effects of dietary oenothera, zizyphus mistol and corn oils, and essential fatty acid deficiency on the progression of a murine mammary gland adenocarcinoma. Nutrition. 1999 Mar;15(3):208-212.

17. Jelinska M, Tokarz A, Oledzka R, Czorniuk-Sliwa A. Effects of dietary linseed, evening primrose or fish oils on fatty acid and prostaglandin E-2 contents in the rat livers and 7,12-dimethylbenz[a]anthracene-induced tumours. Biochim Biophys Acta-Mol Basis Dis. 2003 Apr 17;1637(3):193-9.

18. Sauerwald T, Hachey D, Jensen C, Chen H, Anderson R, Heird W. Effect of dietary alpha-linolenic acid intake on incorporation of docosahexaenoic and arachidonic acids into plasma phospholipids of term infants. Lipids. 1996 Mar;31:S131-5.

19. Chen Q, Nilsson A. Desaturation and chain elongation of N-3 and N-6 polyunsaturated fatty-acids in the human caco-2 cell-line. Biochim Biophys Acta. 1993 Feb 24;1166(2-3):193-201.

20. Emken E, Adlof R, Duval S, Nelson G. Effect of dietary docosahexaenoic acid on desaturation and uptake in vivo of isotope-labeled oleic, linoleic, and linolenic acids by male subjects. Lipids. 1999 Aug;34(8):785-91.

21. Emken E, Adlof R, Gulley R. Dietary linoleic-acid influences desaturation and acylation of deuterium-labeled linoleic and linolenic acids in young-adult males. Biochimica Et Biophysica Acta-Lipids and Lipid Metabolism. 1994 Aug 4;1213(3):277-88.

22. Emken E, Adlof R, Duval S, Nelson G. Effect of dietary arachidonic acid on metabolism of deuterated linoleic acid by adult male subjects. Lipids. 1998 May;33(5):471-80.

23. Grammatikos S, Subbaiah P, Victor T, Miller W. N-3 and N-6 fatty-acid processing and growth effects in neoplastic and non-cancerous human mammary epithelial-cell lines. Br J Cancer. 1994 Aug;70(2):219-27.

24. Sasaki T, Kobayashi Y, Shimizu J, Wada M, In'nami S, Kanke Y, et al. Effects of dietary n-3-to-n-6 polyunsaturated fatty acid ratio on mammary carcinogenesis in rats. Nutrition and Cancer-an International Journal. 1998;30(2):137-43.

25. Wei N, Wang B, Zhang Q, Mi M, Zhu J, Yu X, et al. Effects of different dietary fatty acids on the fatty acid compositions and the expression of lipid metabolic-related genes in mammary tumor tissues of rats. Nutr Cancer. 2008;60(6):810-25.

Chapter Six – C9, t11-CLA on Cell Viability and Phospholipid Composition in Breast Cells

I. Introduction

The major dietary source of CLA is produced by anaerobe *Butyrivibrio fibrisolvens* found in ruminants from ALA, GLA and LA during their hydrogenation to CLA, vaccenic and stearic acid (1, 2). Of the possible isomers produced, the c9, t11-CLA isomer is the most common, and in the rumen can only be produced from the hydrogenation of LA (3). Humans have been found to have microbial species within their intestinal microflora capable of converting ALA and LA to CLA, however only minimal conversion has been observed (4-6). Humans can, however, convert vaccenic acid from the diet to c9, t11-CLA (7).

C9, t11-CLA has been found to have anti-carcinogenic properties in breast cancer *in vitro* and *in vivo* in animal models (8-10). Although c9, t11-CLA has been shown to be incorporated into PL membranes in order to exert their anti-carcinogenic properties, a thorough investigation of the effects of CLA on changes in membrane composition has yet to be done (10). Whether CLA has effects on normal cells has not been well established, nor is it known if incorporation of CLA into membrane PLs displaces other long chain essential fatty acids. Using the most common CLA isomer, c9, t11-CLA, its effects on non-tumorigenic breast cells will be tested, as well as whether c9, t11-CLA incorporation into membranes is balanced by reductions in n-6 fatty acid content.

II. Materials and Methods

Please refer to chapter three, sections I-IV and VI.

III. Results

A. Cell Viability

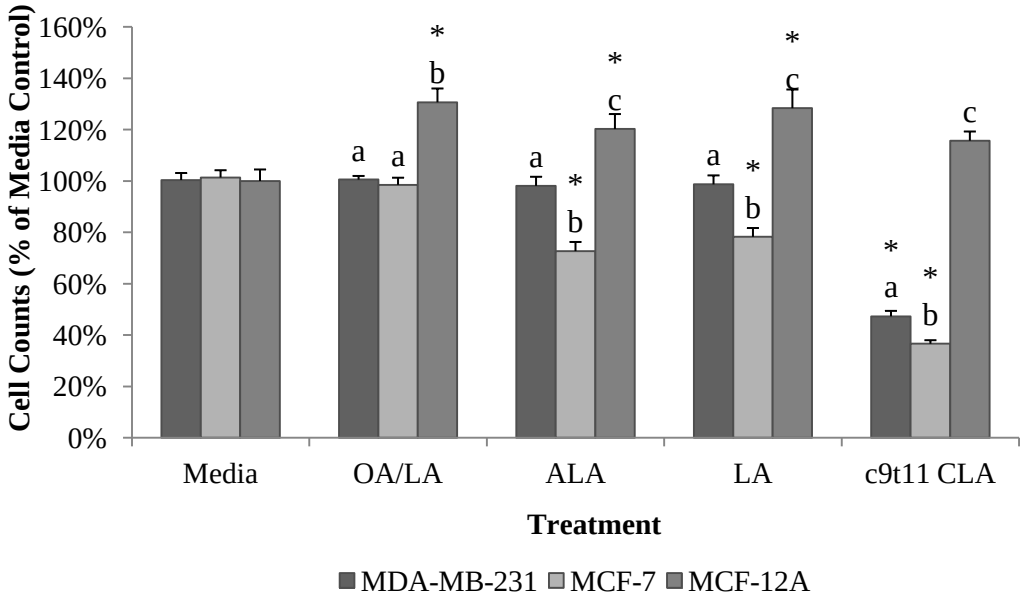

Figure 6.1 Cell Viability of MDA-MB-231, MCF-7 and MCF-12A Cells Incubated with 150μM of ALA, LA or c9, t11-CLA. Values are mean ± SEM (n=3 per cell line per treatment), expressed as percentage of the media only treatment. * indicates a significant difference compared to the cell line's respective media treatment ($p < 0.05$). Different letters within a treatment indicate a significant difference between cell lines ($p < 0.05$).

The potential precursors (by microbes) for CLA, ALA and LA decreased viability in the ER+PR+ MCF-7 cell line, but not in the ER-PR- MDA-MB-231 cell line as illustrated in Figure 6.1. Treatment with c9, t11-CLA significantly suppressed the viability of both MDA-MB-231 and MCF-7 tumorigenic cell lines compared to the media and OA/LA controls. This effect was not observed in the non-tumorigenic MCF-12A cell line with c9, t11-CLA treatment; however, unlike CLA's precursors ALA and LA, c9, t11-CLA did not significantly increase the viability of MCF-12A cells. Between cell lines, MCF-7 cells showed the lowest viability after treatment with ALA, LA and CLA of the three cell lines, while MCF-12A showed the highest viability.

B. Phospholipid Composition

Tables 6.1-6.3 show the whole cell PL composition of each cell line with ALA, LA and CLA treatment, as well as comparisons to OA/LA control and between cell lines. This data will be illustrated in the subsequent sections to compare the effect of fatty acid treatments on fatty acid changes in PLs and to compare the effects on the different cell lines.

Table 6.1 Whole Cell Phospholipid Composition of MDA-MB-231 Cells Incubated with 150μM of ALA, LA or CLA.

Fatty Acid	Fatty Acid OA/LA	ALA	LA	c9, t11-CLA
C14:0	1.22±0.07	1.50±0.14	1.33±0.20	**0.66±0.03** [A]
C16:0	15.1±0.6	16.2±0.8	17.0±1.7	**8.72±0.60** [A]
C16:1n9	1.02±0.16 [A]	0.87±0.12	0.75±0.11 [A]	0.48±0.09 [A]
C17:0	0.64±0.09	1.14±0.35	0.68±0.05	0.38±0.05
C18:0	18.5±0.4 [AC]	**24.3±0.5**[C]	23.0±1.6 [C]	**12.0±1.0**
C18:1t11	3.42±0.44 [AC]	2.94±0.15 [AC]	3.07±0.81	**10.5±0.3** [C]
C18:1n9	29.9±1.2 [A]	**17.9±1.1** [A]	**18.0±1.2**	**12.2±0.7** [A]
C18:1c11	0.19±0.11	0.25±0.11	0.11±0.11	ND
C18:2n6	13.4±1.3 [AC]	15.7±1.7	**26.2±1.2** [AC]	6.09±0.51[AC]
C20:0	0.12±0.07	0.05±0.05	0.10±0.06	ND[C]
C18:3n6	0.60±0.33	0.15±0.15	0.12±0.04	0.04±0.02
C18:3n3	0.46±0.16	**8.67±0.80** [C]	0.28±0.10	0.29±0.18
C18:2c9t11	ND	ND	ND	**37.0±2.9**
C18:4n3	0.11±0.05	0.05±0.05	0.05±0.05	1.22±0.22
C18:2t10c12	ND	ND	ND	**3.3±0.2**[C]
C20:2n6	1.50±0.18 [AC]	0.98±0.21 [A]	**3.43±0.16** [A]	0.79±0.23
C20:3n6	1.47±0.14 [AC]	0.85±0.25	1.55±0.24 [AC]	0.62±0.08
C20:3n3	ND	**1.2±0.4**	ND	ND
C20:4n6	4.26±0.19 [AC]	4.17±0.44 [AC]	3.14±0.22[AC]	3.03±0.47 [C]
C20:4n3	0.10±0.05	**0.54±0.07** [AC]	0.01±0.01	0.67±0.22
C20:5n3	0.06±0.03	0.98±0.17[A]	0.20±0.07	0.02±0.01[C]
C22:3n3	0.99±0.41	1.08±0.35	0.90±0.32	0.02±0.02
C22:4n6	1.65±0.49	1.02±0.22 [C]	0.57±0.25	0.82±0.12 [AC]
C22:1n9	1.48±0.15 [AC]	0.53±0.15[C]	1.57±0.22 [A]	**0.01±0.01**[C]
C22:5n6	0.34±0.12	0.28±0.18	0.70±0.21	0.01±0.01
C22:5n3	0.46±0.01 [C]	1.46±0.19[A]	0.28±0.09 [C]	0.27±0.07 [C]
C22:6n3	0.40±0.10 [A C]	0.14±0.09 [C]	0.30±0.08 [C]	0.20±0.08 [C]
ΣSFA	35.5±0.8[C]	**43.2±0.9**[C]	42.1±3.3[C]	**22.1±1.6**
ΣMUFA	35.1±2.2	22.4±1.1 [A]	24.1±1.4	23.0±1.0
ΣPUFA	26.2±0.7 [C]	**34.5±1.0** [AC]	**37.1±1.5**[C]	**53.9±2.1**
Σn-3	3.32±0.38[A]	**14.1±0.7** [AC]	2.52±0.46	2.44±0.18
Σn-6	23.4±0.8[C]	22.3±0.8[AC]	**35.2±1.1**[C]	**14.2±0.3**[C]

Values are mean percent composition ± SEM (n=5 per cell line per treatment). Bold values indicate a significant difference in comparison to the OA/LA control ($p < 0.05$). [A] and [C] indicate significant differences compared to MCF-7 and MCF-12A cell lines, respectively ($p < 0.05$). ND indicates not detectable.

Table 6.2 Whole Cell Phospholipid Composition of MCF-7 Cells Incubated with 150μM of ALA, LA or CLA.

Fatty Acid	Fatty Acid OA/LA	ALA	LA	c9, t11-CLA
C14:0	1.46±0.19	1.22±0.36	1.53±0.33	0.93±0.05 ^{A}
C16:0	17.1±0.5	17.6±0.4 ^{B}	17.7±0.8	**12.6±0.3** ^{AB}
C16:1n9	1.80±0.12 ^{A}	1.12±0.15	1.24±0.11 ^{A}	**0.95±0.09** ^{A}
C17:0	0.51±0.02	0.67±0.03	0.40±0.14	0.39±0.02
C18:0	16.7±0.5 $^{A B}$	**22.4±1.2** ^{B}	**21.3±0.8** ^{B}	**12.9±0.7**
C18:1t11	5.14±0.46 $^{A B}$	4.50±0.39 $^{A B}$	3.38±0.91	6.90±2.77
C18:1n9	33.8±0.4 $^{A B}$	**23.7±0.9** $^{A B}$	**17.5±0.9**	**16.8±1.0** ^{A}
C18:1c11	0.22±0.14	0.27±0.17	0.13±0.09	0.47±0.20
C18:2n6	18.9±0.2 ^{A}	**14.3±0.4**	**33.3±0.8** ^{A}	10.9±1.6 ^{A}
C20:0	ND	ND	ND	ND
C18:3n6	0.08±0.03	0.27±0.09	0.14±0.07	0.04±0.03
C18:3n3	0.44±0.13	**9.59±0.32** ^{B}	0.35±0.15	0.19±0.09
C18:2c9t11	ND	ND	ND	**28.3±1.3**
C18:4n3	0.02±0.02	0.06±0.05	0.01±0.01	0.81±0.35
C18:2t10c12	ND	ND	ND	**3.31±0.35** ^{B}
C20:2n6	0.80±0.16 $^{A B}$	0.32±0.09 ^{A}	1.40±0.11 $^{A B}$	0.38±0.10
C20:3n6	0.41±0.11 ^{A}	0.51±0.14	0.19±0.05 ^{A}	0.33±0.09 ^{B}
C20:3n3	ND	0.02±0.02	ND	ND
C20:4n6	1.52±0.11 $^{A B}$	1.04±0.24 $^{A B}$	1.21±0.29 $^{A B}$	1.67±0.45 ^{B}
C20:4n3	0.01±0.01	ND ^{AB}	0.10±0.08	0.16±0.07
C20:5n3	0.26±0.12	ND $^{A B}$	0.06±0.04	0.02±0.01 ^{B}
C22:3n3	0.29±0.14	0.32±0.10	0.08±0.08	ND ^{B}
C22:4n6	0.66±0.14	0.55±0.14	0.27±0.13	0.26±0.08 ^{A}
C22:1n9	ND $^{A B}$	0.17±0.16 ^{B}	0.09±0.09 $^{A B}$	0.13±0.07 ^{B}
C22:5n6	0.18±0.09 ^{B}	0.06±0.03	0.30±0.15 ^{B}	0.06±0.04
C22:5n3	0.31±0.10 ^{B}	0.19±0.03 $^{A B}$	0.29±0.07 ^{B}	0.49±0.14 ^{B}
C22:6n3	0.03±0.02 $^{A B}$	0.02±0.02 ^{B}	0.07±0.05 ^{B}	0.25±0.10 ^{B}
ΣSFA	35.8±0.4	40.6±0.8 ^{B}	39.3±0.6	**26.6±1.1**
ΣMUFA	40.8±0.8	**29.7 ±1.2** $^{A B}$	**22.1±1.4**	**24.4±2.0** ^{B}
ΣPUFA	23.8±0.6 ^{B}	27.2±0.9 $^{A B}$	**36.3±0.6** ^{B}	**47.7±2.3** ^{B}
Σn-3	1.55±0.32 ^{AB}	**10.2±0.4** $^{A B}$	0.96±0.24	2.25±0.55
Σn-6	22.5±0.3 ^{B}	**17.0±0.6** ^{AB}	**36.2±0.6** ^{B}	15.2±1.8

Values are mean percent composition ± SEM (n=5 per cell line per treatment). Bold values indicate a significant difference in comparison to the OA/LA control ($p < 0.05$). ^{A} and ^{B} indicate significant differences compared to MDA-MB-231 and MCF-12A cell lines, respectively ($p < 0.05$). ND indicates not detectable.

Table 6.3 Whole Cell Phospholipid Composition of MCF-12A Cells Incubated with 150μM of ALA, LA or CLA.

Fatty Acid	Fatty Acid OA/LA	ALA	LA	c9, t11-CLA
C14:0	1.47±0.05	**1.04±0.05**	**1.02±0.02**	**0.85±0.06**
C16:0	16.9±0.6	**14.3±0.4** ^{B}	**14.2±0.4**	**9.51±0.49** ^{B}
C16:1n9	1.48±0.06	1.13±0.05	**0.96±0.05**	**0.87±0.04**
C17:0	0.51±0.04	**0.67±0.04**	0.64±0.03	**0.38±0.01**
C18:0	13.4±0.1 ^{BC}	**17.6±0.3** ^{BC}	**16.1±0.3** ^{BC}	**11.3±0.1**
C18:1t11	0.11±0.04 ^{BC}	0.22±0.05 ^{BC}	0.17±0.06	0.13±0.11^{C}
C18:1n9	28.7±0.2 ^{B}	**19.1±0.4** ^{B}	**15.5±0.1**	**14.1±0.7**
C18:1c11	0.05±0.00	0.08±0.01	0.07±0.00	0.03±0.02
C18:2n6	20.5±0.8^{C}	**15.1±0.2**	**35.9±1.0**^{C}	**12.2±0.3**^{C}
C20:0	0.07±0.00	0.09±0.00	0.09±0.01	0.03±0.02^{BC}
C18:3n6	0.04±0.00	0.05±0.05	ND	0.09±0.01
C18:3n3	0.79±0.04	**13.5±0.8** ^{BC}	**0.36±0.03**	0.10±0.10
C18:2c9t11	ND	ND	ND	**36.7±2.1**
C18:4n3	0.05±0.01	0.02±0.02	0.03±0.02	0.32±0.04
C18:2t10c12	ND	ND	ND	**1.08±0.05** ^{BC}
C20:2n6	2.25±0.02 ^{BC}	**0.83±0.03**	4.03±0.20^{B}	**0.81±0.07**
C20:3n6	0.83±0.02 ^{C}	0.88±0.00	0.68±0.02^{C}	0.70±0.04 ^{B}
C20:3n3	0.02±0.02	ND	ND	ND
C20:4n6	6.29±0.04 ^{BC}	**9.61±0.25** ^{BC}	**4.65±0.17** ^{BC}	7.19±0.33 ^{BC}
C20:4n3	0.05±0.00	0.30±0.08^{BC}	0.21±0.11	0.10±0.02
C20:5n3	0.32±0.01	0.67±0.10 ^{B}	0.28±0.13	**0.15±0.02** ^{BC}
C22:3n3	0.17±0.14	0.50±0.01	0.02±0.00	0.05±0.01 ^{B}
C22:4n6	0.28±0.13	0.20±0.01^{C}	0.42±0.03	0.07±0.04^{C}
C22:1n9	2.78±0.09 ^{BC}	**1.89±0.07** ^{BC}	**1.84±0.13** ^{B}	**1.15±0.11** ^{BC}
C22:5n6	0.63±0.02 ^{B}	0.49±0.06	**1.30±0.04** ^{B}	**0.02±0.01**
C22:5n3	1.10±0.04 $^{B\,C}$	1.06±0.10 ^{B}	**0.73±0.04** $^{B\,C}$	0.97±0.06 $^{B\,C}$
C22:6n3	1.20±0.09 $^{B\,C}$	**0.74±0.07** $^{B\,C}$	**0.75±0.06** $^{B\,C}$	1.08±0.02 $^{B\,C}$
ΣSFA	32.3±0.5 ^{C}	33.7±0.4 ^{BC}	32.0±0.5 ^{C}	**22.1±0.5**
ΣMUFA	33.1±0.2	**22.4±0.5** ^{B}	**18.6±0.2**	**16.3±0.7** ^{B}
ΣPUFA	34.6±0.7 ^{BC}	**43.9±0.8** ^{BC}	**49.4±0.7** ^{BC}	**61.6±1.1** ^{B}
Σn-3	3.70±0.05 ^{B}	**16.8±0.6** ^{BC}	2.37±0.11	2.77±0.11
Σn-6	30.9±0.7^{BC}	27.2±0.2 ^{BC}	**47.0±0.6** ^{BC}	**22.2±0.5**^{C}

Values are mean percent composition ± SEM (n=3 per cell line per treatment). Bold values indicate a significant difference in comparison to the OA/LA control ($p < 0.05$). ^{B} and ^{C} indicate significant differences compared to MCF-7 and MDA-MB-231 cell lines, respectively ($p < 0.05$). ND indicates not detectable.

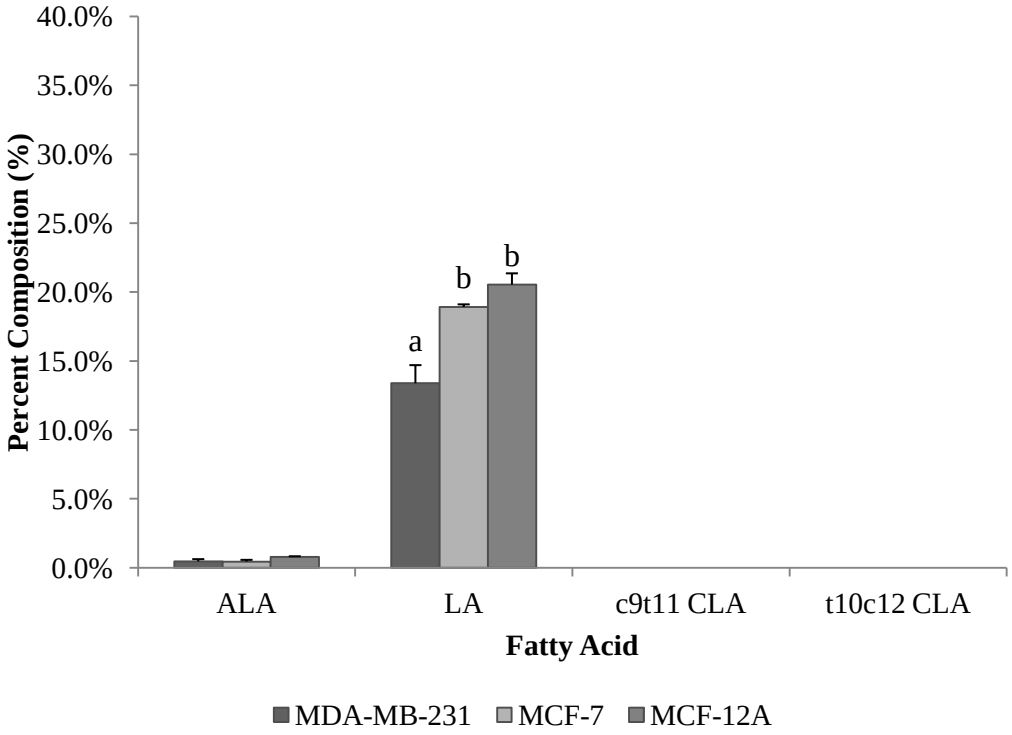

Figure 6.2 ALA, LA and CLA Content in Whole Cell Phospholipids of MDA-MB-231, MCF-7 and MCF-12A Cells After Incubation with OA/LA Control Media. Values are mean percent composition ± SEM (n=3-5 per cell line). Different letters within a fatty acid indicate a significant difference between cell lines ($p < 0.05$).

Cells grown in the OA/LA fatty acid control media have very low levels of ALA, and CLA isomers could not be detected in the PLs (Figure 6.2). LA from this media was readily incorporated into the cell membranes, making up 13.8-20.54% of the PL, where MDA-MB-231 cells had significantly lower levels of LA in comparison to the other two cell lines. This fatty acid profile will be used as the comparison for the subsequent treatments being investigated.

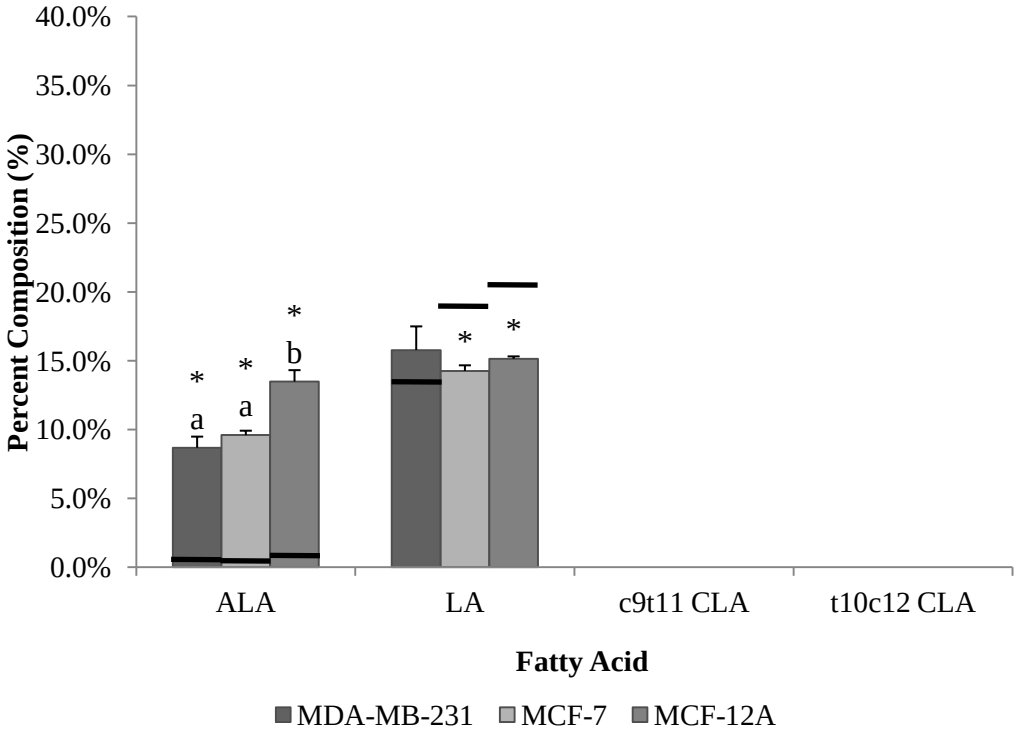

Figure 6.3 ALA, LA and CLA Content in Whole Cell Phospholipids of MDA-MB-231, MCF-7 and MCF-12A Cells After Incubation with 150µM ALA. Values are mean percent composition ± SEM (n=3-5 per cell line). Black horizontal bars indicate the concentration of the fatty acid after treatment with OA/LA (Figure 6.2). * indicate a significant difference ($p<0.05$) in the incorporation into the PLs in comparison to the OA/LA treatment. Different letters within a fatty acid indicate a significant difference between cell lines ($p < 0.05$).

ALA levels significantly increased in comparison to the control values with ALA treatment (Figure 6.3). MCF-12A cells incorporated ALA significantly more than the tumorigenic cell lines. LA levels significantly decreased in comparison to the OA/LA control in the MCF-7 and MCF-12A cell lines. CLA isomers could not be detected with ALA treatment in any cell line.

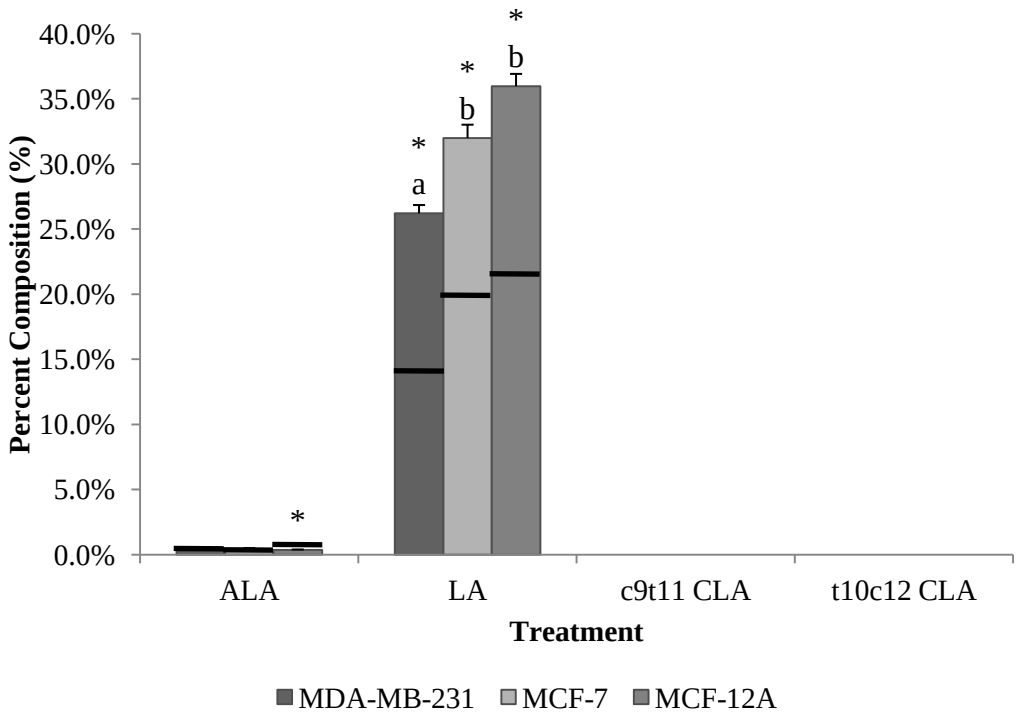

Figure 6.4 ALA, LA and CLA Content in Whole Cell Phopholipids of MDA-MB-231, MCF-7 and MCF-12A Cells After Incubation with 150µM LA. Values are mean percent composition ± SEM (n=3-5 per cell line). Black horizontal bars indicate the concentration of the fatty acid after treatment with OA/LA (Figure 6.2). * indicate a significant difference ($p<0.05$) in the incorporation into the PLs in comparison to the OA/LA treatment. Different letters within a fatty acid indicate a significant difference between cell lines ($p < 0.05$).

Cells incubated with LA showed significant increases in LA content in comparison to the control values in all cell lines (Figure 6.4). ALA content in the PLs remained low, and a significant decrease was observed in MCF-12A cells. CLA isomers could not be detected with LA treatment.

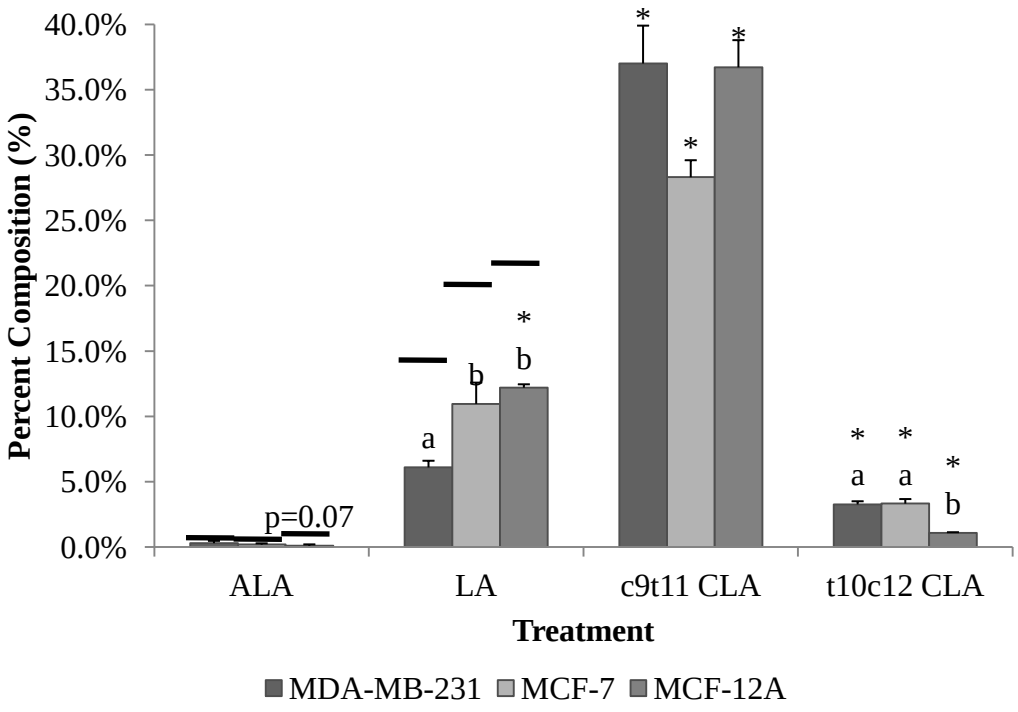

Figure 6.5 ALA, LA and CLA Content in Whole Cell Phospholipids of MDA-MB-231, MCF-7 and MCF-12A Cells After Incubation with 150μM c9, t11-CLA. Values are mean percent composition ± SEM (n=3-5 per cell line). Black horizontal bars indicate the concentration of the fatty acid after treatment with OA/LA (Figure 6.2). * indicate a significant difference ($p<0.05$) in the incorporation into the PLs in comparison to the OA/LA treatment. Different letters within a fatty acid indicate a significant difference between cell lines ($p < 0.05$).

In Figure 6.5, it can be seen that c9, t11-CLA incubation significantly increased both c9, t11-CLA and t10, c12 CLA levels in the PL of all cells lines. The c9, t11-CLA content was higher than t10, c12-CLA content, ranging from about 26-37% of PL compared to 1-3%, respectively. The content of t10, c12 CLA content was much higher in tumorigenic cells than MCF-12A cells, however, this difference was not observed in c9, t11-CLA content. ALA and LA content in the PL decreased after treatment with c9, t11-CLA, however, only the decrease in LA levels in MCF-12A cells reached significance.

C. N-6:N-3 Ratios

Table 6.4 N-6:N-3 Ratios of Whole Cell Phospholipids of MDA-MB-231, MCF-7 and MCF-12A Cells Incubated with 150μM Fatty Acid Treatment.

Treatment	Cell Line		
	MDA-MB-231	MCF-7	MCF-12A
Media	1.55±0.30[a]	2.73±0.32[b]	**1.59±0.03 [ab]**
OA/LA	7.14±1.02	17.5±4.1	8.34±0.38
ALA	1.77±0.14	1.67±0.06	**1.63±0.07**
LA	10.7±0.9[a]	26.9±5.0[b]	**20.0±1.0**[ab]
CLA	5.93±0.53	9.39±1.65	8.02±0.36

Values are means ± SEM (n=3-5 per cell line per treatment). Bold values indicate a significant difference compared to the cell line's respective OA/LA control ($p < 0.05$). Different letters within a treatment indicate a significant difference between cell lines ($p < 0.05$).

From Table 6.4, we can see that the n-6:n-3 ratios did not change significantly with CLA treatment compared to the OA/LA control. The n-6:n-3 ratios did not differ between cell lines with CLA treatment.

IV. Discussion

A. Cell Viability

Of the numerous CLA isomers available, we chose to investigate the effects of the c9, t11-CLA isomer, as it is the most common CLA isomer in the diet (3). C9, t11-CLA was found to inhibit the growth of both our tumorigenic cells lines. Literature reports concentrations from 25-200μM c9, t11-CLA was able to significantly inhibit the growth of MCF-7 cells in a dose dependent manner, but, MDA-MB-231 cell growth was not affected (11-13). However, inhibitory effects of c9, t11-CLA have also been reported on MDA-MB-231 cells (14). Our data confirms the MCF-7 results and agrees with literature indicating an inhibitory effect of this isomer on MDA-MB-231 cells. The discrepancy with previous studies using this cell line may be due to our different culturing conditions, as in our studies we provided sufficient LA which has been found to both limit and enhance tumour growth (15). However, we did observe a significantly greater reduction in cell viability after CLA incubation in MCF-7

cells compared to MDA-MB-231, which agrees with the current literature and suggests a possible effect via the ER in response to CLA (16-19).

Our data suggests that the anti-tumorigenic activity of c9, t11-CLA is different from that of its precursors for bacterial cells LA and ALA, despite common chain length, as non-significant tumour cell inhibition was observed in the MDA-MB-231 cell line with both ALA and LA treatments. LA has two cis-bonds at C9 and C12, while ALA has three cis-bonds at C9, C12 and C15. LA has been previously compared to c9, t11-CLA in terms of effects on cell growth in MCF-7 cells, and results indicate a significant difference between the fatty acid treatments (13). Previous experiments have also found different anti-tumorigenic activities between CLA isomers, as the t10, c12-CLA isomer has been found to inhibit breast tumour growth more than the c9, t11-isomer (20). Therefore, the placements of double bonds along the fatty acid chain and possibly bond configuration (cis- or trans-) have a greater effect on anti-tumorigenic activity than chain length or number of bonds. Which bond locations are the most important for fatty acids to exhibit anti-tumorigenic activity warrants further investigation.

To our knowledge, cell viability has not been previously assessed the MCF-12A cell line. However, in the non-tumorigenic breast MCF-10A cell line, c9, t11-CLA did not inhibit cell viability at any concentration up to 20μM compared to untreated control cells, but at higher concentrations significant inhibition was observed (21). Considering our CLA concentration was much higher, there is a discrepancy between our results. However, these differences may be accounted for by the different cell line, media conditions and that the CLA used in the cited reference was isolated from methyl esters before conjugation to BSA. The method through which fatty acids are delivered to breast cells have been found to affect cell growth differently (15). LA at low dosages under 30μM have also been found to stimulate MCF-10A cell growth by as much as 50%, which could counter some of the inhibitory effects on growth by CLA (15). LA has not been previously supplemented to MCF-12A cells prior to testing the anti-cancer activity of other fatty acids, to our knowledge.

Overall, c9, t11-CLA inhibited the growth of both MDA-MB-231 and MCF-7 tumorigenic cell lines but not the non-tumorigenic MCF-12A cells line like other anti-cancer fatty acids we have tested (ALA, SDA, LA, GLA). Due to this similarity, it is possible that n-3 and n-6 fatty acids and c9, t11-CLA have a common anti-cancer mechanism.

B. Phospholipid Composition

CLA was significantly incorporated into the membranes of both tumorigenic cell lines as well as a non-tumorigenic cell line. Previous findings also show incorporation of CLA into tumorigenic cell lines, however, incorporation into non-tumorigenic breast cell lines has not been previously recorded to our knowledge (10). This study confirmed that human breast cancer cell lines, similar to non-tumorigenic cells, are unable to convert ALA or LA to c9, t11-CLA. This finding is consistent with previous studies from our group in MCF-7 cells (10). We also demonstrated that treatment with c9, t11-CLA significantly increased the content of t10, c12-CLA in all three cell lines, but to a greater extent in the MCF-7 and MDA-MB-231 cells. CLA isomerism has been previously observed for breast cancer and hepatoma cells (10, 22-24). T10, c12-CLA has been found to have anti-tumorigenic effects stronger than c9, t11-CLA on breast cancer cells, so the conversion to this isomer may contribute to the anti-tumorigenic effects of the c9, t11-CLA treatment observed in this study (10, 25).

CLA treatment resulted in lower concentration of LA in MCF-12A cell PLs but not in the other cell lines. These results suggest that CLA may replace LA levels in the PL membrane, similarly to other single n-3 and n-6 fatty acid treatments in MCF-12A cells as discussed in chapter four. However, previous findings have found that CLA did not replace LA in phosphatidylcholine and phosphatidylethanolamine fractions of mammary tissues, indicating that further analysis of our treatments may be required of different PL classes, as they have different functions in the cell (26). No other significant changes in n-3 and n-6 fatty acid levels with CLA treatment on the tumorigenic cell lines indicate the anti-tumorigenic activity may not be determined by LA content in membrane PLs.

Previous studies in MCF-7 cells found decreases in DGLA, AA and DHA content in membranes in a c9, t11-CLA and LA mixture in comparison to untreated cells, however, due to a different background fatty acid mixture being used, our results cannot be compared (10). CLA also did not change the n-6:n-3 ratio in comparison to the OA/LA control for any of the cell lines, possibly due to the wide variability between replicates. Instead, we found that CLA significantly decreased total SFA levels in the membrane, which was not observed with n-3 or n-6 treatments. This difference could contribute to c9, t11-CLA's anti-tumorigenic effects. Also, c9, t11-CLA seemed to be incorporated more readily into the membranes than n-3 and n-6 fatty acids. These changes in membrane composition caused by c9, t11-CLA are different from those that occur with the n-3 and n-6 fatty acid treatments, suggesting that there may be different mechanisms through which these fatty acids act on through changes in the membrane. Future work could isolate PL classes and lipid rafts, which are important in cell signaling.

In MCF-12A cells, significant decreases in various longer fatty acid levels were also found, which is similar to our results in single n-3 and n-6 fatty-acid treatments and the SO treatment, further supporting the suggestion that there may be a fatty acid balance with in the composition of the PL membrane that is more important than the content of the longer-chained fatty acids. This balance may not be maintained in the tumorigenic cells, leading to the anti-carcinogenic effects of bioactive lipids. To our knowledge, the effects of CLA isomers have not been studied on normal breast cells in terms of viability and changes in the PL membrane composition, and these results show the changes normal cells undergo with c9, t11-CLA incubation.

V. Summary

In conclusion, we confirmed that treatment with c9, t11-CLA has inhibitory effects on MDA-MB-231 and MCF-7 cells. These inhibitory effects are accompanied by significant incorporation of c9, t11-CLA into membrane PLs and conversion to and incorporation of t10, c12-CLA. These effects were

different from its 18-carbon precursors ALA and LA, suggesting that bond position and configuration may be important in determining fatty acid anti-tumorigenic activity. Some of the changes in PLs after CLA treatment were similar to those of n-3 and n-6 treatments, but others were unique to c9, t11-CLA treatment. These differences suggest different mechanisms in terms of changes in lipid composition and anti-tumour activity between c9, t11-CLA and n-3 and n-6 fatty acid treatments. We also found that the growth of non-tumorigenic breast cells was not affected by c9, t11-CLA treatment at this dose, which has not been previously studied in this cell line. These cells showed a different response in terms of changes in PL composition, as we observed significant decreases in levels of various longer fatty acid only in MCF-12A cells, and not MDA-MB-231 and MCF-7 cells. These observations were also seen with n-3 and n-6 fatty acids incubation on MCF-12A cells as well, suggesting that normal breast cell membrane homeostasis mechanisms exist that are not in tumorigenic cells. These mechanisms may contribute to the lack of change in cell viability with c9, t11-CLA treatment. By identifying important changes in membrane composition that affects anti-tumorigenic activity, more efficient breast cancer therapies can be identified. However, further investigations into changes in PL classes are required, due to the different functions of different PL classes in cells. Incorporation may be different between classes, which would affect cell signaling.

VI. References

1. Kepler CR, Hirons KP, Mcneill JJ, Tove SB. Intermediates and products of biohydrogenation of linoleic acid by butyrivibrio fibrisolvens. J Biol Chem. 1966 1966;241(6):1350,&.

2. Kemp P, Lander DJ. The hydrogenation of gamma-linolenic acid by pure cultures of 2 rumen bacteria. Biochem J. 1983 1983;216(2):519-22.

3. Schmid A, Collomb M, Sieber R, Bee G. Conjugated linoleic acid in meat and meat products: A review. Meat Sci. 2006 May;73(1):29-41.

4. Herbel BK, McGuire MK, McGuire MA, Shultz TD. Safflower oil consumption does not increase plasma conjugated linoleic acid concentrations in humans. Am J Clin Nutr. 1998 Feb;67(2):332-7.

5. Rosberg-Cody E, Ross RP, Hussey S, Ryan CA, Murphy BP, Fitzgerald GF, et al. Mining the microbiota of the neonatal gastrointestinal tract for conjugated linoleic acid-producing bifidobacteria. Appl Environ Microbiol. 2004 Aug 2004;70(8):4635-41.

6. Coakley M, Ross RP, Nordgren M, Fitzgerald G, Devery R, Stanton C. Conjugated linoleic acid biosynthesis by human-derived bifidobacterium species. J Appl Microbiol. 2003 2003;94(1):138-45.

7. Turpeinen AM, Mutanen M, Aro A, Salminen I, Basu S, Palmquist DL, et al. Bioconversion of vaccenic acid to conjugated linoleic acid in humans. Am J Clin Nutr. 2002 Sep 2002;76(3):504-10.

8. De La Torre A, Debiton E, Durand D, Chardigny JM, Berdeaux O, Loreau O, et al. Conjugated linoleic acid isomers and their conjugated derivatives inhibit growth of human cancer cell lines. Anticancer Res. 2005 Nov-Dec 2005;25(6B):3943-9.

9. Ip C, Chin SF, Scimeca JA, Pariza MW. Mammary cancer prevention by conjugated dienoic derivative of linoleic acid. Cancer Res. 1991 Nov 15;51(22):6118-24.

10. Amaru DL, Field CJ. Conjugated linoleic acid decreases mcf-7 human breast cancer cell growth and insulin-like growth factor-1 receptor levels. Lipids. 2009 May;44(5):449-58.

11. Tanmahasamut P, Liu J, Hendry LB, Sidell N. Conjugated linoleic acid blocks estrogen signaling in human breast cancer cells. J Nutr. 2004 Mar;134(3):674-80.

12. Chujo H, Yamasaki M, Nou S, Koyanagi N, Tachibana H, Yamada K. Effect of conjugated linoleic acid isomers on growth factor-induced proliferation of human breast cancer cells. Cancer Lett. 2003 Dec 8;202(1):81-7.

13. Durgam VR, Fernandes G. The growth inhibitory effect of conjugated linoleic acid on MCF-7 cells is related to estrogen response system. Cancer Lett. 1997 Jun 24;116(2):121-30.

14. Miglietta A, Bozzo F, Bocca C, Gabriel L, Trombetta A, Belotti S, et al. Conjugated linoleic acid induces apoptosis in MDA-MB-231 breast cancer cells through ERK/MAPK signalling and mitochondrial pathway. Cancer Lett. 2006 Mar 28;234(2):149-57.

15. Grammatikos S, Subbaiah P, Victor T, Miller W. N-3 and N-6 fatty-acid processing and growth effects in neoplastic and non-cancerous human mammary epithelial-cell lines. Br J Cancer. 1994 Aug;70(2):219-27.

16. Choi Y, Park Y, Storkson JM, Pariza MW, Ntambi JM. Inhibition of stearoyl-CoA desaturase activity by the cis-9, trans-11 isomer and the trans-10,cis-12 isomer of conjugated linoleic acid in MDA-MB-231 and MCF-7 human breast cancer cells. Biochem Biophys Res Commun. 2002 Jun 21;294(4):785-90.

17. Bocca C, Bozzo F, Cannito S, Colombatto S, Miglietta A. CLA reduces breast cancer cell growth and invasion through ER alpha and PI3K/Akt pathways. Chem Biol Interact. 2010 Jan 5;183(1):187-93.

18. Wang L, Huang Y, Liu S, Yan P, Lin YC. Conjugated linoleic acid induces apoptosis through estrogen receptor alpha in human breast tissue. BMC Cancer. 2008 Jul 24;8:208.

19. Majumder B, Wahle K, Moir S, Schofield A, Choe S, Farquharson A, et al. Conjugated linoleic acids (CLAs) regulate the expression of key apoptotic genes in human breast cancer cells. Faseb Journal. 2002 Jul;16(9):1447,+.

20. Lau DSY, Archer MC. The 10t,12c isomer of conjugated linoleic acid inhibits fatty acid synthase expression and enzyme activity in human breast, colon, and prostate cancer cells. Nutr Cancer. 2010;62(1):116-21.

21. Rakib MA, Kim YS, Jang WJ, Choi BD, Kim JO, Kong IK, et al. Attenuation of 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced gap junctional intercellular communication (GJIC) inhibition in MCF-10A cells by c9, t11-conjugated linoleic acid. J Agric Food Chem. 2010 Nov 24;58(22):12022-30.

22. Huot PS, Sarkar B, Ma DW. Conjugated linoleic acid alters caveolae phospholipid fatty acid composition and decreases caveolin-1 expression in MCF-7 breast cancer cells. Nutr Res. 2010 Mar;30(3):179-85.

23. Ma D, Field C, Clandinin M. An enriched mixture of trans-10,cis-12-CLA inhibits linoleic acid metabolism and PGE(2) synthesis in NWA-MB-231 cells. Nutrition and Cancer-an International Journal. 2002;44(2):202-12.

24. Igarashi M, Miyazawa T. The growth inhibitory effect of conjugated linoleic acid on a human hepatoma cell line, HepG2, is induced by a change in fatty acid metabolism, but not the facilitation of lipid peroxidation in the cells. Biochimica Et Biophysica Acta-Molecular and Cell Biology of Lipids. 2001 Feb 26;1530(2-3):162-71.

25. Drouin G, Douillette A, Lacasse P, Paquette B. Radiosensitizing effect of conjugated linoleic acid in MCF-7 and MDA-MB-231 breast cancer cells. Can J Physiol Pharmacol. 2004 Feb;82(2):94-102.

26. Ip C, Briggs SP, Haegele AD, Thompson HJ, Storkson J, Scimeca JA. The efficacy of conjugated linoleic acid in mammary cancer prevention is independent of the level or type of fat in the diet. Carcinogenesis. 1996 May 1996;17(5):1045-50.

Chapter Seven – General Discussion and Conclusions

I. Summary of Results

A. N-3 and N-6 Fatty Acids

In this thesis, the effects of n-3 and n-6 fatty acids and their intermediates on two human tumorigenic breast cell lines, ER-PR- MDA-MB-231 and ER+PR+ MCF-7, and one non-tumorigenic breast cell line, MCF-12A were studied. After 48 hours of incubation with 150μM fatty acid, we assessed the treatment's effects on the cell viability and whole cell PLs compared to a no fatty acid or OA/LA control treatment. From our experiments we wanted to determine if:

> ***i.*** *n-3 and n-6 fatty acids (n-3: ALA, SDA, ETA, EPA and DHA; n-6: LA, GLA, DGLA and AA) will decrease the growth of tumorigenic cells without affecting the growth of non-tumorigenic cells*

First investigating cell viability, we found that n-3 and n-6 metabolic pathway intermediates SDA, ETA, GLA and DGLA inhibited the viability of two tumorigenic breast cell lines. SDA and GLA did not negatively affect the viability of a non-tumorigenic cell line, as hypothesized. ALA and LA only inhibited MCF-7 cell viability, while AA only inhibited MDA-MB-231 cell viability. As expected, EPA and DHA inhibited the growth of both tumorigenic cell lines.

> ***ii.*** *individual n-3 and n-6 fatty acids will be incorporated into the PLs of all cells. The proportion of n-3 and n-6 fatty acids incorporated into membrane PLs will be greater in tumorigenic cells than non-tumorigenic cells after incubations*

In the whole cell PL, we found that each of the five n-3 treatment fatty acids (ALA, SDA, ETA, EPA and DHA) and four n-6 fatty acids (LA, GLA, DGLA, and AA) were incorporated into the membranes of both tumorigenic cell lines, which the exception of SDA in MDA-MB-231 cells, possibly due to its immediate conversion to ETA (Figure 7.1-2). Similar PL incorporation was

observed in MCF-12A cells with ALA, SDA, LA and GLA treatments (Figure 7.3).

iii. *n-3 and n-6 fatty acid intermediate supplementation (SDA, ETA, GLA and DGLA) will result in their conversion to their longer-chained counterparts and will be reflected in the fatty acid content of the cell PL*

Examining changes in n-3 and n-6 fatty acid content in breast cells by fatty acid intermediates in the n-3 and n-6 fatty acid pathway, we did not find significant increases in levels of pathway intermediates and products after ALA and LA incubation most likely due to a low catalytic activity of Δ-6 desaturase, as summarized in Figures 7.1-3. However, increases in Δ8-desaturase substrates C20:3n3 and C20:2n6 were found, suggesting the use of an alternate pathway. Treatments with n-3 pathway intermediates SDA and ETA showed significant increases in the ETA and EPA content in MDA-MB-231 cell PL, while they only accumulated in MCF-7 cell PLs. SDA treatment accumulated ETA, EPA and DPA in MCF-12A cells. EPA treatment only increased DPA and DHA content in both tumorigenic cell lines. N-6 intermediate GLA treatment resulted in increases in DGLA PL content, while AA treatment increased levels of C22:4n6. Although conversions of fatty acids through the catalytic activity of the Δ6-desaturase were observed, it was not to pathway endpoints DHA and AA, as expected. We also found significant increases in C20:3n3 and C20:2n6 levels with ALA and LA treatment, respectively, in MDA-MB-231 cells, suggesting the use of an alternate pathway involving a Δ8-desaturase enzyme for metabolism to longer chain intermediates.

We also examined n-6:n-3 ratios in whole cell PL, and found that none of the n-6:n-3 ratios in breast cell PLs were significantly different from control OA/LA treatment in tumorigenic cells; however, ratios of all treatments with MCF-12A cells were significantly different from the control (decreased with n-3 ALA and SDA treatment, and increased with n-6 LA and GLA treatment). These changes in ratios did not match the results on cell viability, suggesting that this ratio in whole cell PLs is not a useful estimation for tumour viability. It also suggests that tumour cells compensate differently than non-tumorigenic cells

when n-3 and n-6 fatty acids are incorporated into the membrane PLs. In addition, we estimated desaturase activity based on whole cell PLs and found it was low in all cell lines. Comparison of estimated activities between two different concentrations of LA suggested increased activity with a lower concentration of LA.

Overall, we found that n-3 and n-6 fatty acid intermediates inhibited breast tumour cell growth without negatively affecting normal cell growth, and that these intermediates have anti-cancer effects themselves, independent of their conversion and incorporation into pathway endpoints in PLs.

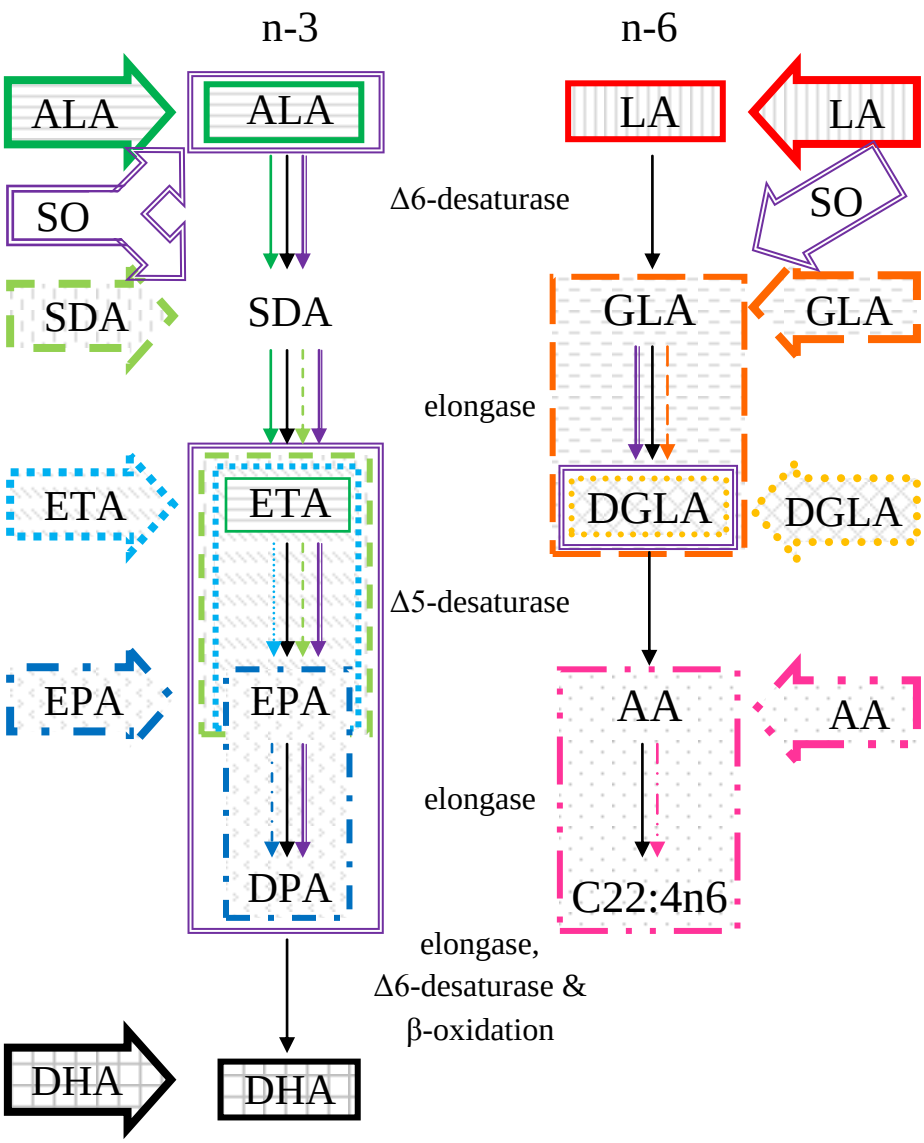

Figure 7.1 Fatty Acid Conversion of N-3 and N-6 Fatty Acid and SO Treatment Incorporated into MDA-MB-231 Cell Phospholipids. Large arrows pointing at fatty acids in the pathway indicate treatment fatty acid. Small solid arrows between pathway fatty acids indicate the main metabolic pathway in humans, while patterned arrows indicate conversion of treatment fatty acids. Boxes indicate significant fatty acid incorporation into PLs.

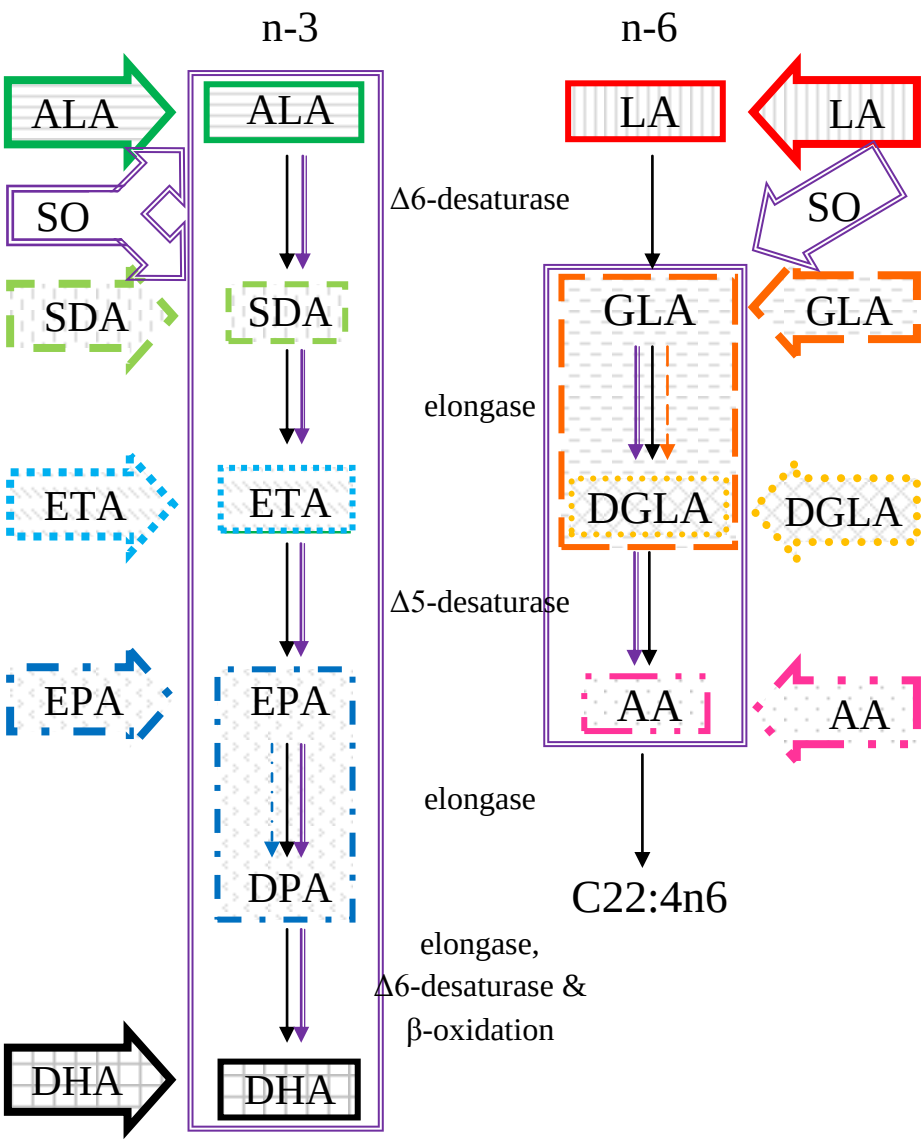

Figure 7.2 Fatty Acid Conversion of N-3 and N-6 Fatty Acid and SO Treatment Incorporated into MCF-7 Cell Phospholipids. Large arrows pointing at fatty acids in the pathway indicate treatment fatty acid. Small solid arrows between pathway fatty acids indicate the main metabolic pathway in humans, while patterned arrows indicate conversion of treatment fatty acids. Boxes indicate significant fatty acid incorporation into PLs.

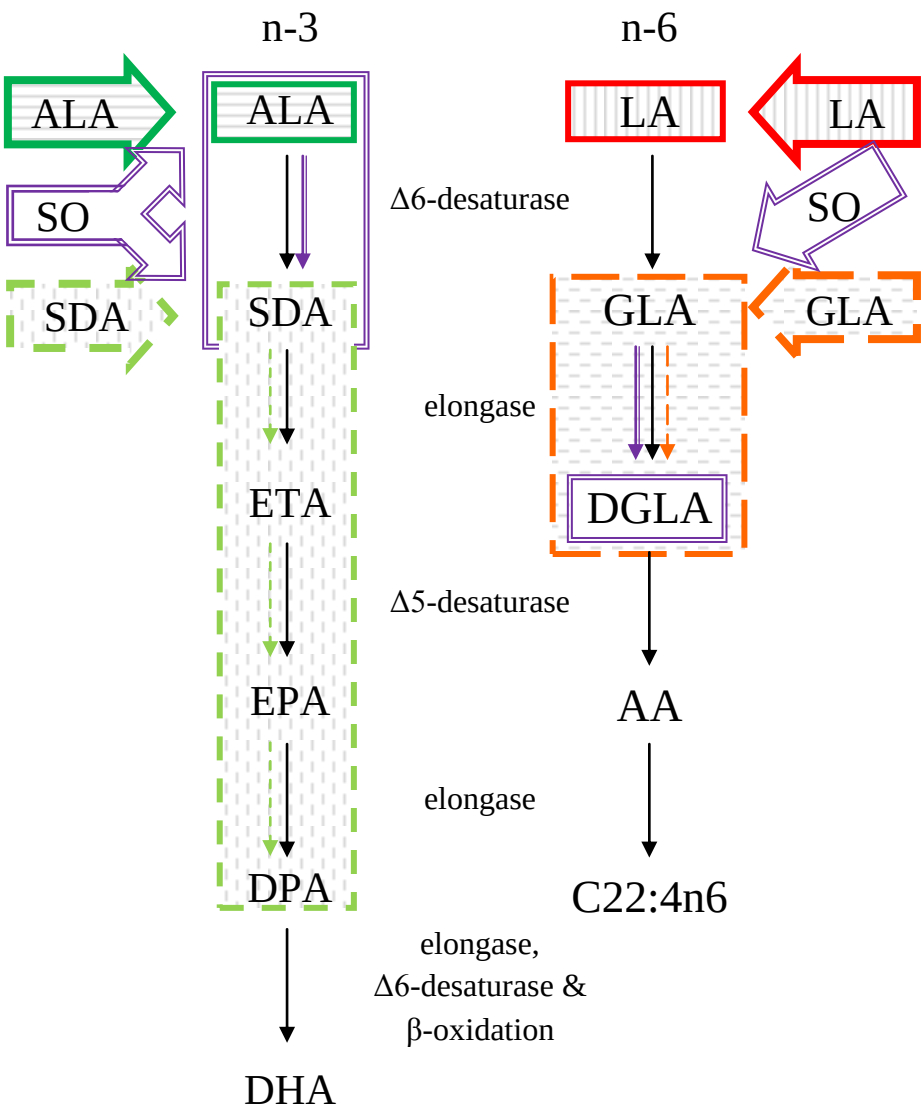

Figure 7.3 Fatty Acid Conversion of N-3 and N-6 Fatty Acid and SO Treatment Incorporated into MCF-12A Cell Phospholipids. Large arrows pointing at fatty acids in the pathway indicate treatment fatty acid. Small solid arrows between pathway fatty acids indicate the main metabolic pathway in humans, while patterned arrows indicate conversion of treatment fatty acids. Boxes indicate significant fatty acid incorporation into PLs.

B. SDA-Enriched Flax Seed Oil Mixture

This thesis also investigated the effects of a fatty acid mixture mimicking the fatty acid content of a novel SDA-enriched flax oil (SO). This oil is mainly composed of 2 n-3 and a n-6 fatty acid: ALA, SDA and GLA. Using the same cell lines and treatments conditions in the investigation of the effects of n-3 and n-

6 pathway intermediates, cell viability and whole cell PLs were assessed in cells treated with this mixture. Through these experiments, we wanted to determine whether:

i. *a mixture of fatty acids representative of the composition of an SDA-enriched flax seed oil will reduce cell growth in tumorigenic but not non-tumorigenic human breast cells*

As predicted, SO treatment showed similar inhibitory effects on the viability of tumorigenic cell MDA-MB-231 and MCF-7 cells, as compared to providing its main components ALA, SDA and GLA. This same mixture, however, did not negatively affect the growth of normal MCF-12A cells, in agreement with the effects of its main components.

ii. *fatty acids within an oil that represents the composition of an SDA-enriched flax seed oil will exhibit fatty acid conversion to their longer-chained counterparts and be incorporated into the PLs to a greater extent in tumorigenic cells than non-tumorigenic cells*

Changes in the membrane PLs were similar to that of the individual fatty acid treatments of its major components: ALA, SDA and GLA. SDA was converted to EPA and GLA was converted to DGLA, in MDA-MB-231 cells, while conversion of GLA to DGLA was observed in MCF-7 cells with SO treatment (Figures 7.1-2). ALA and SDA from the SO were converted further to DPA in MDA-MB-231 cells and DHA in the MCF-7 cells. Significant conversion of GLA to AA was also observed with SO treatment. In MCF-12A cell lines, incorporation of converted fatty acids was less extensive, only increasing levels of ALA, SDA and DGLA in PLs (Figure 7.3). Therefore, there was less incorporation of converted SO fatty acids in the PLs of MCF-12A cells, compared to the tumorigenic cell lines. This difference in PL incorporation may relate to their anti-growth activity in tumorigenic cells.

Overall, we found that SO has inhibitory effects on cell viability specific to tumorigenic breast cell lines, similarly to its main components ALA, SDA and GLA. This inhibitory effect was accompanied by fatty acid conversion and

incorporation into whole cell membrane PLs further down the n-3 and n-6 metabolic pathways than its main individual components, suggesting that providing mixtures of fatty acids alters the ability of cells to convert and incorporate the downstream longer chain products of reactions involving desaturase and elongase enzymes.

C. C9, t11-CLA

The effects of another anti-carcinogenic lipid, c9, t11-CLA on cell viability and whole cell PLs was also investigated in this thesis, using the same cell lines and treatment conditions as the n-3 and n-6 fatty acid experiments. In these experiments, we wanted to ascertain whether:

i. *c9, t11-CLA will not negatively affect the growth of non-tumorigenic breast cells*

C9, t11-CLA treatment was found to inhibit the viability of both tumorigenic cell lines. As hypothesized, incubation with c9, t11-CLA did not inhibit the viability of non-tumorigenic MCF-12A breast cells.

ii. *c9, t11-CLA will alter fatty acid tumour PL composition, replacing n-6 fatty acids in the PL*

In whole cell PL, we examined CLA and n-3 and n-6 fatty acid content with c9, t11-CLA treatment. We found c9, t11-CLA content increased in all cell lines with c9, t11-CLA treatment. Unexpectedly, increases in t10, c12-CLA levels, and decreases in total SFA (not observed with n-3 and n-6 fatty acid treatments in tumorigenic cells) were observed with c9, t11-CLA treatment in all cell lines. Decreases in LA content in tumorigenic MDA-MB-231, and decreased LA and EPA content in non-tumorigenic MCF-12A cells were also observed compared to OA/LA control treatments within the same cell line. Compared to tumorigenic cells, MCF-12A cells also showed significant decreases in the content of EPA, DPA, DHA and AA into the PLs with c9, t11-CLA treatment like with n-3 and n-6 fatty acid treatments. This finding suggests there are specific compensations for the incorporation of this lipid into PLs. Similar to our

observations with n-3 and n-6 fatty acid treatments, the n-6:n-3 ratios did not change significantly in tumour cells, and did not correlate with viability results. In summary, c9, t11-CLA treatment selectively decreased cell viability of tumorigenic cells, possibly due to CLA isomerisation.

II. Implications

Previously, it was shown that dietary fat can affect the progression of breast cancer, especially n-3 fatty acids EPA and DHA, n-6 fatty acid AA, and the two major CLA isomers (1-5). Mechanisms through which these fatty acids act include interference with the cell cycle, apoptosis induction, and interference with growth factor signaling, as well as incorporation into cell membrane PLs (6, 7). Changes in tumour cell membrane composition from these fatty acids have been previously associated with inhibitory effects of breast cancer tumours *in vitro* and are suggested to be a mechanism for their anti-cancer effects (8-12).

Despite their benefits, intake of the essential fatty acids (greater than 18 carbons), with demonstrated anti-cancer properties, is low in the North American diet (1). Although humans can convert the dietary available essential fatty acids ALA to EPA and DHA, and LA to AA, conversion rates in the body have been found to be limited by low catalytic activity of the Δ6-desaturase enzyme (13-15). However, we have shown that by bypassing this desaturase enzyme and directly providing pathway intermediates SDA and GLA, significant conversion of these fatty acids to longer-chained counterparts and incorporation of these fatty acids into the membrane PLs can occur, and these changes were associated with decreased tumour cell but not normal cell viability at the same concentration. Interestingly, providing intermediates in the n-6 and n-3 pathway upstream from DHA and AA did not increase the membrane content of DHA or AA—two fatty acids with established anti-tumorigenic properties. These results suggest that these intermediates have anti-tumorigenic activity independent of their conversion and incorporation to biological pathway endpoints in PLs. These results may spark more interest in these fatty acid intermediates as a potential anti-cancer

therapies and their incorporation into current therapies, as EPA and DHA have been found to enhance chemotherapy toxicity in breast cancer patients (16, 17).

Our findings suggesting the use of a Δ8-desaturase pathway in tumour cells is also of interest, as it is not a pathway that has been well studied in humans. To our knowledge, our research is the first to identify the potential presence of an active Δ8-desaturase pathway in breast tumour cells. Depending on the activity of this enzyme in tumorigenic cells compared to the Δ6-desaturase enzyme, other intermediates, C20:2n6 and C20:3n3, may also have anti-tumorigenic activities or can be converted to anti-tumorigenic fatty acids, such as the intermediates investigated in this study.

Despite the observed benefits of n-3 and n-6 metabolic pathway intermediates, there are few sources of these fatty acids in our current food supply. Therefore, by using a novel oil that has higher levels of these intermediates, it may be possible to increase the intake of these intermediates and to provide more potent anti-cancer activity. Our results show that our fatty acid mixture representing an SDA-enriched flax oil has potential anti-tumorigenic effects on breast cancer cells *in vitro* without affecting the viability of normal cells. These results not only demonstrate the potential efficacy of this novel oil on breast cancer cells, but also confirm that there are no negative effects *in vitro* on non-tumorigenic cells. Our results will be useful for determining the commercial potential of this plant oil. This oil appears to have beneficial effects, similar to that of DHA, on breast cancer cells. The incorporation of converted long chain essential fatty acids was higher in cells incubated with this fatty acid mixture than single fatty acids in tumorigenic cells, suggesting that these fatty acids may be more effective when provided in a mixture of fatty acids than individually in a higher dose in terms of fatty acid conversion.

We also observed that changes in the n-6:n-3 ratios in the membranes of tumour cells did not predict changes in cell viability. Therefore, the n-6:n-3 ratio in membrane PLs may not a good predictor of breast tumour growth, as previously suggested (18, 19). As we observed that not all n-3 and n-6 fatty acids have the same inhibitory effect on the same cells, future studies should investigate

which fatty acid is provided and in what mixture when attempting to optimize the retardation of tumour progression.

Through our study with c9, t11-CLA, we found that normal breast cell viability is not affected by c9, t11-CLA, confirming the safety of using this fatty acid as a possible breast cancer treatment. Some of the effects of c9, t11-CLA on the n-3 and n-6 membrane PLs were similar to the effects of n-3 and n-6 fatty acids, like decreasing long chain PUFA content. Therefore, c9, t11-CLA may induce the same changes as n-3 and n-6 fatty acids to exhibit their anti-tumorigenic effects. However, changes in SFA levels and isomerisation to t10, c12-CLA were also observed with c9, t11-CLA treatment. These unique changes may also contribute to the anti-tumorigenic activity of c9, t11-CLA, as SFA has been implicated in breast cancer progression and t10, c12-CLA has been shown to be more potent than c9, t11-CLA on breast cell viability (20, 21). These unique changes suggest a mechanism different from n-3 and n-6 fatty acid treatment on cell viability. Despite these changes in membrane composition, similar PL changes were observed in non-tumorigenic cells, suggesting changes in whole cell PLs may not be a good indicator of anti-tumorigenic activity. Future studies should investigate the differences in treatment fatty acid localization in PL classes and lipid raft-based cell signaling on tumorigenic and non-tumorigenic cells. Through these experiments, which changes in the membrane are the most important in determining anti-cancer effects have yet to be elucidated and need to be investigated.

Our study investigated the effects of our treatment fatty acids in a more physiological environment than previous studies, with concentrations of OA and LA added into the cell culture media that mimic physiological membrane composition. We investigated the effects of the n-3 and n-6 metabolic pathway intermediates as well as a novel SDA oil on breast cancer, which have not been well studied, if at all. We also specifically studied the incorporation of converted fatty acids as a potential anti-cancer mechanism, which has not been previously studied. A strength of our study is the use of a control cell line, rarely used in other cell culture studies, as well as two tumorigenic cell lines to enable a wider

application of the studied treatment fatty acids. Our study revealed that the use of an n-6:n-3 ratio in whole cell PLs to predict breast cancer cell viability to be ineffective, which has been debated in literature, and determined estimated desaturase enzyme activity, which has not been investigated thoroughly in breast cancer cells. From these points, new knowledge is added to the existing pool of literature.

III. Future Directions

The effects of n-3 and n-6 fatty acid intermediates SDA, ETA, GLA and DGLA, and novel plant oils on chronic disease progression are fairly new concepts and have not been investigated thoroughly, nor have the effects of novel plant oils on chronic disease. Because of these new ideas, there are many avenues through which these fatty acids and oils can be investigated.

Our experiments are somewhat limited in that only a single concentration was tested on the cell lines. With only one concentration, it could not be determined whether the n-3 and n-6 fatty acid intermediates exhibited a dose response relationship with cell viability, similar to EPA and DHA, as previously determined (22). By examining multiple concentrations, we would be able to determine the potency and acceptable dosage of these fatty acids on breast tumour cells. Literature has suggested dose-response relationships exist with other fatty acids such as EPA, DHA, LA and AA (4, 23, 24). We also did not study PL classes in the membrane lipids, nor did we study lipid raft composition, which may be important in determining the changes in cell signaling that leads to decreased cell viability in tumour cells, yet no negative changes in normal breast cells. Previous studies have examined the effects of dietary fatty acids on different PL classes and lipid rafts and have found that changes in their composition result in different protein localizations that affect cell signaling (2, 11).

As we focused on the n-3 and n-6 pathway products, we used membrane incorporation as predictors of desaturase activity rather than measuring actual enzyme activity and protein levels with our various treatments. Future studies

should confirm our predictions by determining whether there are decreases in desaturase protein levels and use radio-labeled fatty acids to determine actual fatty acid conversion. Not only have the activity and protein levels of Δ5- and Δ6-desaturase enzymes yet to be investigated, but the Δ8-desaturase should also be investigated. It would be of interest especially when using the intermediates as an anti-cancer therapy to determine mechanisms through which it acts. Our results suggest that total membrane PL incorporation may not be a strong predictor of the effect of these fatty acids on tumour cell growth. It is very possible that the fatty acids or the downstream product were not incorporated into the PL, and directly or indirectly affect mechanisms like eicosanoids production (prostaglandin E_2 and cyclooxygenase-2), chemokine receptor 4 levels, epidermal growth factor receptor delocalization from lipid rafts, and tumour suppressor syndecan-1 that can affect tumour growth, metastasis and apoptosis (2, 11, 12, 25, 26).

Although we studied two tumorigenic cell lines representing the most common types of breast cancer, it is possible that our results only hold *in vitro*. The use of cell lines is the first step in determining the anti-cancer activity of a dietary treatment. The next step would be to move to use an appropriate animal model and determine whether the effects seen *in vitro* are similar to those in a living system, where circulating hormones and eicosanoids, and the diet (including carbohydrates, proteins and other fats) may play a role. Also, through using an animal model, fatty acids can be fed as triglycerides, the major form of dietary fat. The most accurate way to represent physiological conditions in the way fatty acids are delivered to the body has been contested in literature. By eliminating this variable and using an animal model, the animal's natural digestion, absorption and processing of the fat would be the best way to deliver fatty acids in the most accurate physiological method. Animal models would also expose tumours to other factors like circulating hormones and immune system modulators in the body which may have an effect on the progression of cancer. Circulating hormones are of special interest in breast cancer due to the presence of estrogen and progesterone receptors on some types of breast cancer. These

hormones can aggravate the growth and progression of breast cancers when these receptors are present and are proposed to be one of the mechanisms by which n-3 fatty acids mediate their effect on tumour growth/death (27, 28). Desaturase activity is also dependent on circulating estrogen levels, and would affect fatty acid conversion (29). Pro-inflammatory cytokines have also been implicated in the progression of breast cancer (30, 31). Since many of the fatty acids investigated are eicosanoids precursors, it would be interesting to evaluate whether the fatty acid intermediates induce changes in the immune system in order to activate their anti-carcinogenic effects. Previous studies with the more common fatty acids EPA and DHA have found that these fatty acids change the inflammatory state of the body (31).

In this thesis, we studied the fatty acid composition of one novel plant oil. It would be interesting to see if other novel plants with enriched SDA as well as plants enriched with other n-3 and n-6 fatty acid intermediates have similar effects on the viability and PL composition of breast cells. Whether it was the mixture we used that was effective or if any plant oil enriched with intermediates have similar effectiveness on breast cancer viability has not been tested. It would also be important to test this oil within a mixture of fats that are more representative of what is consumed by the population, as people usually do not limit their diet to a single plant oil. When a variety of oils mix within a diet, not only may the proportional dosage of this oil may be considerably lower but other oils may negate the positive effects of this novel oil on breast cancer.

From our observations, CLA and n-3 and n-6 fatty acids exhibit both similar and different effects on the cell membrane PL composition. It may be interesting to see if there is a specific change in the composition that causes changes in tumour viability and will not affect normal cells, as a possible target for dietary intervention. We also determined that bond positioning may be important in terms of anti-tumorigenic activity, therefore, studying at other isomers like t10, c12-CLA may also be of interest. As we only investigated one CLA isomer, examining different isomers could be interesting to see if there are certain bonds or combination of bonds important for anti-tumorigenic effects.

IV. References

1. Macronutrients,A Report of the Panel on, Intakes,Subcommittees on Upper Reference Levels of Nutrients and Interpretation and Uses of Dietary Reference, Intakes,Standing Committee on the Scientific Evaluation of Dietary Reference. Dietary reference intakes for energy, carbohydrate, fiber, fat, fatty acids, cholesterol, protein, and amino acids (macronutrients). The National Academies Press; 2005.

2. Schley PD, Brindley DN, Field CJ. (n-3) PUFA alter raft lipid composition and decrease epidermal growth factor receptor levels in lipid rafts of human breast cancer cells. J Nutr. 2007 Mar;137(3):548-53.

3. Schonberg S, Krokan HE. The inhibitory effect of conjugated dienoic derivatives (cla) of linoleic-acid on the growth of human tumor-cell lines is in part due to increased lipid-peroxidation. Anticancer Res. 1995 Jul-Aug 1995;15(4):1241-6.

4. Bocca C, Bozzo F, Martinasso G, Canuto RA, Miglietta A. Involvement of PPAR alpha in the growth inhibitory effect of arachidonic acid on breast cancer cells. Br J Nutr. 2008 Oct;100(4):739-50.

5. Ritzenthaler KL, McGuire MK, Falen R, Shultz TD, Dasgupta N, McGuire MA. Estimation of conjugated linoleic acid intake by written dietary assessment methodologies underestimates actual intake evaluated by food duplicate methodology. J Nutr. 2001 May;131(5):1548-54.

6. Sawyer MB, Field CJ. Possible mechanisms of omega-3 PUFA anti-tumour action. Dietary Omega-3 Polyunsaturated Fatty Acids and Cancer. 2010;1:3-38.

7. Amaru DL, Biondo PD, Field CJ. The role of conjugated linoleic acid in breast cancer growth and development. The Open Nutraceuticals Journal. 2010;3:30-46.

8. Grammatikos S, Subbaiah P, Victor T, Miller W. N-3 and N-6 fatty-acid processing and growth effects in neoplastic and non-cancerous human mammary epithelial-cell lines. Br J Cancer. 1994 Aug;70(2):219-27.

9. Schley P, Jijon H, Robinson L, Field C. Mechanisms of omega-3 fatty acid-induced growth inhibition in MDA-MB-231 human breast cancer cells. Breast Cancer Res Treat. 2005 Jul;92(2):187-95.

10. Byon CH, Hardy RW, Ren C, Ponnazhagan S, Welch DR, McDonald JM, et al. Free fatty acids enhance breast cancer cell migration through plasminogen activator inhibitor-1 and SMAD4. Lab Invest. 2009 Nov;89(11):1221-8.

11. Rogers KR, Kikawa KD, Mouradian M, Hernandez K, McKinnon KM, Ahwah SM, et al. Docosahexaenoic acid alters epidermal growth factor receptor-related signaling by disrupting its lipid raft association. Carcinogenesis. 2010 Sep;31(9):1523-30.

12. Sun H, Berquin IM, Owens RT, O'Flaherty JT, Edwards IJ. Peroxisome proliferator-activated receptor gamma-mediated up-regulation of syndecan-1 by n-3 fatty acids promotes apoptosis of human breast cancer cells. Cancer Res. 2008 Apr 15;68(8):2912-9.

13. Yamazaki K, Fujikawa M, Hamazaki T, Yano S, Shono T. Comparison of the conversion rates of alpha-linolenic acid (18:3(n - 3)) and stearidonic acid (18:4(n - 3)) to longer polyunsaturated fatty acids in rats. Biochim Biophys Acta. 1992 Jan 3;1123(1):18-26.

14. Burdge GC, Wootton SA. Conversion of alpha-linolenic acid to eicosapentaenoic, docosapentaenoic and docosahexaenoic acids in young women. Br J Nutr. 2002 Oct;88(4):411-20.

15. Pawlosky RJ, Hibbeln JR, Novotny JA, Salem N,Jr. Physiological compartmental analysis of alpha-linolenic acid metabolism in adult humans. J Lipid Res. 2001 Aug;42(8):1257-65.

16. Menendez J, Barbacid M, Montero S, Sevilla E, Escrich E, Solanas M, et al. Effects of gamma-linolenic acid and oleic acid on paclitaxel cytotoxicity in human breast cancer cells. Eur J Cancer. 2001 Feb;37(3):402-13.

17. Menendez J, Ropero S, del Mar Barbacid M, Montero S, Solanas M, Escrich E, et al. Synergistic interaction between vinorelbine and gamma-linolenic acid in breast cancer cells. Breast Cancer Res Treat. 2002 Apr;72(3):203-19.

18. Ge Y, Chen Z, Kang ZB, Cluette-Brown J, Laposata M, Kang JX. Effects of adenoviral gene transfer of C. elegans n-3 fatty acid desaturase on the lipid profile and growth of human breast cancer cells. Anticancer Res. 2002 Mar-Apr;22(2A):537-43.

19. Maillard V, Bougnoux P, Ferrari P, Jourdan ML, Pinault M, Lavillonniere F, et al. N-3 and N-6 fatty acids in breast adipose tissue and relative risk of breast cancer in a case-control study in tours, france. Int J Cancer. 2002 Mar 1;98(1):78-83.

20. Chan PC, Didato F, Cohen LA. High dietary fat, elevation of rat serum prolactin and mammary cancer. Proc Soc Exp Biol Med. 1975 May;149(1):133-5.

21. Lau DSY, Archer MC. The 10t,12c isomer of conjugated linoleic acid inhibits fatty acid synthase expression and enzyme activity in human breast, colon, and prostate cancer cells. Nutr Cancer. 2010;62(1):116-21.

22. Ewaschuk JB, Newell M, Field CJ. Docosahexanoic acid improves chemotherapy efficacy by inducing CD95 translocation to lipid rafts in breast cancer cells. Lipids. In press .

23. Chamras H, Ardashian A, Heber D, Glaspy J. Fatty acid modulation of MCF-7 human breast cancer cell proliferation, apoptosis and differentiation. J Nutr Biochem. 2002 Dec;13(12):711-6.

24. Rose D, Connolly J, Liu X. Effects of linoleic-acid and gamma-linolenic acid on the growth and metastasis of a human breast-cancer cell-line in nude-mice and on its growth and invasive capacity in-vitro. Nutrition and Cancer- An International Journal. 1995;24(1):33-45.

25. Horia E, Watkins B. Comparison of stearidonic acid and alpha-linolenic acid on PGE(2) production and COX-2 protein levels in MDA-MB-231 breast cancer cell cultures. J Nutr Biochem. 2005 Mar;16(3):184-92.

26. Altenburg JD, Siddiqui RA. Omega-3 polyunsaturated fatty acids down-modulate CXCR4 expression and function in MDA-MB-231 breast cancer cells. Molecular Cancer Research. 2009 Jul;7(7):1013-20.

27. Huang Y, Li X, Muyan M. Estrogen receptors similarly mediate the effects of 17 beta-estradiol on cellular responses but differ in their potencies. Endocrine. 2011 FEB 2011;39(1):48-61.

28. Kenny F, Gee J, Nicholson R, Ellis I, Morris T, Watson S, et al. Effect of dietary GLA plus /-tamoxifen on the growth, ER expression and fatty acid profile of ER positive human breast cancer xenografts. International Journal of Cancer. 2001 May 1;92(3):342-347.

29. Ottosson UB, Lagrelius A, Rosing U, Vonschoultz B. Relative fatty-acid composition of lecithin during postmenopausal replacement therapy - a comparison between ethinyl estradiol and estradiol valerate. Gynecol Obstet Invest. 1984 1984;18(6):296-302.

30. Lewis GP. Immunoregulatory activity of metabolites of arachidonic acid and their role in inflammation. Br Med Bull. 1983 Jul;39(3):243-8.

31. Lands W. Biochemistry and physiology of N-3 fatty-acids. FASEB J. 1992 May;6(8):2530-6.