Determination of the Relative Efficacy of Docosahexaenoic Acid and Eicosapentaenoic Acid in Two-Dimensional and Three-Dimensional Cell Culture Models of Human Breast Cancer

by

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Abstract

Omega-3 long chain polyunsaturated fatty acids (n-3 LCPUFA), docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), decrease breast cancer cell viability. DHA, EPA, and dietary relevant DHA:EPA mixtures have not been systematically investigated in two-dimensional (2D) cell culture models of human breast cancer and compared to three-dimensional (3D) cell culture models, which recapitulate the tumour microenvironment. The overall objectives of this thesis were to use MDA-MB-231 (triple negative (estrogen receptor-, progesterone receptor-, human epidermal growth factor receptor (Her2)-) and SK-BR-3 (Her2+) human breast cancer cells to: 1) determine if differences exist between DHA, EPA, and DHA:EPA mixtures on cell viability, tumour fatty acid composition, and proteins related to cell death and growth pathways in 2D culture and 2) determine if the effects are maintained in 3D culture. In 2D culture, cells were incubated with 100, 150, or 200 μM DHA, EPA, or DHA:EPA mixtures (1:1 or 2:1) with a background fatty acid mixture (oleic/linoleic acid). In MDA-MB-231 cells all treatments decreased cell viability to the same extent at 100 and 150 μM (25-29% and 19-26%, respectively, $p<0.05$). DHA was more efficacious than other treatments at 200 μM (59% vs. 36-44%, $p<0.05$). Relative EPA+docosapentaenoic acid (DPA) and DHA content (%w/w) in total phospholipids (PL) and PL classes differed between DHA, EPA, and 2:1 treatments and the 1:1 mixture (EPA+DPA≈DHA vs. EPA+DPA>DHA, $p<0.05$). Similar decreases in cell content of apoptotic proteins RIPK1 (16%-28%, $p<0.05$), FADD (14%-31%, $p<0.05$), and increases in phosphorylated epidermal growth factor receptor (84%-96%, $p<0.05$) with all treatments may account for the similar effects on cell viability at

100 and 150 μM. In SK-BR-3 cells, EPA decreased cell viability to the greatest extent at each dose tested (35-47% vs. 17-39%, $p<0.05$). The relative EPA+DPA content in total PL and PL classes differed with EPA, DHA, and 1:1 treatments compared to the 2:1 mixture (EPA+DPA≈DHA vs. DHA>EPA+DPA, $p<0.05$). Increases in CD95 death receptor and decreased FADD content (14 and 22%, $p<0.05$) may explain the effect of EPA. In 3D culture, changes in EPA+DPA and DHA content with n-3 LCPUFA treatments in whole cell fatty acids were consistent with 2D culture. However, there were increases in MDA-MB-231 spheroid growth (26%, $p<0.05$) and SK-BR-3 aggregate formation (38-62%, $p<0.05$), suggesting these indices are not appropriate for studying the anti-cancer effects of n-3 LCPUFA established in 2D culture and animal feeding trials. Collectively, this research is important for using n-3 LCPUFA mixtures to target breast cancer tumours.

Preface

Chapter 3 of this thesis is an adapted version of a review that has been published as L. VanderSluis, V.C. Mazurak, S. Damaraju, and C.J. Field, "Determination of the Relative Efficacy of Eicosapentaenoic Acid and Docosahexaenoic Acid for Anti-Cancer Effects in Human Breast Cancer Models," *International Journal of Molecular Sciences*, vol. 18, issue 12, 2607. Laura VanderSluis and Catherine J. Field designed the review; Laura VanderSluis wrote the draft of the manuscript; Vera C. Mazurak, Sambasivarao Damaraju and Catherine J. Field reviewed drafts and edited the manuscript.

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Abbreviations

2D	Two-dimensional
3D	Three-dimensional
AA	Arachidonic acid
AG	Aggregate
CD95	Cluster of differentiation 95
DISC	Death inducing signaling complex
DHA	Docosahexaenoic acid
DPA	Doscosapentaenoic acid
EGFR	Epidermal growth factor receptor
EPA	Eicosapentaenoic acid
FADD	Fas-associated protein with death domain
Her2	Human epidermal growth factor receptor 2
LA	Linoleic acid
MUFA	Monounsaturated fatty acid
n-3 LCPUFA	Omega-3 long chain polyunsaturated fatty acids
OA	Oleic acid
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
pEGFR	Phosphorylated epidermal growth factor receptor
PI	Phosphatidylinositol
PL	Phospholipid
PUFA	Polyunsaturated fatty acid
RIPK1	Receptor-interacting serine/threonine-protein kinase 1
SFA	Saturated fatty acid

Chapter One-Introduction

1. Breast cancer and n-3 long chain polyunsaturated fatty acids

Breast cancer is the leading cause of cancer in Canadian women. It is estimated that 1 in 8 Canadian women will be develop breast cancer in their lifetime and 1 in 31 will die from the disease [1]. Breast cancer is a heterogeneous disease that is stratified into histological and molecular subtypes [2, 3]. Histological subtypes stratify breast cancer based on the growth patterns and tissue architecture [3]. Histological subtypes can be defined as either in situ carcinomas (ex. ductal or lobular) or invasive carcinomas (ex. infiltrating ductal, invasive lobular, ductal/lobular, tubular, mucinous, medullary, and papillary). Molecular subtypes are based on the expression of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor (Her2) [2]. Based on differential expression of these receptors, there at least 5 molecular subtypes. These histological and molecular subtypes have distinct responses to treatment and therefore prognosis [2, 4]. The prevalence and heterogeneity of breast cancer has prompted researchers to investigate potential therapeutics for disease prevention and treatment.

Prospective cohort studies have shown that increased fish oil consumption is associated with a decreased risk of breast cancer [5, 6]. Fish oil is rich in n-3 long chain polyunsaturated fatty acids (n-3 LCPUFA), docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA). These n-3 LCPUFA also demonstrate anti-cancer effects in human breast cancer cell lines and *in vivo* in rodent mammary tumour models (reviewed in [7] and [8]). Polyunsaturated fatty acids (PUFA) are carboxylic acids with hydrocarbon chains that have two or more double bonds [9]. PUFA are furthered classified by the length of their hydrocarbon chain (>12 carbons are long-chain FA) and the location of the first double bond from the methyl end of the fatty acid (n-3 or n-6) [9]. It is well established that EPA and DHA exert anti-cancer effects and research is being done to define the pleiotropic effects of these n-3 LCPUFA. The beneficial effects of the n-3 LCPUFA DHA (22:6n-3) and EPA (20:5n-3) are thought to be attributed to their affect at the plasma membrane (reviewed in [10] and [11]).

2. *The plasma membrane and n-3 LCPUFA*

The plasma membrane is critical for the compartmentalization of organelles and control of the spatiotemporality of biochemical reactions [12]. The plasma membrane is comprised of lipids and embedded proteins that are important for cell signaling [12]. Cells maintain their structural integrity by regulating the lipid composition of the membrane [13]. Within a cell, there are several sources of lipids including: triglycerides, phospholipids (PL), PL classes, and cholesterol [14]. The major membrane lipids are PL, which have a hydrophilic phosphate head and hydrophobic tail comprised of two fatty acid chains that form the lipid bilayer [13]. The hydrophobic core of the lipid bilayer allows for the lateral movement of PL and membrane associated proteins [13]. There are 6 types of glycerophospholipids: phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine, phosphoglycerol, and phosphatidic acid. Each glycerophospholipid has a glycerol backbone attached to two FA and a different polar head group, and as a result, each PL has unique functions [15]. PC and PE are the most abundant PL classes in the plasma membrane [15]. PI is found in much smaller amounts in the plasma membrane, yet is crucial for many signal transduction pathways and membrane trafficking [16]. De novo synthesis of PL is regulated by the Kennedy (de novo) pathway and the fatty acid composition of PL is regulated by the Lands cycle [17]. In the Lands cycle, fatty acids are cleaved from PL at the sn-2 position by phospholipase A_2 [18]. The acyl chains of PL in the membrane may be altered based on changes in lipid metabolism, which may impact the structure of the lipid bilayer and lipid-protein interactions [19]. In the membrane, most PUFA are esterified into PC and PE at the sn-2 position [19].

Cancer cells have been shown to have altered or reprogrammed lipid metabolism resulting in increased lipogenesis [20-22]. Lipid analysis of breast cancer cells is typically conducted using chromatographic methods (thin layer chromatography, gas chromatography, high-performance lipid chromatography) or mass spectrometry (reviewed in [23, 24]). Although the lipid composition of breast cancer cells are not well characterized, increased amounts of PC, PE, PI, and sphingolipids (sphingomyelin and ceramide) have been found in

tumour tissue and are associated with poor survival [21]. The effect of DHA in glycerophospholipids and subsequent effects on physicochemical membrane properties has been explored in the retina, testes, brain, heart, and skeletal muscle (reviewed in [25]). When provided to human breast cancer cells, DHA and/or EPA are incorporated readily into whole cell FA [26], membrane PL [27], PL classes [26] and have been shown to affect both breast cancer cell death and growth signaling pathways [26-31]. DHA and EPA are also incorporated into the FA and PL fraction of specialized microdomains of the plasma membrane called lipid rafts [30], which are important sites for membrane-associated receptors [32].

3. Membrane-mediated effects of n-3 LCPUFA on cell death and growth pathways in vitro

In non-malignant cells, apoptosis is a tightly regulated process that results in cell death [33]. The regulation of this pathway is lost in cancer cells and they become resistant to apoptosis [34]. There are two main apoptotic pathways: 1) extrinsic apoptosis, which is mediated by membrane-associated proteins and 2) intrinsic apoptosis, or the mitochondrial pathway [35]. To determine the effect of treatment on breast cancer cell death, the trypan blue exclusion assay is used most commonly to detect changes in membrane integrity (reviewed in [36]). Intracellular metabolic activity is also assessed in breast cancer cells using colorimetric assays with tetrazolium salts or analogues (MTT, MTS, WST-1) [36]. Exposure of breast cancer cells to n-3 LCPUFA has been shown to decrease cell viability using both trypan blue exclusion [27-29, 37, 38] and colorimetric assays [26, 29, 31, 32, 39-44].

Extrinsic apoptosis is mediated by a family of membrane associated death receptors [35], one of the most well characterized being cluster of differentiation 95 (CD95 also known as APO-1 or Fas)[45]. CD95 is present in homotrimers in the plasma membrane and, upon ligand binding to Fas ligand (FasL), the death domain of CD95 recruits Fas-associated protein with death domain (FADD), procaspase-8, procaspase-10, and c-FLIP to form the death inducing signaling complex (DISC). This complex activates effector caspases that induce apoptosis [46]. Human breast cancer cells are resistant to CD95 and FasL and are able to evade cell death even though they express these proteins [47]. Not only do cancer cells resist

cell death, they are also able to evade growth suppressors and sustain proliferation by enabling replicative immorality [34]. ErbB growth factor receptors are a family of transmembrane proteins [48] that regulate growth signaling pathways as well as apoptosis, migration, adhesion, and differentiation [49]. There are four ErbB receptors: EGFR/ERBB1, ERBB2/Her2, ERBB3, and ERBB4 that are able to bind a variety of ligands (ex. epidermal growth factor (EGF), Transforming growth factor-α (TGF-α)) (reviewed in [50]and [49]). Upon ligand binding, these receptors form homo-or hetero-dimers at the cell surface in the plasma membrane promoting autophosphorylation through the intrinsic kinase domain [50-52]. The phosphorylated residues allow interaction with proteins downstream, initiating signal transduction [50]. The formation of these dimers is influenced by the lipid environment in the membrane, ligand concentration, and receptor expression [53]. In breast cancer, EGFR is over expressed in all breast cancer subtypes [54], while Her2 is overexpressed exclusively in Her2 breast cancer subtypes [55]. The overexpression of these receptors promotes tumour growth and is associated with a poor prognosis [54, 56].

The effect of n-3 LCPUFA on plasma membrane incorporation and changes in CD95 [40], EGFR [26, 28, 32, 39], and Her2 [32, 57, 58] has been studied in breast cancer cells. Changes in these proteins with treatment can be detected directly using immunoblotting and flow cytometry to determine changes in these proteins at the level of the whole cell and cell surface, respectively (reviewed in [36]). Using a combination of these techniques, DHA has been shown to increase the amount of CD95 found in the lipid raft and the translocation to the cell surface for apoptotic signaling [40]. EPA has been shown to decrease whole cell phosphorylated EGFR (pEGFR) [26], while DHA has been shown to decrease whole cell pEGFR [26] and EGFR [26, 32]. EPA:DHA mixtures have been shown to increase pEGFR and have no effect on whole cell EGFR [28]. Taken together, this suggests that EPA and/or DHA have distinct effects on cell growth pathways and highlights the need for research to be conducted on the relative efficacy of DHA and/or EPA on tumour cell death *in vitro*.

4. Three-dimensional (3D) in vitro cell culture techniques and breast cancer

Human breast cancer cell lines representing commonly diagnosed breast cancer subtypes are used as preclinical models to test the efficacy and safety of potential therapeutic drugs and/or nutraceuticals [59]. Human breast cancer cells are ideal preclinical models for the preliminary stage of drug testing as they are self-replicating, easily cultured, and inexpensive compared to other preclinical models such as animal feeding studies [60, 61]. Traditional cell culture techniques grow human breast cancer cells in a two-dimensional (2D) plane, which does not recapitulate the tumour microenvironment [62-65]. Researchers have turned to 3D models of human breast cancer that grow breast cancer cells (monotypic) or xenografts (mouse or patient derived) to more closely the resemble the tissue architecture of the mammary gland [63, 66]. Human breast cancer cells have been established in several 3D cell culture models in the presence of a matrigel to promote tumour formation including embedded, on-top, drip, or high throughput models [63, 67]. Matrigel is a solubilized extract from an Engelbreth-Holm-Swarm (EHS) mouse sarcoma, containing proteins from the extracellular matrix [68]. Each of these models differs with respect to the amount of matrigel used, cell culture maintenance, and suitability for phase-contrast or fluorescence imaging [63]. Studying these 3D models provides many other advantages over 2D cell culture models including: cell-to-cell and extracellular matrix-to-cell interactions, non-uniform exposure to nutrients and/or drugs [69], and the presence of physiologically relevant gradients (proliferation [69, 70], oxygen [70, 71], ATP and glucose distribution [70], and apoptosis [70]).

The effect of treatment in 3D cell culture models can be tested in several ways. Microscopy is often used to characterized the effect of treatment on cell growth parameters including invasiveness [69], spheroid or aggregate size [69, 72], tumour area [73], cell number [74], cell count [75], and the number of cells per spheroid [72]. Confocal microscopy with nuclear staining can also be used as a more accurate measure of tumour cell death [76]. This type of microscopy may also be used for immunostaining for visualization of proteins of interest [67]. Cell viability can be assessed in 3D models using specific 3D specific colorimetric (MTS [69] or MTT [77]) or luminescent assays [69]. Of note, one of the challenges or

limitations with 3D cell culture work is the reproducibility of the several 3D cell culture models and analysis techniques [61]. In addition, a gold standard 3D cell culture model and analysis technique has yet to be identified for each cell type. The effects of treatment on human breast cancer cells grown in 2D cell culture have been compared and contrasted to that of 3D cell culture models [78]. Researchers have shown that breast cancer cell lines forming dense spheroids in 3D were more resistant to treatment than in 2D cell culture, while breast cancer cell lines that formed less dense 3D structures displayed more similar responses to 2D cell culture models. [78]. This highlights the importance of studying distinct breast cancer cell subtypes to determine the suitability of 3D cell culture models.

Chapter Two- Rationale

1. Rationale

Approximately 1 in 8 Canadian women will develop breast cancer in their lifetime and 1 in 31 will die from the disease [1]. Increased consumption of fish oil has been associated with a lower incidence of breast cancer [5, 6]. Fish and fish oils contain high amounts of the omega-3 LCPUFA, DHA and EPA [79]. DHA and EPA have demonstrated anticancer effects in human breast cancer cell lines and rodents implanted with human tumours (reviewed in [7, 8]); however, the mechanisms explaining these effects have not been fully characterized.

The mechanisms by which DHA and EPA exert anti-cancer effects have been hypothesized to be related to their effect at the cell membrane (reviewed in [7, 11, 80]). It is well established that EPA and DHA are readily incorporated into whole cell lipids [26, 29], membrane PL [27, 28, 31], and lipid microdomains in the plasma membrane (lipid rafts [28, 30]). Incorporation of EPA and DHA into the plasma membrane is associated with structural alterations in the lipid bilayer [81]. Researchers have found that this incorporation has subsequent effects on the translocation and abundance of membrane-associated cell death (CD95)[40] and cell growth (EGFR and Her2 [28]) receptors as well as changes in apoptosis and proliferation [26, 31, 37, 39, 82]. This is of particular relevance as breast cancer is a heterogenous disease that can be stratified by histological and molecular subtypes [3].

DHA and EPA are often considered together as n-3 LCPUFA in the context of breast cancer. These n-3 LCPUFA are rarely considered separately in breast cancer literature despite their structural differences that may result in distinct effects on membrane fluidity and as a result, have unique anti-cancer properties [83]. Studies conducted by our group have found that when compared directly, DHA caused a greater decrease in human breast cancer cell viability than EPA at the same dose and is more effective at increasing the translocation of CD95 to the cell surface to initiate apoptotic signal transduction [40]. Little research has been done in human breast cancer models to compare the anti-cancer effect of DHA and EPA with dietary relevant ratios of these n-3 LCPUFA present in foods and supplements. As a result, the relative efficacy of these n-3 LCPUFA on plasma membrane fatty acid composition and the

effect on membrane associated cell growth and death receptors in common breast cancer subtypes is not clear. This work is critical to understanding the predictability of DHA:EPA mixtures present in the diet of Canadian women on breast cancer cell incorporation and subsequent effect on tumour growth.

To determine the effect and efficacy of potential preventative agents or therapeutics for breast cancer, *in vitro* mammary epithelial cells are often grown in a 2D monolayer and exposed to the potential agent or drug of interest [59, 70, 84]. This traditional cell culture technique, although useful at establishing many mechanisms, may not represent the dynamics between the basement membrane, extracellular matrix, and stromal cells in the initiation, promotion, and progression of breast cancer [85]. In addition, 2D models are not able to take into account cell-cell or cell-microenvironment interactions, tissue architecture polarity, or oncogene expression (reviewed in [59, 69, 84, 86]), which are critical components of breast cancer pathogenesis and may be affected by n-3 LCPUFA. To circumvent the inherent limitations of 2D cell culture techniques, 3D cell culture models of breast cancer have been a focus of research [65] and have proved to be a valuable tool and for some types of therapeutic interventions results have been found to be more translatable to the patient [78]. To date, research has not yet investigated the effect of n-3 LCPUFA treatments in a 3D cell culture model of human breast cancer. This presents an opportunity to determine the value of a 3D model for this work and to determine the effect of n-3 LCPUFA treatments on a tumour grown in a microenvironment that might be more representative of the heterogenicity of a breast tumour. The investigation of the optimal ratio of EPA and DHA required to decrease breast cancer cell viability and research done in 3D cell culture will be useful to help in the translation of *in vitro* work to pre-clinical (animal) and human trials.

2. *Objectives and hypotheses*

The overall aim of this research is to determine the relative efficacy of DHA and EPA in human breast cancer models on changes in tumour fatty acid composition of whole cell fatty acids, total PL, PL classes and subsequent effects on cell growth and death pathways. This aim was achieved by addressing the following four objectives:

Objective 1: Determine state of knowledge on the specific effects of DHA, EPA, and DHA:EPA mixtures on incorporation into cellular lipids, cell growth, and death in human breast cancer models.

It was hypothesized that:

1. In vitro and animal feeding models of breast cancer that have compared DHA to EPA and/or DHA:EPA mixtures will observe differences in relative efficacy of these n-3 LCPUFA on incorporation into cellular lipids, cell growth, and death.
2. Studies investigating the effect of DHA:EPA mixtures with more DHA than EPA will demonstrate greater anti-cancer effects.

Objective 2: Determine if differences in cell viability exist between DHA, EPA, and DHA:EPA mixtures when provided at the same total concentration on MDA-MB-231 and SK-BR-3 human breast cancer cell lines and determine if changes in tumour fatty acid composition explains these differences.

It is hypothesized that:

1. Treatment of breast cancer cells with different amounts of DHA and/or EPA will have distinct effects on tumour cell viability.
2. The effects of the n-3 LCPUFA treatments on tumour cell viability and fatty acid composition and will differ between breast cancer cells representing distinct breast cancer subtypes.
3. The increase in n-3 LCPUFA content in tumour cells when cells are incubated with n-3 LCPUFA will result in a lower content of arachidonic acid (AA).

4. The amount and relative incorporation of the different n-3 LCPUFA into breast cancer cells will explain the effects on tumour cell viability.

Objective 3: To identify potential membrane-associated receptors that could explain the effects of n-3 LCPUFA on cell death and growth pathways in MDA-MB-231 & SK-BR-3 human breast cancer cells.

It is hypothesized that:

1. Consistent with the effects on viability, treatment of breast cancer cells with DHA and/or EPA will increase the amount or activation of proteins associated with cell death proteins and decrease those associated with cell growth.

Objective 4: Determine if the effects of DHA and/or EPA, on cell viability and lipids in 2D culture are consistent when MDA-MB-231 and SK-BR-3 breast cancer cells are grown in a 3D on-top cell culture model.

It is hypothesized that:

3. The same effects and differences between n-3 LCPUFA observed in 2D cell culture will be seen in 3D cell culture.

3. Chapter format

The above objectives and hypotheses were studied/tested in a series of experiments and the results are compiled into chapters.

Chapter 1 addressed objective 1 by conducting an introduction on the topics investigated in the present thesis.

Chapter 2 presents the research question of the present thesis, including the knowledge gaps that exist in the present literature. This chapter also summarizes the objectives that were used to address the overall research question.

Chapter 3 addresses objective 1 by providing a literature review entitled, "Determination of the Relative Efficacy of Eicosapentaenoic Acid and Docosahexaenoic Acid for Anti-Cancer Effects in Human Breast Cancer Models" that summarizes the current state of evidence on the relative efficacy of DHA, EPA, and DHA:EPA mixtures in breast cancer models on tumour cell viability, apoptosis, and proliferation and incorporation into cellular lipids.

Chapter 4 provides details on the methods used to test hypotheses.

Chapter 5.1 addresses objective 2 by examining the effect of DHA, EPA, and DHA:EPA mixtures on human breast cancer cell viability and changes in the fatty acid content of tumour whole cell FA, total PL, and PL classes. It is hypothesized that the amount and relative incorporation of the different n-3 LCPUFA into breast cancer cells will predict the effects on tumour cell viability.

Chapter 5.2 addresses objective 3 by investigating the effect of DHA, EPA, and DHA:EPA mixtures on whole cell and cell surface expression of membrane-associated proteins related to cell growth and cell death pathways implicated in human breast cancer. It is

hypothesized that DHA and/or EPA will increase the amount or activation of proteins associated with cell death proteins and decrease those associated with cell growth.

Chapter 5.3 addresses objective 4 by establishing a novel 3D on-top cell culture model of human breast cancer and treating tumours grown in 3D with DHA and/or EPA. Subsequent analyses were conducted on tumour growth parameters and whole cell FA composition. It is hypothesized that the same effects and differences between n-3 LCPUFA observed in 2D cell culture will be seen in 3D cell culture.

Chapter 6 summarizes the thesis findings related to the objectives and hypothesizes. This chapter contains an integrative discussion of the results and their implications for future work with n-3 LCPUFA and human breast cancer models.

Chapter Three – Determination of the relative efficacy of EPA and DHA for anti-cancer effects in human breast cancer models[1]

1. Systematic analysis of current literature

To determine the state of knowledge on the specific effects of DHA, EPA, and DHA:EPA mixtures on survival of human breast cancer models, the present critical review took a systematic approach to analyzing the literature and included studies that met the following criteria: 1) *in vitro* studies that compared the effect of DHA to EPA and/or different EPA:DHA mixtures on anti-cancer outcomes in human breast cancer cell lines, or 2) feeding studies that compared the effect of supplementing the diet with EPA, DHA, or different EPA:DHA mixtures in rats with induced mammary carcinogenesis or mice bearing human breast cancer tumours. A literature search of *in vitro* and feeding studies was conducted in Medline/OVID database on 20 June 20 2017 and Elton B. Stephens (EBSCO) host database on 1-4 July 2017 using the following terms including: "fatty acids, omega-3, polyunsaturated fatty acids, docosahexaenoic acids, DHA, eicosapentaenoic acid, EPA, marine oil, fish oil(s)". Keywords including "anticancer; anti-cancer; breast or mammary neoplasms; experimental; carcinoma, ductal; triple negative breast neoplasms; cell line, tumour; MCF-7 cells; SK-BR-3; MDA-MB-231; neoplasms; heterografts; triple negative or HER2 positive; Mammary Neoplasms, Experimental"/ci [Chemically Induced]; rats, transgenic or Sprague Dawley; mice, transgenic or nude or knockout or athymic" were used to capture relevant breast cancer literature. No restriction was made on publication date. The search was rerun on 26 September 2017 in both databases to ensure relevant articles were included.

2. Characteristics of included studies

In total, 21 studies met the search criteria including 16 *in vitro* studies and 5 feeding studies. Of the included *in vitro* studies, 15 directly compared EPA to DHA [26, 27, 29-32, 37-43, 82, 88], while 3 analyzed different EPA:DHA mixtures [28, 29, 37]. Of the three studies

[1] This chapter is an adapted version of 87. VanderSluis, L., et al., *Determination of the Relative Efficacy of Eicosapentaenoic Acid and Docosahexaenoic Acid for Anti-Cancer Effects in Human Breast Cancer Models.* Int J Mol Sci, 2017. **18**(12).

that used EPA:DHA mixtures, none of these studies compared the effect of these combination treatments to EPA and DHA alone. Fatty acid concentrations used in these studies are of physiological relevance as fish oil supplementation in non-small cell lung cancer patients resulted in plasma PL EPA levels equivalent to approximately 88 μM [89]. Of the included feeding studies, three compared the effect of feeding EPA and DHA [90-92], while two compared mixtures [93, 94]. One of the studies comparing a EPA alone diet and DHA alone diet also examined a 1:1 EPA:DHA diet [92].

3. Data extraction and standardization

For each of the studies included, data was extracted on study design (breast cancer subtype, breast cancer model, treatment groups, concentration of EPA and/or DHA, exposure period, assays) and effect on anti-cancer outcome measures (plasma membrane incorporation, cell growth and viability, EGFR, apoptosis, and phosphoinositide-3-kinase/protein kinase B (PI3K/Akt) signaling). To synthesize the literature in a clear, concise, and consistent manner, data from included studies was standardized after analysis of reported tables and graphs. To standardize the method of reporting concentrations of EPA and/or DHA, concentrations were standardized to micromolar (μM) from *in vitro* studies and g/100 g diet weight/weight (w/w) for feeding studies. To standardize the data related to anti-cancer outcome measures, data from *in vitro* and feeding studies were standardized to fold-change or percent-change, as appropriate.

4. The incorporation of EPA and DHA into tumour cell lipids

EPA and DHA are readily incorporated into tumour lipids, [29], PL [26-28, 30, 31] and plasma membrane raft PL [28, 30] of triple negative (ER-, PR-, HER2-) MDA-MB-231 [26-31] and ER+ MCF-7 breast cancer cells [26, 27, 29-31]. An increase in EPA and/or DHA into tumour cell lipids and PL was found to decrease cell survival, as determined by decreased in cell viability [27-29, 37, 38, 82, 88] and proliferation [26, 29, 31, 32, 39-43] as well as increased apoptosis [26, 31, 32, 37, 39, 42, 43, 82].

The relative increase of EPA and DHA with EPA:DHA mixtures into the plasma membrane has been assessed in MDA-MB-231 [28, 29] and MCF-7 [29] breast cancer cells exposed to a 1.5:1 EPA:DHA ratio. The fold increase in EPA was more than DHA in whole

cell PL and lipid raft PL [28, 29]. If EPA and DHA were equally incorporated, it could be predicted that the amount of EPA in the membrane would be approximately 1.5 times that of DHA in a 1.5:1 EPA:DHA mixture. However, researchers found that the fold increase in EPA was more than twice that of DHA in whole cell and lipid raft PL in both breast cancer subtypes, showing that there is preferential uptake of EPA (Table 3.4.1). The fold increase in DHA in MDA-MB-231 breast cancer membrane lipids was reported to be higher than EPA when AA was provided at 140 μM in the media [29]. EPA and AA compete for D5-desaturase [95] and PL uptake into the plasma membrane [80], which may explain why EPA was not preferentially taken up in the presence of a high concentration of AA. Additionally, in this study the fold increase of EPA in MCF-7 breast cancer membrane lipids was greater than that of MDA-MB-231 breast cancer cells with the same EPA:DHA mixtures [29].

A feeding study by Yuri et al. [92] with a 1:1 EPA:DHA mixture found the fold increase of DHA was approximately double of EPA (23 vs. 14 fold increase), which is higher than what would be predicted if equivalent uptake into the membrane occurred. However, when a n-3 LCPUFA diet with more EPA than DHA (1:0.75 EPA:DHA) was fed to rats with induced mammary carcinogenesis, the fold increase in EPA in tumour lipids was greater than what would be predicted [94]. Wei et al. [93] compared the effects of feeding five EPA:DHA diets to rats with induced mammary carcinogenesis. All diets had a 1:5.5 EPA:DHA ratio, but varied in the total concentration (w/w) of EPA and DHA (Table 3.4.2). When the total concentration of EPA+DHA (w/w) was low, the amount of EPA in tumour lipids was greater than predicted [93], whereas feeding the diet with the highest concentration of EPA+DHA (w/w) resulted in more DHA in tumour lipids. Collectively, these studies suggest that EPA is preferentially incorporated with EPA:DHA mixtures. It is likely that Yuri et al. [92] and Wei et al. [93] saw more DHA in the membrane due to a concentration effect, as these researchers used much higher concentrations of EPA and DHA (w/w) in their EPA:DHA diets than other studies included in the present review (9.5 g/100 g and 7.6 g/100 g w/w, respectively) (Table 3.4.2).

When comparing EPA and DHA directly at the same concentration, preferential uptake into tumour lipids or PL differs between tumour cell membrane location (whole cell lipids or

lipid raft) and breast cancer subtype (Table 3.4.3). In MDA-MB-231 breast cancer cells, more EPA was found in whole cell lipids [26] and PL [27, 31], while DHA appears to be more concentrated into lipid rafts [30]. In MCF-7 breast cancer cells, the fold increase of EPA is similar to DHA in whole cell lipids [26] and PL [27], while the amount of EPA is greater than DHA in lipid rafts [30]. This shows that there are distinctions between breast cancer subtypes and that measurement of whole cell PL may not be reflective of changes in lipid raft PL. In feeding studies, the amount of DHA found in tumour cell lipids is greater than that of EPA at same concentration [90, 91] (Table 3.4.4). DHA has also been shown to increase to a greater extent than EPA in tumour PL after long-term feeding (13 weeks), but this was not apparent in short-term feeding (1 week) [91], showing that the exposure period to DHA and EPA is an important consideration to determine the relative efficacy of fold increases into tumour PL. EPA and DHA are enzymatically cleaved from the plasma membrane by phospholipase A_2 under inflammatory stimuli [80]. EPA's hydrocarbon backbone is the same length as that of AA (20:5n-3)[80]; therefore, EPA acts as a substrate for cyclooxygenase (COX) in the eicosanoid synthesis pathway and produces prostaglandin (PGE_3)[7, 96]. DHA is a longer n-3 LCPUFA than EPA (22:6n-3) and cannot act as a substrate for COX, although it is able to bind and inhibit COX [96]. Therefore, it could be hypothesized that since EPA, and not DHA, is readily cleaved and used as a substrate for eicosanoid synthesis there appears to be less EPA than DHA in tumour PL.

In summary, *in vitro* studies with strictly controlled environmental conditions show that the fold increase of EPA in plasma membrane is greater than DHA when provided as a single n-3 LCPUFA and in EPA:DHA mixtures, providing mechanistic evidence for preferential incorporation. In feeding studies, DHA appears to increase to a greater extent into tumour lipid and PL fractions, while EPA is preferentially incorporated in mixtures. It is possible that in feeding studies when EPA is combined with DHA, EPA's effect on membrane-mediated processes is altered.

Table 3.4.1: Incorporation of EPA and DHA measured in the plasma membrane with EPA:DHA mixtures in human breast cancer cell lines.

Citation	Cell Line	Concentration of EPA or DHA (μM)	Ratio EPA:DHA	Lipid Fraction	Fold Change in Incorporation*	
					EPA	DHA
Schley, Brindley [28]	MDA-MB-231	60 EPA + 40 DHA	1.5:1	Whole cell PL	↑157	↓0.2
				Lipid raft PL	↑73	↑8
		45 EPA+ 30 DHA + 75 LA	1.5:1	Whole cell PL	↑49	↑2
				Lipid raft PL	↑21	↑3
Mansara, Deshpande [29]	MDA-MB-231	84 EPA+ 56 DHA + 140 AA	1.5:1	Whole cell total lipids	↑0	↑1
		120 EPA + 80 DHA + 80 AA			↑1	↑0.2
		134 EPA+ 90 DHA+ 56 AA			↑1	↑1
		140 EPA+ 93 DHA + 47 AA			↑2	↑1
		153 EPA + 102 DHA+ 25 AA			↑4	↑1
	MCF-7	84 EPA+ 56 DHA + 140 AA	1.5:1	Whole cell total lipids	↑1	↑0.3
		120 EPA + 80 DHA + 80 AA			↑2	↑1
		134 EPA+ 90 DHA+ 56 AA			↑3	↑1
		140 EPA+ 93 DHA + 47 AA			↑3	↑1
		153 EPA + 102 DHA+ 25 AA			↑5	↑1

EPA=eicosapentaenoic acid, DHA=docosahexaenoic acid; LA=linoleic acid; AA= arachidonic acid; PL=phospholipid; FA=fatty acids; "↑" denotes significant increase ($p<0.05$); "↓" denotes significant decrease ($p<0.05$). *Fold change relative to control conditions.

Table 3.4.2: **Incorporation of EPA and DHA measured in the plasma membrane when feeding EPA:DHA mixtures in rodent models.**

Citation	Method Used to Induce Mammary Carcinogenesis	Experimental Diets	Concentration of EPA or DHA (w/w diet, g/100g)	Ratio EPA:DHA	Lipid Fraction	Exposure (weeks)	Fold change in Incorporation*	
							EPA	DHA
Yuri, Danbara [92]	MNU administration in rats	EPA	9.5 EPA	1:0	Mammary tissue total lipids	20	↑31	↑0.5
		DHA	9.5 DHA	0:1			↑2	↑30
		EPA+DHA	4.75 EPA +4.75 DHA	1:1			↑14	↑23
Wei, Wang [93]	MNU administration in rats	SFA	0 EPA+ 0 DHA	0:0	Tumour total lipids	18	ND	↑0.04
		MUFA	0 EPA+ 0 DHA	0:0			ND	↑0.5
		n-6 PUFA	0 EPA+ 0 DHA	0:0			ND	↓0.2
		n-3 LCPUFA	1 EPA + 5.6 DHA	1: 5.5			↓0.3	↑0.3
		1:1 (n-6:n-3)	0.5 EPA + 2.8 DHA				↑0.04	↓0.07
		1:2:1 S/M/P 1:1 (n-6:n-3)	0.2 EPA+ 1.1 DHA				↑0.1	↑0.04
		5:1 (n-6:n-3)	0.16 EPA + 0.9 DHA				↑0.5	↓0.05
		10:1 (n-6:n-3)	0.09 EPA + 0.49 DHA				↑0.07	↓0.06
Rose, Rayburn [94]	Xenograft in mammary fat pad using MDA-MB-435 in nude mice	11.5% MO+11.5% CO	0.42 EPA+0.32 DHA	1: 0.75	Tumour PL	12	↑1	↑0.2
		18% MO+5% CO	0.66 EPA + 0.55 DHA				↑3	↑0.4

Italicized numbers represents fatty acid composition of the mammary tissue and not fold-increase in incorporation, as this study did not have a control group. MNU= N-methyl-N-nitrosourea; EPA=eicosapentaenoic acid, DHA=docosahexaenoic acid; SFA=saturated fatty acid; MUFA=monounsaturated fatty acid; n-6=omega-6; PUFA=polyunsaturated fatty acids; n-3=omega-3; PUFA=polyunsaturated fatty acid; S/M/P=saturated/monounsaturated/polyunsaturated; MO=menhaden oil; CO=corn oil; PL=phospholipid; "↑" denotes significant increase ($p<0.05$); ND=not determined; "↓" denotes significant decrease ($p<0.05$). *Fold change relative to control conditions.

Table 3.4.3: Incorporation of EPA and DHA measured in the plasma membrane when comparing EPA to DHA in human breast cancer cell lines.

Citation	Cell Line	Concentration of EPA or DHA (μM)	Lipid Fraction	Fold Change in Incorporation*	
				EPA	DHA
Corsetto, Montorfano [26]	MDA-MB-231	230 EPA	Whole cell total lipids	↑15	↓0.2
		200 DHA	Whole cell total lipids	↓0.8	↑7
	MCF-7	230 EPA	Whole cell total lipids	↑10	↑0.5
		200 DHA	Whole cell total lipids	↓0.6	↑9
Corsetto, Cremona [30]	MDA-MB-231	230 EPA	Lipid raft PL	↑7	↑1
		200 DHA	Lipid raft PL	↑0.6	↑11
	MCF-7	230 EPA	Lipid raft PL	↑16	↑6
		200 DHA	Lipid raft PL	↓0.3	↑6
Yu [27]	MDA-MB-231	150 EPA+ 40 OA+ 40 LA	Whole cell PL	↑31	↑1.5
		150 DHA + 40 OA+ 40 LA	Whole cell PL	↓0.5	↑11
	MCF-7	150 EPA+ 40 OA+ 40 LA	Whole cell PL	↑10	↑0.1
		150 DHA + 40 OA+ 40 LA	Whole cell PL	↓0.5	↑7
Barascu, Besson [31]	MDA-MB-231	30 EPA	Whole cell PL	↑13	↑2
		30 DHA	Whole cell PL	↑0.2	↑3

EPA=eicosapentaenoic acid, DHA=docosahexaenoic acid; OA=oleic acid; LA=linoleic acid; FA=fatty acids; PL=phospholipid; "↑" denotes significant increase ($p<0.05$); "↓" denotes significant decrease ($p<0.05$). *Fold change relative to control conditions.

Table 3.4.4: Incorporation of EPA and DHA measured in the plasma membrane when comparing EPA to DHA in rodent models of human breast cancer.

Citation	Method Used to Induce Mammary Carcinogenesis	Experimental Diets	Concentration of EPA or DHA (w/w diet, g/100g)	Lipid Fraction	Exposure Period (weeks)	Fold change in Incorporation*	
						EPA	DHA
Rose, Connolly [90]	Xenograft in mammary fat pad using MDA-MB-435 in nude mice	4% EPA	0.7 EPA	Tumour PL	13	↑54	↑26
		4% DHA	0.7 DHA			↑15	↑107
		8% EPA	1.5 EPA			↑104	↑18
		8% DHA	1.5 DHA			↑36	↑127
Rose, Connolly [91]	Xenograft in mammary fat pad using MDA-MB-435 in nude mice	4% EPA	0.7 EPA	Tumour PL	1	↑3	↑1
		4% DHA	0.7 DHA			↑1	↑5
		8% EPA	1.5 EPA			↑5	↑1
		8% DHA	1.5 DHA			↑1	↑5

EPA=eicosapentaenoic acid, DHA=docosahexaenoic acid; PL=phospholipid; "↑" denotes significant increase ($p<0.05$); "↓" denotes significant decrease ($p<0.05$). *Fold change relative to control conditions.

5. Effect of DHA and EPA on tumour cell survival

Cell growth and viability

There is considerable evidence that exposing breast cancer cells to EPA and DHA significantly reduces survival (Table 3.5.1). In these studies growth and viability was measured using a number of different methods, including trypan blue exclusion [27, 32, 37, 38] colony formation assays [82], and changes in MTS [41], MTT [26, 29, 31, 39, 42, 43], and WST-1 [40] measures of metabolic activity, which likely contributes to the wide range in efficacy reported. Studies have also reported decreases in the activation of the PI3K/Akt proliferative signaling pathway [32, 39] and phosphorylation of EGFR [26, 32]. EPA and DHA also increase proteins involved in apoptotic signaling [26, 31, 39, 82].

Although both EPA and DHA alter viability, their incorporation into tumours is not the same and likely their mechanisms are different. Few researchers have attempted to find the ratio and concentration of EPA and DHA that optimally reduces breast cancer cell survival (Table 3.5.1). Mansara et al. [29] and Schley et al. in 2005 and 2007 [28, 37] examined the effect of EPA and DHA at a ratio of 1.5:1 (EPA:DHA). Both studies reported decreases in breast cancer cell viability in experiments that ensured a sufficient n-6 fatty acid supply by providing either linoleic acid (LA) [28, 37] or AA [29] in the media.

The majority of studies directly comparing the relative efficacy of DHA to EPA have shown that DHA decreases cell viability to a greater extent in MDA-MB-231 [31, 32, 41, 88], MCF-7 [26, 27, 37, 42, 43, 82], SK-BR-3 [40] and 4HT1 [43] breast cancer cells (Table 3.5.2). No studies found that DHA and EPA increase cell viability [26, 29, 38]. The greater anti-cancer effect of DHA occurred in most of these studies despite EPA being incorporated into tumour lipids and PL fractions to a greater extent than DHA in MDA-MB-231 [26, 27, 31] breast cancer cells and similar incorporation to that of EPA in MCF-7 breast cancer cells [26, 27]. This suggests that DHA alters tumour cell survival differently than EPA and that simply measuring the relative amount that is incorporated into lipids does not explain the difference in efficacy. EPA and DHA are established precursors for anti-inflammatory lipid mediators [7, 11, 96]. Lipoxygenase and COX pathways use EPA as a substrate for the synthesis of E-series

resolvins and DHA is used to produce D-series resolvins, protectins, and maresins [7, 80]. These lipid mediators are cytoprotective in normal cells [7, 97]. The role of resolvin and protectins in cancer has not been fully elucidated [98, 99]. Due to their potent anti-inflammatory properties, it has been hypothesized that resolvins attenuate inflammation-related carcinogenesis [98]. Although not yet investigated in BC [100], it is possible that in E-and D-series resolvins may have distinct effects on cytotoxicity and may account for differences in cell viability.

There is conflicting evidence surrounding the relative efficacy of DHA and EPA on breast cancer cell survival. Das et al. [38] showed that EPA decreases cell viability to a significantly greater extent than DHA after 3 days in luminal B (ER+ PR-/+ HER2+) ZR-75-1 breast cancer cells. DHA and EPA have been shown to be equally efficacious in MDA-MB-231 breast cancer cells using a oleic acid (OA)/LA FA background mixture [27]. Researchers have observed concentration dependent effects of EPA and DHA on survival in MDA-MB-231 [26, 40, 42], MDA-MB-435s [42], and MCF-7 breast cancer cells [39, 40]. Ewaschuk et al. [40] identified a concentration gradient in MCF-7 cells, where DHA decreased cell viability to a greater extent at lower concentrations and EPA was more efficacious at killing breast cancer cells at higher concentrations [26, 40]. However, Ewaschuk et al. [40] did not statistically examine the differences in the effects of DHA and EPA. More efficacious killing was observed when DHA was provided in low amounts, suggesting that DHA is more potent. Triple negative breast cancer cells have also been reported to have concentration gradients; however, there is conflicting evidence on the relative efficacy of EPA and DHA at high and low concentrations [26, 29, 40, 42]. The difference in the relative efficacy seen in these studies is likely due to the way EPA and DHA were delivered to the tumour cells as some deliver the n-3 LCPUFA bound to either bovine serum albumin (BSA) [27, 40], delipidated endotoxin free BSA [29], or dissolved in ethanol [26, 31, 32, 37, 38, 42, 88]. FA that are dissolved in ethanol are not bound to protein and are more readily accessible for incorporation into breast cancer cells, which may induce cytotoxic effects [101].

In summary, EPA and DHA when provided alone or in a mixture, reduce survival of triple negative, ER+, luminal B, and HER2+ breast cancer cells *in vitro*, although when compared at the same dose, DHA appears to be more efficacious. This might be explained by the structural differences between DHA and EPA. DHA (C22:6n-3) has one more double bond than EPA (20:5n-3) and a longer hydrocarbon chain, giving DHA a distinct 3D conformation that may disrupt the highly ordered cellular membrane to a greater extent [83, 102].

Table 3.5.1: Comparison of EPA:DHA mixtures on cell viability in human breast cancer cell lines.

Citation	Cell Line	Concentration of EPA or DHA (μM)	Ratio EPA:DHA	Assay	Change in Cell Viability*	Exposure (hours)	Form of n-3 LCPUFA
Schley, Jijon [37]	MDA-MB-231	60 EPA + 40 DHA	1.5:1	TBE	↓40%	72	Dissolved in ethanol
		45 EPA + 30 DHA + 75 LA			↓31%		
Schley, Brindley [28]	MDA-MB-231	60 EPA + 40 DHA	1.5:1	TBE	↓62%	72	Dissolved in ethanol
		45 EPA + 30 DHA + 75 LA			↓48%		
Mansara, Deshpande [29]	MDA-MB-231	84-153 EPA+56-102 DHA+ 25-140 AA	1.5:1	TBE	↓ 54%-↓82%	24	Conjugated to delipidated, endotoxin free BSA
				MTT	↓ 15%-↓30%		
	MCF-7	84-153 EPA+56-102 DHA+ 25-140 AA	1.5:1	TBE	↓ 38%-↓81%	24	Conjugated to delipidated, endotoxin free BSA
				MTT	↓ 20%-↓30%		

EPA=eicosapentaenoic acid, DHA=docosahexaenoic acid; AA= arachidonic acid; TBE= Trypan Blue Exclusion; MTT= 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; "↓" denotes significant decrease ($p<0.05$); BSA=bovine serum albumin. *Fold change relative to control conditions.

Table 3.5.2: Comparison of EPA and DHA on cell growth & viability in human breast cancer cell lines.

Citation	Cell Line	Concentration of EPA or DHA (μM)	Assay	Change in Cell Viability*	Exposure (hours)	Form of n-3 LCPUFA	Conclusion on Relative Efficacy
Schley, Jijon [37]	MDA-MB-231	100 EPA	TBE	↓42%	72	Dissolved in ethanol	DHA>EPA
		75 EPA + 75 LA		↓30%			
		100 DHA		↓65%			
		75 DHA+ 75 LA		↓58%			
Lee, Yun [32]	MDA-MB-231	5, 10, 30, & 50 EPA	MTS	↓15%-↓ 20%	24	Dissolved in ethanol	DHA>EPA
		5, 10, 30, & 50 DHA		↓20%-↓ 45 %			
Corsetto, Montorfano [26]	MDA-MB-231	50-300 EPA	MTT	NS Δ-↓88%	72	Dissolved in ethanol	DHA>EPA (200-260 μM) & EPA>DHA (>260 μM)
		50-300 DHA		NS Δ-↓75%			
	MCF-7	50-300 EPA	MTT	NS Δ-↓75%	72	Dissolved in ethanol	DHA>EPA
		50-300 DHA		NS Δ-↓75%			
Cao, Ma [39]	MCF-7	30, 60, 90, 140 EPA	MTT	↓ 2%-↓45%	72	Dissolved in ethanol	DHA=EPA with exception of DHA>EPA (at 90 μM) •
		30, 60, 90, 140 DHA		↓ 2%-↓45%			
Ewaschuk, Newell [40]	MDA-MB-231	50, 100 EPA	WST-1	↓ 5%-↓100%	72	Conjugated to BSA	DHA>EPA (<95 μM) & EPA>DHA(>95 μM) •
		50, 100, 150, 200 DHA		↓ 45%-↓90%			
	MCF-7	50, 100 EPA	WST-1	↓ 25%-↓100%	72	Conjugated to BSA	

Citation	Cell Line	Concentration of EPA or DHA (μM)	Assay	Change in Cell Viability*	Exposure (hours)	Form of n-3 LCPUFA	Conclusion on Relative Efficacy
		50, 100, 150 DHA		↓ 40%-↓100%			DHA>EPA (<95 μM) & EPA>DHA(>95 μM) •
	SK-BR-3	50, 100, 150 EPA	WST-1	↓ 5%-100%	72	Conjugated to BSA	DHA>EPA•
		50, 100 DHA		↓80%-↓100%			
Rahman, Veigas [41]	MDA-MB-231	50, 100 EPA	MTS	NS Δ-↓58%	48	No information given	DHA>EPA
		50, 100 DHA		↓ 26%-↓74%			
Mansara, Deshpande [29]	MDA-MB-231	40-320 EPA	MTT	NS Δ	24	Conjugated to delipidated, endotoxin free BSA	DHA=EPA (<280 μM) & DHA>EPA (≥ 280 μM)
		40-320 DHA		NS Δ-↓25%			
	MCF-7	40-320 EPA	MTT	NS Δ -↓20%	24	Conjugated to delipidated, endotoxin free BSA	DHA=EPA (<200 μM), DHA>EPA (≥ 200 μM)
		40-320 DHA		NS Δ -↓22%			
Rose and Connolly [88]	MDA-MB-231	1.7-8.3 EPA	[^{3}H] Inc.	NS Δ -↓29%	144	Dissolved in ethanol	DHA>EPA
		1.5-7.6 DHA		NS Δ-↓64%			
Barascu, Besson [31]	MDA-MB-231	10-100 EPA	MTT	NS Δ -↓75%	96	Dissolved in ethanol	DHA>EPA
		10-100 DHA		NS Δ -↓85%			
		12.5-200 EPA	MTT	↓0%-↓17%	72		

Citation	Cell Line	Concentration of EPA or DHA (μM)	Assay	Change in Cell Viability*	Exposure (hours)	Form of n-3 LCPUFA	Conclusion on Relative Efficacy
Kang, Wang [42]	MDA-MB-231	12.5-200 DHA		↓0%-↓87%		Dissolved in ethanol	DHA=EPA (<50 μM) & DHA>EPA (>50 μM) •
	MCF-7	6.25-200 EPA	MTT	↑5%-↓95%	72	Dissolved in ethanol	DHA>EPA
		6.25-200 DHA		↓5%-↓95%			
	MDA-MB-435s	12.5-200 EPA	MTT	↓0%-↓50%	72	Dissolved in ethanol	DHA=EPA (<50 μM) & DHA>EPA (>50 μM) •
		12.5-200 DHA		↓0%-↓87%			
Xue, Wang [43]	MCF-7	25, 50, 100 EPA	MTT	↓15%, ↓25%, ↓40%	72	No information given	DHA>EPA
		25, 50, 100 DHA		↓20%, ↓33%, ↓48%			
	4T1	25, 50, 100 EPA	MTT	↓20%, ↓35%, ↓55%	72	No information given	DHA>EPA
		25, 50, 100 DHA		↓25%, ↓45%, ↓83%			
Yu [27]	MDA-MB-231	150 EPA + 40 OA+40 LA	TBE	↓40%	48	Conjugated to BSA	DHA=EPA
		150 DHA+ 40 OA+40 LA		↓50%			
	MCF-7	150 EPA + 40 OA+40 LA	TBE	↓45%	48	Conjugated to BSA	DHA>EPA
		150 DHA+ 40 OA+40 LA		↓58%			
Das [38]	ZR-75-1	66 EPA	TBE	↓10%	72		EPA>DHA

Citation	Cell Line	Concentration of EPA or DHA (μM)	Assay	Change in Cell Viability*	Exposure (hours)	Form of n-3 LCPUFA	Conclusion on Relative Efficacy
		61 DHA		NS Δ		Dissolved in ethanol	
Chamras, Ardashian [82]	MCF-7	100 EPA	Cell count	↓30%	120	No information given	DHA>EPA●
		100 DHA		↓50%			
	MCF-7	1, 10, 100 EPA	Colony Formation	↓18%, ↓35%, ↓75%	2 weeks	No information given	DHA>EPA
		1, 10, 100 DHA		↓30%, ↓38%, ↓82%			
Yun, Song [44]	MDA-MB-231	1-50 EPA	MTT	NS-↓55%	24	No information given	DHA>EPA
		1-50 DHA		NS-↓80%			
	MDA-MB-231	25 EPA	MTT	↓25%	36	No information given	DHA>EPA●
		25 DHA		↓60%			
	T47D	1-50 EPA	MTT	NS-↓20%	24	No information given	DHA>EPA
		1-50 DHA		NS-↓30%			

EPA=eicosapentaenoic acid, DHA=docosahexaenoic acid; OA=oleic acid; LA=linoleic acid; TBE= Trypan Blue Exclusion; MTT= 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MTS=(3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium); [^{3}H] Inc.=Thymidine Incorporation; WST-1= Water-soluble Tetrazolium salt; PL=phospholipid; "↑" denotes significant increase ($p<0.05$); "↓" denotes significant decrease ($p<0.05$); NS Δ=no significant change; "●"= statistical significance was not assessed; BSA=bovine serum albumin. *Fold change relative to control conditions.

EGFR

Of the many receptors involved in growth, the EGFR has been studied the most in n-3 LCPUFA studies. The EGFR is an important membrane receptor that regulates growth and possibly apoptosis in breast cancer cells [26, 28, 32, 39]. EGFR is activated by phosphorylation [103] and both EPA and DHA have been shown to alter EGFR phosphorylation in human breast cancer cells [26]. A study conducted by Schley et al. [28] reported that EPA:DHA mixtures increased whole cell pEGFR and decreased lipid raft EGFR. There was no significant change in whole cell EGFR, implying that EPA:DHA mixtures changed the activation and translocation of EGFR but not total levels of EGFR (Table 3.5.3). Increased pEGFR is typically associated with increases in proliferation [104]; however, Schley et al. [28] also observed a decrease in cell viability and an increase in phosphorylated p38 MAPK in cells incubated with this EPA:DHA mixture, which is proposed to promote apoptosis by phosphorylating Bim_{EL}, a pro-apoptotic Bcl-2 protein [105, 106].

In Corsetto et al. 2011 [26], treatment with DHA decreased whole cell EGFR and pEGFR to a greater extent than EPA in MDA-MB-231 breast cancer cells [26]. When 0.01 μM EGF was added to the media with EPA or DHA treatments, EPA decreased pEGFR to a greater extent and the effect of DHA on EGFR was blunted (Table 3.5.4). Lee et al. [32] compared the effect of DHA and EPA on whole cell EGFR in MDA-MB-231 breast cancer cells. Western Blot Analysis showed that DHA decreased amount of EGFR, while EPA did not have a visible effect. Unfortunately, these researchers did not quantify the effect on EGFR. Collectively, this data suggests that DHA is more efficacious than EPA when provided as a single fatty acid in triple negative breast cancer. In MCF-7 breast cancer cells, treatment with either DHA or EPA did not significantly change the ratio of whole cell pEGFR:EGFR. [39].

To summarize, EPA:DHA mixtures significantly increase whole cell pEGFR and decrease lipid raft EGFR in MDA-MB-231 breast cancer cells. DHA decreases whole cell EGFR and pEGFR to a greater extent than EPA when provided as a single FA in these breast cancer cells, suggesting that the effects of EPA:DHA mixtures are attributable to the presence of DHA. The EGFR typically partitions into the lipid raft; however, changes in the lipid bilayer are associated

with decreases in EGFR [107]. Since MDA-MB-231 breast cancer cells favour incorporation of DHA into the lipid raft, it is plausible that DHA disrupts the structural integrity of the lipid raft and affects EGFR localization and phosphorylation status. In ER+ MCF-7 breast cancer cells, DHA and EPA act differently and there is not an effect in ER+ cells (MCF-7) on EGFR. This could be attributed to the preferential uptake of EPA into lipid rafts compared to DHA [30], which does not have the same spatial conformation as DHA [83] and, as a result, may not affect receptors found in the lipid raft.

Table 3.5.3: Change in total amounts of EGFR and pEGFR in whole cell lipids and lipid rafts with EPA:DHA mixtures in human breast cancer cell lines.

Citation	Cell Line	Concentration of EPA or DHA (μM)	Exposure (hours)	Change in EGFR *
Schley, Brindley [28]	MDA-MB-231	60 EPA + 40 DHA	72	NS Δ in whole cell EGFR** ↑50% whole cell pEGFR ↓lipid raft EGFR**
		45 EPA + 30 DHA + 75 LA		NS Δ in whole cell EGFR** ↑21% whole cell pEGFR ↓lipid raft EGFR**

EPA=eicosapentaenoic acid, DHA=docosahexaenoic acid; LA=linoleic acid; EGFR= epidermal growth factor receptor; pEGFR= phosphorylated EGFR; NS Δ=no significant change; "↑" denotes significant increase ($p<0.05$); "↓" denotes significant decrease ($p<0.05$); **Researchers did not quantify bands from Western Blot Analysis. *Fold change relative to control conditions.

Table 3.5.4: Comparison of DHA and EPA on total amounts of EGFR and pEGFR in whole cell lipids and lipid rafts in human breast cancer cell lines.

Citation	Cell Line	Concentration of EPA or DHA (μM)	Exposure (hours)	Change in EGFR*
Cao, Ma [39]	MCF-7	90 EPA	24	NS Δ in whole cell pEGFR:EGFR
		90 DHA		NS Δ in whole cell pEGFR:EGFR
Corsetto, Montorfano [26]	MDA-MB-231	230 EPA	72	NS **Δ** in whole cell EGFR; ↓10% whole cell pEGFR
		230 EPA + 0.01 EGF		NS **Δ** in whole cell EGFR; ↓52% whole cell pEGFR
		200 DHA		↓20% whole cell EGFR; ↓100% whole cell pEGFR
		200 DHA + 0.01 EGF		NS **Δ** in whole cell EGFR; ↓100% whole cell pEGFR
Lee, Yun [32]	MDA-MB-231	30, 50 EPA	24	NS **Δ** in whole cell EGFR●
		30, 50 DHA		↓whole cell EGFR●

EPA=eicosapentaenoic acid, DHA=docosahexaenoic acid; EGF=epidermal growth factor; EGFR= epidermal growth factor receptor; pEGFR= phosphorylated EGFR; NS Δ=no significant change; "↓" denotes significant decrease ($p<0.05$); "●"= statistical significance was not assessed. *Fold change relative to control conditions.

6. Effect of DHA and EPA on tumour cell death

Apoptosis

EPA and/or DHA have pro-apoptotic effects in both triple negative [26, 31] and ER+ [26, 39, 82] breast cancer subtypes. The pro-apoptotic effects occur with increases in plasma membrane incorporation [26, 31] and decreases in cell viability [26, 31, 39, 82], PI3K/Akt activation, [39], and pEGFR activation [26]. These data suggest that EPA and DHA may affect multiple steps in apoptosis.

Mixtures of EPA and DHA with or without LA have been shown to significantly increase apoptosis of MDA-MB-231 breast cancer cells as indicated by an increase in activated caspases [37](Table 3.6.1). The presence of LA in the media blunted the observed increase in activated caspases as well as decreases in cell viability and Akt phosphorylation, suggesting that the efficacy of EPA:DHA combination treatments is dependent upon the presence of other FA.

In triple negative breast cancer cell lines, DHA caused greater decreases in total amounts of Bcl-2 and procaspase 8 [26] as well as larger increases in single stranded DNA when compared to EPA [31](Table 3.6.2). These data suggest that DHA is a more efficacious inducer of apoptosis, which may be related to the preferential incorporation of DHA into lipid rafts and its more potent effect on decreasing cell viability and whole cell EGFR [32] and pEGFR [26].

In Corsetto et al. [26], researchers examined changes in Bcl-2 and procaspase 8 in ER+ MCF-7 breast cancer cells. DHA had more of an effect on decreasing procaspase 8 and EPA had a larger effect on decreasing Bcl-2. Of note, these endpoints are not valid markers of apoptosis in MCF-7 breast cancer cells, which do not express caspase-3 [108], a critical effector caspase in the apoptosis signaling cascade [109]. These breast cancer cells rely on caspases 6, 7, and 9 to initiate apoptosis [108]; therefore, procaspase 8 is not a central part in the initiation of MCF-7 breast cancer cells. In addition, MCF-7 breast cancer cells are associated with increased Bcl-2; therefore, it may be easier to see differences than with a cell line that does not overexpress this protein [110]. Chamras et al. [82] found significant increases in the number of apoptotic cells with EPA and DHA treatments when provided alone in MCF-7 breast cancer cells; however, these increases were not different between treatment groups.

In summary, the published data suggests that DHA and EPA have similar effects on apoptotic signaling in ER+ breast cancer cells. This phenomenon may also be explained by previous work conducted in breast cancer cells with DHA, and cell death receptors. In Ewaschuk et al. [40], treatment of MDA-MB-231 breast cancer cells with DHA caused the CD95 death receptor to be translocated to lipid rafts for apoptosis induction. This demonstrates that DHA regulates membrane-associated proteins associated with extrinsic apoptosis. The reliance of MCF-7 breast cancer cells on the intrinsic pathway through the initiation of capsase-9 suggest that the effect of DHA on the membrane and subsequent effects on cell death membrane receptors in triple negative breast cancer would not impact apoptosis to the same extent in ER+ breast cancer.

Researchers have yet to compare and contrast the effect of DHA and EPA on autophagy, a conserved process that involves the sequestration and degradation of cellular components [111]. Jing et al. [111] reported that in MCF-7 BC cells, DHA induces AMPK phosphorylation, and a decrease in p53 expression and mTOR signaling. mTOR is a negative regulator of autophagy; therefore, DHA promotes autophagy, decreases cell viability, and increases tumour cell susceptibility to apoptosis [111] .

A decrease in pAkt is reported upon incubation of MCF-7 BC cells with DHA (see PI3K/Akt section).When Akt is activated, it removes the inhibition of TSC1/2 on Rheb, facilitating the activation of the mTORC1 complex (Raptor and mTOR), which promotes protein synthesis and cell growth [112]. It is plausible that the observed decrease in Akt activation with DHA is a consequence of inhibition of the activation of mTOR in the mTORC1 complex through p53 (as seen in Jing et al. [111]). Increases in apoptosis and decreases in cell viability were also found in MCF-7 BC cells, consistent with the induction of autophagy and promotion of apoptosis as reported by Jing et al. [111].

Table 3.6.1: Change in apoptotic markers with EPA:DHA mixtures in human breast cancer cells.

Citation	Cell Line	Concentration of EPA or DHA (µM)	Assay	Exposure (hours)	Effect on Markers of Apoptosis*
Schley, Jijon [37]	MDA-MB-231	60 EPA + 40 DHA	Caspase Detection Kit	72	↑29% activated caspases
		45 EPA + 30 DHA + 75 LA			↑22% activated caspases

EPA=eicosapentaenoic acid, DHA=docosahexaenoic acid; LA= linoleic acid; "↑" denotes significant increase ($p<0.05$). *Fold change relative to control condition

Table 3.6.2: Comparison of EPA and DHA on changes in apoptotic markers in human breast cancer cells.

Citation	Cell Line	Concentration of EPA or DHA (µM)	Assay	Exposure (hours)	Effect on Markers of Apoptosis*
Cao, Ma [39]	MCF-7	90 EPA	Flow Cytometry (Annexin V/PI)	12	↑11% apoptotic cells•
			TUNEL		↑11% TUNEL positive cells•
		90 DHA	Flow Cytometry (Annexin V/PI)	12	↑10% apoptotic cells•
			TUNEL		↑9% TUNEL positive cells•
Corsetto, Montorfano [26]	MDA-MB-231	230 EPA	Western Blot	72	NS Δ in Bcl2; NS Δ in procaspase 8
		200 DHA			↓100% Bcl2; ↓ 45% procaspase 8
	MCF-7	230 EPA	Western Blot	72	↓100% Bcl2; ↓20% procaspase 8
		200 DHA			NS Δ in Bcl2; ↓35% procaspase 8
Barascu, Besson [31]	MDA-MB-231	10, 30, 50 EPA	Flow Cytometry (ssDNA)	24	↑0.6%; ↑39%; ↑79%
		10, 30, 50 DHA			↑27%; ↑63%; ↑246%
Chamras, Ardashian [82]	MCF-7	100 EPA	Diff-Quik Stain Set	120	NS Δ in % apoptotic cells
		100 DHA			NS Δ in % apoptotic cells

EPA=eicosapentaenoic acid, DHA=docosahexaenoic acid; PI=Propidium Iodide; TUNEL= Terminal deoxynucleotidyl transferase (TdT) dUTP Nick-End Labeling; ssDNA=single stranded DNA; NS Δ=no significant change; "↑" denotes significant increase ($p<0.05$); "↓" denotes significant decrease ($p<0.05$); "•"= statistical significance was not assessed. *Fold change relative to control conditions.

The PI3K/Akt pathway

The PI3K/Akt signaling pathway is a proliferative signaling pathway that has been implicated in breast cancer pathogenesis [113]. EPA and DHA have been investigated for their ability to regulate the phosphorylation and activation of Akt, a serine/threonine kinase that regulates cell survival, growth, and transcription [114, 115]. One study by Schley et al. [37] examined the effect of a EPA:DHA mixture on the PI3K/Akt pathway (Table 3.6.3). Researchers compared the effects of EPA and DHA with or without LA in the media on phosphorylated Akt (pAkt) and Akt in MDA-MB-231 breast cancer cells. They observed a 47% decrease of pAkt; however, this decrease was blunted by the presence of LA. This demonstrates that the presence of n-6 FA may decrease the effect of EPA and DHA on Akt phosphorylation. No significant changes in Akt were observed, suggesting that the combination of DHA and EPA decreases phosphorylation but not total Akt.

Lee et al. [32] saw a decrease in total Akt with DHA and not EPA in MDA-MB-231 breast cancer cells. This may be due to higher lipid raft PL incorporation of DHA [30], a greater decrease in cell viability [31, 32, 37, 41, 88], higher apoptosis [26, 31], and lower levels of pEGFR [26] and EGFR [32] in tumour cells with DHA treatment compared to MDA-MB-231 breast cancer cells treated with EPA. It is likely that higher lipid raft PL incorporation of DHA decreased EGFR and resulted in a decreased activation of the downstream PI3K/Akt pathway. This may have blunted or removed Akt's inhibitory effect on Bad and the intrinsic apoptotic signaling cascade [109].

Cao et al. [39] found that EPA and DHA decrease the ratio of pAkt:Akt in MCF-7 breast cancer cells to the same extent, albeit statistical analysis was not performed in this study (Table 3.6.4). Western blot analysis showed that these EPA and DHA decrease the pAkt:Akt ratio by decreasing pAkt and had no effect on total Akt [39]. This may be explained by the lack of effect of EPA and DHA on total EGFR in these breast cancer cells [39]. PI3K and Akt are kinases that are subsequently activated in a signal cascade upon binding of EGF to EGFR, receptor dimerization, and EGFR activation by phosphorylation [116]. In controlled *in vitro* conditions,

if EGFR phosphorylation status does not change with exposure to EPA and/or DHA, neither will Akt unless activated by another stimuli.

The effect of feeding EPA and DHA on Akt phosphorylation status has been examined by Chen et al. [117]. These authors intragastrically delivered either a low EPA:DHA diet (0.42 g/100 g diet EPA and 0.38 g/100 g diet DHA) or a high EPA:DHA diet (3.12 g/ 100 g diet EPA and 1.58 g/ 100 g diet DHA) to Sprague Dawley Rats bearing N-Nitroso-N-methylurea (MNU) induced mammary carcinogenesis. Rats provided the high EPA:DHA diet experienced a decrease in tumour size and multiplicity compared to the low EPA:DHA group, albeit no statistical analysis was conducted [117]. Western blot analysis revealed no significant differences in Akt between groups; however, a significantly lower level of pAkt (S473) was observed in the tumours of rats fed the high EPA:DHA diet [117]. Researchers did not find a significant difference between groups on the phosphorylation status of the T308 residue of Akt [117]. Phosphorylation of both S473 and T308 are required for full Akt activation [118]; therefore, the decrease in S473 observed with the high n-3 diet most likely affected the function of pAkt, but did not inhibit pAkt activation. The results of this study demonstrating that feeding DHA and EPA can decrease Akt activation in tumours is consistent with the results of the *in vitro* breast cancer cell studies. This study also shows that western blot analysis of membrane receptors and phosphorylation status of a single residue may not directly translate to protein activity; therefore, functional assays should also be considered.

Table 3.6.3: Change in Akt and pAkt with EPA:DHA mixtures in human breast cancer cell lines.

Citation	Cell Line	Concentration of EPA or DHA (μM)	Assay	Exposure (hours)	Effect on Akt and pAkt*
Schley, Jijon [37]	MDA-MB-231	60 EPA + 40 DHA	Western Blot	72	↓47% pAkt NS Δ in Akt
		45 EPA + 30 DHA + 75 LA			↓27% pAkt NS Δ in Akt

EPA=eicosapentaenoic acid, DHA=docosahexaenoic acid; "↓" denotes significant decrease ($p<0.05$); NS Δ=no significant change; Akt=protein kinase B; pAkt=phosphorylated Akt. *Fold change relative to control conditions

Table 3.6.4: Comparison of EPA and DHA on Akt and pAkt in human breast cancer cell lines.

Citation	Cell Line	Concentration of EPA or DHA (μM)	Assay	Exposure (hours)	Effect on Akt and pAkt*
Cao, Ma [39]	MCF-7	90 EPA	Western Blot	24	↓27 pAkt:Akt •
		90 DHA			↓33% pAkt:Akt•
Lee, Yun [32]	MDA-MB-231	30, 50 EPA	Western Blot**	24	NS Δ in Akt•
		30, 50 DHA			↓ Akt•

EPA=eicosapentaenoic acid, DHA=docosahexaenoic acid; "↓" denotes significant decrease ($p<0.05$); "•"= statistical significance was not assessed; NS Δ=no significant change; Akt=protein kinase B; pAkt=phosphorylated Akt. *Fold change relative to control conditions. **Researchers did not quantify bands from Western Blot Analysis.

7. Summary, conclusions and future directions

EPA and DHA have demonstrated anti-cancer effects across a variety of in cancer types (reviewed in [7]). Currently, it is not known if EPA and DHA have similar effects on breast cancer tumours and if mixtures alter their effect on tumour cell viability, apoptosis, proliferation and incorporation into cellular lipids. Research conducted in BC cell lines and animal models provide essential evidence for changing BC treatments. However, the findings from these studies need to be validated in clinical trials before they can be used to change recommendations or treatment of BC patients.

In the present review, EPA and DHA were compared at the same concentration, DHA had higher anti-cancer activity in triple negative breast cancer cells. This was explained by greater decreases in cell viability [31, 32, 37, 41, 88], EGFR [26, 32], pEGFR [26], apoptosis, [26, 31] and Akt [32] with DHA compared to EPA (Figure 3.7.1). This effect was not predicted by whole cell lipid incorporation as EPA was incorporated more [26], but is related to lipid raft incorporation where DHA was preferentially incorporated over EPA [30]. The data suggests that DHA's spatial conformation disrupts the organization and fluidity of the lipid raft bilayer in breast cancer cells, effecting membrane receptors involved in proliferative signalling pathways. In MCF-7 breast cancer cells, DHA caused greater decreases in cell viability than EPA when provided at the same concentration. However, unlike triple negative breast cancer this could not be explained by changes in EGFR and pEGFR [39], Akt [39], or apoptosis [39]. We hypothesized that this was due to preferential incorporation of EPA into lipid rafts of MCF-7 breast cancer cells [30]. EPA has a smaller, more rigid spatial conformation [83] and as a result, may not affect receptors and proteins involved in proliferative and apoptotic signaling to the same extent as DHA but may affect other proteins. The production of distinct lipid mediators from EPA and DHA (E- and D-series resolvins) may also account for differences in cytotoxicity; however, this remains to be tested in human BC cells representing distinct BC subtypes.

Few studies have attempted to compare and contrast the effect of feeding EPA and DHA alone [90-92]. In contrast to *in vitro* studies, feeding diets supplemented with DHA, compared

to EPA resulted in higher membrane incorporation of DHA [90-92]. This discrepancy between preferential incorporation of EPA in *in vitro* and DHA in feeding studies may be due either to an interaction between EPA and DHA at the level of the membrane, decreasing availability for membrane uptake or it could be due to inherent differences between cells *in vitro* and tumour models in animals. *In vitro* studies allow for the strict control of experimental conditions as well as the precise and accurate delivery of EPA and/or DHA. The presence of the gastrointestinal and hepatic portal vein systems in feeding trials effects how n-3 LCPUFA are absorbed and distributed, therefore; the concentrations of EPA and DHA presented to the tumour in feeding studies may differ compared to what is in the experimental diet.

EPA:DHA mixtures have been studied at a ratio of 1.5:1 and decrease cell viability in both MDA-MB-231 and MCF-7 breast cancer cells [28, 29]. There was more EPA *in vitro* than predicted by this 1.5:1 ratio into whole cell lipids, PL, and lipid raft PL [28, 29], again demonstrating that the ratio of EPA:DHA provided in the diet does not predict membrane incorporation. EPA:DHA mixtures in feeding studies [92-94] also did not predict membrane incorporation. Despite this, in MDA-MB-231 breast cancer cells, 1.5:1 EPA:DHA mixtures decreased proliferative signaling by decreasing Akt activation [37] and increased apoptosis through caspase activation [37] and phosphorylation of whole cell EGFR [28]. *In vitro* [28, 29] studies have not compared mixtures to EPA and DHA alone on proliferative and apoptotic signaling pathways, making it difficult to determine if the anti-cancer effect of mixtures can be predicted on these endpoints. However, a feeding study conducted by Yuri et al. [92] found that a 1:1 EPA:DHA diet and EPA alone decreased tumour multiplicity (number of carcinomas per effective rat) to a similar extent (1.67 and 1.59, respectively) but to a lesser extent than DHA alone (0.23). This study also found that DHA was preferentially incorporated into mammary tissue lipids to a greater extent than predicted, confirming that the dietary ratio does not predict the relative amount of DHA in the membrane or changes in tumour cell proliferation [92].

Presently there is a great deal of heterogeneity found in the literature that make it challenging for researchers to directly compare and contrast findings from studies. The

exposure period, ratios, and concentrations of EPA and/or DHA used *in vitro* and in feeding studies differ between studies. Few *in vitro* studies include a control fatty acid condition [27, 28, 37] or use background fatty acids that are of physiological relevance [27-29, 37]. Similarly, few feeding studies used a basal diet to compare the effects of EPA and/or DHA diets [90, 91]. In addition, a wide range of assays each with their unique set of strengths and limitations are used across studies to determine the effect of EPA and/or DHA on breast cancer cell viability (Table 3.5.2) and apoptosis (Table 3.6.2).

Oftentimes, feeding studies did not record food intake [91, 92, 94], which is critical to determine if animals consumed a sufficient amount of the experimental diet to be exposed to the intended concentration of EPA and DHA. In the present review, feeding studies either induced mammary carcinogenesis by administering MNU [92, 93, 117] or implanted MDA-MB-435 human breast cancer cell lines in the mammary fat pad of rodents [90, 91, 94]. Both of these models have inherent limitations. The carcinogenicity of MNU can vary based on the route of administration, timing of exposure, and dose [119], which varied between studies. Xenograft models that inject human breast cancer cells into the mammary tissue more closely represent the tumour microenvironment and breast cancer tumour progression; however, the MDA-MB-435 cells used by Rose et al. [90, 91, 94] originated from melanoma cell line and are spontaneously metastatic [2] and, as a result, may not accurately represent breast cancer pathogenesis.

Several knowledge gaps exist in the current literature that need to be addressed before the pleiotropic effects and relative efficacy of EPA and DHA in breast cancer subtypes are fully characterized. In the present review, studies that investigated the anti-cancer effects of EPA, DHA, or EPA:DHA mixtures *in vitro* were primarily studied in MDA-MB-231 and MCF-7 breast cancer cells. Other breast cancer subtypes including Her2+ and luminal B breast cancer have not been studied. There is also a lack of feeding studies that verify the mechanistic data from *in vitro* studies findings. The present review showed that preferential incorporation of EPA and DHA differed between *in vitro* and some feeding models and as the membrane changes (fluidity) may be important in driving the mechanism(s) of cellular

phenotypes (apoptosis, proliferation etc.). It would be beneficial to further examine EPA, DHA, and EPA:DHA mixtures in well-designed pre-clinical models. This could include either: (1) *in vitro* models that mimic the *in vivo* tumour microenvironment (such as 3D cell culture; reviewed in [63, 84]) or (2) animal models that represent the heterogeneity of human tumours (such as patient derived xenografts implanted into mammary tissue; reviewed in [120, 121]). HER2 is another ErbB receptor that is commonly truncated or overexpressed in breast cancer [103, 122]. Evidence exists for a beneficial effect of DHA [32, 57, 58] on Her2. However, to date, there are no studies that have systematically compared and contrasted the effect of EPA, DHA, and EPA:DHA mixtures in Her2 overexpressing human breast cancer cell lines, warranting further research. Lastly, the effect of EPA and DHA on autophagy should be examined in MCF-7 BC cells as DHA has been shown to promote autophagy and apoptosis through p53 in these cells [111]. Research could also be done to identify if there is an effect of EPA and/or DHA on autophagy in MDA-MB-231 BC cells, a cell line with a mutated p53 gene [123].

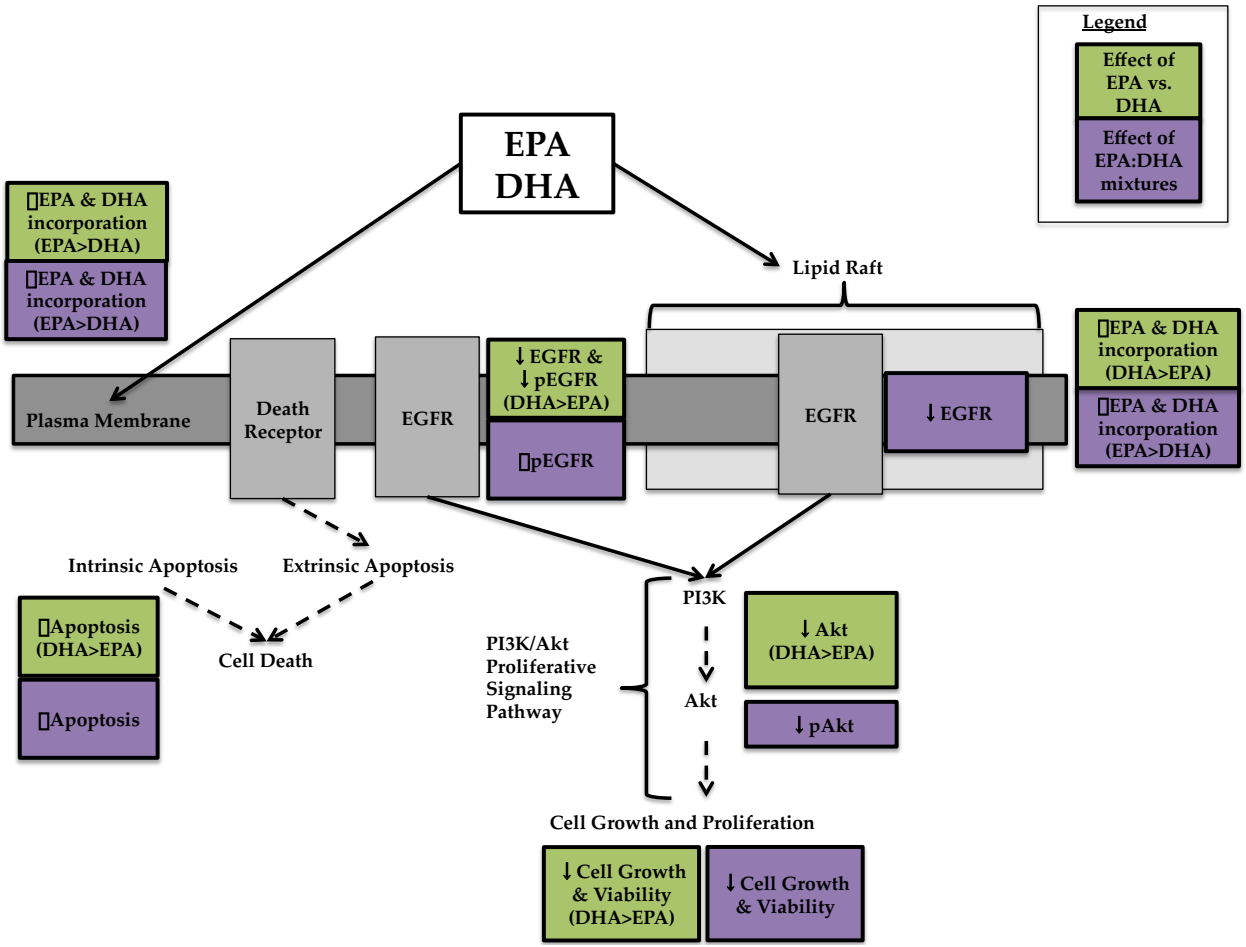

Figure 3.7.1: Schematic illustrating the pleiotropic effects and relative efficacy of EPA and/or DHA in MDA-MB-231 human breast cancer cells. EGFR=Epidermal Growth Factor Receptor; pEGFR=Phosphorylated EGFR; PI3K= Phosphoinositide-3-kinase; Akt=Protein Kinase B; pAkt=Phosphorylated Akt.

Chapter Four-Methods

1. Introduction

Similar methodologies were used in the included studies; therefore, the methods were compiled into a chapter. This chapter includes the following methodologies: 2D and 3D cell culture techniques, preparation of conjugated fatty acids, cell viability assays (trypan blue exclusion and WST-1 assays), analysis of whole cell, total PL, and PL class fatty acid composition, flow cytometry, western blotting, and analysis of 3D phase-contrast images.

2D Cell Culture Maintenance

MDA-MB-231 and SK-BR-3 human breast cancer cell lines were used to model mammary tumorigenesis. MDA-MB-231 breast cancer cells are triple negative (ER-, PR-, HER2-). SK-BR-3 breast cancer cells are HER2+. SK-BR-3 paclitaxel resistant derived sub-lines were studied in auxiliary studies that were not part of the thesis objectives (see Appendix 1).

MDA-MB-231 breast cancer cells (epithelial adenocarcinoma [124]) were obtained from American Type Culture Centre (ATCC, Cedarlane, Burlington, ON) and SK-BR-3 parental (epithelial adenocarcinoma [124]) and resistant cells were kindly provided by Dr. Sambasivarao Damaraju's Lab in the Department of Laboratory Medicine and Pathology at the University of Alberta. Cells were maintained in media as previously described [27, 28, 37, 40]. Briefly, MDA-MB-231 cells were maintained in Iscove's Modified Dulbecco's Medium (Life Technologies, Burlington, ON) supplemented with 1% v/v antibiotic-antimycotic solution (100X; Fisher Scientific, Edmonton, AB) and 5% v/v fetal calf serum (FCS) (Fisher Scientific, Edmonton, AB). SK-BR-3 parental and resistant cells were maintained in McCoy's 5a Medium Modified (Life Technologies, Burlington, ON) supplemented with 1% v/v antibiotic-antimycotic solution and 10% v/v fetal calf serum. Cells were grown at 37°C and 5% v/v CO2 at 98% relative humidity. MDA-MB-231 breast cancer cells were maintained in

75 cm^2 culture flasks and SK-BR-3 breast cancer cells in 25 cm^2 flasks purchased from Fisher Scientific (Edmonton, AB). Media was changed twice weekly and passaged regularly at 80-90% confluence. Cells were trypsinized from flasks using 5 mL HyClone Trypsin 0.25% w/v (Fisher Scientific, Edmonton, AB, Canada). Trypsinization was stopped with Iscoves and McCoy's, respectively, after which cells were centrifuged for 5 minutes at 1500 rpm at 4°C. Cells were resuspended in fresh media and then used for cell line maintenance or for experiments.

Separate growth experiments were conducted in 24 well plates to obtain cells for lipid analysis (whole cell fatty acids, total PL, or PL class composition), determination of changes in cell viability, and changes in cell surface expression of membrane-associated proteins (CD95 and EGFR). Separate growth experiments were also done in cell culture flasks to obtain cells for western blot analysis of proteins related to cell death (CD95, RIPK1, FADD) and growth (EGFR, pEGFR, Her2).

Preparation of Conjugated Fatty Acids

EPA, DHA, OA, and LA were purchased from Matreya LLC (Matreya LLC, Brockville, ON). EPA, DHA, OA, and LA were dissolved in hexane (100mg/10 mL) and stored at -20°C. Fatty acids were bound to BSA (Fisher Scientific, Edmonton, AB) as follows: fatty acids were solubilized in 10mg/mL ethanol, dried under nitrogen gas and resuspended in 1 mL potassium hydroxide (0.1 M and incubated and 50 °C for 10 min). 7.5% w/v BSA was prepared in sterile double distilled water. The 7.5% BSA solution was filtered through a 0.2 micron bottle-top filter (Fisher Scientific, Edmonton, AB). Nine mL of the solution was added drop-wise to the fatty acid-potassium hydroxide mixture and was left at room temperature for 3 hours and then incubated overnight at 4°C. FA were aliquoted into 2 mL sterile Eppendorf tubes (Fisher Scientific, Edmonton, AB) and stored at -20°C.

2. Objective 2: Compare anti-cancer effects of DHA, EPA, & DHA:EPA mixtures in MDA-MB-231 & SK-BR-3 human breast cancer cells.

Trypan Blue Exclusion

In the present thesis, changes in cell viability were determined by counting the number of live cells after exposure to treatment using Trypan Blue Exclusion. A WST-1 assay was also used to confirm that typan blue exclusion was an appropriate measure of cell viability. For the Trypan Blue Exclusion assay, cells were detached from 75 cm^2 flasks as described previously and plated in replicates of four at a density of 20 000 cells/mL for MDA-MB-231 cells, 25 000 cells/mL for SK-BR-3 cells, and 30 000 cells/mL for SK-BR-3 resistant cells in a 24 well flat-bottomed cell culture plate with 2 mL of their respective media for 72 hours. Following this incubation period, cells were incubated with either DHA, EPA or a mixture of DHA and EPA at a ratio of 1:1 DHA:EPA or 2:1 DHA:EPA to cells in quadruplicate at a concentration of 100, 150, or 200 μM of n-3 LCPUFA. All treatments also contained 40 μM OA and 40 μM LA (referred to as 80 μM OA/LA throughout the present thesis) in addition to the n-3 LCPUFA (see Table 4.2.1 for outline of all treatments and doses). To ensure that the effects of the n-3 LCPUFA treatments were due to the anti-cancer effects of the treatments themselves and not a concentration-dependent effect of fatty acids, a fatty acid cytotoxic control consisting of equal parts OA and LA was used for each dose accounting for the concentration of the n-3 LCPUFA treatments (100, 150, or 200 μM) and background fatty acids (80 μM OA/LA) combined (see Appendix 2 for justification for control fatty acid treatment). For 100 μM a fatty acid control of 180 μM was used (90 μM OA/90 μM LA), for 150 μM treatments 230 μM OA/LA was used (115 μM OA/115 μM LA), for 200 μM treatments 280 OA/LA was used (140 μM OA/140 μM LA).

Every 24 hours for 48 hours, the media was replaced with fresh treatment media. After 72 hours, cells were detached with 500 μL of trypsin for 5 minutes (MDA-MB-231) or 10 minutes (SK-BR-3). Trypsinization was stopped with Iscoves and McCoy's, for MDA-MB-

231 and SK-BR-3 breast cancer cells, respectively after which cells were centrifuged for 5 minutes at 1500 rpm at 4°C in a sterile 15 mL Falcon tube. Cells were resuspended in media and a 20 μL aliquot was taken and mixed with 20 μL of a 1:1 Water:Trypan Blue solution. Viable cells were counted using a hemocytometer under a light microscope as previously described [27, 28, 37]. Cell counts for each treatment were converted to a ratio of the control treatment (80 μM OA/LA).

WST-1 assay

MDA-MB-231 breast cancer cells were detached from 75 cm^2 flasks as described previously, and plated at a density of 7.5 $X10^3$ cells/mL in a 96 well flat-bottomed culture plate (Fisher Scientific, Edmonton, AB, Canada) with 200 μL of 5% Iscoves media for 48 hours. One column contained 200 μL of fresh media without breast cancer cells as a control. Media was replaced every 24 hours for 48 hours with 200 μL fresh media containing 40 μM OA and 40 μM LA with or without 100 μM of either DHA, EPA, 1:1 DHA:EPA, 2:1 DHA:EPA. A 90 μM OA/90 μM LA mixture was also used as a FA concentration control treatment. Each treatment was tested in replicates of 8 (n=2 separate passages). After the 72-hour exposure period, the media was replenished with 100 μL of 5% Iscoves (without FA) and 10 μL of WST-1 reagent (Roche, Mississauga, ON) was added to each well. Cells were incubated with the fresh media and WST-1 reagent at 37°C for 1 hour as previously described [40]. The absorbance was recorded at 440 nm on a Spectra Max Plus 384 Microplate reader, (Molecular Devices, San Jose, CA, USA). The absorbance for each treatment were converted to a ratio of the control treatment (80 μM OA/LA).

Table 4.2.1: Doses of EPA and/or DHA (μM) in each n-3 LCPUFA treatment at each dose used in the present thesis for assays in 2D and 3D cell culture.

			Treatments				
			EPA+ 80 μM OA/LA	**DHA+ 80 μM OA/LA**	**1:1 DHA:EPA+ 80 μM OA/LA**	**2:1 DHA:EPA+80 μM OA/LA**	
Total Dose of n-3 LCPUFA (μM)	100 μM	DHA	0	100	50	66	**Dose of DHA and/or EPA (μM) in each treatment**
		EPA	100	0	50	33	
	150 μM	DHA	0	150	75	100	
		EPA	150	0	75	50	
	200 μM	DHA	0	200	100	133	
		EPA	200	0	100	67	

Note: Each n-3 LCPUFA treatment also had 80 μM OA/LA as a fatty acid background. For treatments with a total of 100, 150 and 200 μM DHA and/or EPA the total fatty acid concentration with the background fatty acid was 180, 230, and 280 μM, respectively.

Lipid Analysis

To determine the effect of n-3 LCPUFA treatments on human breast cancer cells, lipids from whole cells were extracted using a modified Folch procedure [1 mL of 0.1M KCl and 4 mL of 2:1 Chloroform: Methanol [27, 28]. The lower chloroform phase of the extract was dried under nitrogen gas and suspended in hexane. Subsequent analyses were conducted on these samples to determine the composition of one of the following: whole cell FA, total PL, or PL classes.

Isolation of whole cell FA

Whole cell fatty acids were isolated using modified Folch method [28, 125]. Briefly, fatty acid methyl esters (FAME) were prepared (1.5 mL of BF_3, 1.5 mL hexane, 1 mL ddH_2O) left overnight at 4 °C and dried the next day under nitrogen gas [27, 28]. Samples were resuspended in 200 μL of hexane and were separated and quantified by Gas-Liquid Chromatography (GLC) as described below (*see Fatty acid analysis)*.

Total PL composition

The total PL fraction was separated from Folched samples using silica "G" thin layer chromatography (TLC) plates (Fisher Scientific, Edmonton, AB) as previously described [126]. Briefly, lipids were spotted on the TLC plate and the other lipids removed using a ratio of petroleum either: diethyl ether: glacial acetic acid (80:20:1 v/v/v) and the PL band was visualized under ultraviolet light using 8-anilino-1-napthalenesulphonic acid (ANSA). FAME were prepared as described above and GLC analysis was used to identify the relative fatty acid composition of total PL.

Total PL class composition

PL classes were separated from Folched samples using silica "H" TLC plates (Fisher

Scientific, Edmonton, AB) using a ratio of chloroform: methanol: 2-propanol-triethylamine: KCl (0.25%) (60:18:50:36:12 v/v/v/v/v) and the following bands were visualized under UV light using ANSA: PC, PE , and PI [127]. FAME were prepared as described above and GLC analysis was used to identify the relative FA composition of PL classes.

Fatty acid analysis

FAME were separated using a 7890A gas-chromatograph with a fused-silica CP-Sil 88 capillary column (Agilent Technologies, Mississauga, ON) and identified as described by Cruz-Herenandez et al. [27, 127, 128]. Briefly, prepared FAME were heated to 250°C to volatilize fatty acid. Hydrogen gas (mobile phase) was used to carry the volatilized FAME through the capillary column (highly polar stationary phase). As volatilized FAME move through the column, the fatty acid constituents of the sample separate on the basis of boiling point, polarity, and degree of saturation. As the sample runs through the column, the fatty acids run past a detector and a chromatogram is generated. This schematic generates peaks corresponding to the intensity and retention time (time elapsed from sample being injected to running past detector) of each fatty acid. The different peaks were identified using commercial standards. The area under these peaks is proportional to the relative amount of fatty acid in a sample and was used to determine differences in the relative composition of fatty acid (whole cell fatty acid, total PL, and PL classes) between treatment groups. All lipids were expressed as a percent (%w/w) of total identified fatty acids.

Selection of n-3 LCPUFA treatments

The 1:1 ratio of DHA:EPA was selected to directly compare and contrast the anti-cancer effects of DHA and EPA alone and in combination with one another. To date, researchers have not studied this ratio of DHA:EPA and compared it to these n-3 LCPUFA alone *in vitro* on human breast cancer cell viability (see Chapter 3). Our lab group has previously investigated

the effects of a 1:2 DHA:EPA mixture [28, 37], which is a ratio found most often in fish oil supplements [11]. For the present study, we selected a 2:1 DHA:EPA ratio for two reasons: 1) our lab group has previously found that DHA has been shown to exert a greater anti-cancer effect than EPA at the same concentration [40]; therefore, we wanted to determine if providing more DHA in a mixture enhanced its anti-cancer effect and 2) The 2:1 DHA:EPA ratio is found in Atlantic salmon [129], which was the most commonly consumed seafood contributing to total n-3 LCPUFA intake in a cohort of pregnant Albertan women as determined by the Alberta Pregnancy Outcomes and Nutrition (APRoN)[130] .

Our lab group found that DHA and EPA, when provided alone, had a significant effect on cell killing at 100 and 150 μM [40]. We selected a minimum dose of 100 μM as most researchers have investigated the effect of DHA and/or EPA on cell viability concurrently with other anti-cancer assays (i.e. flow cytometry, western blot analysis on proteins related to cell growth and death) (see Chapter 3) at a total dose of 100 μM of n-3 LCPUFA. This concentration has applicability to human intakes as 2.2 g/day of EPA supplementation in non-small cell lung cancer patients resulted in plasma PL EPA levels equivalent to approximately 88 μM [89]. Doses of 150 and 200 μM were also selected to determine if there was a dose-dependent effect of these n-3 LCPUFA alone or in mixtures on tumour cell incorporation and anti-cancer mechanisms.

3. Objective 3: Compare DHA, EPA, & DHA:EPA mixtures on cell death & growth pathways.

Flow Cytometry

MDA-MB-231 and SK-BR-3 human breast cancer cells were treated with DHA, EPA, 1:1 DHA:EPA, and 2:1 DHA:EPA at 100 μM and a 180 μM FA control (90 μM OA/90 μM LA) for 72 hours in a 24 well plate and harvested with trypsin as previously described. Treatments were done in quadruplicate and when harvested, cells from the same treatment

group were pooled to ensure there was a sufficient amount of cells for analysis (100 000 cells/mL). Following extraction, cells were washed twice in 2 mL of sterile 1% FCS-PBS and cells were counted using a hemocytometer under a light microscope. The calculated volume of cells was added to 1000 μL of sterile 1% FCS-PBS in IF tubes (VWR, Edmonton, AB). The suspension was centrifuged for 5 minutes at 1500 rpm at 4°C in a sterile 15 mL Falcon tube. Cells were incubated with their respective antibody or isotype control at 4°C for 1 hour and cells were washed twice with 1000 μL of sterile 1% FCS-PBS. MDA-MB-231 breast cancer cells were incubated with one of the following: diluted anti-Fas antibody [DX2] APC (ab25290), anti-EGFR antibody [ICR10] FITC (Abcam, ab11400), or isotype control (BD Pharmigen, FITC Mouse IgG2a, K isotype control) all at a dilution of 1:20 (in 1% FCS-PBS). SK-BR-3 breast cancer cells were incubated with diluted anti-Fas antibody [DX2] APC (ab25290) at a dilution of 1:20 (in 1% FCS-PBS). Following incubation, cells were fixed in 300 μL of 1% paraformaldehyde and analyzed using a FACSCantoII (Becton Dickinson, Mississauga ON) equipped with: an air-cooled 405-nm solid state diode, 30mW fibre power output violet laser, with 450/50 and 510/50 band pass (bp) filters; a 488-nm solid state, 20-mW blue laser with 530/30 bp, 585/42 bp, 670 long pass (lp) and 780/60 bp filters; and a 633-nm HeNe, 17-mW red laser with 660/20 and 780/60 filters. Calibration was performed with CaliBRITE Beads (Becton Dickenson, Mississauga ON). Analysis was performed using FlowJo© software v10 (Tree Star, Ashland, OR, USA, Version 10.0.8r1). There was no difference between the percentage of stained and unstained cells with staining; therefore, median fluorescence intensity was used to determine the effect of treatment on cell surface proteins related to cell death and growth. Gates were set on stained cells in dot plots of side scatter area (SSC-A) vs. forward scatter area FSC-A (see Appendix 3) and the median fluorescence of cells within these gates were quantified. The median fluorescence intensity for each treatment were converted to a ratio of the control treatment (180 μM OA/LA).

Western Blotting

Cells were harvested from the flasks (as described) with trypsin. Whole cell protein lysates were prepared from fatty-acid treated cells by the addition of lysis buffer consisting of: 1 mL of 1M Tris–HCl pH 7.4, 1.37 mL of 5M NaCl, 5mL glycerol, 0.5 mL of Nonidet P-40, 200 microlitres of 0.5 M EDTA, 41.93 mL of dd H2O with freshly added protease and phosphatase inhibitor cocktails (1:100 of each; PIC P8340; PPIC P2850; PPIC II P5726) (Sigma-Aldrich Canada Ltd., Oakville, Ontario, Canada). Lysates were incubated on ice for 30 minutes, then centrifuged at 4 °C for 20 min at 13,300 rpm, and supernatants were aliquoted and stored at -20 °C. The protein concentration of cell lysates was determined using a Pierce Bicinchoninic Acid (BCA) protein assay kit (Fisher Scientific, Edmonton, AB). Equal amounts of protein from each treatment were separated by SDS-PAGE on 10% polyacrylamide gels. Precision Plus Protein™ All Blue Standards (BioRad; Fisher Scientific, Edmonton, AB) were used to monitor protein separation. Proteins were electrophoretically transferred to nitrocellulose membranes (BioRad; Fisher Scientific, Edmonton, AB). Even protein loading and transfer was confirmed by staining with Ponceau S solution P7170 (Sigma-Aldrich Canada Ltd., Oakville, Ontario, Canada). Membranes were blocked for one hour at room temperature with TBST (0.01 M Tris–HCl pH 7.4, 0.15 M NaCl, 0.1% Tween-20) and 5% w/v powdered milk. Membranes were washed 1 x 15 and 2 x 5 minutes with TBST. Primary antibodies to CD95 (Cell Signaling, 4233S), EGFR (Cell Signaling, 4267S), pEGFR (Cell Signaling, CS 3777S), FADD (Cell Signaling, 2782S) were diluted 1:1000 in TBST containing 5% w/v BSA. Antibodies to Her2 (Cell Signalling, 2165S) and RIPK1 (Abcam, ab72139) were diluted 1:1000 in TBST containing 5% w/v powdered milk. All primary antibodies were incubated with membranes overnight at 4 °C. Parallel blots were probed under the same conditions with primary antibody for GAPDH (Cell Signaling, 2118S diluted 1:50 000) or B-tubulin (Cell Signalling 2128S, 1:50 000) to confirm even protein loading. After incubation overnight at 4ºC, membranes were washed with TBST for 1 x 15 minutes and then 2 x 5 minutes. All

membranes were incubated with secondary antibodies for one hour at room temperature. For HRP-conjugated anti-mouse secondary antibody (Cell Signaling, 7074S) was used at 1:2000 dilution in TBST containing 5% w/v BSA. For GAM (for RIP; ab205719) was used at 1:2000 in 5% w/v powdered milk. Membranes were washed with TBST 1 x 15 min and 2 x 10 minutes. Membranes were developed using an enhanced chemiluminescence (ECLTM) Western Blotting detection kit (GE Healthcare; Buckinghamshire UK Amersham; RPN2235). The relative intensities of band signals were visualized using laser densitometry Amersham (GE) Typhoon 9400 Variable Mode Imager (Edmonton, AB) and analyzed using ImageQuant TL Toolbox Version 7.0 (GE, Edmonton, AB). The band density for each treatment were converted to a ratio of the control treatment (180 μM OA/LA).

4. Objective 4: Determine the effects of DHA, EPA, & DHA:EPA mixtures on cell growth parameters in 3D on-top Cell Culture model of human breast cancer.

3D on-top cell culture model for human breast cancer cells

A 3D on-top Cell Culture model was established with modifications of the method reported by from Lee et al. [67]. MDA-MB-231 and SK-BR-3 cells were seeded between two layers of a Matrigel Basement Membrane (Matrix Growth Factor Reduced, Fisher Scientific, Edmonton, AB). This particular 3D model and matrigel were selected to model work previously done Dr. Mina Bissell and her laboratory group at the Lawerence Berkeley National Laboratory in California. This group has successfully established the growth of several breast cancer cell lines representing a variety of subtypes [131-134].

Before use, the matrigel was thawed overnight at 4°C. Matrigel gelatinizes promptly upon heat, so all manipulation steps with the matrigel were performed on ice using chilled pipette tips (stored at 4°C). To establish the bottom layer of the Matrigel, a chilled 1000 μL pipette tip was used to pipette 240 μL of thawed Matrigel, coating the bottom of a well in a 24 well flat-bottomed plate. The 24-well plate was placed in incubator at 37°C and 5% v/v CO_2 at

98% relative humidity for 15 minutes to allow solidification of this layer. After the bottom layer of the gel had been solidified, MDA-MB-231 and SK-BR-3 cells that were detached from 75 cm^2 flasks (as described previously) were resuspended in 500 μL of fresh growth media and slowly added on top of the solidified bottom layer of matrigel. MDA-MB-231 breast cancer cells were seeded at 10 000 cells/mL and SK-BR-3 at 15 000 cells/mL. The 24-well plate was placed in incubator at 37°C and 5% v/v CO2 at 98% relative humidity for 15 minutes to allow the breast cancer cells to settle in the gel. After this incubation period, the top layer (500 μL of a 10% media-matrigel mixture) was slowly added to the top of each well. Cells were incubated in this 3D on-top Model for 24 hours before being treated with FA. Cells were maintained in media for 96 hours. Media was aspirated slowly using a chilled 1000 μL pipette tip and 1000 μL of a fresh 10% matrigel-media-n-3 LCPUFA fresh treatment containing 80 μM of OA/LA (control, 40 μM OA and 40 μM LA) with or without 150 μM of DHA, EPA, or a 1:1 DHA:EPA mixture (75 μM DHA and 75 μM EPA) was added every 48 hours. The control condition (80 μM OA/LA) was done in triplicate and n-3 LCPUFA (DHA, EPA, or 1:1 DHA:EPA) treatments in duplicate.

Phase-contrast imaging of cells in 3D model

Phase-contrast images of cells grown in our 3D on-top Cell Culture model were taken after a 72-hour exposure period to either 80 μM OA/LA with or without n-3 LCPUFA treatments (150 μM DHA, EPA, or 1:1 DHA:EPA) in a flat bottomed 24 well plate. Phase-contrast microscopy is a commonly used technique that was developed to maximize the visibility of small, transparent (unstained) specimens by transforming irregularities in wave front and amplitude to differences in transparency (or image contrast) [135]. 2D phase-contrast images were captured with a Zeiss Axiovert 200M Microscope (Lens: 10x/0.3NA Plan-NEOFLUAR ph1) using a CoolsnapHQ camera from Roper Scientific. The captured images were processed in Metamorph (Version 7.7.13.0, Molecular Devices).

Analysis of phase-contrast images in 3D

There is a great deal of heterogeneity in the literature regarding the terminology and definitions of terms used to describe tumour cells grown in 3D cell culture (reviewed in [136]). Examples include spheroids, mammospheres, oncospheres, and tumourspheres (reviewed in [136]). Oftentimes this terminology is used interchangeably; however, there are studies that have shown that this may not be appropriate. For example, SK-BR-3 breast cancer cells may not able be able to form mammospheres because they do not express E-cadherin [137]. In this thesis, human breast cancers cells grown in 3D cell culture will be referred to as spheroids (spherical structures) and disorganized groupings of spheroids in 3D cell culture will be referred to as aggregates (AG) [136].

Due to intrinsic differences in cell morphology of MDA-MB-231 and SK-BR-3 breast cancer cells, distinct approaches were used to determine the effect of control and n-3 LCPUFA treatments. MDA-MB-231 breast cancer cells have a stellate morphology when grown in 2D models of human breast cancer. Grown in 3D cell culture, these cells readily form spheroids rather than AG. To assess the effect of our treatments in this cell line, we were interested on the effect on the morphology of the spheroids (average pixel area, shape factor, and breadth) as well as the ratio of spheroids:AG. SK-BR-3 breast cancer cells have a grape-like morphology and have a propensity towards AG formation when grown in a 3D on-top cell culture model. Therefore, in this cell line, we were interested in the effect of our treatments on AG formation (pixel area) and the morphology of these AG (length, breadth, and shape factor). It was rare to see spheroids in this cell line when grown in our model. To determine if our n-3 LCPUFA treatments had an effect on the presence of spheroids, an estimate for the number of spheroids was determined by dividing AG area by that of spheroids found in each image. The average number of spheroids/AG was also calculated.

Analysis of phase-contrast images were conducted in MetaXpress (Version 6.2.1.704 , Molecular Devices) using a customized script (journal). Briefly, the images were field-

corrected for uneven illumination. A top-hat filter was used to enhance the feature contrast and, then, the resulted images were analyzed using a threshold mask with visual quality controls for measuring the sizes of the spheroids . Visual inspection of the threshold mask was conducted to ensure proper shading of spheroids and AG. Spheroids and/or AG on the edges of phase-contrast images were excluded from analyses (out of field of view). In each cell line, for each treatment group (DHA, EPA, or 1:1 DHA:EPA) a total of 12 images (from 3 separate passages) were analyzed while 18 images were analyzed for each 80 μM OA/LA control group (also from 3 separate passages). There are several distinct types of gels and culture conditions that can be used in 3D cell culture models and, as a result, this presents many challenges when deciding the most appropriate imaging modality [138]. When analyzing phase-contrast images of 3D cell culture models researchers have often looked at growth parameters including: pixel area [139], AG size [140], cell number [140], and the number of cells per spheroid [72].

In both breast cancer cell lines, the following attributes were captured using MetXpress: count, total pixel area (number of pixels in the object), length (the span of the longest chord through the object), breadth (the caliper width of the object, perpendicular to the longest chord), and shape factor (value from 0 to 1 representing how closely the object represents a circle, 0 a flattened object and 1 a perfect circle) of spheroids and AG in MDA-MB-231 and SK-BR-3 breast cancer cells. The journal did not always reliably determine counts due to differences in spheroids and/or cell morphology and AG formation; therefore, all photos analyzed using the journal in MetaXpress were cross-referenced with original phase-contrast images to ensure the reliability of counts and pixel area of spheroids, and AG. All data was exported into Microsoft excel spreadsheets before statistical analysis. Once the analyzed images were cross-referenced with phase-contrast images to ensure that the accurate number of spheroids and AG were counted, the total and average pixel area (for either spheroids+ AG, spheroids only, or AG only) was determined for each treatment in each breast cancer cell line.

Extraction of cells from 3D on-top cell culture model

Cells were extracted from the 3D on-top Cell Culture Model using a method adapted from Lee et al. [67]. All steps were conducted at 4°C (in cold room). Media was aspirated off using a chilled 1000 μL pipette tip. Ice cold phosphate-buffered saline (PBS)(130 mM NaCl, 13 mM Na_2HPO_4, 3.5 mM NaH_2PO_4(pH 7.4)) (500 μL) and PBS- Ethylenediaminetetraacetic acid (EDTA)(5 mM EDTA, 1 mM NaVO4, 1.5 mM NaF in PBS)(1000 μL) was then added to each well. The PBS/PBS-EDTA mixture was used to degrade the structural integrity of the matrigel and rinse the well. The plate containing the Matrigel/breast cancer cell/PBS/PBS-EDTA mixtures were put on a plate shaker (The Belly Dancer, Stovall Life Science Incorporation, Greensboro NC USA) for 30 minutes. Mixtures from each well in the same treatment group were pooled together and transferred into 15 mL falcon tubes. The wells were rinsed with 1000 μL of PBS-EDTA to ensure the gel was degraded from each well and all cells were extracted. The extracted Matrigel/breast cancer cell/PBS/PBS-EDTA mixtures in falcon tubes were placed on a tube rotator (Labnet International Inc, Labnet Mini Labroller) for 10 minutes and subsequently centrifuged at 4°C at 1000 rpm for 10 minutes (Beckman J2-HC Centrifuge, USA). The supernatant was aspirated and pellets isolated for whole cell fatty acid analysis.

Fatty acid analysis of cell from 3D model

Cells were isolated from 3D on-top cell culture model as described above. Lipids from whole cells were extracted using a modified Folch procedure [500 μL of 0.1M KCl and 2 mL of 2:1 Chloroform: Methanol]. FAME were prepared (1.5 mL of BF_3, 1.5 mL hexane, 1 mL ddH_2O) left overnight at 4 °C and dried the next day under nitrogen. Samples were resuspended in 75 μL of hexane and were separated and quantified by GLC as previously described.

5. Statistical analysis

Statistical analyses were conducted using STATA/IC, version 15.0. When possible to test for a normal distribution, this was done. For parameters where normality could be assumed (cell viability, membrane n-3 LCPUFA incorporation, and 3D analysis), a one-way analysis of variance was used to determine differences between control and treatment groups. When a significant difference between groups was found ($p<0.05$), a Bonferroni test for post-hoc analysis was conducted for multiple comparisons. When parameters were not normally distributed (median fluorescence intensity (flow cytometry) and band intensity (western blotting) relative to control), a Kruskal Wallis test was used to determine differences between control and treatment groups. When a significant difference was determined between groups ($p<0.05$), a Mann Whitney U test for post-hoc analysis was conducted for multiple comparisons. OA/LA (180 μM) was used as a control treatment for experiments that added 100 μM of the fatty acid treatment to the 80 μM OA/LA background. This includes the flow cytometry and western blot analysis. Control (80 μM OA/LA) was used as control in cell viability and n-3 LCPUFA incorporation into tumour cells as there was no difference between the 80 and 180 μM OA/LA treatments. 80 μM OA/LA was also used as control in 3D culture analysis of growth parameters. Data is presented as means ± SEM.

Chapter Five-Results

1. Cell viability

Effect of n-3 LCPUFA treatments on cell viability in MDA-MB-231 human breast cancer cells

In MDA-MB-231 human breast cancer cells, no significant differences in cell viability were observed between the control (80 μM OA/LA) and the fatty acid dose controls (180, 230, or 280 μM OA/LA) (Figure 5.1.1). Therefore, the 80 μM OA/LA was used as the comparison in the statistical analysis. At 100 μM, all n-3 LCPUFA treatments decreased cell viability, to the same extent, relative to the 80 μM OA/LA control (25-29%, $p<0.05$; Figure 5.1.1A). The observed decrease in cell viability with 100 μM n-3 LCPUFA treatments was also associated with a decrease in metabolic activity, as determined by a WST-1 assay (Figure 5.1.1B). In this assay, all n-3 LCPUFA treatments significantly decreased the metabolic activity of MDA-MB-231 breast cancer cells relative to the 80 μM OA/LA control (28-41%, $p<0.05$). Using trypan blue exclusion at 150 μM, all n-3 LCPUFA treatments decreased cell viability to the same extent (19-26% decrease, $p<0.05$; Figure 5.1.1C). At 200 μM, all n-3 LCPUFA treatments decreased cell viability; however, DHA decreased viability to a greater extent than EPA, 1:1, and 2:1 DHA:EPA treatments (59% compared to 37%-44%, $p<0.05$; Figure 5.1.1D).

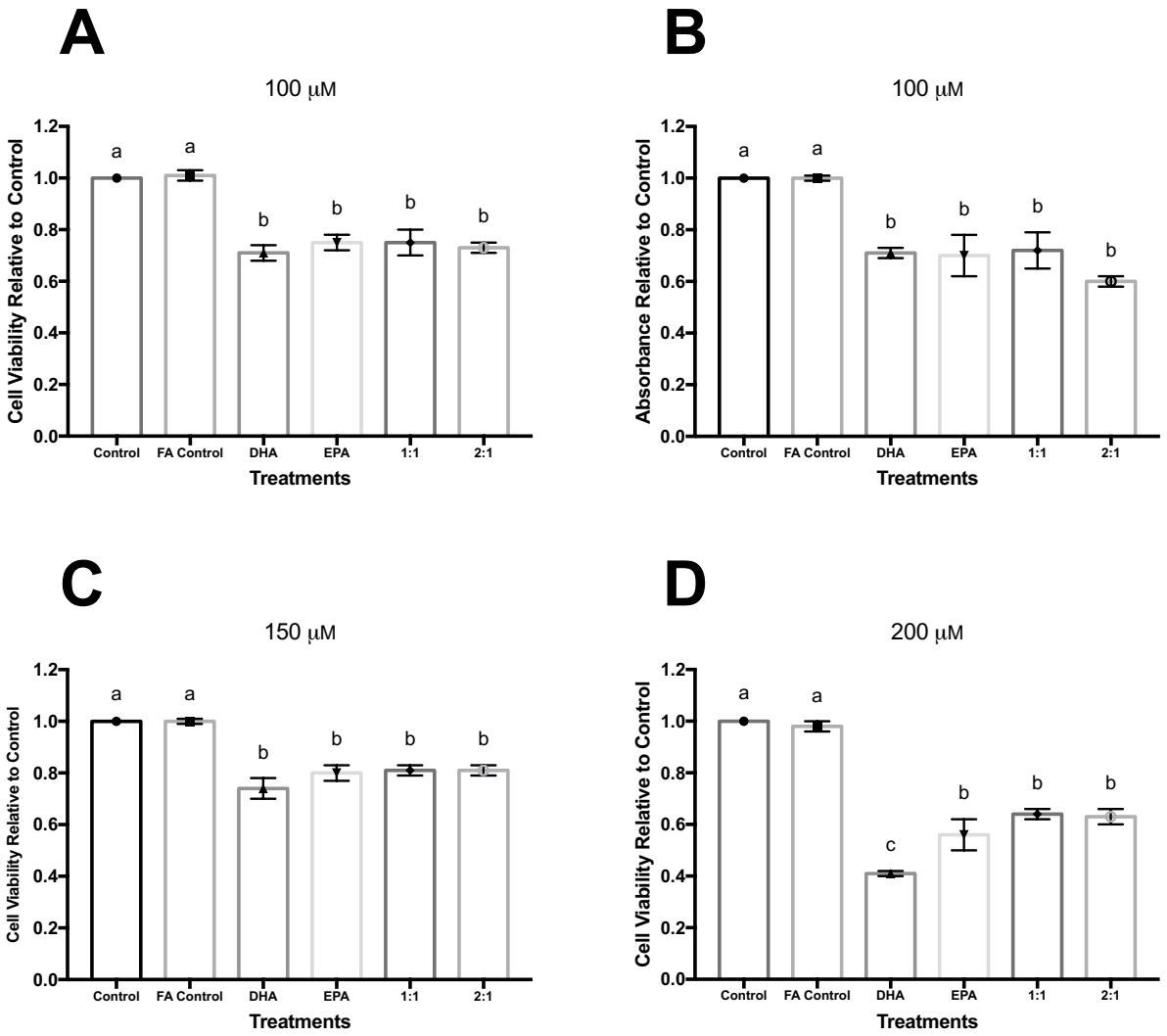

Figure 5.1.1 Cell viability of MDA-MB-231 human breast cancer cells incubated with n-3 LCPUFA treatments. Measured by counting cells (A, C-D) or WST-1 assay (B)) at the following total concentrations with 80 μM OA/LA background: (A) 100 μM (n=3 separate passages), (B) 100 μM (n=2 separate passages); (C) 150 μM (n=3 separate passages); or (D) 200 μM (n=6 separate passages). Bars represent the mean ± SEM for MDA-MB-231 breast cancer cells. Bars that do not share a common letter are significantly different ($p<0.05$). "FA control" =fatty acid control.

Post-hoc analysis was conducted to determine if there was an effect of dose on the anti-cancer effect of each n-3 LCPUFA treatment (Figure 5.1.2). There was no significant difference in the mean percent decrease in breast cancer cell viability from 100 μM to 150 μM for any of the n-3 LCPUFA treatments ($p>0.05$). The mean percent decrease in breast cancer

cell viability was significantly higher for all n-3 LCPUFA treatments from 150 μM to 200 μM ($p<0.05$) (Figure 5.1.2).

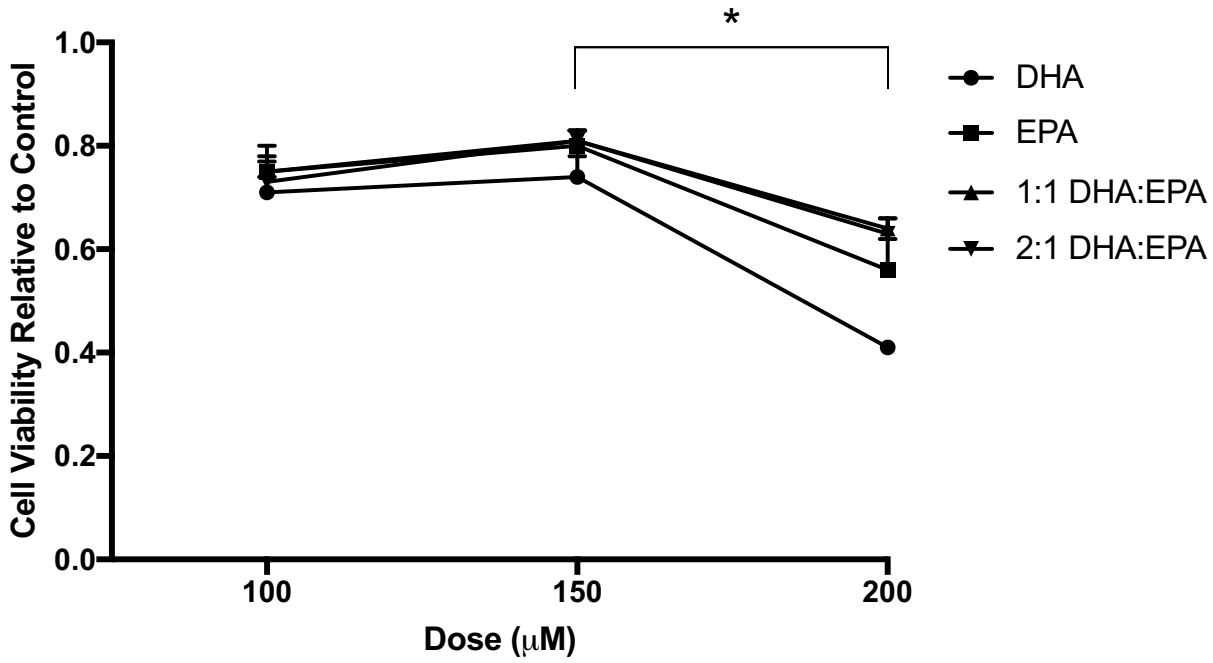

Figure 5.1.2: Dose effect of n-3 LCPUFA treatments on cell viability of MDA-MB-231 human breast cancer cells incubated with n-3 LCPUFA treatments at 100 μM (n=3 separate passages); 150 μM (n=3 separate passages); and 200 μM (n=6 separate passages) with 80 μM OA/LA background. Dots represent the mean ± SEM for MDA-MB-231 breast cancer cells. "*" = significant difference in cell viability from 150 to 200 μM for each respective n-3 LCPUFA ($p<0.05$).

Effect of n-3 LCPUFA treatments on cell viability in SK-BR-3 human breast cancer cells

In SK-BR-3 human breast cancer cells, no significant differences were observed between the control (80 μM OA/LA) and the fatty acid controls at each dose tested (180, 230, or 280 μM OA/LA) (Figure 5.1.3). Therefore, the 80 μM OA/LA treatment was used as the control. Across doses, all n-3 LCPUFA decreased cell viability relative to the 80 μM OA/LA control and EPA decreased cell viability to a significantly greater extent than DHA, 1:1, or 2:1 DHA:EPA mixtures. EPA when provided at 100 μM decreased cell viability by 35% ($p<0.05$). When DHA was provided with the 100 μM and 2:1 DHA:EPA (66 μM DHA) treatments cell

viability was decreased to the same, but lesser extent than EPA (22% and 24% decrease, respectively, $p<0.05$). The 1:1 DHA:EPA treatment (50 μM DHA and 50 μM EPA) resulted in the smallest observed decrease in cell viability (17% , $p<0.05$). This suggests that low doses of DHA (50 μM) can blunt the effect of EPA on cell death. This coincides with changes in side scatter area (SSC-A), a measure of granularity which was assessed using flow cytometry (Figure 5.1.4). Cells become more granular when undergoing apoptosis [141]; therefore, this parameter confirms that EPA causes a greater decrease in cell viability. In SK-BR-3 breast cancer cells, 100 μM EPA alone significantly increased granularity relative to the 180 μM OA/LA FA control (7%, $p<0.05$), while DHA and DHA:EPA did not significantly increase granularity compared to 180 μM OA/LA. At 150 μM, EPA decreased viability by 44% ($p<0.05$), while DHA and DHA:EPA mixtures decreased viability to the same extent (31-37%, $p<0.05$). At 200 μM, EPA decreased cell viability by 47% ($p<0.01$), while DHA and DHA:EPA treatments decreased viability to the same extent (33-39%, $p<0.05$).

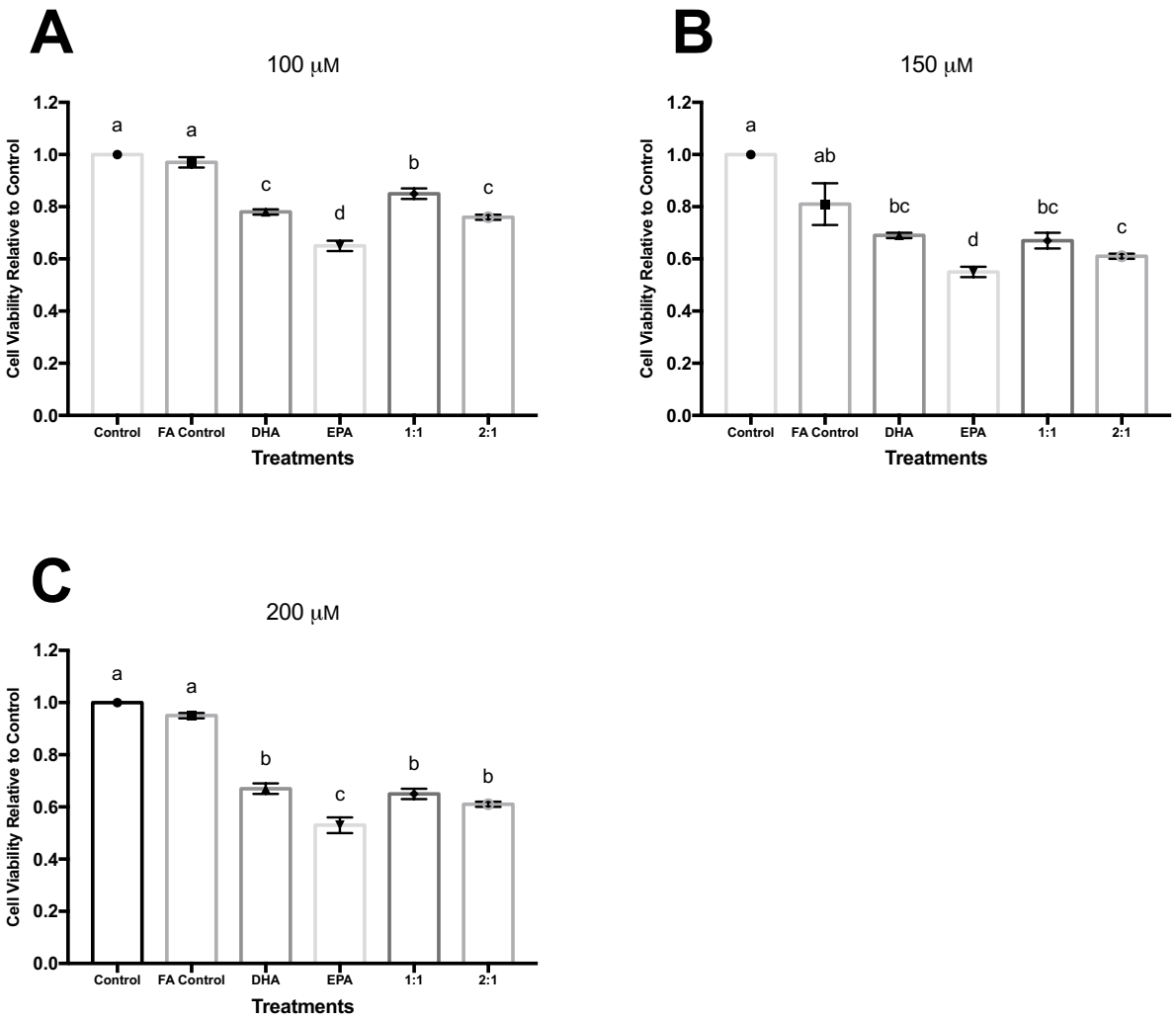

Figure 5.1.3: Cell viability (measured by counting cells) of SK-BR-3 human breast cancer cells incubated with n-3 LCPUFA treatments. The following concentrations were used with 80 μM OA/LA background: (A) 100 μM (n=6 separate passages); (B) 150 μM (n=3 separate passages); or (C) 200 μM (n=4 separate passages) with 80 μM OA/LA background. Bars represent the mean ± SEM for SK-BR-3 breast cancer cells. Bars that do not share a common letter are significantly different ($p<0.05$). "FA control" =fatty acid control.

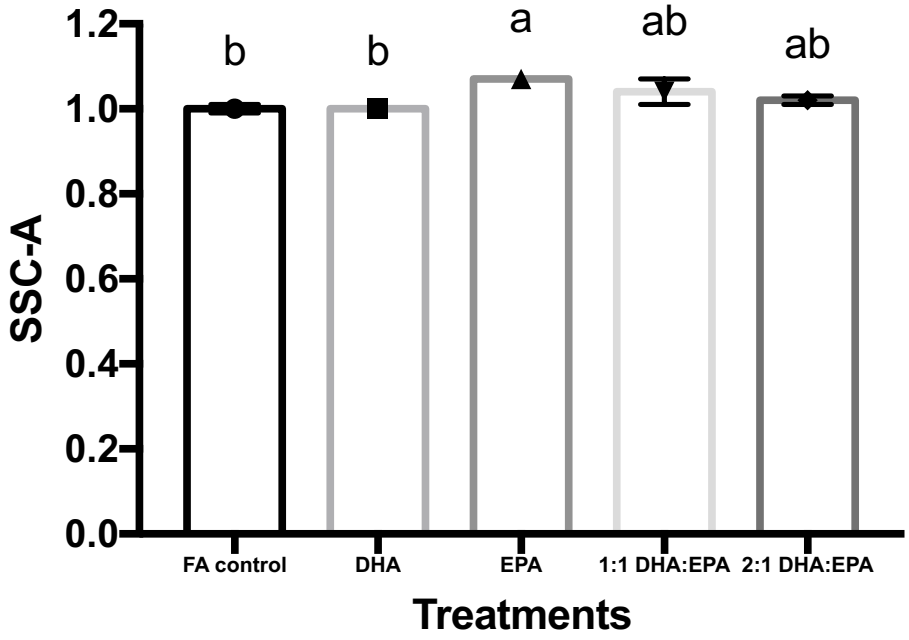

Figure 5.1.4: Side scatter area (SSC-A) of SK-BR-3 human breast cancer cells incubated with 100 μM n-3 LCPUFA treatments (n=3 separate passages) relative to 180 μM OA/LA fatty acid (FA) control. Bars represent the mean ± SEM for SK-BR-3 breast cancer cells. Bars that do not share a common letter are significantly different ($p<0.05$).

Post-hoc analysis was conducted to determine if there was an effect of dose on the anti-cancer effect of each n-3 LCPUFA treatment (Figure 5.1.5). The mean percent decrease in breast cancer cell viability was significantly higher for all n-3 LCPUFA treatments from 100 μM to 150 μM ($p<0.05$) except for EPA ($p>0.05$) (Figure 5.1.5). There was no significant difference in the mean percent decrease in breast cancer cell viability from 150 μM to 200 μM for any of the n-3 LCPUFA treatments ($p>0.05$).

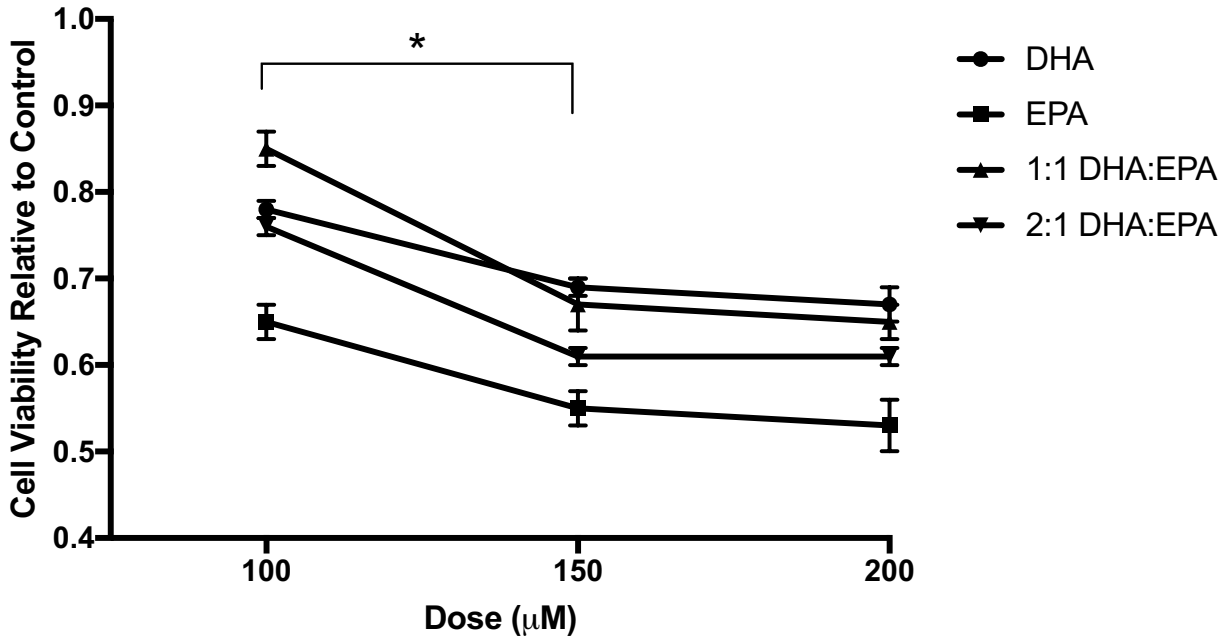

Figure 5.1.5: Dose effect of n-3 LCPUFA treatments on cell viability of SK-BR-3 human breast cancer cells incubated with n-3 LCPUFA treatments at 100 μM (n=6 separate passages); 150 μM (n=3 separate passages); and 200 μM (n=4 separate passages) with 80 μM OA/LA background. Dots represent the mean ± SEM for SK-BR-3 cells. "*" = significant difference in cell viability from 100 to 150 μM for each respective n-3 LCPUFA ($p<0.05$).

2. Lipids

Effect of n-3 LCPUFA treatments on fatty acid content of whole cell total PL and PL classes in MDA-MB-231 breast cancer cells grown in 2D cell culture

Selective fatty acids profiles for whole cell PL and PL classes are shown in Tables 5.2.1-5.2.9 for MDA-MB-231 breast cancer cells (see Appendix 4 for complete fatty acid profiles). The effect of fatty acid treatments on changes in EPA, docosapentaenoic acid (DPA), DHA, and AA on whole cell total PL and PL classes from these tables are summarized in Figures 5.2.1 and 5.2.2. The fatty acid profiles for whole cell PL and PL classes were also analyzed to compare the following: 1) relative EPA+DPA content (%w/w) with the EPA treatment to that of DHA content (%w/w) with the DHA treatment; 2) relative EPA+DPA content (%w/w) compared to DHA content (%w/w) with the 1:1 DHA:EPA treatment; 3) relative EPA+DPA content (%w/w) compared to DHA content (%w/w) with the 2:1 DHA:EPA treatment; and 4) the predictability of the ratio in DHA:EPA mixtures on EPA+DPA and DHA content (%w/w) in human breast cancer cells. The results are summarized in Table 5.2.10.

Table 5.2.1: Change in selected fatty acids in the PL fraction of MDA-MB-231 breast cancer cells incubated with control or 100 μM n-3 LCPUFA treatments

Fatty Acid	80 μM OA/LA	DHA	EPA	1:1 DHA:EPA	2:1 DHA:EPA	180 μM OA/LA
18:1n-9	8.64±0.26^{bc}	**10.34±0.24^{a}**	7.96±0.05^{c}	9.62±0.15^{ab}	9.04±0.12^{abc}	8.17±0.55^{bc}
18:2n-6	35.36±2.13^{ab}	**23.78±0.27^{c}**	34.97±0.33^{ab}	**25.99±0.24^{c}**	30.71±0.47^{b}	37.65±2.12^{a}
20:4n-6	2.85±0.31^{a}	2.04±0.07^{abc}	**1.07±0.02^{c}**	**1.42±0.03^{c}**	**1.77±0.03^{ab}**	2.46±0.33^{ab}
20:5n-3	0.08±0.03^{c}	0.48±0.02^{c}	**5.77±0.23^{a}**	**5.03±0.22^{a}**	**2.40±0.14^{b}**	0.05±0.02^{c}
22:5n-3	0.29±0.06^{d}	0.42±0.55^{d}	**6.56±0.26^{a}**	**5.03±0.23^{b}**	**2.62±0.17^{c}**	0.26±0.07^{d}
22:6n-3	0.23±0.07^{c}	**13.04±0.05^{a}**	0.19±0.06^{c}	**5.92±0.36^{b}**	**5.13±0.44^{b}**	0.19±0.05^{c}
ΣEPA+DPA	0.37±0.08^{d}	0.90±0.05^{d}	**12.33±0.50^{a}**	**10.06±0.45^{b}**	**5.02±0.31^{c}**	0.31±0.09^{d}
ΣSFA	38.00±0.77^{b}	**42.86±0.31^{a}**	36.92±0.56^{b}	40.24±0.97^{ab}	40.46±1.04^{ab}	36.65±0.83^{b}
ΣMUFA	11.35±0.42^{a}	11.69±0.27^{a}	**9.06±0.10^{b}**	10.87±0.23^{ab}	10.27±0.14^{ab}	10.62±0.73^{ab}
ΣPUFA	50.64±1.19^{ab}	45.45±0.45^{b}	54.02±0.68^{a}	48.88±0.95^{ab}	49.26±1.13^{ab}	52.22±2.07^{a}
Σn-3	0.90±0.19^{c}	**14.15±0.57^{a}**	**12.62±0.45^{b}**	**16.13±0.80^{a}**	**10.36±0.71^{b}**	0.74±0.15^{c}
Σn-6	49.74±1.38^{a}	**31.30±0.12^{c}**	**41.40±0.23^{b}**	**32.76±0.18^{c}**	**38.91±0.43^{b}**	51.48±2.00^{a}

Values are mean percent composition ± SEM (n=3). "OA/LA"=oleic acid (18:1n-9)/linoleic acid (18:2n-6);"EPA=eicosapentaenoic acid (20:5n-3); "DHA"=docosahexaenoic acid (22:6n-3); "ΣEPA+DPA"=summation of EPA and docosapentaenoic acid (22:5n-3); "SFA"=saturated fatty acids; "MUFA"=monounsaturated fatty acids; "PUFA"=polyunsaturated fatty acids. For each FA identified, **bold means** indicate significant difference compared to control (80 μM OA/LA) and means that do not share a common letter are significantly different ($p<0.05$).

Table 5.2.2: Change in selected fatty acids in the PL fraction of MDA-MB-231 breast cancer cells incubated with control or 150 μM n-3 LCPUFA treatments

Fatty Acid	80 μM OA/LA	DHA	EPA	1:1 DHA:EPA	2:1 DHA:EPA	230 μM OA/LA
18:1n-9	11.79±0.10^{a}	**10.53±0.10^{b}**	**10.16±0.09^{bc}**	**9.50±0.18^{c}**	**9.55±0.04^{c}**	11.70±0.14^{a}
18:2n-6	30.83±1.11^{a}	**19.50±0.12^{c}**	**23.78±0.21^{b}**	**22.56±0.56^{bc}**	**21.27±0.27^{bc}**	32.59±1.28^{a}
20:4n-6	3.80±0.45^{a}	**1.49±0.01^{b}**	**1.07±0.01^{b}**	**1.13±0.01^{b}**	**1.20±0.00^{b}**	3.30±0.38^{a}
20:5n-3	0.06±0.01^{e}	**0.57±0.05^{d}**	**8.73±0.14^{a}**	**5.87±0.03^{b}**	**4.15±0.11^{c}**	0.03±0.01^{e}
22:5n-3	0.32±0.02^{d}	0.54±0.07^{d}	**9.34±0.12^{a}**	**5.42±0.05^{b}**	**3.65±0.11^{c}**	0.29±0.01^{d}
22:6n-3	0.23±0.02^{d}	**18.29±0.67^{a}**	0.46±0.09^{d}	**7.73±0.28^{c}**	**11.55±0.17^{b}**	0.34±0.07^{d}
ΣEPA+DPA	0.38±0.02^{d}	1.11±0.12^{d}	**18.07±0.26^{a}**	**11.29±0.07^{a}**	**7.80±0.23^{c}**	0.33±0.01^{d}
ΣSFA	38.69±0.19^{b}	**43.37±0.92^{a}**	40.43±0.62^{ab}	41.58±0.78^{ab}	**42.81±0.12^{a}**	38.03±0.97^{b}
ΣMUFA	14.96±0.30^{a}	**11.96±0.08^{b}**	**11.56±0.10^{bc}**	**10.85±0.22^{c}**	**10.93±0.06^{c}**	14.60±0.16^{a}
ΣPUFA	46.35±0.44^{a}	44.67±0.86^{a}	48.00±0.54^{a}	47.57±0.59^{a}	46.27±0.18^{a}	47.37±1.07^{a}
Σn-3	1.06±0.08^{b}	**19.58±0.77^{a}**	**18.66±0.26^{a}**	**19.16±0.34^{a}**	**19.48±0.37^{a}**	1.04±0.08^{b}
Σn-6	45.29±0.52^{a}	**25.09±0.19^{d}**	**29.34±0.42^{b}**	**28.42±0.64^{c}**	**26.78±0.21^{bcd}**	46.33±1.01^{a}

Values are mean percent composition ± SEM (n=3). "OA/LA"=oleic acid (18:1n-9)/linoleic acid (18:2n-6);"EPA=eicosapentaenoic acid (20:5n-3); "DHA"=docosahexaenoic acid (22:6n-3); "ΣEPA+DPA"=summation of EPA and docosapentaenoic acid (22:5n-3); "SFA"=saturated fatty acids; "MUFA"=monounsaturated fatty acids; "PUFA"=polyunsaturated fatty acids. For each FA identified, **bold means** indicate significant difference compared to control (80 μM OA/LA) and means that do not share a common letter are significantly different ($p<0.05$).

Table 5.2.3: Change in selected fatty acids in the PL fraction of MDA-MB-231 breast cancer cells incubated with control or 200 μM n-3 LCPUFA treatments

Fatty Acid	80 μM OA/LA	DHA	EPA	1:1 DHA:EPA	2:1 DHA:EPA	280 μM OA/LA
18:1n-9	14.32±0.13^{a}	**11.10±0.32^{bc}**	**10.65±0.21^{c}**	**10.52±0.29^{c}**	**9.94±0.18^{c}**	**11.94±0.10^{b}**
18:2n-6	24.81±0.18^{b}	**19.46±0.57^{d}**	**17.81±0.27^{e}**	**16.13±0.18^{f}**	21.10±0.26^{c}	**34.90±0.05^{a}**
20:4n-6	4.62±0.06^{a}	**1.00±0.07^{c}**	**0.83±0.02^{c}**	**0.91±0.04^{c}**	**0.95±0.04^{c}**	**2.32±0.07^{b}**
20:5n-3	0.12±0.08^{d}	0.45±0.09^{d}	**11.34±0.38^{a}**	**7.15±0.37^{b}**	**5.26±0.29^{c}**	0.47±0.01^{d}
22:5n-3	0.37±0.06^{d}	0.35±0.06^{d}	**11.05±0.42^{a}**	**6.60±0.28^{b}**	**4.63±0.27^{c}**	0.24±0.01^{d}
22:6n-3	0.32±0.13^{c}	**15.98±1.22^{a}**	0.22±0.05^{c}	**9.06±0.64^{b}**	**10.90±0.84^{b}**	0.15±0.02^{c}
ΣEPA+DPA	0.49±0.14^{d}	0.80±0.12^{d}	**22.38±0.80^{a}**	**13.76±0.66^{b}**	**9.90±0.56^{c}**	0.71±0.01^{d}
ΣSFA	42.04±0.70^{a}	45.85±1.16^{a}	42.98±0.43^{a}	44.71±062^{a}	41.95±1.18^{a}	**37.15±0.24^{b}**
ΣMUFA	17.68±0.14^{a}	**12.43±0.55^{c}**	**12.10±0.23^{c}**	**11.97±0.31^{c}**	**11.33±0.18^{c}**	**14.27±0.16^{b}**
ΣPUFA	40.28±0.56^{c}	41.71±1.16^{c}	44.92±0.57^{abc}	43.32±0.92^{bc}	**46.72±1.35^{ab}**	**48.58±0.26^{a}**
Σn-3	1.37±0.28^{c}	**16.96±1.28^{b}**	**22.79±0.75^{a}**	**23.01±1.30^{a}**	**20.98±1.39^{ab}**	1.28±0.03^{c}
Σn-6	38.91±0.50^{b}	**24.75±0.82^{c}**	**22.13±0.32^{d}**	**20.31±0.38^{d}**	**25.74±0.09^{c}**	**47.30±0.28^{a}**

Values are mean percent composition ± SEM (n=3). "OA/LA"=oleic acid (18:1n-9)/linoleic acid (18:2n-6);"EPA=eicosapentaenoic acid (20:5n-3); "DHA"=docosahexaenoic acid (22:6n-3); "ΣEPA+DPA"=summation of EPA and docosapentaenoic acid (22:5n-3); "SFA"=saturated fatty acids; "MUFA"=monounsaturated fatty acids; "PUFA"=polyunsaturated fatty acids. For each FA identified, **bold means** indicate significant difference compared to control (80 μM OA/LA) and means that do not share a common letter are significantly different ($p<0.05$).

Table 5.2.4: Change in selected fatty acids of PC in MDA-MB-231 breast cancer cells incubated with control or 100 μM of n-3 LCPUFA treatments

Fatty Acid	80 μM OA/LA	DHA	EPA	1:1 DHA:EPA	2:1 DHA:EPA	180 μM OA/LA
18:1n-9	27.62±0.44^{a}	**24.70±0.72^{b}**	27.91±0.64^{a}	27.73±0.20^{a}	28.44±0.22^{a}	**23.93±0.48^{b}**
18:2n-6	27.49±0.29^{b}	25.64±0.62^{bc}	**22.98±0.28^{d}**	**24.30±0.23^{cd}**	25.59±0.16^{bc}	**36.14±0.44^{a}**
20:4n-6	1.37±0.25^{a}	1.06±0.09^{ab}	**0.53±0.07^{b}**	**0.64±0.05^{b}**	**0.70±0.01^{b}**	0.83±0.06^{ab}
20:5n-3	0.23±0.04^{d}	0.44±0.02^{d}	**4.73±0.50^{a}**	**2.72±0.24^{b}**	**1.91±0.03^{c}**	0.23±0.02^{d}
22:5n-3	0.18±0.03^{d}	0.41±0.05^{d}	**4.53±0.56^{a}**	**2.50±0.35^{b}**	**1.54±0.13^{c}**	0.14±0.06^{d}
22:6n-3	0.14±0.03^{c}	**6.76±0.84^{a}**	0.13±0.04^{c}	**2.61±0.40^{b}**	**2.95±0.26^{b}**	0.08±0.03^{c}
ΣEPA+DPA	0.40±0.00^{d}	0.84±0.07^{d}	**9.27±1.06^{a}**	**5.22±0.59^{b}**	**3.46±0.14^{c}**	0.37±0.06^{d}
ΣSFA	36.82±1.34^{a}	38.19±2.04^{a}	37.89±0.71^{a}	36.83±1.48^{a}	35.86±0.42^{a}	32.43±1.28^{a}
ΣMUFA	28.22±0.48^{a}	**25.15±0.74^{b}**	28.28±0.63^{a}	28.06±0.20^{a}	28.79±0.18^{a}	**24.39±0.49^{b}**
ΣPUFA	34.96±0.86^{ab}	35.24±1.29^{ab}	31.78±1.29^{b}	35.11±1.28^{ab}	35.34±0.61^{ab}	43.18±0.82^{a}
Σn-3	1.49±0.09^{b}	**8.00±0.80^{a}**	**6.63±1.03^{a}**	**8.21±0.99^{a}**	**6.86±0.41^{a}**	1.25±0.12^{b}
Σn-6	33.47±0.78^{b}	**29.45±0.73^{c}**	**25.15±0.36^{d}**	**26.90±0.34^{cd}**	**28.49±0.20^{c}**	**41.93±0.70^{a}**

Values are mean percent composition ± SEM (n=2-3). "OA/LA"=oleic acid (18:1n-9)/linoleic acid (18:2n-6);"EPA=eicosapentaenoic acid (20:5n-3); "DHA"=docosahexaenoic acid (22:6n-3); "ΣEPA+DPA"=summation of EPA and docosapentaenoic acid (22:5n-3); "SFA"=saturated fatty acids; "MUFA"=monounsaturated fatty acids; "PUFA"=polyunsaturated fatty acids. For each FA identified, **bold means** indicate significant difference compared to control (80 μM OA/LA) and means that do not share a common letter are significantly different ($p<0.05$).

Table 5.2.5: Change in selected fatty acids of PE in MDA-MB-231 breast cancer cells incubated with control or 100 μM of n-3 LCPUFA treatments

Fatty Acid	80 μM OA/LA	DHA	EPA	1:1 DHA:EPA	2:1 DHA:EPA	180 μM OA/LA
18:1n-9	28.05±0.21^{a}	24.00±1.21^{a}	25.12±0.72^{a}	22.26±3.09^{a}	27.75±0.74^{a}	25.77±0.53^{a}
18:2n-6	14.44±1.15^{b}	13.44±1.28^{b}	11.05±0.28^{b}	9.78±1.48^{b}	11.88±1.16^{b}	**20.57±0.58^{a}**
20:4n-6	7.87±1.66^{a}	**3.10±0.50^{bc}**	**2.18±0.13^{c}**	**2.29±0.32^{c}**	**2.73±1.17^{c}**	**4.80±0.32^{b}**
20:5n-3	0.30±1.14^{c}	0.70±0.04^{c}	**9.45±0.68^{a}**	**5.75±0.86^{b}**	**4.33±1.30^{b}**	0.40±0.08^{c}
22:5n-3	0.61±0.75^{c}	0.34±0.10^{c}	**9.91±0.78^{a}**	**4.70±0.65^{b}**	**3.28±0.91^{b}**	0.54±0.01^{c}
22:6n-3	0.45±0.06^{c}	**15.58±3.02^{a}**	0.36±0.05^{c}	**6.89±0.70^{b}**	**10.05±1.20^{b}**	0.46±0.30^{c}
ΣEPA+DPA	0.92±1.88^{c}	1.04±0.09^{c}	**19.36±1.45^{a}**	**10.45±1.51^{b}**	**7.61±2.20^{b}**	0.95±0.06^{c}
ΣSFA	34.37±0.79^{a}	28.42±2.01^{a}	37.52±0.67^{a}	43.63±5.49^{a}	35.22±1.99^{a}	35.68±1.44^{a}
ΣMUFA	33.96±1.69^{a}	25.45±1.29^{ab}	26.47±0.66^{ab}	**23.85±2.93^{b}**	29.02±0.27^{ab}	29.78±0.59^{a}
ΣPUFA	31.67±2.23^{a}	31.54±3.26^{a}	36.01±1.33^{a}	30.48±2.69^{a}	35.75±2.43^{a}	34.10±0.96^{a}
Σn-3	2.58±1.50^{b}	**17.16±3.04^{a}**	**20.10±1.44^{a}**	**18.39±2.02^{a}**	**18.09±5.34^{a}**	2.63±0.21^{b}
Σn-6	29.09±3.73^{a}	**19.58±0.62^{b}**	**15.92±0.12^{c}**	**15.97±0.77^{c}**	**17.66±3.33^{c}**	31.74±0.81^{a}

Values are mean percent composition ± SEM (n=2-3). "OA/LA"=oleic acid (18:1n-9)/linoleic acid (18:2n-6);"EPA=eicosapentaenoic acid (20:5n-3); "DHA"=docosahexaenoic acid (22:6n-3); "ΣEPA+DPA"=summation of EPA and docosapentaenoic acid (22:5n-3); "SFA"=saturated fatty acids; "MUFA"=monounsaturated fatty acids; "PUFA"=polyunsaturated fatty acids. For each FA identified, **bold means** indicate significant difference compared to control (80 μM OA/LA) and means that do not share a common letter are significantly different ($p<0.05$).

Table 5.2.6: Change in selected fatty acids of PI in MDA-MB-231 breast cancer cells incubated with control or 100 μM of n-3 LCPUFA treatments

Fatty Acid	80 μM OA/LA	DHA	EPA	1:1 DHA:EPA	2:1 DHA:EPA	180 μM OA/LA
18:1n-9	14.23±0.11^{a}	13.82±0.39^{a}	13.08±0.64^{a}	13.20±0.62^{a}	13.96±0.38^{a}	12.30±0.29^{a}
18:2n-6	11.79±0.23^{cd}	**13.92±0.62^{b}**	10.22±0.26^{d}	11.51±0.23^{cd}	12.53±0.48^{bc}	**17.42±0.38^{a}**
20:4n-6	12.13±1.23^{a}	**6.67±0.58^{bc}**	**5.61±0.14^{c}**	**6.71±0.31^{bc}**	**6.92±0.46^{bc}**	9.34±0.43^{ab}
20:5n-3	0.14±0.08^{d}	0.61±0.02^{d}	**9.78±0.53^{a}**	**5.68±0.31^{b}**	**3.83±0.33^{c}**	0.14±0.02^{d}
22:5n-3	0.28±0.02^{c}	0.63±0.07^{c}	**7.04±0.63^{a}**	**4.86±0.39^{b}**	**3.49±0.31^{b}**	0.18±0.01^{c}
22:6n-3	0.30±0.05^{c}	**9.43±3.35^{a}**	0.35±0.07^{c}	**3.93±0.53^{b}**	**4.90±0.49^{b}**	0.15±0.01^{c}
ΣEPA+DPA	0.43±0.07^{c}	1.24±0.09^{c}	**16.83±1.11^{a}**	**10.53±0.71^{b}**	**7.31±0.65^{b}**	0.32±0.01^{c}
ΣSFA	50.03±1.72^{a}	45.25±3.67^{a}	49.82±1.63^{a}	48.77±1.07^{a}	48.10±2.68^{a}	50.00±1.11^{a}
ΣMUFA	14.58±0.05^{a}	13.99±0.36^{a}	13.27±0.62^{a}	13.39±0.61^{a}	14.71±0.67^{a}	12.52±0.26^{a}
ΣPUFA	35.26±1.72^{a}	34.34±3.33^{a}	36.91±1.02^{a}	37.85±1.32^{a}	37.19±2.21^{a}	37.47±1.29^{a}
Σn-3	1.30±0.18^{c}	7.83±3.42^{bc}	**17.31±1.10^{a}**	**14.73±1.20^{ab}**	**12.49±1.12^{ab}**	1.08±0.05^{c}
Σn-6	33.96±1.87^{a}	**26.51±0.54^{b}**	**19.61±0.12^{c}**	**23.12±0.26^{bc}**	**24.70±1.10^{bc}**	36.39±1.31^{a}

Values are mean percent composition ± SEM (n=2-3). "OA/LA"=oleic acid (18:1n-9)/linoleic acid (18:2n-6);"EPA=eicosapentaenoic acid (20:5n-3); "DHA"=docosahexaenoic acid (22:6n-3); "ΣEPA+DPA"=summation of EPA and docosapentaenoic acid (22:5n-3); "SFA"=saturated fatty acids; "MUFA"=monounsaturated fatty acids; "PUFA"=polyunsaturated fatty acids. For each FA identified, **bold means** indicate significant difference compared to control (80 μM OA/LA) and means that do not share a common letter are significantly different ($p<0.05$).

Table 5.2.7: Change in selected fatty acids of PC in MDA-MB-231 breast cancer cells incubated with control or 200 μM of n-3 LCPUFA treatments

Fatty Acid	80 μM OA/LA	DHA	EPA	1:1 DHA:EPA	2:1 DHA:EPA	280 μM OA/LA
18:1n-9	28.09±1.75^{b}	24.97±0.42^{b}	27.86±1.10^{b}	26.77±0.35^{b}	25.94±0.22^{b}	**37.33±0.39^{a}**
18:2n-6	21.99±2.39^{ab}	20.99±0.24^{b}	20.00±0.54^{b}	19.89±0.22^{b}	19.52±0.09^{b}	28.31±1.83^{a}
20:4n-6	1.08±0.17^{a}	0.81±0.02^{ab}	**0.42±0.01^{b}**	**0.45±0.01^{b}**	**0.53±0.03^{b}**	**0.51±0.06^{b}**
20:5n-3	0.44±0.10^{c}	0.32±0.02^{c}	**5.59±0.21^{a}**	**3.63±0.11^{b}**	**2.92±0.20^{b}**	0.52±0.36^{c}
22:5n-3	0.42±0.07^{c}	0.36±0.03^{c}	**4.32±0.06^{a}**	**2.74±0.11^{b}**	**2.27±0.20^{b}**	0.29±0.19^{c}
22:6n-3	0.34±0.01^{d}	**8.07±0.48^{a}**	0.28±0.16^{d}	**2.68±0.11^{c}**	**4.10±0.35^{b}**	0.29±0.21^{d}
ΣEPA+DPA	0.79±0.09^{c}	0.68±0.04^{c}	**9.91±0.26^{a}**	**6.37±0.19^{b}**	**5.19±0.37^{b}**	0.81±0.54^{c}
ΣSFA	41.04±4.34^{a}	40.92±0.55^{a}	38.31±1.63^{a}	40.49±0.28^{a}	41.37±0.76^{a}	**26.25±1.38^{b}**
ΣMUFA	29.72±1.85^{b}	26.51±0.36^{b}	29.68±0.90^{b}	28.49±0.27^{b}	27.63±0.30^{b}	**39.13±0.99^{a}**
ΣPUFA	29.24±2.57^{a}	32.57±0.37^{a}	32.00±0.75^{a}	31.02±0.04^{a}	31.00±0.81^{a}	34.62±0.80^{a}
Σn-3	1.48±0.24^{b}	**8.87±0.52^{a}**	**10.29±0.22^{a}**	**9.17±0.28^{a}**	**9.42±0.70^{a}**	1.56±1.14^{b}
Σn-6	27.76±2.60^{ab}	23.70±0.26^{b}	21.72±0.54^{b}	21.85±0.27^{b}	21.58±0.12^{b}	33.06±1.30^{a}

Values are mean percent composition ± SEM (n=3). "OA/LA"=oleic acid (18:1n-9)/linoleic acid (18:2n-6);"EPA=eicosapentaenoic acid (20:5n-3); "DHA"=docosahexaenoic acid (22:6n-3); "ΣEPA+DPA"=summation of EPA and docosapentaenoic acid (22:5n-3); "SFA"=saturated fatty acids; "MUFA"=monounsaturated fatty acids; "PUFA"=polyunsaturated fatty acids. For each FA identified, **bold means** indicate significant difference compared to control (80 μM OA/LA) and means that do not share a common letter are significantly different ($p<0.05$).

Table 5.2.8: Change in selected fatty acids of PE in MDA-MB-231 breast cancer cells incubated with control or 200 μM of n-3 LCPUFA treatments

Fatty Acid	80 μM OA/LA	DHA	EPA	1:1 DHA:EPA	2:1 DHA:EPA	280 μM OA/LA
18:1n-9	28.53±0.88^{ab}	**19.06±0.68^{c}**	22.99±1.15^{bc}	**19.90±1.38^{c}**	**20.25±1.65^{c}**	34.32±0.52^{a}
18:2n-6	14.86±0.55^{b}	**8.11±0.43^{c}**	**9.44±0.63^{c}**	**7.67±0.93^{c}**	**7.90±0.49^{c}**	**18.65±0.34^{a}**
20:4n-6	4.49±0.43^{a}	**1.37±0.11^{b}**	**1.06±0.04^{b}**	**0.99±0.06^{b}**	**1.04±0.18^{b}**	**2.11±0.22^{b}**
20:5n-3	0.31±0.13^{c}	0.67±0.26^{c}	**6.48±0.80^{a}**	**3.60±0.45^{b}**	**3.12±0.38^{b}**	0.30±0.04^{c}
22:5n-3	0.43±0.09^{bc}	1.11±0.54^{bc}	**5.64±0.88^{a}**	2.82±0.40^{b}	2.58±0.27^{bc}	0.35±0.04^{c}
22:6n-3	0.40±0.00^{c}	**10.54±1.86^{a}**	0.68±0.20^{c}	5.03±1.12^{bc}	**9.67±1.22^{ab}**	0.31±0.11^{c}
ΣEPA+DPA	0.74±0.17^{d}	1.79±0.79^{cd}	**12.12±1.67^{a}**	**6.42±0.84^{b}**	**5.69±0.61^{bc}**	0.65±0.07^{d}
ΣSFA	44.43±1.00^{b}	**51.02±0.54^{a}**	47.85±1.18^{ab}	**52.60±1.31^{a}**	49.73±1.92^{ab}	**37.79±0.56^{c}**
ΣMUFA	29.45±0.60^{b}	**21.17±0.14^{d}**	**25.69±0.41^{c}**	**22.31±1.26^{d}**	**22.38±0.52^{cd}**	**35.32±0.28^{a}**
ΣPUFA	26.11±1.26^{a}	27.81±0.43^{a}	26.45±1.45^{a}	25.09±0.13^{a}	27.89±1.91^{a}	26.90±0.32^{a}
Σn-3	1.50±0.30^{b}	**13.52±0.77^{a}**	**13.30±1.51^{a}**	**12.47±1.30^{a}**	**15.74±1.76^{a}**	1.20±0.05^{b}
Σn-6	24.62±0.97^{a}	**14.29±0.85^{b}**	**13.15±0.31^{b}**	**12.61±1.27^{b}**	**12.15±0.53^{b}**	25.70±0.32^{a}

Values are mean percent composition ± SEM (n=3). "OA/LA"=oleic acid (18:1n-9)/linoleic acid (18:2n-6);"EPA=eicosapentaenoic acid (20:5n-3); "DHA"=docosahexaenoic acid (22:6n-3); "ΣEPA+DPA"=summation of EPA and docosapentaenoic acid (22:5n-3); "SFA"=saturated fatty acids; "MUFA"=monounsaturated fatty acids; "PUFA"=polyunsaturated fatty acids. For each FA identified, **bold means** indicate significant difference compared to control (80 μM OA/LA) and means that do not share a common letter are significantly different ($p<0.05$).

Table 5.2.9: Change in selected fatty acids of PI in MDA-MB-231 breast cancer cells incubated with control or 200 μM of n-3 LCPUFA treatments

Fatty Acid	80 μM OA/LA	DHA	EPA	1:1 DHA:EPA	2:1 DHA:EPA	280 μM OA/LA
18:1n-9	14.34±1.46^{ab}	13.29±0.61^{ab}	10.85±1.76^{b}	11.01±1.05^{b}	12.70±0.17^{ab}	18.03±0.46^{a}
18:2n-6	11.57±0.97^{b}	12.28±0.45^{b}	**7.49±1.07^{c}**	9.20±1.06^{bc}	10.66±0.28^{bc}	**17.10±0.08^{a}**
20:4n-6	6.31±0.26^{a}	**3.03±0.02^{bc}**	**2.45±0.26^{c}**	**2.73±0.36^{c}**	**3.20±0.23^{bc}**	**4.16±0.33^{b}**
20:5n-3	0.41±0.13^{d}	0.53±0.08^{d}	**8.85±2.45^{a}**	**7.21±0.88^{b}**	**5.55±0.33^{c}**	0.18±0.03^{d}
22:5n-3	0.41±0.07^{b}	0.45±0.03^{b}	**3.62±0.97^{a}**	**3.34±0.55^{a}**	**2.88±0.18^{a}**	0.24±0.06^{b}
22:6n-3	0.32±0.08^{c}	**7.73±0.76^{a}**	1.51±1.05^{bc}	2.58±0.72^{bc}	**4.60±0.37^{ab}**	0.14±0.01^{c}
ΣEPA+DPA	0.81±0.18^{b}	0.97±0.10^{b}	**12.47±3.42^{a}**	**10.55±1.43^{a}**	8.42±0.51^{ab}	0.42±0.06^{b}
ΣSFA	53.00±0.79^{b}	**65.67±1.08^{a}**	58.71±3.10^{ab}	**62.00±0.79^{a}**	**62.81±0.39^{a}**	52.31±0.39^{b}
ΣMUFA	17.53±0.51^{ab}	14.30±0.79^{b}	13.44±0.55^{b}	14.41±1.51^{b}	13.81±0.09^{b}	19.09±0.51^{a}
ΣPUFA	30.20±0.53^{a}	28.21±1.00^{a}	32.98±3.01^{a}	29.51±2.16^{a}	30.86±0.91^{a}	28.99±0.47^{a}
Σn-3	1.74±0.46^{b}	**9.10±0.86^{a}**	**15.74±1.19^{a}**	**13.80±1.81^{a}**	**13.37±0.87^{a}**	0.80±0.10^{b}
Σn-6	28.46±0.14^{a}	**19.11±0.38^{b}**	**17.24±3.88^{b}**	**15.72±0.36^{b}**	**17.49±0.22^{b}**	28.19±0.40^{a}

Values are mean percent composition ± SEM (n=3). "OA/LA"=oleic acid (18:1n-9)/linoleic acid (18:2n-6);"EPA=eicosapentaenoic acid (20:5n-3); "DHA"=docosahexaenoic acid (22:6n-3); "ΣEPA+DPA"=summation of EPA and docosapentaenoic acid (22:5n-3); "SFA"=saturated fatty acids; "MUFA"=monounsaturated fatty acids; "PUFA"=polyunsaturated fatty acids. For each FA identified, **bold means** indicate significant difference compared to control (80 μM OA/LA) and means that do not share a common letter are significantly different ($p<0.05$).

In MDA-MB-231 breast cancer cell lines, treatment with EPA and/or DHA resulted in a significant increase in EPA, DPA, and/or DHA content (%w/w) in whole cell total PL and PL classes (Tables 5.2.1-5.2.9 and Figure 5.2.1).

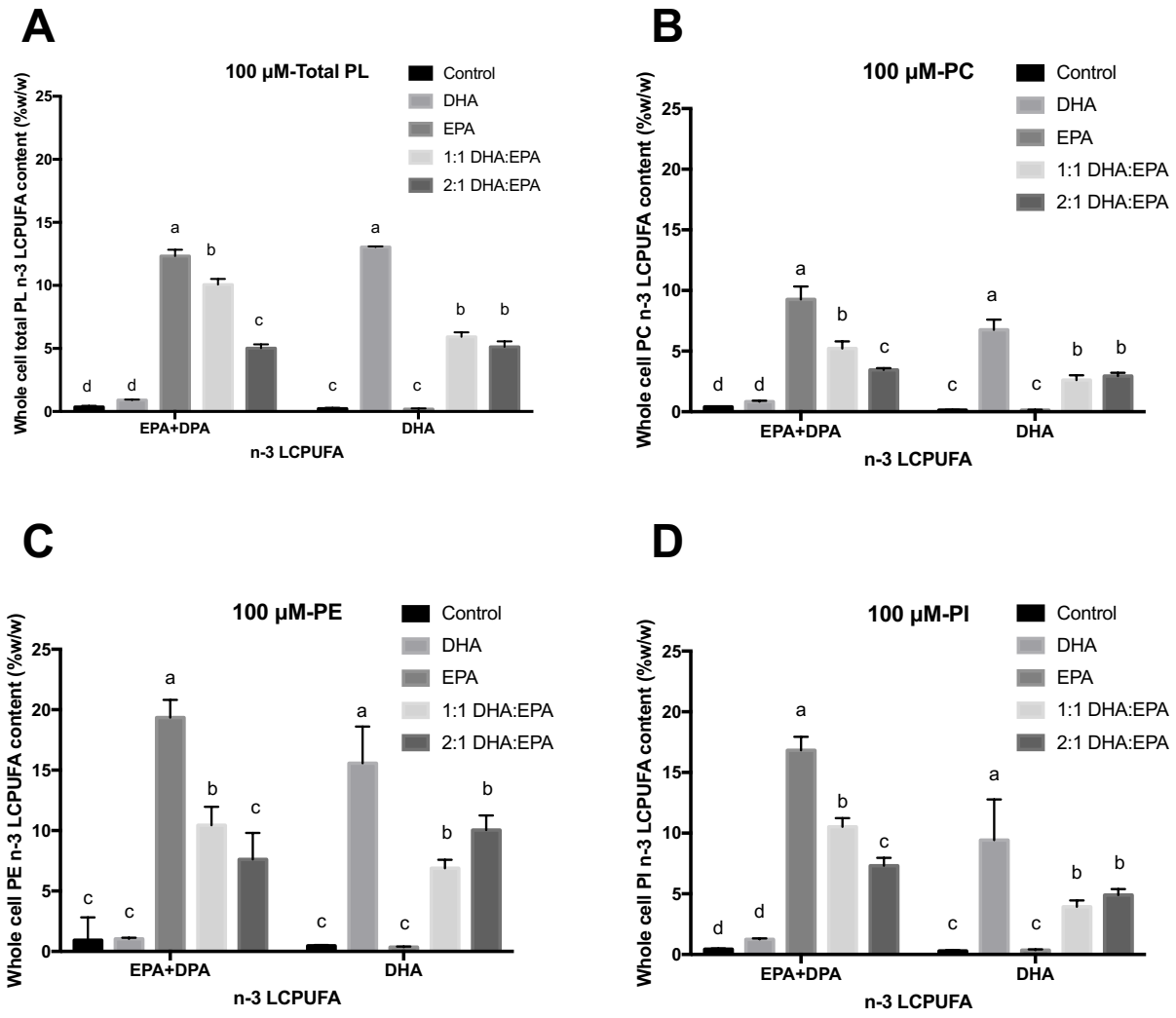

Figure 5.2.1: Effect of 100 µM n-3 LCPUFA treatments in MDA-MB-231 breast cancer cells on whole cell total PL content (%w/w) in (A) total PL; (B) PC; (C) PE; (D) PI. Bars represent the mean ± SEM for MDA-MB-231 breast cancer cells (n=3 separate experiments and passages). Differences in of EPA+DPA and DHA content with each n-3 LCPUFA treatment relative to control were tested using a one-way ANOVA. Bars that do not share a common letter are significantly different ($p<0.05$).

When EPA was provided with or without DHA, there was a significant increase in both EPA and DPA content in total PL and PL classes relative to control. When discussing the effect

of EPA containing treatments on n-3 LCPUFA content in PL and PL classes, the summation of EPA+DPA content will be considered. Across doses and PL fractions (whole cell PL, PC, PE, and PI), EPA+DPA increased to the greatest extent with the EPA treatment. The only exceptions were in total PL at 150 μM and PI at 200 μM, where EPA+DPA content was the same with EPA and the 1:1 DHA:EPA mixture. Total PL EPA+DPA increased significantly, relative to control, with 1:1 and 2:1 DHA:EPA mixtures; however, this was to a lesser extent than the EPA treatment. At all doses in total PL and at 100 μM in PC, EPA+DPA content was higher with the 1:1 than 2:1 mixture. In most lipid fractions, EPA+DPA and DHA content was not predicted by the ratio of DHA:EPA in the mixture (Table 5.2.10). The incorporation of EPA+DPA and DHA was predicted by the ratio in the mixture(s) in whole cell PL with the 1:1 mixture at 150 μM (11.29% EPA+ DPA compared to 7.73% DHA) and with both the 1:1 (6.42% EPA+DPA and 5.03% DHA) and 2:1 mixtures (5.69% EPA+DPA and 9.67%) at 200 μM in PE.

The total PL content of EPA+DPA increased with increasing dose of EPA in n-3 LCPUFA treatments (Figure 5.2.2B). The presence of DHA did not appear to affect PL EPA content. At the same time, AA decreased in the presence of EPA in n-3 LCPUFA treatments (Figure 5.2.2C). There was not an effect of dose on the observed decrease in AA, as PL AA was limited to ~1-2% with any dose of EPA.

Table 5.2.10: Comparison of the relative EPA+DPA and DHA content in whole cell PL and PL classes with n-3 LCPUFA treatments in MDA-MB-231 breast cancer cells.

	Dose (μM)	% w/w EPA+DPA with EPA vs. % w/w DHA with DHA	Ratio	% w/w EPA+DPA vs. % w/w DHA with 1:1 DHA:EPA	Ratio	Predict-able based on 1:1 ratio?	% w/w EPA+DPA vs. % w/w DHA with 2:1 DHA:EPA	Ratio	Predict-able based on 2:1 ratio?
Total PL	**100**	EPA+DPA≈DHA	1	EPA+DPA>DHA	2*	NO	EPA+DPA≈DHA	1	NO
	150	EPA+DPA≈DHA	1	EPA≈DPA>DHA	1	YES	EPA+DPA≈DHA	1	NO
	200	EPA+DPA≈DHA	1	EPA+DPA>DHA	2*	NO	EPA+DPA≈DHA	1	NO
PC	**100**	EPA+DPA≈DHA	1	EPA+DPA>DHA	2*	NO	EPA+DPA≈DHA	1	NO
PE		EPA+DPA≈DHA	1	EPA+DPA>DHA	2*	NO	EPA+DPA≈DHA	1	NO
PI		EPA+DPA>DHA	2*	EPA+DPA>DHA	3*	NO	EPA+DPA≈DHA	1	NO
PC	**200**	EPA+DPA≈DHA	1	EPA+DPA>DHA	2*	NO	EPA+DPA≈DHA	1	NO
PE		EPA+DPA≈DHA	1	EPA≈DPA>DHA	1	YES	DHA>EPA+DPA	2*	YES
PI		EPA+DPA>DHA	2*	EPA+DPA>DHA	4*	NO	EPA+DPA>DHA	2*	NO

"≈"=approximately equal to; ">" significantly more than ($p<0.05$); %w/w=percent weight/weight; PL=phospholipid; EPA=eicosapentaenoic acid; DPA=docosapentaenoic acid; DHA=docosahexaenoic acid; n-3 LCPUFA=n-3 long chain polyunsaturated fatty acids; "PC"=phosphatidylcholine; "PE"=phosphatidyl ethanolamine; "PI"=phosphatidylinositol. EPA+DPA considered together with EPA treatment due to large increases in both fatty acids with EPA treatment. Ratios calculated by dividing the n-3 LCPUFA with the higher % w/w by the n-3 LCPUFA with the lower %w/w (i.e. if DHA> EPA+DPA to get ratio divide %w/w DHA/% w/w EPA+DPA). "*"=significant difference in the relative % w/w of EPA+DPA and DHA detected using unpaired t-test ($p<0.05$).

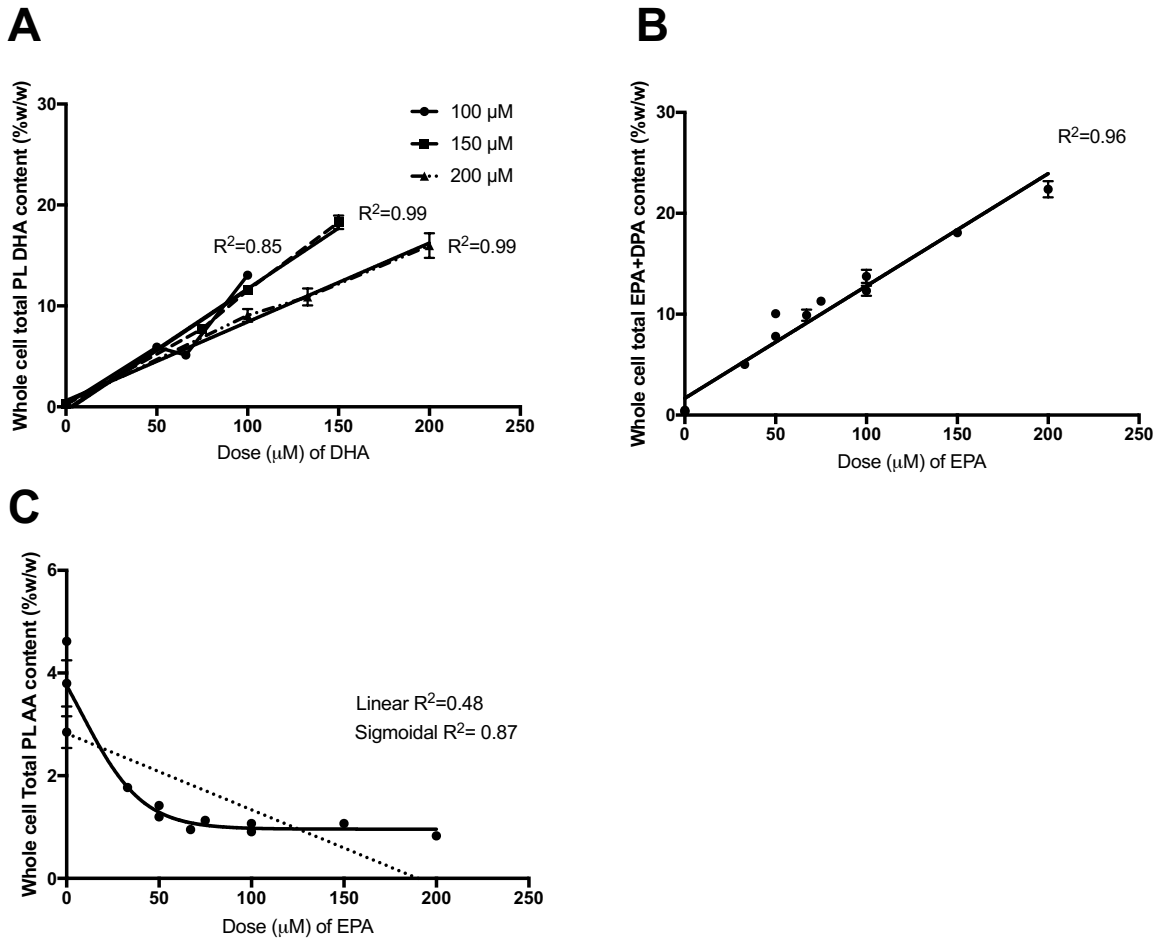

Figure 5.2.2: Dose-effect of the following n-3 LCPUFA treatments in MDA-MB-231 breast cancer cells on whole cell total PL FA content (%w/w): (A) total PL DHA with increasing DHA; (B) total PL EPA+DPA with increasing EPA; (C) total PL AA with increasing EPA. See Tables 5.2.1-5.2.9 for comparison to control treatment.

When the DHA treatment was provided to MDA-MB-231 breast cancer cells, using PL fatty acid composition one can assume that there was no retro-conversion to EPA and/or DPA across doses and lipid fractions studied, as EPA and DPA did not significantly increase relative to control. Across most doses and PL fractions (whole cell PL, PC, PE, and PI), DHA content increased to the greatest extent with the DHA treatment (Figure 5.2.1). Total PL DHA content increased significantly, relative to control, to the same extent, with 1:1 and 2:1 DHA:EPA mixtures. However, this was to a lesser extent than the DHA treatment. DHA content was

higher with the 2:1 compared to the 1:1 mixture in whole cell PL at 150 μM and PC at 200 μM. The presence of EPA in DHA:EPA mixtures appeared to limit total PL DHA with 100 μM treatments as DHA incorporation was limited to ~5-6% (Figure 5.2.2A). However, at 150 and 200 μM, EPA did not affect DHA incorporation. At these doses DHA content increased with increasing dose of DHA. Of note, there appeared to be a maximum amount of DHA in whole cell total PL that was achieved at 150 μM, as the magnitude of increase in total PL DHA was lower with treatments at 200 μM.

To determine if the magnitude of total PL EPA+DPA content differs from that of DHA content in the cells with n-3 LCPUFA treatments, total EPA+DPA content (%w/w) with the EPA treatment was compared to that of PL DHA content (%w/w) with the DHA treatment (Table 5.2.10). Across doses and PL fractions EPA+DPA and DHA content were similar with EPA and DHA treatments, respectively. The only exception was PI, where the magnitude of EPA+DPA with the EPA treatment was greater than that of DHA with the DHA treatment.

Effect of n-3 LCPUFA treatments on fatty acid content of whole cell total PL and PL classes in SK-BR-3 breast cancer cells grown in 2D cell culture

Selective fatty acids profiles for whole cell total PL and PL classes are shown in Tables 5.2.11-5.2.18 for SK-BR-3 breast cancer cells (see Appendix 4 for complete fatty acid profiles). The effect of n-3 LCPUFA treatments on changes in EPA, DPA, DHA, and AA on whole cell total PL and PL classes are summarized in Figures 5.2.3 and 5.2.4. The FA profiles for whole cell PL and PL classes were analyzed to compare the following: 1) relative EPA+DPA content (%w/w) with the EPA treatment to that of DHA (%w/w) content with the DHA treatment; 2) relative EPA+DPA content (%w/w) compared to DHA content (%w/w) with the 1:1 DHA:EPA treatment; 3) relative EPA+DPA content (%w/w) compared to DHA content (%w/w) with the 2:1 DHA:EPA treatment; and 4) the predictability of the ratio in DHA:EPA mixtures on

relative EPA+DPA and DHA content in human breast cancer cells. The results are summarized in Table 5.2.19.

Table 5.2.11: Change in selected fatty acids in the PL fraction of SK-BR-3 breast cancer cells incubated with control or 100 μM n-3 LCPUFA treatments

Fatty Acid	80 μM OA/LA	DHA	EPA	1:1 DHA:EPA	2:1 DHA:EPA	180 μM OA/LA
18:1n-9	26.81±0.84^{ab}	23.00±0.81^{b}	25.63±0.20^{b}	24.26±0.07^{b}	25.40±0.69^{b}	30.50±1.18^{a}
18:2n-6	13.73±0.64^{b}	14.87±0.34^{ab}	15.05±0.51^{ab}	15.25±0.52^{ab}	14.59±0.57^{ab}	**16.92±0.83^{a}**
20:4n-6	5.59±0.38^{a}	**2.74±0.09^{c}**	**2.00±0.10^{c}**	**2.14±0.06^{c}**	**2.58±0.12^{c}**	**4.31±0.27^{b}**
20:5n-3	0.06±0.01^{c}	0.11±0.02^{c}	**3.04±0.27^{a}**	**2.25±0.18^{b}**	**1.71±0.02^{b}**	0.12±0.05^{c}
22:5n-3	0.42±0.02^{cd}	0.33±0.04^{d}	**2.59±0.37^{a}**	**1.72±0.25^{ab}**	1.37±0.16^{bc}	0.36±0.02^{cd}
22:6n-3	0.19±0.01^{d}	**6.97±0.28^{a}**	0.21±0.04^{d}	**3.17±0.05^{c}**	**4.73±0.37^{b}**	0.18±0.01^{d}
ΣEPA+DPA	0.49±0.03^{c}	0.44±0.06^{c}	**5.63±0.65^{a}**	**3.97±0.43^{ab}**	**3.07±0.17^{b}**	0.48±0.03^{c}
ΣSFA	44.37±0.78^{ab}	45.70±0.99^{a}	45.25±1.24^{a}	44.73±0.86^{a}	43.42±0.77^{ab}	39.77±0.93^{b}
ΣMUFA	30.22±1.19^{ab}	**25.00±0.99^{c}**	28.66±0.23^{abc}	27.32±0.25^{bc}	28.00±0.70^{bc}	32.92±1.36^{a}
ΣPUFA	25.42±0.48^{a}	29.30±0.49^{a}	26.09±1.05^{a}	27.95±1.11^{a}	28.58±1.07^{a}	27.31±0.61^{a}
Σn-3	1.31±0.14^{b}	**7.77±0.25^{a}**	**6.25±0.62^{a}**	**7.46±0.38^{a}**	**8.25±0.49^{a}**	1.49±0.22^{b}
Σn-6	21.67±0.47^{ab}	19.67±0.29^{b}	19.03±0.37^{b}	19.35±0.70^{b}	19.05±0.59^{b}	23.91±0.64^{a}

Values are mean percent composition ± SEM (n=3). "OA/LA"=oleic acid (18:1n-9)/linoleic acid (18:2n-6);"EPA=eicosapentaenoic acid (20:5n-3); "DHA"=docosahexaenoic acid (22:6n-3); "ΣEPA+DPA"=summation of EPA and docosapentaenoic acid (22:5n-3); "SFA"=saturated fatty acids; "MUFA"=monounsaturated fatty acids; "PUFA"=polyunsaturated fatty acids. For each FA identified, **bold means** indicate significant difference compared to control (80 μM OA/LA) and means that do not share a common letter are significantly different ($p<0.05$).

Table 5.2.12: Change in selected fatty acids in the PL fraction of SK-BR-3 breast cancer cells incubated with control or 200 μM n-3 LCPUFA treatments

Fatty Acid	80 μM OA/LA	DHA	EPA	1:1 DHA:EPA	2:1 DHA:EPA	280 μM OA/LA
18:1n-9	24.93±0.97^{b}	**19.84±0.62^{c}**	23.37±0.36^{b}	23.29±0.40^{b}	22.63±0.09^{bc}	**29.63±0.33^{a}**
18:2n-6	14.65±0.72^{b}	15.39±0.32^{b}	16.94±0.68^{b}	16.05±0.37^{b}	14.94±0.14^{b}	**19.62±0.27^{a}**
20:4n-6	5.72±0.25^{a}	**1.69±0.17^{c}**	**1.25±0.13^{c}**	**1.60±0.09^{c}**	**1.75±0.08^{c}**	**3.97±0.18^{b}**
20:5n-3	0.06±0.03^{d}	0.16±0.03^{d}	**4.99±0.30^{a}**	**3.42±0.12^{b}**	**2.36±0.11^{c}**	0.10±0.02^{d}
22:5n-3	0.54±0.04^{c}	0.20±0.05^{c}	**4.20±0.41^{a}**	**2.73±0.19^{b}**	**1.84±0.13^{b}**	0.45±0.04^{c}
22:6n-3	0.29±0.00^{d}	**11.25±0.64^{a}**	0.26±0.04^{d}	**5.04±0.11^{c}**	**6.90±0.02^{b}**	0.20±0.02^{d}
ΣEPA+DPA	0.59±0.07^{d}	0.36±0.08^{d}	**9.20±0.61^{a}**	**6.15±0.06^{b}**	**4.21±0.07^{c}**	0.55±0.02^{d}
ΣSFA	42.57±0.74^{b}	**45.86±1.04^{a}**	43.08±0.67^{ab}	42.07±0.23^{b}	43.90±0.11^{ab}	**36.23±0.35^{c}**
ΣMUFA	29.64±1.18^{b}	**21.70±0.72^{d}**	26.45±0.56^{bc}	**25.89±0.59^{c}**	**24.98±0.14^{cd}**	**33.23±0.34^{a}**
ΣPUFA	27.78±1.20^{b}	**32.44±0.91^{a}**	30.48±1.02^{ab}	32.04±0.49^{ab}	31.12±0.23^{ab}	30.54±0.43^{ab}
Σn-3	1.51±0.04^{c}	**11.93±0.66^{a}**	**9.75±0.62^{b}**	**11.54±0.13^{ab}**	**11.40±0.06^{ab}**	1.29±0.04^{c}
Σn-6	26.28±1.24^{a}	**20.51±0.32^{b}**	**20.72±0.50^{b}**	**20.50±0.40^{b}**	**19.72±0.23^{b}**	29.26±0.39^{a}

Values are mean percent composition ± SEM (n=3). "OA/LA"=oleic acid (18:1n-9)/linoleic acid (18:2n-6);"EPA=eicosapentaenoic acid (20:5n-3); "DHA"=docosahexaenoic acid (22:6n-3); "ΣEPA+DPA"=summation of EPA and docosapentaenoic acid (22:5n-3); "SFA"=saturated fatty acids; "MUFA"=monounsaturated fatty acids; "PUFA"=polyunsaturated fatty acids. For each FA identified, **bold means** indicate significant difference compared to control (80 μM OA/LA) and means that do not share a common letter are significantly different ($p<0.05$).

Table 5.2.13: Change in selected fatty acids of PC in SK-BR-3 breast cancer cells incubated with control or 100 μM of n-3 LCPUFA treatments

Fatty Acid	80 μM OA/LA	DHA	EPA	1:1 DHA:EPA	2:1 DHA:EPA	180 μM OA/LA
18:1n-9	27.03±1.51^{a}	**21.66±0.73^{b}**	26.37±0.56^{a}	24.97±0.41^{ab}	24.79±0.48^{ab}	28.11±0.54^{a}
18:2n-6	14.59±2.73^{a}	16.06±1.01^{a}	17.13±1.25^{a}	16.44±0.86^{a}	17.02±1.50^{a}	18.25±1.66^{a}
20:4n-6	2.52±0.75^{a}	1.32±0.10^{ab}	**0.96±0.07^{b}**	**1.11±0.11^{b}**	1.18±0.15^{ab}	2.09±0.31^{ab}
20:5n-3	0.14±0.11^{c}	0.13±0.01^{c}	**2.30±0.30^{a}**	**1.78±0.22^{ab}**	**1.20±0.09^{b}**	0.12±0.03^{c}
22:5n-3	0.14±0.03^{c}	0.16±0.02^{c}	**1.26±0.10^{a}**	**1.16±0.12^{a}**	**0.73±0.05^{b}**	0.16±0.03^{c}
22:6n-3	0.14±0.01^{d}	**6.29±0.73^{a}**	0.23±0.03^{cd}	**2.05±0.24^{bc}**	**3.13±0.39^{b}**	0.11±0.03^{d}
ΣEPA+DPA	0.30±0.12^{c}	0.29±0.02^{c}	**3.56±0.40^{a}**	**2.94±0.31^{ab}**	**1.93±0.14^{b}**	0.28±0.05^{c}
ΣSFA	45.80±3.06^{a}	48.26±2.43^{a}	44.55±2.33^{a}	44.27±2.59^{a}	45.28±2.65^{a}	43.01±3.01^{a}
ΣMUFA	31.33±0.78^{a}	**24.22±0.85^{b}**	30.70±0.55^{a}	29.00±0.73^{a}	28.28±0.50^{ab}	31.32±0.57^{a}
ΣPUFA	22.87±2.29^{a}	27.52±1.78^{a}	24.75±1.78^{a}	26.71±2.03^{a}	26.44±2.15^{a}	25.67±2.47^{a}
Σn-3	1.16±0.07^{b}	**6.94±0.73^{a}**	**4.12±0.47^{a}**	**5.66±0.68^{a}**	**5.40±0.55^{a}**	0.93±0.10^{b}
Σn-6	21.70±2.27^{a}	20.58±1.24^{a}	20.63±1.35^{a}	21.05±1.36^{a}	21.05±1.60^{a}	24.74±2.37^{a}

Values are mean percent composition ± SEM (n=2-3). "OA/LA"=oleic acid (18:1n-9)/linoleic acid (18:2n-6);"EPA=eicosapentaenoic acid (20:5n-3); "DHA"=docosahexaenoic acid (22:6n-3); "ΣEPA+DPA"=summation of EPA and docosapentaenoic acid (22:5n-3); "SFA"=saturated fatty acids; "MUFA"=monounsaturated fatty acids; "PUFA"=polyunsaturated fatty acids. For each FA identified, **bold means** indicate significant difference compared to control (80 μM OA/LA) and means that do not share a common letter are significantly different ($p<0.05$).

Table 5.2.14: Change in selected fatty acids of PE in SK-BR-3 breast cancer cells incubated with control or 100 μM of n-3 LCPUFA treatments

Fatty Acid	80 μM OA/LA	DHA	EPA	1:1 DHA:EPA	2:1 DHA:EPA	180 μM OA/LA
18:1n-9	28.81±0.60^{a}	**21.57±1.23^{b}**	25.10±0.78^{ab}	23.15±2.06^{ab}	23.05±1.19^{ab}	28.72±0.97^{a}
18:2n-6	9.76±0.74^{a}	8.74±0.16^{a}	9.50±0.06^{a}	9.04±1.12^{a}	8.21±0.62^{a}	11.02±0.41^{a}
20:4n-6	9.41±0.38^{a}	**6.07±0.37^{b}**	**3.89±0.22^{c}**	**4.77±0.05^{bc}**	**4.74±0.31^{bc}**	7.99±0.21^{a}
20:5n-3	0.13±0.01^{c}	0.43±0.14^{c}	**6.96±0.98^{a}**	**4.87±0.39^{ab}**	**3.40±0.02^{b}**	0.37±0.21^{c}
22:5n-3	1.29±0.01^{c}	0.67±0.07^{c}	**9.56±1.58^{a}**	**5.25±0.69^{b}**	3.33±0.09^{bc}	1.48±0.05^{c}
22:6n-3	0.57±0.02^{c}	**16.33±2.54^{a}**	0.45±0.14^{c}	**8.05±1.25^{b}**	**10.03±0.47^{b}**	0.51±0.06^{c}
ΣEPA+DPA	1.42±0.01^{c}	1.11±0.20^{c}	**16.52±2.56^{a}**	**10.12±1.08^{b}**	6.74±0.07^{bc}	1.85±0.26^{c}
ΣSFA	38.46±1.70^{a}	39.31±0.24^{a}	38.01±0.72^{a}	38.72±1.40^{a}	41.18±2.81^{a}	36.75±2.56^{a}
ΣMUFA	34.85±0.60^{a}	**23.35±1.46^{b}**	**27.62±1.03^{b}**	**24.71±2.10^{b}**	**24.94±1.05^{b}**	34.94±1.12^{a}
ΣPUFA	26.70±1.16^{b}	**37.34±1.70^{a}**	34.37±1.75 ^{ab}	**36.60±0.72^{a}**	33.88±1.76 ^{ab}	27.72±1.14^{b}
Σn-3	2.76±0.04^{b}	**18.09±2.15^{a}**	17.48±2.27^{ab}	**18.61±2.27^{a}**	17.17±0.59^{ab}	3.13±0.29^{b}
Σn-6	23.94±1.15^{a}	19.25±0.45^{a}	16.88±0.52^{a}	17.99±1.55^{a}	16.71±1.17^{a}	24.59±0.87^{a}

Values are mean percent composition ± SEM (n=2-3). "OA/LA"=oleic acid (18:1n-9)/linoleic acid (18:2n-6);"EPA=eicosapentaenoic acid (20:5n-3); "DHA"=docosahexaenoic acid (22:6n-3); "ΣEPA+DPA"=summation of EPA and docosapentaenoic acid (22:5n-3); "SFA"=saturated fatty acids; "MUFA"=monounsaturated fatty acids; "PUFA"=polyunsaturated fatty acids. For each FA identified, **bold means** indicate significant difference compared to control (80 μM OA/LA) and means that do not share a common letter are significantly different ($p<0.05$).

Table 5.2.15: Change in selected fatty acids of PI in SK-BR-3 breast cancer cells incubated with control or 100 μM of n-3 LCPUFA treatments

Fatty Acid	80 μM OA/LA	DHA	EPA	1:1 DHA:EPA	2:1 DHA:EPA	180 μM OA/LA
18:1n-9	16.74±0.85^{a}	18.61±1.16^{a}	18.86±1.48^{a}	17.90±0.81^{a}	20.30±0.78^{a}	20.90±1.51^{a}
18:2n-6	10.41±0.99^{a}	13.10±0.99^{a}	12.33±1.05^{a}	11.72±0.72^{a}	13.55±0.50^{a}	12.49±1.13^{a}
20:4n-6	7.23±1.15^{a}	**3.26±0.38^{b}**	3.12±0.54^{ab}	**3.06±0.46^{b}**	4.03±0.56^{ab}	4.44±0.59^{ab}
20:5n-3	0.30±0.08^{d}	0.37±0.05^{cd}	**2.48±0.25^{a}**	**1.41±0.10^{b}**	**1.19±0.16^{bc}**	0.53±0.21^{d}
22:5n-3	0.35±0.05^{b}	0.38±0.04^{b}	**1.11±0.18^{a}**	**1.08±0.13^{a}**	0.89±0.23^{ab}	0.56±0.07^{ab}
22:6n-3	0.35±0.14^{b}	**1.68±0.31^{a}**	0.43±0.07^{b}	0.75±0.05^{b}	1.34±0.22^{ab}	0.35±0.01^{b}
ΣEPA+DPA	0.65±0.11^{c}	0.74±0.10^{c}	**3.59±0.43^{a}**	**2.49±0.18^{b}**	2.07±0.39^{bc}	1.09±0.26^{c}
ΣSFA	56.32±3.05^{a}	54.38±1.87^{a}	54.80±2.86^{a}	54.49±0.57^{a}	50.66±1.13^{a}	51.68±2.80^{a}
ΣMUFA	18.51±0.94^{a}	20.24±1.10^{a}	21.29±1.15^{a}	20.77±0.58^{a}	22.35±0.20^{a}	22.99±1.46^{a}
ΣPUFA	25.17±2.12^{a}	25.38±0.84^{a}	23.91±1.72^{a}	23.45±1.04^{a}	27.00±0.93^{a}	24.95±1.15^{a}
Σn-3	1.43±0.18^{d}	**2.96±0.17^{bc}**	**4.53±0.18^{a}**	**3.99±0.22^{b}**	**3.97±0.51^{ab}**	2.04±0.29^{cd}
Σn-6	23.74±2.17^{a}	22.42±0.74^{a}	19.38±1.53^{a}	19.46±1.18^{a}	23.03±0.42^{a}	22.90±1.11^{a}

Values are mean percent composition ± SEM (n=2-3). "OA/LA"=oleic acid (18:1n-9)/linoleic acid (18:2n-6);"EPA=eicosapentaenoic acid (20:5n-3); "DHA"=docosahexaenoic acid (22:6n-3); "ΣEPA+DPA"=summation of EPA and docosapentaenoic acid (22:5n-3); "SFA"=saturated fatty acids; "MUFA"=monounsaturated fatty acids; "PUFA"=polyunsaturated fatty acids. For each FA identified, **bold means** indicate significant difference compared to control (80 μM OA/LA) and means that do not share a common letter are significantly different ($p<0.05$).

Table 5.2.16: Change in selected fatty acids of PC in SK-BR-3 breast cancer cells incubated with control or 200 μM of n-3 LCPUFA treatments

Fatty Acid	80 μM OA/LA	DHA	EPA	1:1 DHA:EPA	2:1 DHA:EPA	280 μM OA/LA
18:1n-9	21.87±1.47^{ab}	15.21±1.27^{b}	21.26±0.58^{ab}	20.66±1.08^{ab}	20.48±1.59^{ab}	26.39±0.21^{a}
18:2n-6	19.60±1.50^{a}	17.16±1.39^{a}	21.10±1.71^{a}	19.57±1.91^{a}	18.61±2.15^{a}	26.22±0.67^{a}
20:4n-6	2.87±0.11^{a}	**0.78±0.07^{b}**	**0.57±0.03^{b}**	**0.70±0.14^{b}**	**0.79±0.18^{b}**	1.56±0.03^{a}
20:5n-3	0.02±0.01^{c}	0.14±0.03^{bc}	**4.20±1.02^{a}**	**2.68±0.72^{ab}**	1.95±0.53^{abc}	0.02±0.01^{bc}
22:5n-3	0.15±0.01^{b}	0.12±0.02^{b}	**2.10±0.57^{a}**	1.64±0.48^{ab}	1.25±0.38^{ab}	0.10±0.01^{b}
22:6n-3	0.13±0.00^{b}	**12.09±1.86^{a}**	0.29±0.11^{b}	3.94±1.46^{ab}	6.34±2.11^{ab}	0.08±0.00^{b}
ΣEPA+DPA	0.17±0.01^{b}	0.26±0.05^{b}	**6.30±1.58^{a}**	4.32±1.20^{ab}	3.20±0.91^{ab}	0.12±0.01^{b}
ΣSFA	45.06±1.36^{a}	49.68±4.21^{a}	44.00±2.29^{a}	45.25±3.59^{a}	45.19±3.90^{a}	37.54±1.09^{a}
ΣMUFA	25.36±1.67^{a}	**16.97±1.36^{b}**	25.22±0.69^{ab}	23.77±1.21^{ab}	23.10±1.81^{ab}	28.72±0.22^{a}
ΣPUFA	29.51±1.66^{a}	33.36±3.46^{a}	30.77±2.98^{a}	30.97±4.80^{a}	31.72±5.71^{a}	33.74±0.87^{a}
Σn-3	0.98±0.03^{b}	**12.60±1.91^{a}**	**6.92±1.43^{a}**	**8.54±2.65^{a}**	**9.84±3.03^{a}**	0.71±0.00^{b}
Σn-6	28.53±1.69^{ab}	20.76±1.59^{b}	23.85±1.55^{b}	22.43±2.15^{b}	21.87±2.68^{b}	33.03±0.86^{a}

Values are mean percent composition ± SEM (n=2-3). "OA/LA"=oleic acid (18:1n-9)/linoleic acid (18:2n-6);"EPA=eicosapentaenoic acid (20:5n-3); "DHA"=docosahexaenoic acid (22:6n-3); "ΣEPA+DPA"=summation of EPA and docosapentaenoic acid (22:5n-3); "SFA"=saturated fatty acids; "MUFA"=monounsaturated fatty acids; "PUFA"=polyunsaturated fatty acids. For each FA identified, **bold means** indicate significant difference compared to control (80 μM OA/LA) and means that do not share a common letter are significantly different ($p<0.05$).

Table 5.2.17: Change in selected fatty acids of PE in SK-BR-3 breast cancer cells incubated with control or 200 μM of n-3 LCPUFA treatments

Fatty Acid	80 μM OA/LA	DHA	EPA	1:1 DHA:EPA	2:1 DHA:EPA	280 μM OA/LA
18:1n-9	22.93±0.79^{ab}	**16.14±0.64^{b}**	20.54±0.49^{ab}	**18.99±1.21^{b}**	**15.82±3.09^{b}**	27.20±1.26^{a}
18:2n-6	12.59±0.67^{a}	9.48±0.77^{a}	11.27±0.86^{a}	9.96±0.41^{a}	11.45±1.81^{a}	14.20±1.12^{a}
20:4n-6	11.36±1.19^{a}	**3.97±0.38^{b}**	**2.40±0.21^{b}**	**3.34±0.31^{b}**	**2.80±0.62^{b}**	**5.94±0.32^{b}**
20:5n-3	0.07±0.01^{c}	0.37±0.06^{c}	**8.69±0.81^{a}**	**6.71±0.47^{a}**	**3.53±0.64^{b}**	0.09±0.03^{c}
22:5n-3	1.44±0.19^{d}	0.54±-.08^{d}	**10.71±1.11^{a}**	**7.12±0.60^{b}**	**3.64±0.48^{c}**	0.95±0.06^{d}
22:6n-3	0.80±0.07^{c}	**18.25±0.22^{a}**	0.49±0.06^{c}	**10.05±0.81^{b}**	**10.72±0.33^{b}**	0.42±0.06^{c}
ΣEPA+DPA	1.52±0.18^{d}	0.91±0.12^{d}	**19.40±1.92^{a}**	**13.84±1.06^{b}**	**7.17±1.12^{c}**	1.04±0.04^{d}
ΣSFA	35.51±2.53^{a}	43.41±3.38^{a}	41.32±2.64^{a}	38.81±1.32^{a}	47.04±3.30^{a}	38.38±0.77^{a}
ΣMUFA	30.83±1.02^{a}	**17.93±0.54^{b}**	**22.31±0.56^{b}**	**20.70±1.38^{b}**	**16.98±3.38^{b}**	32.70±1.22^{a}
ΣPUFA	33.66±1.99^{a}	30.65±5.01^{a}	36.37±3.21^{a}	40.49±2.70^{a}	35.82±0.09^{a}	28.92±0.63^{a}
Σn-3	2.99±0.25^{b}	**19.49±0.42^{a}**	**20.25±1.88^{a}**	**24.26±1.89^{a}**	**18.31±1.43^{a}**	2.18±0.14^{b}
Σn-6	30.67±1.74^{a}	**17.18±1.18^{b}**	**16.12±1.33^{b}**	**16.23±0.81^{b}**	**17.51±1.35^{b}**	26.74±0.68^{a}

Values are mean percent composition ± SEM (n=2-3). "OA/LA"=oleic acid (18:1n-9)/linoleic acid (18:2n-6);"EPA=eicosapentaenoic acid (20:5n-3); "DHA"=docosahexaenoic acid (22:6n-3); "ΣEPA+DPA"=summation of EPA and docosapentaenoic acid (22:5n-3); "SFA"=saturated fatty acids; "MUFA"=monounsaturated fatty acids; "PUFA"=polyunsaturated fatty acids. For each FA identified, **bold means** indicate significant difference compared to control (80 μM OA/LA) and means that do not share a common letter are significantly different ($p<0.05$).

Table 5.2.18: Change in selected fatty acids of PI in SK-BR-3 breast cancer cells incubated with control or 200 μM of n-3 LCPUFA treatments

Fatty Acid	80 μM OA/LA	DHA	EPA	1:1 DHA:EPA	2:1 DHA:EPA	280 μM OA/LA
18:1n-9	12.03±1.02^{b}	13.82±0.88^{b}	18.45±2.56^{ab}	15.51±0.21^{ab}	16.51±1.43^{ab}	**22.45±0.89^{a}**
18:2n-6	13.03±0.62^{a}	16.41±1.04^{a}	12.75±0.23^{a}	13.48±1.88^{a}	13.70±1.09^{a}	16.04±0.41^{a}
20:4n-6	9.06±0.81^{a}	**2.33±0.27^{b}**	**1.75±0.21^{b}**	**2.41±0.47^{b}**	**2.58±0.13^{b}**	**3.55±0.41^{b}**
20:5n-3	0.14±0.04^{d}	0.12±0.02^{d}	**3.43±0.07^{a}**	**2.28±0.13^{b}**	**1.61±0.15^{c}**	0.12±0.00^{d}
22:5n-3	0.19±0.01^{b}	0.14±0.01^{b}	**1.36±0.07^{a}**	**1.02±0.19^{a}**	**0.90±0.12^{a}**	0.20±0.04^{b}
22:6n-3	0.14±0.02^{c}	**2.16±0.15^{a}**	0.24±0.04^{c}	0.73±0.17^{bc}	**1.07±0.19^{b}**	0.13±0.01^{c}
ΣEPA+DPA	0.34±0.05^{c}	0.26±0.04^{c}	**4.79±0.14^{a}**	**3.30±0.32^{b}**	**2.51±0.26^{b}**	0.33±0.04^{c}
ΣSFA	56.45±1.18^{a}	58.22±2.55^{a}	57.20±1.74^{a}	58.61±3.03^{a}	62.73±4.40^{a}	50.63±1.10^{a}
ΣMUFA	13.28±1.12^{b}	14.86±0.81^{ab}	19.65±2.46^{ab}	16.93±0.01^{ab}	17.56±1.62^{ab}	**23.48±1.01^{a}**
ΣPUFA	30.27±1.53^{a}	27.01±1.83^{a}	23.15±0.72^{a}	24.46±3.02^{a}	24.58±2.09^{a}	25.73±0.43^{a}
Σn-3	0.73±0.08^{d}	**2.73±0.09^{c}**	**5.44±0.02^{a}**	**4.39±0.46^{ab}**	**3.86±0.50^{bc}**	0.93±0.05^{d}
Σn-6	29.54±1.56^{a}	24.28±1.74^{a}	17.71±0.73^{a}	20.07±2.56^{a}	20.71±1.59^{a}	24.88±0.37^{a}

Values are mean percent composition ± SEM (n=3-4). "OA/LA"=oleic acid (18:1n-9)/linoleic acid (18:2n-6);"EPA=eicosapentaenoic acid (20:5n-3); "DHA"=docosahexaenoic acid (22:6n-3); "ΣEPA+DPA"=summation of EPA and docosapentaenoic acid (22:5n-3); "SFA"=saturated fatty acids; "MUFA"=monounsaturated fatty acids; "PUFA"=polyunsaturated fatty acids. For each FA identified, **bold means** indicate significant difference compared to control (80 μM OA/LA) and means that do not share a common letter are significantly different ($p<0.05$).

In SK-BR-3 breast cancer cells, when EPA was provided with or without DHA, there was a significant increase in both EPA and DPA content in total PL relative to control. When discussing the effect of EPA containing treatments on whole cell fatty acids in total PL and PL classes, the summation of EPA+DPA content will be considered.

Across doses and PL fractions (whole cell PL, PC, PE, and PI), cell EPA+DPA content increased to the greatest extent with the EPA treatment (Figure 5.2.3). EPA+DPA content increased significantly, relative to control, with 1:1 and 2:1 DHA:EPA mixtures; however, this was to a lesser extent than the EPA treatment. The only exceptions were total PL at 100 μM and PC at 100 and 200 μM, where EPA+DPA content was the same with EPA and 1:1 DHA:EPA treatments. With most lipid fractions and doses the 1:1 and 2:1 mixtures increased EPA+DPA to the same extent. The only exceptions were total PL and PE at 200 μM where the 1:1 mixture caused a greater increase in EPA+DPA content. In total PL and PL classes EPA+DPA and DHA content was predicted by the ratio of DHA:EPA in the 1:1 mixture (Table 5.2.19), with the exception of PI at 100 and 200 μM where EPA+DPA content was higher than DHA. With the 2:1 mixture, the ratio was predictive of relative EPA+DPA and DHA incorporation in total PL at both doses tested. The 2:1 mixture was also predictable at in both doses in PC and 200 μM in PE (ratio of PL class content DHA:EPA+DPA was 2:1); however, the ratio of DHA:EPA+DPA content did not reach statistical significance. The ratio in the 2:1 mixture was not predictive for PI at either of the doses tested.

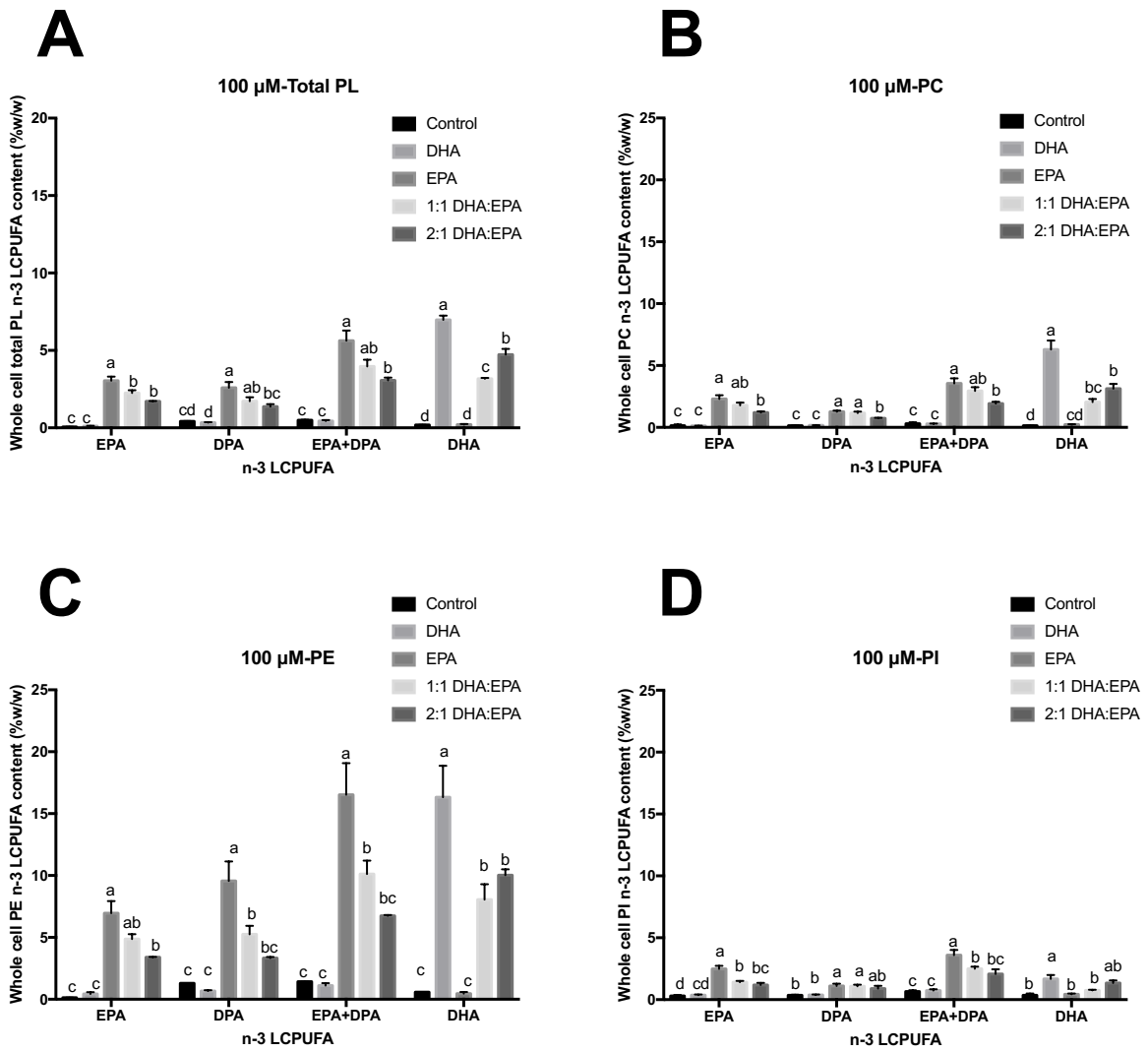

Figure 5.2.3: Effect of 100 µM n-3 LCPUFA treatments in SK-BR-3 breast cancer cells on whole cell total PL n-3 LCPUFA content (% w/w) in (A) total PL; (B) PC; (C) PE; (D) PI. Bars represent the mean ± SEM for SK-BR-3 breast cancer cells (n=3 separate experiments and passages). Bars that do not share a common letter are significantly different ($p<0.05$).

Table 5.2.19: Comparison of the relative EPA+DPA and DHA content in whole cell total PL and PL classes with n-3 LCPUFA treatments in SK-BR-3 breast cancer cells.

	Dose (μM)	% w/w EPA+DPA with EPA vs. % w/w DHA with DHA	Ratio	% w/w EPA+DPA vs. % w/w DHA with 1:1 DHA:EPA	Ratio	Predict-able % w/w of EPA, DPA, & DHA based on 1:1 ratio?	% w/w EPA+DPA vs. % w/w DHA with 2:1 DHA:EPA	Ratio	Predict-able % w/w of EPA, DPA, & DHA based on 2:1 ratio?
Total PL	100	EPA+DPA≈DHA	1	EPA+DPA≈DHA	1	YES	DHA>EPA+DPA	2*	YES
	200	EPA+DPA≈DHA	1	EPA+DPA≈DHA	1	YES	DHA>EPA+DPA	2*	YES
PC	100	DHA>EPA+DPA	2*	EPA+DPA≈DHA	1	YES	DHA>EPA+DPA	2	YES
PE		EPA+DPA≈DHA	1	EPA+DPA≈DHA	1	YES	EPA+DPA≈DHA	1	NO
PI		EPA+DPA>DHA	2*	EPA+DPA>DHA	3*	NO	EPA+DPA>DHA	2	NO
PC	200	DHA>EPA+DPA	2	EPA+DPA≈DHA	1	YES	DHA>EPA+DPA	2	YES
PE		EPA+DPA≈DHA	1	EPA+DPA≈DHA	1	YES	DHA>EPA+DPA	2	YES
PI		EPA+DPA>DHA	2*	EPA+DPA>DHA	5*	NO	EPA+DPA>DHA	2*	NO

"≈"=approximately equal to; ">" significantly more than ($p<0.05$); %w/w=percent weight/weight; PL=phospholipid; EPA=eicosapentaenoic acid; DPA=docosapentaenoic acid; DHA=docosahexaenoic acid; n-3 LCPUFA=n-3 long chain polyunsaturated fatty acids; "PC"=phosphatidylcholine; "PE"=phosphatidyl ethanolamine; "PI"=phosphatidylinositol. EPA+DPA considered together with EPA treatment due to large increases in both fatty acids with EPA treatment. Ratios calculated by dividing the n-3 LCPUFA with the higher % w/w by the n-3 LCPUFA with the lower %w/w (i.e. if DHA> EPA+DPA to get ratio divide %w/w DHA/% w/w EPA+DPA). "*"=significant difference in the relative % w/w of EPA+DPA and DHA detected using unpaired t-test ($p<0.05$)

The whole cell content of EPA+DPA increased with increasing dose of EPA in n-3 LCPUFA treatments in total PL (Figure 5.2.4D). The presence of DHA did not appear to affect EPA incorporation. At the same time, AA decreased when EPA was present in the n-3 LCPUFA treatments (Figure 5.2.4E). There was not an effect of EPA dose on the decrease in AA, as PL content of AA was only ~1-3%.

When DHA was provided to SK-BR-3 breast cancer cells, there appeared to be no retro-conversion across doses and lipid fractions studied, as EPA and DPA did not significantly increase in total PL relative to the control treatment. Across doses and PL fractions (whole cell PL, PC, PE, and PI), PL DHA increased to the greatest extent with the DHA treatment compared to the mixtures (Figure 5.2.3). PL DHA content was significantly higher with the 2:1 mixture than the 1:1 mixture in whole cell PL at both doses. In PL classes the amount of DHA content with 1:1 and 2:1 treatments were not significantly different. The content of DHA increased with increasing dose of DHA in n-3 LCPUFA treatments (Figure 5.2.4A). Of note, there appeared to be a maximum amount of DHA in cell PL that was achieved at 100 μM, as the magnitude of increase in DHA was lower with treatments at 200 μM.

To determine if the magnitude of PL EPA+DPA content differs from that of DHA content in whole cell total PL and PL classes with n-3 LCPUFA treatments, the relative EPA+DPA content with the EPA treatment was compared to that of DHA content with the DHA treatment (Table 5.2.19). In total PL and PE, the content of EPA+DPA and DHA were similar at both doses tested. However, with PC there was more DHA at both doses and more EPA+DPA in PI at both doses.

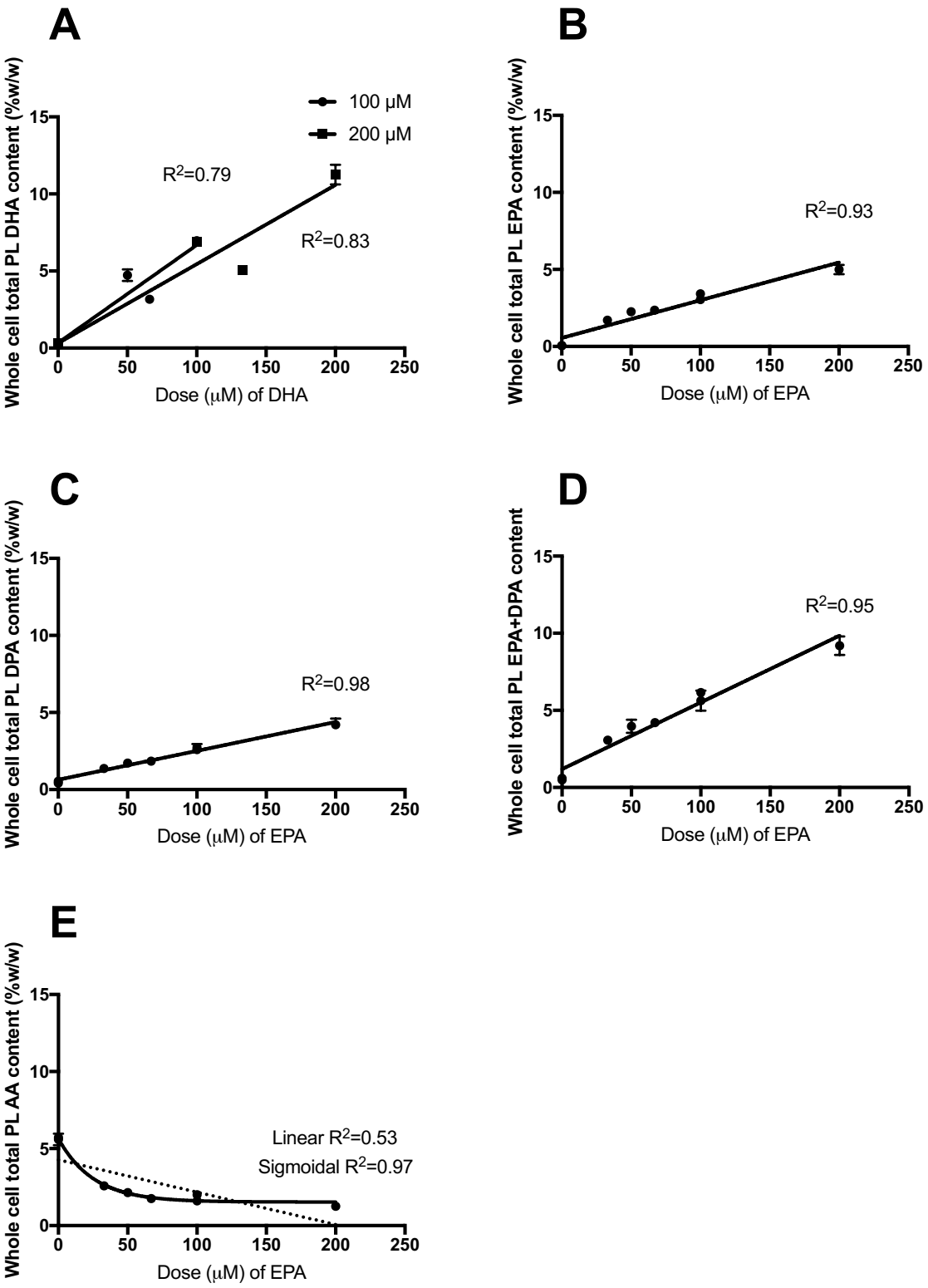

Figure 5.2.4: Dose-effect of n-3 LCPUFA treatments in SK-BR-3 breast cancer cells on total PL content (%w/w): (A) total PL DHA with increasing DHA; (B) total PL EPA with increasing EPA; (C) total PL DPA with increasing EPA; (D) total PL EPA+DPA with increasing EPA; (E) total PL AA with increasing EPA. See Tables 5.2.11-5.2.18 for comparison to control treatment.

Effect of n-3 LCPUFA treatments on whole cell fatty acid content in MDA-MB-231 and SK-BR-3 breast cancer cells grown in 2D and 3D cell culture models

To determine if the same effects and differences in n-3 LCPUFA observed in 2D cell culture are seen in 3D cell culture, whole cell fatty acid content of MDA-MB-231 and SK-BR-3 breast cancer cells were examined following n-3 LCPUFA exposure. Selective fatty acids profiles for whole cell FA are shown in Tables 5.2.20-5.2.22 for MDA-MB-231 breast cancer cells in 2D and 3D cell culture models and Tables 5.2.23-5.2.25 for SK-BR-3 breast cancer cells in 2D and 3D cell culture models (see Appendix 4 for complete fatty acid profiles). The effect of n-3 LCPUFA treatments on changes in EPA, DPA, and DHA content in total PL and PL classes are summarized in Figure 5.2.5. The fatty acid profiles for whole cell fatty acids were analyzed to compare the following: 1) relative EPA+DPA content (%w/w) with the EPA treatment to that of DHA content (%w/w) with the DHA treatment; 2) relative EPA+DPA content (%w/w) compared to DHA content (%w/w) with the 1:1 DHA:EPA treatment; 3) relative EPA+DPA content (%w/w) compared to DHA content (%w/w) with the 2:1 DHA:EPA treatment; and 4) the predictability of the ratio in DHA:EPA mixtures on whole cell fatty acid EPA+DPA and DHA content (%w/w) and EPA+DPA and DHA content (%w/w) in 2D and 3D cell culture models. The results are summarized in Table 5.2.26 and Table 5.2.27.

Table 5.2.20: Change in selected fatty acids in whole cell FA of MDA-MB-231 breast cancer cells incubated with control or 100 μM n-3 LCPUFA treatments

Fatty Acid	80 μM OA/LA	DHA	EPA	1:1 DHA:EPA	2:1 DHA:EPA	180 μM OA/LA
18:1n-9	10.36±0.83^{c}	8.86±0.13^{c}	7.43±0.05^{c}	8.86±0.46^{c}	**28.96±0.23^{b}**	**37.54±0.22^{a}**
18:2n-6	44.45±2.37^{a}	39.72±0.58^{a}	**31.37±2.59^{b}**	**28.99±0.71^{b}**	**20.82±0.17^{c}**	45.54±0.27^{a}
20:4n-6	2.29±0.11^{a}	**1.17±0.06^{b}**	**0.69±0.00^{c}**	**0.84±0.07^{c}**	**0.80±0.02^{c}**	**0.78±0.04^{c}**
20:5n-3	0.50±0.09^{c}	0.41±0.01^{c}	**17.27±2.55^{a}**	**11.00±0.53^{b}**	**6.81±0.04^{b}**	0.05±0.00^{c}
22:5n-3	0.23±0.01^{b}	0.81±0.05^{b}	**15.71±2.89^{a}**	11.66±0.54^{ab}	6.79±0.01^{b}	0.10±0.00^{b}
22:6n-3	0.12±0.02^{c}	**22.56±1.03^{a}**	0.17±0.02^{c}	**16.15±1.17^{b}**	**19.28±0.17^{ab}**	0.05±0.00^{c}
ΣEPA+DPA	0.73±0.08^{c}	1.22±0.04^{c}	**32.97±5.44^{a}**	**22.66±1.06^{ab}**	**13.60±0.05^{b}**	0.14±0.00^{c}
ΣSFA	27.71±3.11^{a}	19.24±1.00^{a}	22.97±7.85^{a}	18.28±1.28^{a}	12.59±0.32^{a}	**7.96±0.23^{b}**
ΣMUFA	11.57±0.81^{c}	9.96±0.16^{c}	**8.42±0.15^{a}**	9.94±0.53^{c}	**29.95±0.21^{a}**	**38.30±0.04^{b}**
ΣPUFA	60.73±2.30^{a}	70.80±1.11^{a}	68.62±7.99^{a}	71.78±1.74^{a}	57.46±0.31^{a}	53.74±0.25^{a}
Σn-3	1.98±0.33^{c}	**24.07±1.02^{b}**	**33.33±5.44^{ab}**	**39.05±2.24^{a}**	**33.42±0.16^{ab}**	1.45±0.01^{c}
Σn-6	58.75±2.38^{a}	**46.73±0.49^{b}**	**35.29±2.55^{c}**	**32.73±0.70^{c}**	**24.04±0.15^{d}**	52.29±0.25^{ab}

Values are mean percent composition ± SEM (n=3). "OA/LA"=oleic acid (18:1n-9)/linoleic acid (18:2n-6);"EPA=eicosapentaenoic acid (20:5n-3); "DHA"=docosahexaenoic acid (22:6n-3); "ΣEPA+DPA"=summation of EPA and docosapentaenoic acid (22:5n-3); "SFA"=saturated fatty acids; "MUFA"=monounsaturated fatty acids; "PUFA"=polyunsaturated fatty acids. For each FA identified, **bold means** indicate significant difference compared to control (80 μM OA/LA) and means that do not share a common letter are significantly different ($p<0.05$).

Table 5.2.21: Change in selected fatty acids in whole cell FA of MDA-MB-231 breast cancer cells incubated with control or 150 μM n-3 LCPUFA treatments

Fatty Acid	80 μM OA/LA	DHA	EPA	1:1 DHA:EPA	2:1 DHA:EPA	230 μM OA/LA
18:1n-9	35.23±2.85^{a}	**25.18±0.94^{b}**	**23.88±0.66^{b}**	**24.73±0.31^{b}**	24.32±0.98^{a}	**32.31±2.83^{b}**
18:2n-6	40.17±6.02^{a}	**22.32±0.49^{b}**	**22.43±0.09^{b}**	**24.04±0.32^{b}**	**22.86±0.40^{b}**	46.32±6.67^{a}
20:4n-6	1.85±0.66^{a}	0.74±0.18^{a}	0.52±0.01^{a}	0.56±0.01^{a}	0.66±0.01^{a}	1.02±0.17^{a}
20:5n-3	0.21±0.02^{d}	0.50±0.02^{d}	**24.75±0.93^{a}**	**11.37±0.01^{b}**	**8.79±0.42^{c}**	0.30±0.06^{d}
22:5n-3	0.15±0.03^{d}	0.63±0.01^{d}	**15.49±0.48^{a}**	**9.51±0.15^{b}**	**7.82±0.32^{c}**	0.12±0.02^{d}
22:6n-3	0.08±0.01^{d}	**37.15±2.10^{a}**	0.07±0.01^{d}	**16.02±0.34^{c}**	**22.39±1.27^{b}**	0.08±0.01^{d}
ΣEPA+DPA	0.36±0.05^{d}	1.14±0.01^{d}	**40.24±1.33^{a}**	**20.88±0.16^{b}**	**16.61±0.74^{c}**	0.42±0.08^{d}
ΣSFA	12.16±1.80^{a}	10.10±0.81^{a}	10.01±1.00^{a}	10.48±0.28^{a}	9.74±0.63^{a}	10.59±2.99^{a}
ΣMUFA	36.31±3.04^{a}	**25.93±0.99^{b}**	**24.53±0.64^{b}**	**25.46±0.31^{b}**	**25.09±1.00^{b}**	33.18±2.97^{ab}
ΣPUFA	51.53±4.74^{a}	63.97±1.74^{a}	65.46±1.39^{a}	64.06±0.58^{a}	65.18±1.55^{a}	56.23±5.95^{a}
Σn-3	1.98±0.29^{b}	**38.73±2.09^{a}**	**40.64±1.32^{a}**	**37.30±0.48^{a}**	**39.44±1.96^{a}**	1.81±0.30^{b}
Σn-6	49.54±5.01^{a}	**25.24±0.41^{b}**	**24.81±0.08^{b}**	**26.76±0.28^{b}**	**25.73±0.41^{b}**	54.42±6.25^{a}

Values are mean percent composition ± SEM (n=3). "OA/LA"=oleic acid (18:1n-9)/linoleic acid (18:2n-6);"EPA=eicosapentaenoic acid (20:5n-3); "DHA"=docosahexaenoic acid (22:6n-3); "ΣEPA+DPA"=summation of EPA and docosapentaenoic acid (22:5n-3); "SFA"=saturated fatty acids; "MUFA"=monounsaturated fatty acids; "PUFA"=polyunsaturated fatty acids. For each FA identified, **bold means** indicate significant difference compared to control (80 μM OA/LA) and means that do not share a common letter are significantly different ($p<0.05$).

Table 5.2.22: Change in selected fatty acids in whole cell FA of MDA-MB-231 breast cancer cells grown in 3D on-top cell culture model incubated with control or 150 μM n-3 LCPUFA treatments

Fatty Acid	80 μM OA/LA	DHA	EPA	1:1 DHA:EPA
18:1n-9	19.77±3.93[a]	19.36±1.03[a]	17.03±0.53[a]	17.33±0.77[a]
18:2n-6	20.24±3.87[a]	18.23±1.28[a]	17.25±0.59[a]	18.26±0.62[a]
20:4n-6	3.26±0.18[a]	**1.35±0.30[b]**	**0.94±0.09[b]**	**1.14±0.06[b]**
20:5n-3	0.15±0.03[c]	0.27±0.09[c]	**20.71±0.89[a]**	**12.88±0.16[b]**
22:5n-3	0.43±0.12[b]	0.82±0.09[b]	**11.55±2.31[a]**	**10.38±1.22[a]**
22:6n-3	0.35±0.07[c]	**29.72±4.17[a]**	0.62±0.45[c]	**10.23±2.53[b]**
ΣEPA+DPA	0.58±0.09[c]	1.09±0.05[c]	**32.25±2.20[a]**	**23.27±1.31[b]**
ΣSFA	46.75±8.73[a]	25.99±1.34[a]	27.24±3.52[a]	26.00±2.77[a]
ΣMUFA	21.85±3.89[a]	20.33±0.90[a]	18.26±0.38[a]	18.37±0.76[a]
ΣPUFA	31.40±5.00[b]	**53.64±2.13[a]**	**54.47±3.34[a]**	**55.60±2.36[a]**
Σn-3	2.13±0.69[b]	**31.13±4.19[a]**	**33.07±2.41[a]**	**33.72±2.04[a]**
Σn-6	29.27±4.83[a]	22.51±2.13[a]	21.40±1.03[a]	21.88±0.81[a]

Values are mean percent composition ± SEM (n=3-5). "OA/LA"=oleic acid (18:1n-9)/linoleic acid (18:2n-6);"EPA=eicosapentaenoic acid (20:5n-3); "DHA"=docosahexaenoic acid (22:6n-3); "ΣEPA+DPA"=summation of EPA and docosapentaenoic acid (22:5n-3); "SFA"=saturated fatty acids; "MUFA"=monounsaturated fatty acids; "PUFA"=polyunsaturated fatty acids. For each FA identified, **bold means** indicate significant difference compared to control (80 μM OA/LA) and means that do not share a common letter are significantly different ($p<0.05$).

Table 5.2.23: Change in selected fatty acids in whole cell FA of SK-BR-3 breast cancer cells incubated with control or 100 μM n-3 LCPUFA treatments

Fatty Acid	80 μM OA/LA	DHA	EPA	1:1 DHA:EPA	2:1 DHA:EPA	180 μM OA/LA
18:1n-9	37.48±3.43^{b}	36.54±1.40^{b}	40.08±1.02^{b}	38.56±1.13^{b}	34.65±0.30^{b}	**41.34±0.72^{a}**
18:2n-6	13.04±3.45^{b}	11.78±0.09^{b}	13.69±0.04^{b}	12.30±0.17^{b}	18.50±0.21^{b}	**31.04±0.68^{a}**
20:4n-6	3.39±1.10^{a}	1.48±0.09^{ab}	**0.83±0.11^{b}**	0.93±0.04^{ab}	1.48±0.18^{ab}	2.42±0.25^{ab}
20:5n-3	0.42±0.13^{c}	0.24±0.05^{c}	**5.62±0.40^{a}**	**3.28±0.18^{b}**	**2.70±0.27^{b}**	0.17±0.04^{c}
22:5n-3	0.20±0.09^{c}	0.26±0.06^{c}	**3.25±0.31^{a}**	**2.66±0.21^{ab}**	**2.20±0.21^{b}**	0.14±0.03^{c}
22:6n-3	0.07±0.01^{c}	**5.23±0.72^{a}**	0.13±0.01^{c}	**2.63±0.23^{b}**	**2.05±0.16^{b}**	0.12±0.04^{c}
ΣEPA+DPA	0.62±0.21^{c}	0.50±0.10^{c}	**8.87±0.66^{a}**	**5.94±0.38^{b}**	**4.91±0.46^{b}**	0.31±0.05^{c}
ΣSFA	34.70±2.96^{a}	35.60±0.64^{a}	30.28±0.77^{a}	32.86±0.56^{a}	30.43±1.39^{a}	**16.54±0.80^{b}**
ΣMUFA	40.47±3.75^{a}	39.23±1.44^{a}	42.98±1.00^{a}	41.29±1.03^{a}	37.27±0.29^{a}	42.69±0.76^{a}
ΣPUFA	24.83±3.93^{b}	25.17±0.87^{b}	26.74±0.81^{b}	25.85±0.66^{b}	**32.30±1.23^{a}**	**40.77±0.87^{a}**
Σn-3	2.51±0.28^{c}	**6.67±0.64^{b}**	**9.71±0.66^{a}**	**9.33±0.56^{ab}**	**7.67±0.62^{ab}**	0.99±0.06^{c}
Σn-6	22.32±3.88^{b}	18.49±0.23^{b}	17.03±0.16^{b}	16.52±0.10^{b}	24.63±0.61^{b}	**39.78±0.89^{a}**

Values are mean percent composition ± SEM (n=3). "OA/LA"=oleic acid (18:1n-9)/linoleic acid (18:2n-6);"EPA=eicosapentaenoic acid (20:5n-3); "DHA"=docosahexaenoic acid (22:6n-3); "ΣEPA+DPA"=summation of EPA and docosapentaenoic acid (22:5n-3); "SFA"=saturated fatty acids; "MUFA"=monounsaturated fatty acids; "PUFA"=polyunsaturated fatty acids. For each FA identified, **bold means** indicate significant difference compared to control (80 μM OA/LA) and means that do not share a common letter are significantly different ($p<0.05$).

Table 5.2.24: Change in selected fatty acids in whole cell FA of SK-BR-3 breast cancer cells incubated with control or 150 μM n-3 LCPUFA treatments

Fatty Acid	80 μM OA/LA	DHA	EPA	1:1 DHA:EPA	2:1 DHA:EPA	280 μM OA/LA
18:1n-9	32.60±1.35^{a}	26.58±2.74^{a}	34.72±3.75^{a}	32.17±4.60^{a}	30.85±6.47^{a}	31.73±5.55^{a}
18:2n-6	19.67±0.26^{ab}	17.13±1.47^{b}	10.00±1.00^{b}	11.34±1.21^{b}	9.19±1.77^{b}	28.05±4.14^{a}
20:4n-6	2.73±0.10^{a}	**0.94±0.16^{bc}**	**0.59±0.11^{c}**	**0.93±0.07^{bc}**	**0.81±0.19^{c}**	**1.75±0.25^{b}**
20:5n-3	0.42±0.09^{b}	0.21±0.05^{b}	**6.39±0.81^{a}**	**4.56±0.43^{a}**	2.35±0.48^{b}	0.10±0.02^{b}
22:5n-3	0.18±0.02^{b}	0.25±0.04^{b}	**3.00±0.35^{a}**	**2.97±0.30^{a}**	**1.87±0.33^{a}**	0.27±0.13^{b}
22:6n-3	0.06±0.00^{c}	**6.85±0.47^{a}**	0.17±0.02^{c}	**2.02±0.27^{b}**	**2.61±0.45^{b}**	0.10±0.02^{c}
ΣEPA+DPA	0.60±0.06^{c}	0.47±0.09^{c}	**9.39±1.14^{a}**	**7.53±0.71^{ab}**	**4.22±0.81^{b}**	0.37±0.12^{c}
ΣSFA	32.31±1.25^{a}	32.02±3.96^{a}	29.80±3.44^{a}	29.34±3.87^{a}	30.06±6.17^{a}	21.20±5.28^{a}
ΣMUFA	35.38±1.38^{a}	28.34±2.95^{a}	37.37±4.00^{a}	34.60±4.87^{a}	33.18±6.92^{a}	32.90±5.52^{a}
ΣPUFA	32.31±0.39^{a}	30.87±2.28^{a}	22.87±2.66^{a}	25.36±2.44^{a}	20.15±3.80^{a}	34.66±4.49^{a}
Σn-3	1.52±0.13^{b}	**7.89±0.47^{a}**	**10.11±1.26^{a}**	**10.12±0.97^{a}**	**7.42±1.38^{a}**	1.19±0.45^{b}
Σn-6	30.80±0.40^{a}	22.98±1.90^{ab}	**12.76±1.41^{b}**	**15.25±1.57^{b}**	**12.72±2.47^{b}**	33.47±4.21^{a}

Values are mean percent composition ± SEM (n=3). "OA/LA"=oleic acid (18:1n-9)/linoleic acid (18:2n-6);"EPA=eicosapentaenoic acid (20:5n-3); "DHA"=docosahexaenoic acid (22:6n-3); "ΣEPA+DPA"=summation of EPA and docosapentaenoic acid (22:5n-3); "SFA"=saturated fatty acids; "MUFA"=monounsaturated fatty acids; "PUFA"=polyunsaturated fatty acids. For each FA identified, **bold means** indicate significant difference compared to control (80 μM OA/LA) and means that do not share a common letter are significantly different ($p<0.05$).

Table 5.2.25: Change in selected fatty acids in whole cell FA of SK-BR-3 breast cancer cells grown in 3D on-top cell culture model incubated with control or 150 μM n-3 LCPUFA treatments

Fatty Acid	80 μM OA/LA	DHA	EPA	1:1 DHA:EPA
18:1n-9	16.43±2.38[a]	13.63±2.66[a]	16.03±1.58[a]	15.87±1.97[a]
18:2n-6	11.18±1.48[a]	13.47±2.68[a]	17.09±1.15[a]	16.93±0.56[a]
20:4n-6	4.11±0.79[a]	6.73±4.88[a]	3.90±1.79[a]	2.27±0.45[a]
20:5n-3	1.23±0.75[c]	0.20±0.07[c]	**14.88±0.72[a]**	**7.23±1.08[b]**
22:5n-3	0.26±0.07[b]	0.25±0.07[b]	**1.27±0.23[a]**	**0.91±0.12[a]**
22:6n-3	0.21±0.08[b]	**12.73±1.36[a]**	0.25±0.06[b]	**8.32±1.72[a]**
ΣEPA+DPA	1.49±0.77[c]	0.45±0.14[c]	**16.15±0.94[a]**	**8.14±1.02[b]**
ΣSFA	57.54±4.50[a]	43.74±6.25[a]	41.90±4.92[a]	42.96±0.86[a]
ΣMUFA	19.09±2.95[a]	15.28±2.99[a]	17.78±1.69[a]	17.75±2.13[a]
ΣPUFA	23.38±2.04[b]	**40.98±3.35[a]**	**40.32±3.23[a]**	**39.30±01.26[a]**
Σn-3	3.40±1.30[b]	**13.43±1.51[a]**	**16.69±0.96[a]**	**16.78±2.68[a]**
Σn-6	19.98±2.50[a]	27.55±3.52[a]	23.63±2.89[a]	22.52±1.77[a]

Values are mean percent composition ± SEM (n=3). "OA/LA"=oleic acid (18:1n-9)/linoleic acid (18:2n-6);"EPA=eicosapentaenoic acid (20:5n-3); "DHA"=docosahexaenoic acid (22:6n-3); "ΣEPA+DPA"=summation of EPA and docosapentaenoic acid (22:5n-3); "SFA"=saturated fatty acids; "MUFA"=monounsaturated fatty acids; "PUFA"=polyunsaturated fatty acids. For each FA identified, **bold means** indicate significant difference compared to control (80 μM OA/LA) and means that do not share a common letter are significantly different ($p<0.05$).

Effect of n-3 LCPUFA treatments on whole cell FA profile in MDA-MB-231 and SK-BR-3 breast cancer cells

MDA-MB-231 breast cancer cells

In both 2D and 3D cell culture models when EPA was provided with or without DHA, there was a significant increase in both EPA and DPA in cells relative to control. When discussing the effect of EPA containing treatments, the summation of EPA+DPA content will be considered (Tables 5.2.20-5.2.22). Whole cell fatty acid DHA content (%w/w) with DHA and 1:1 treatments and EPA+DPA content (%w/w) with EPA and 1:1 treatments were compared between 2D cell culture (100 and 150 μM treatments) and 3D cell culture models (150 μM treatments) (Table 5.2.26). In MDA-MB-231 cells, whole cell content of DHA and EPA+DPA with each n-3 LCPUFA treatment were not significantly different between the 3D cell culture model and 2D cell culture model at either dose tested. Whole cell FA content of EPA+DPA increased significantly with EPA containing treatments in both models (Figure 5.2.5A&B). At 150 μM in 2D and 3D cell culture models, EPA+DPA content was significantly higher with the EPA treatment than the 1:1 treatment. The relative whole cell fatty acid content of EPA+DPA and DHA with the 1:1 DHA:EPA mixture was predictable in 2D cell culture at both doses tested. However, it was not predictable in 3D cell culture as EPA+DPA content was greater than DHA content (Table 5.2.27). There was little AA in these breast cancer cells in both models (Tables 5.2.20-5.2.25); however, whole cell AA content decreased in 2D at 100 μM and 3D cell culture models with n-3 LCPUFA treatments. In 2D at 100 μM and 3D cell culture models, AA content decreased to the same extent with EPA and 1:1 treatments. When DHA was provided to MDA-MB-231 breast cancer cells, there was no retro-conversion in both models as EPA and DPA did not significantly increase relative to control. Whole cell DHA content increased to the greatest extent with the DHA treatment compared to the 1:1 mixture in both cell culture models (Figure 5.2.5A&B).

SK-BR-3 breast cancer cells

In 2D cell culture, when EPA was provided to SK-BR-3 breast cancer cells with or without DHA, there was a significant increase in whole cell FA content of EPA and DPA relative to control (Tables 5.2.23-5.2.25). Whole cell fatty acid DHA content with DHA and 1:1 treatments and EPA+DPA content with EPA and 1:1 treatments were compared between 2D cell culture (100 and 150 μM treatments) and 3D cell culture models (150 μM treatments) (Table 5.2.26). Whole cell DHA content (%w/w) with DHA and 1:1 treatments and EPA+DPA content (%w/w) with the EPA treatment was slightly higher in 3D cell culture (6-7% increase in EPA+DPA and/or DHA content with n-3 LCPUFA treatments in 3D cell culture, $p<0.05$). There was no difference between models in EPA+DPA content (%w/w) with the 1:1 DHA:EPA treatment. In 3D cell culture, whole cell EPA content increased significantly with EPA containing treatments; however, the increase in DPA content with EPA treatments was lower (3% DPA in 2D cell culture at both doses vs. 1% in 3D cell culture (150 μM)). In 2D cell culture at 100 μM and in 3D cell culture, whole cell content of EPA+DPA was significantly higher with the EPA treatment than the 1:1 treatment. Whole cell EPA+DPA content with the 1:1 DHA:EPA mixture was predictable in 3D cell culture; however, it was not predictable in 2D cell culture as there was a higher amount of EPA+DPA than DHA (Table 5.2.27). Whole cell AA content decreased significantly with EPA at 100 μM and at 150 μM. EPA and 1:1 DHA:EPA decreased AA content to the same extent. There was more AA in whole cell fatty acids in the 3D cell culture model. In 3D there was no significant changes in AA content with n-3 LCPUFA treatments. When DHA was provided to SK-BR-3 breast cancer cells, there was no retro-conversion in either cell culture model as EPA and DPA did not significantly increase relative to control. Whole cell DHA content increased to a greater extent with the DHA treatment than the 1:1 mixture in 2D cell culture and to the same extent in 3D cell culture (Figure 5.2.5C&D).

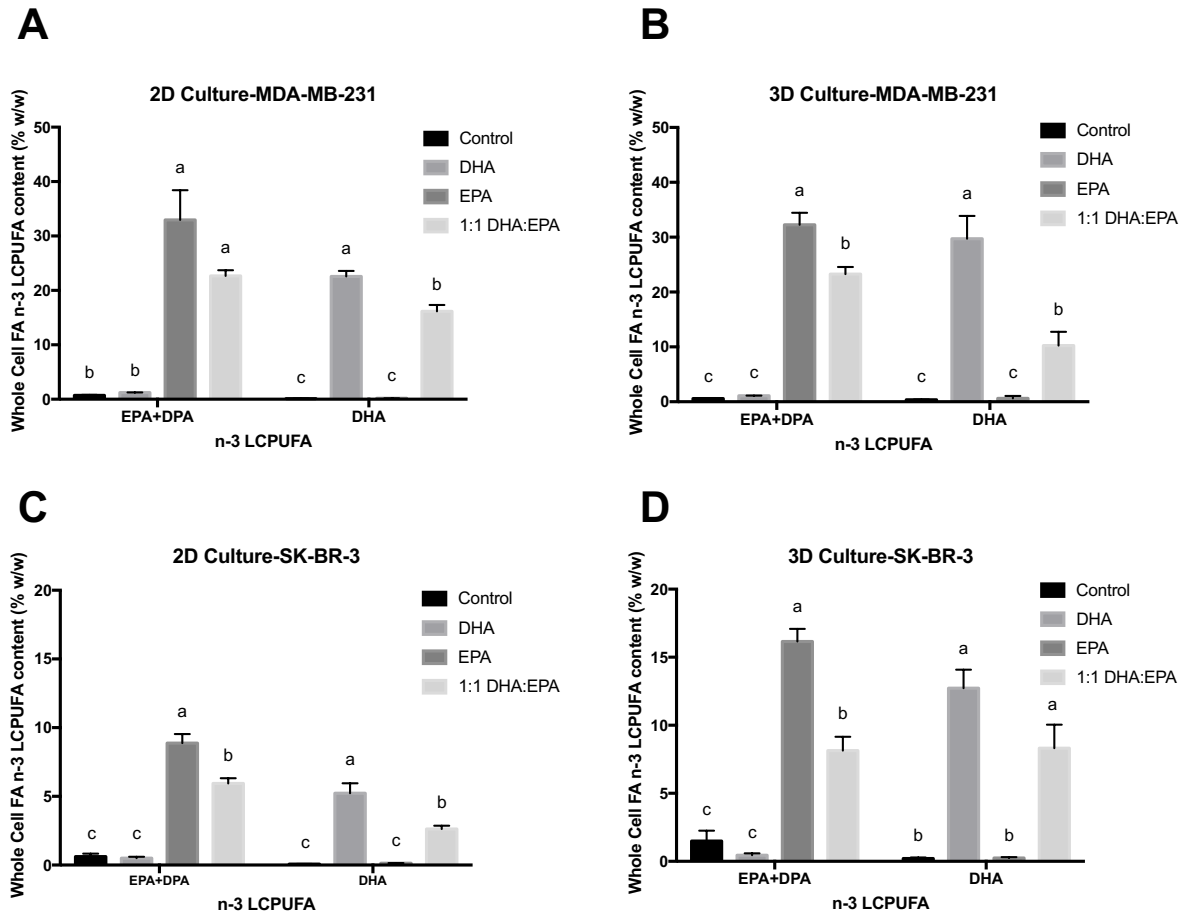

Figure 5.2.5: Effect of n-3 LCPUFA treatments on whole cell FA content (%w/w) in (A) in MDA-MB-231 cells grown in 2D cell culture (100 μM treatments); (B) in MDA-MB-231 breast cancer grown in 3D cell culture (150 μM treatments); (C) in SK-BR-3 breast cancer grown in 2D cell culture (100 μM treatments); (D) in SK-BR-3 breast cancer grown in 3D cell culture (150 μM treatments). Bars represent the mean ± SEM for breast cancer cells (n=3 separate experiments and passages). Bars that do not share a common letter are significantly different ($p<0.05$).

Table 5.2.26: Summary of EPA+DPA and DHA content in whole cell FA with n-3 LCPUFA treatments in MDA-MB-231 and SK-BR-3 breast cancer cells grown in 2D and 3D cell culture.

Breast Cancer Cell Line	Cell Culture Model (2D or 3D)	Dose (μM)	% w/w DHA with DHA	% w/w EPA+DPA with EPA	% w/w DHA with 1:1 DHA:EPA	% w/w EPA+DPA with 1:1 DHA:EPA
MDA-MB-231	**3D**	**150**	**29.72±4.17^{ab}**	**32.25±2.20^{a}**	**10.23±2.53^{a}**	**23.27±1.31^{a}**
	2D	100	22.56±1.03^{b}	32.97±5.44^{a}	16.15±1.17^{a}	22.66±1.06^{a}
		150	37.15±2.10^{a}	40.24±1.33^{a}	16.02±0.34^{a}	20.88±0.16^{a}
SK-BR-3	**3D**	**150**	**12.73±1.36^{a}**	**16.15±0.94^{a}**	**8.32±1.72^{a}**	**8.14±1.02^{a}**
	2D	100	5.23±0.72^{b}	8.87±0.66^{b}	2.63±0.23^{b}	5.94±0.38^{a}
		150	6.85±0.47^{b}	9.39±1.14^{b}	2.02±0.27^{b}	7.53±0.71^{a}

Values are mean percentages ± SEM. “EPA=eicosapentaenoic acid (20:5n-3); “DHA”=docosahexaenoic acid (22:6n-3); docosapentaenoic acid (22:5n-3). For each cell line, within a column, values that do not share a common letter are significantly different ($p>0.05$). See Tables 5.2.20-5.2.25 for comparison to control.

Table 5.2.27: Comparison of the relative EPA+DPA and DHA content (%w/w) in whole cell FA with n-3 LCPUFA treatments in MDA-MB-231 and SK-BR-3 breast cancer cells grown in 2D and 3D cell culture.

Breast Cancer Cell Line	Cell Culture Model (2D or 3D)	Dose (μM)	% w/w EPA+DPA with EPA vs. % w/w DHA with DHA	Ratio	% w/w EPA+DPA vs. % w/w DHA with 1:1 DHA:EPA	Ratio	Predictable % w/w of EPA, DPA, & DHA based on 1:1 ratio?
MDA-MB-231	3D	150	EPA+DPA≈DHA	1	EPA+DPA>DHA	2*	NO
	2D	100	EPA+DPA≈DHA	1	EPA+DPA≈DHA	1	YES
		150	EPA+DPA≈DHA	1	EPA+DPA≈DHA	1	YES
SK-BR-3	3D	150	EPA+DPA≈DHA	1	EPA+DPA≈DHA	1	YES
	2D	100	EPA+DPA>DHA	2*	EPA+DPA>DHA	2*	NO
		150	EPA+DPA≈DHA	1	EPA+DPA>DHA	4*	NO

"≈"=approximately equal to; ">" significantly more than ($p<0.05$); %w/w=percent weight/weight; PL=phospholipid; EPA=eicosapentaenoic acid; DPA=docosapentaenoic acid; DHA=docosahexaenoic acid; n-3 LCPUFA=n-3 long chain polyunsaturated fatty acids; "PC"=phosphatidylcholine; "PE"=phosphatidyl ethanolamine; "PI"=phosphatidylinositol. EPA+DPA considered together with EPA treatment due to large increases in both FA with EPA exposure. Ratios calculated by dividing the n-3 LCPUFA with the higher % w/w by the n-3 LCPUFA with the lower %w/w (i.e. if DHA> EPA+DPA to get ratio divide %w/w DHA/% w/w EPA+DPA). "*"=significant difference in the relative % w/w of EPA+DPA and DHA detected using unpaired t-test ($p<0.05$).

3. Western blot analysis and flow cytometry

Effect of n-3 LCPUFA treatments on membrane-associated receptors related to cell death in MDA-MB-231 human breast cancer cells

All mechanistic experiments (western blot analysis, flow cytometry) were done at 100 μM. This was to study the effect of n-3 LCPUFA when there was an approximate 25%-29% decrease in cell viability (Figure 5.1.1A). With the 100 μM n-3 LCPUFA treatments there were increases in the relative amounts of DHA and EPA in whole cell FA, total PL, and PL classes when compared to both 80 μM and 180μM OA/LA treatments (Tables 5.2.1,5.2.4-5.2.6,5.2.20). For western blot and flow cytometry experiments, 180 μM OA/LA was used as a control (fatty acid (FA) control) to control for the same total concentration of fatty acid in the treatment (100 μM of n-3 LCPUFA + 80 μM OA/LA background).

DHA alone had no significant effect on whole cell CD95 in MDA-MB-231 breast cancer cells ($p>0.05$) (Figure 5.3.1A). All of the treatments containing EPA resulted in a significant increase in the whole cell content of CD95 ($p<0.05$). There was significantly more CD95 in the cells treated with EPA (100 μM) compared to the 2:1 DHA:EPA mixture (containing 33 μM EPA), suggesting a dose effect of EPA. DHA alone (100 μM) and the 2:1 DHA:EPA mixture (66 μM of DHA) resulted in a small, yet significant increase in cell surface CD95 relative to control (8% increases, $p<0.05$)(Figure 5.3.1B). EPA had no significant effect on cell surface CD95 ($p>0.05$).

Effect of n-3 LCPUFA treatments on apoptotic proteins in MDA-MB-231 human breast cancer cells

All n-3 LCPUFA caused a statistically significant, yet small, decrease in whole cell FADD and Receptor-interacting serine/threonine-protein kinase 1 (RIPK1) (14%-31% decrease, $p<0.05$ and 16%-28% decrease, $p<0.05$, respectively) (Figure 5.3.1 C&D). Of note,

the observed small decreases in FADD and RIPK1 were not statistically different among n-3 LCPUFA treatment groups.

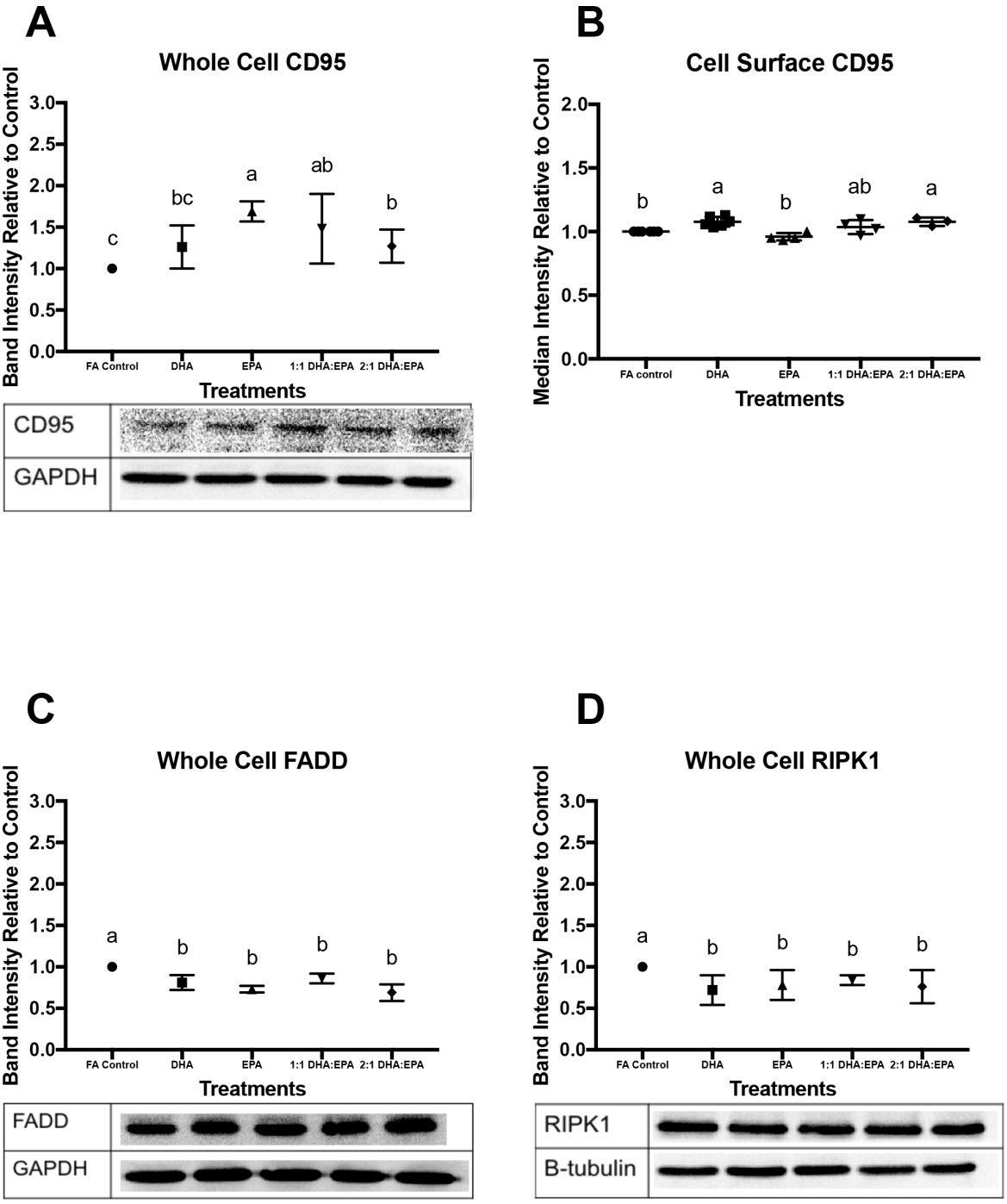

Figure 5.3.1: Effect of 100 μM n-3 LCPUFA treatments in MDA-MB-231 breast cancer cells on proteins related to cell death including (A) whole cell CD95 content (n=4 separate blots and passages); (B) cell surface CD95 (n=3 separate experiments and passages); (C) whole cell FADD content (n=3 separate blots and passages) and (D) whole cell RIPK1 content (n=4 separate blots and passages). Bars represent the mean ± SEM for MDA-MB-231 breast cancer cells. Bars that do not share a common letter are significantly different ($p<0.05$). Representative blots shown below A, C, & D. "FA control" =fatty acid control (180 μM OA/LA).

Effect of n-3 LCPUFA treatments on membrane-associated receptors related to cell growth in MDA-MB-231 human breast cancer cells

In the present study, DHA, EPA, and the 2:1 DHA:EPA mixture had no significant effect on whole cell EGFR ($p>0.05$)(Figure 5.3.2A). However, whole cell EGFR significantly increased relative to control with the 1:1 DHA:EPA mixture (50 μM DHA and 50 μM EPA)(19%, $p<0.05$), but this did not differ significantly from the other n-3 LCPUFA treatments. Whole cell pEGFR was significantly higher relative to control to the same extent with all n-3 LCPUFA treatments (84%-96% increase, $p<0.05$)(Figure 5.3.2B).

At 100 μM, DHA alone had no significant effect on cell surface EGFR, relative to control ($p>0.05$) (Figure 5.3.2C). EPA alone (100 μM) significantly decreased cell surface EGFR, to a small extent (11%, $p<0.05$). The presence of DHA significantly decreased cell surface EGFR in the 2:1 mixture (66 μM DHA) (7% decrease, $p<0.05$); however, this was not observed with the 1:1 mixture (2% decrease, $p>0.05$). This indicates that EPA alone has a greater effect than DHA alone on the translocation of EGFR to the cell surface. In addition, these data suggest that the ratio of these n-3 LCPUFA does not predict the effect of EPA and DHA on cell surface EGFR, as the mixture with more EPA (50 μM in the 1:1 mixture) had less of an effect than the mixture with less EPA (33 μM in the 2:1 mixture).

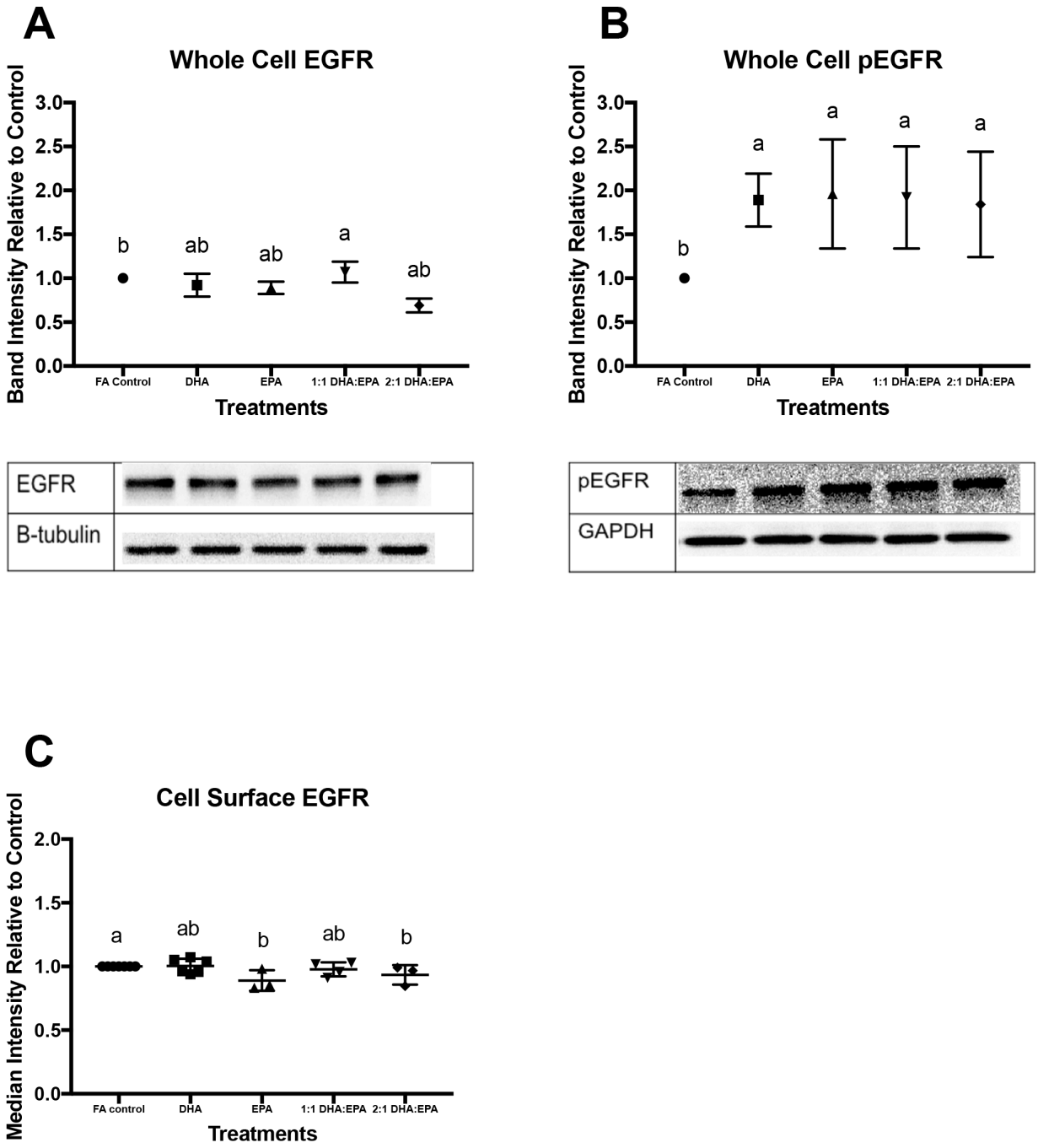

Figure 5.3.2: Effect of 100 μM n-3 LCPUFA treatments in MDA-MB-231 breast cancer cells on proteins related to cell growth including (A) whole cell EGFR content (n=5 separate blots and passages); (B) whole cell surface pEGFR content (n=4 separate blots and passages); (C) cell surface EGFR (n=3 separate experiments and passages). Bars represent the mean ± SEM for MDA-MB-231 breast cancer cells. Bars that do not share a common letter are significantly different ($p<0.05$). Representative blots shown below A & B. "FA control" =fatty acid control (180 μM OA/LA).

Effect of n-3 LCPUFA treatments on membrane-associated receptors related to cell death in SK-BR-3 human breast cancer cells

DHA alone (100 μM) significantly decreased whole cell CD95 (14% decrease, $p<0.05$), while EPA alone (100 μM) significantly increased whole cell CD95 (14% increase, $p<0.05$) (Figure 5.3.3A). With the 1:1 mixture (50 μM DHA and 50 μM EPA) there was no significant effect on whole cell CD95 relative to control. However, the 2:1 ratio mixture (66 μM DHA and 33 μM EPA) significantly decreased whole cell CD95 (28% decrease, $p<0.05$). Collectively, this suggests the presence of EPA blunts the effect of DHA on whole cell CD95 unless DHA is provided at a dose of 66 μM in a mixture. DHA alone (100 μM) and the 2:1 mixture (66 μM DHA), but not the 1:1 mixture (50 μM DHA) significantly increased cell surface CD95 expression relative to control (13% $p<0.05$ and 11% $p<0.05$, respectively). EPA alone nor the 1:1 mixture had no significant effect on cell surface CD95. This also suggests that the presence of EPA blunts the effect of DHA on cell surface CD95 unless DHA is provided at a dose of 66 μM in a 2:1 ratio of DHA:EPA.

Effect of n-3 LCPUFA treatments on apoptotic proteins in SK-BR-3 human breast cancer cells

Only EPA when provided at 100 μM caused a statistically significant, yet small, decrease in whole cell FADD relative to control (22% decrease, $p<0.05$) (Figure 5.3.3C). DHA containing treatments (DHA alone, 1:1, and 2:1 DHA:EPA mixtures) had no significant effect on whole cell FADD ($p>0.05$). DHA and EPA alone had no significant effect on whole cell RIPK1 relative to control (2% $p>0.05$ and 17% $p>0.05$ increase, respectively) (Figure 5.3.3D). However, 1:1 and 2:1 DHA:EPA mixtures significantly increased whole cell RIPK1 relative to control to the same extent (36%, $p<0.05$ and 19%, $p<0.05$, respectively).

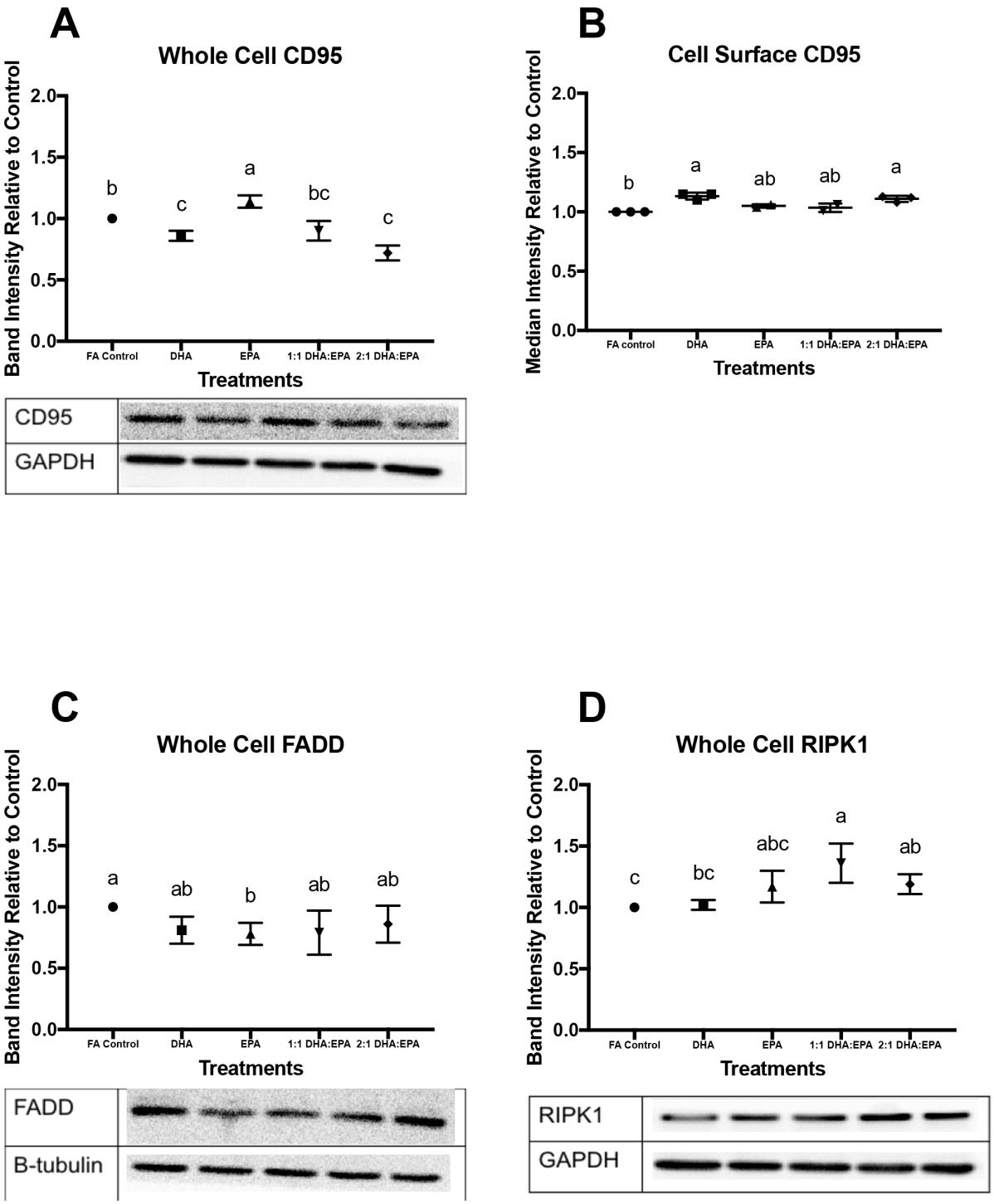

Figure 5.3.3: Effect of 100 μM n-3 LCPUFA treatments in SK-BR-3 breast cancer cells on proteins related to cell death including (A) whole cell CD95 content (n=3 separate blots and passages); (B) cell surface CD95 content (n=3 separate experiments and passages); (C) whole cell FADD content (n=4 separate blots and passages) and (D) whole cell RIPK1 content (n=3 separate blots and passages). Bars represent the mean ± SEM for SK-BR-3 breast cancer cells. Bars that do not share a common letter are significantly different ($p<0.05$). Representative blots shown below A, C, & D**.** "FA control"=fatty acid control (180 μM OA/LA).

Effect of n-3 LCPUFA treatments on membrane-associated receptors related to cell growth in SK-BR-3 human breast cancer cells

Whole cell Her2 was significantly increased with DHA alone (100 μM) (22% increase, $p<0.05$) (Figure 5.3.4) and decreased with EPA (15% decrease); however, this did not reach statistical significance ($p>0.05$). None of the other n-3 LCPUFA treatments significantly changed whole cell Her2.

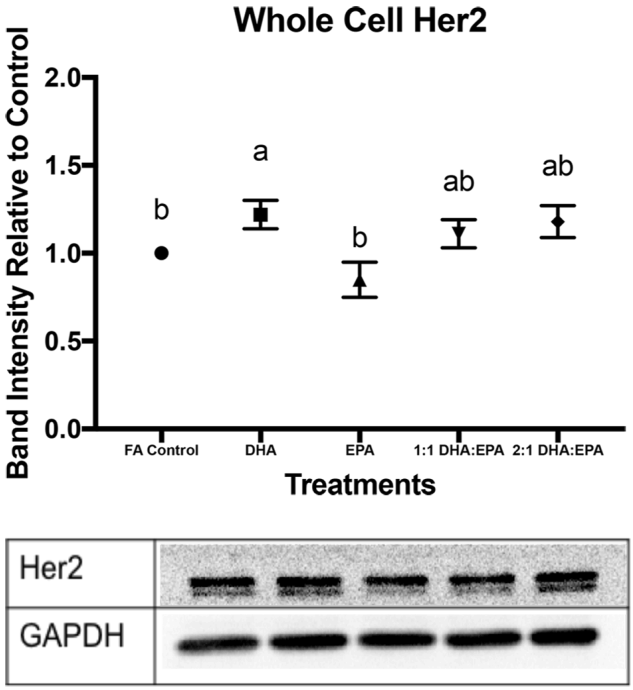

Figure 5.3.4: Effect of 100 μM n-3 LCPUFA treatments in SK-BR-3 breast cancer cells on whole cell Her2 (n=3 separate blots and passages). Bars represent the mean ± SEM for SK-BR-3 breast cancer cells. Bars that do not share a common letter are significantly different ($p<0.05$). Representative blot shown below. "FA control"=fatty acid control (180 μM OA/LA).

4. 3D on-top cell culture model

Effect of n-3 LCPUFA treatment on growth parameters in MDA-MB-231 breast cancer cells

There was no difference in in the following growth parameters amongst n-3 LCPUFA treatments in MDA-MB-231 breast cancer cells grown in a 3D on-top cell culture model: the length, shape factor, average pixel area, and breadth of spheroids (Table 5.4.1 and Figure 5.4.1).

Table 5.4.1: Effect of 150 µM n-3 LCPUFA treatments on growth parameters of spheroids formed by MDA-MB-231 breast cancer cells grown in a 3D on-top cell culture model of breast cancer

Treatment	Length of Spheroids (Pixels)	Shape Factor of Spheroids	Average Pixel Area of Spheroid	Breadth of spheroids (Pixels)
80 µM OA/LA	85±1.84 [a]	0.79±0.01 [a]	4946±280 [a]	75±1.9 [a]
DHA	87±5.41 [a]	0.75±0.02 [a]	4457±381 [a]	71±2.7 [a]
EPA	83±1.29 [a]	0.77±0.03 [a]	4246±202 [a]	73±2.8 [a]
1:1 DHA:EPA	89±4.51 [a]	0.79±0.05 [a]	4895±368 [a]	80±3.3 [a]

"OA" =oleic acid; "LA"=linoleic acid; "DHA"= docosahexaenoic acid; "EPA"=eicosapentaenoic acid; Length of spheroids was measured using the longest chord of the object. Shape factor is a value from 0 to 1 that represents how closely an object represents a circle (shape factor $=\frac{4\pi A}{P^2}$ where A= area and P=perimeter of spheroid). Breadth of spheroid is length perpendicular to the longest chord. Values represent the mean±SEM for MDA-MB-231 breast cancer cells (n=3 separate passages). Within a column, means that do not share a letter are significantly different ($p<0.05$).

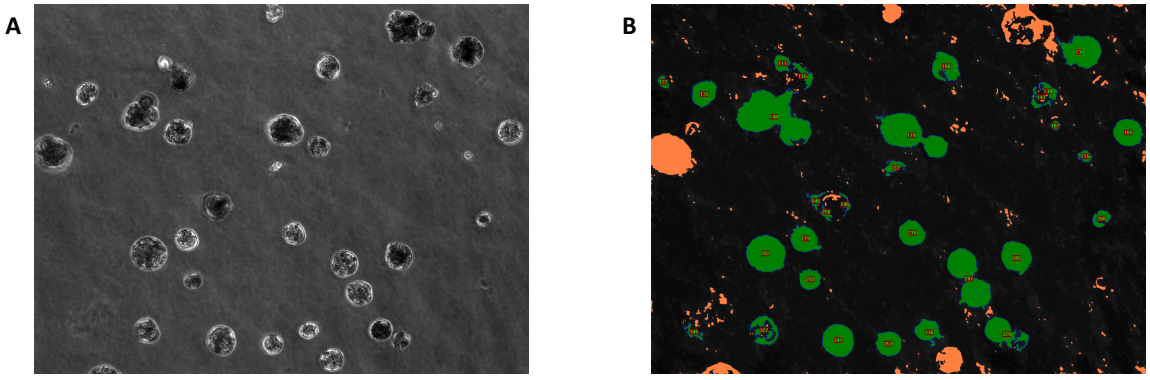

Figure 5.4.1: (A) Phase-contrast image of MDA-MB-231 breast cancer cells treated with 150 µM EPA+ 80 µM OA/LA and (B) corresponding image analyzed using top-hat journal with threshold mask described in chapter 4.

Average number of spheroids and AG

DHA when provided at 150 µM or in the 1:1 mixture (75 µM DHA) had no significant effect on the number of spheroids or AG (Figure 5.4.1). EPA when provided at 150 µM resulted in a higher number of spheroids (26% higher (24 vs. 19) $p<0.05$) but not the number of AG. There were no significant differences of any of the n-3 LCPUFA treatments on the total pixel area (spheroids+AG) (Table 5.4.2).

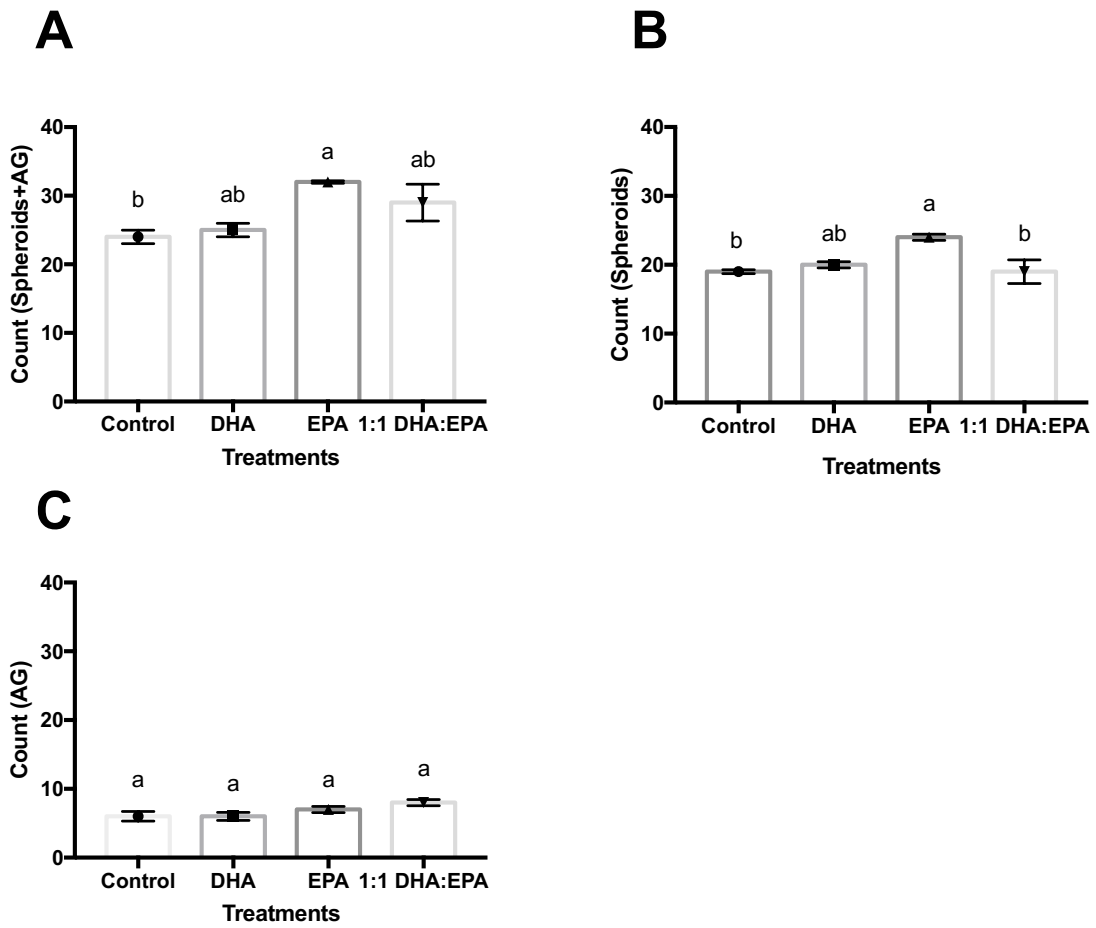

Figure 5.4.2: The effect of n-3 LCPUFA treatments on MDA-MB-231 spheroids grown in a 3D on-top cell culture model on (A) count (spheroids+AG); (B) count (spheroids); and (C) count (AG) (n=3 separate passages). Bars represent the mean±SEM for MDA-MB-231 breast cancer cells. Bars that do not share a letter are significantly different ($p<0.05$).

Table 5.4.2: Effect of 150 µM n-3 LCPUFA treatments on the total pixel area (spheroids+AG) of MDA-MB-231 breast cancer cells grown in a 3D on-top cell culture model of breast cancer.

Treatment	Total Pixel Area (spheroids+AG)
80 µM OA/LA	128497±6058 [a]
DHA	126983±5510 [a]
EPA	153843±12163 [a]
1:1 DHA:EPA	173078±25360 [a]

"OA"=oleic acid; "LA"=linoleic acid; "DHA"=docosahexaenoic acid; "EPA"=eicosapentaenoic acid; "AG"=aggregate. Values represent the mean±SEM for MDA-MB-231 breast cancer cells (n=3 separate passages). Within a column, means that do not share a letter are significantly different ($p<0.05$).

Effect of n-3 LCPUFA treatment on growth parameters in SK-BR-3 breast cancer cells

There was no effect of n-3 LCPUFA on the following growth parameters in SK-BR-3 breast cancer cells grown in a 3D on-top Cell culture model: total pixel area or the following characteristics of AG: pixel area, breadth, length, and shape factor (Table 5.4.3 and Figure 5.4.3).

Table 5.4.3: Effect of 150 µM n-3 LCPUFA treatments on growth parameters on SK-BR-3 breast cancer cells grown in a 3D on-top cell culture model of breast cancer.

Treatment	Total Pixel Area	Pixel Area of AG	Breadth of AG (Pixels)	Length of AG (Pixels)	Shape Factor of AG
80 µM OA/LA	403344±28375 [a]	418737±28162 [a]	102±9.3[a]	142±15.3[a]	0.43±0.02 [a]
DHA	384435±5823 [a]	378741±4747 [a]	77±9.2[a]	95±14.4[a]	0.40±0.03 [a]
EPA	400470±11181 [a]	392019±9562 [a]	73±4.2[a]	105±4.1[a]	0.44±0.06 [a]
1:1 DHA:EPA	418556±48585 [a]	410961±49101 [a]	71±8.3[a]	91±2.4[a]	0.42±0.05 [a]

"DHA"= docosahexaenoic acid; "EPA"=eicosapentaenoic acid; "AG"=aggregate. Breadth of AG is length perpendicular to the longest chord Length of AG was measured using the longest chord of the object. Shape factor is a value from 0 to 1 that represents how closely an object represents a circle (shape factor $=\frac{4\pi A}{P^2}$ where A= area and P=perimeter of AG). Values represent the mean±SEM for SK-BR-3 breast cancer cells (n=3 separate passages). Within a column, means that do not share a letter are significantly different ($p<0.05$).

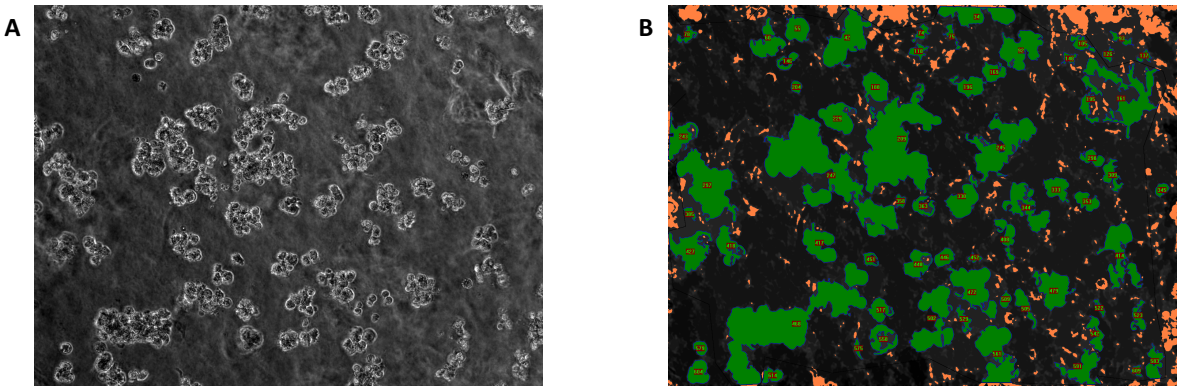

Figure 5.4.3: (A) Phase-contrast image of SK-BR-3 breast cancer cells treated with 150 µM EPA+ 80 µM OA/LA and (B) corresponding image analyzed using top-hat journal with threshold mask described in chapter 4.

Average number of AG and spheroids

In SK-BR-3 breast cancer cells, EPA at 150 μM significantly increased the average total number of objects (spheroids+AG) ((64 vs. 39) 64% increase, $p<0.05$) (Figure 5.4.2A). DHA when provided at 150 μM increased the number of spheroids+AG ((54 v. 39) 38% increase, $p<0.05$) while the 1:1 mixture (75 μM DHA) had no significant effect on the total number of spheroids+AG. DHA and EPA significantly increased the number of AG to the same extent ((47 vs. 34) 38% increase, $p<0.05$ and (55 vs. 34) 62% increase, $p<0.05$, respectively). There was no significant effect of the 1:1 mixture on the number of AG. There was no significant effect of any n-3 LCPUFA treatment on the number of spheroids (Figure 5.4.2C).

Since DHA and EPA at 150 μM significantly increased the number of AG, the effect of treatment on AG characteristics (the number of spheroids/AG) was examined (Figure 5.4.2D). DHA had no significant effect on the number of spheroids/AG, relative to control. When EPA was provided at 150 μM and in the 1:1 mixture (75 μM EPA) there was a significant decrease in the number of spheroids/AG ((8 vs. 18) 56% decrease, $p<0.05$ and ((10 vs. 18) 44% decrease, $p<0.05$, respectively).

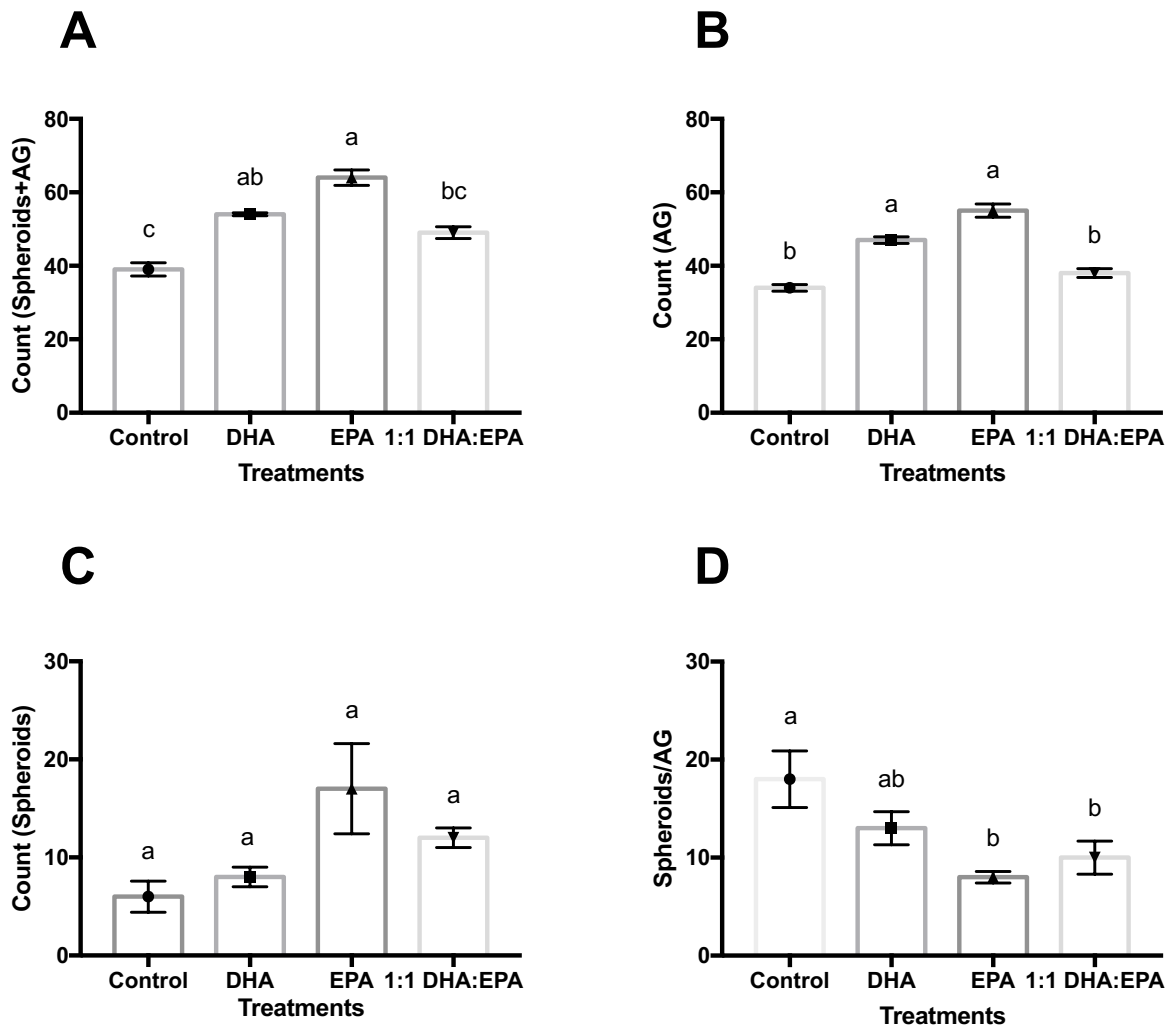

Figure 5.4.4: The effect of n-3 LCPUFA treatments on SK-BR-3 AG and spheroids grown in a 3D on-top Cell Culture Model on (A) average number of AG and spheroids, (B) average number of AG, (C) average number of spheroids; and (D) average number of spheroids/AG (n=3 separate passages and experiments). Bars represent the mean±SEM for SK-BR-3 breast cancer cells. Bars that do not share a letter are significantly different ($p<0.05$)

Chapter Six-Discussion and Future Directions

1. Summary of results

Objective 1: Determine state of knowledge on the specific effects of DHA, EPA, and DHA:EPA mixtures on survival in human breast cancer models.

When DHA and EPA were compared directly *in vitro* at the same concentration, DHA had a greater effect than EPA on decreasing cell growth (see Table 6.1.1 for relative efficacy in each breast cancer model studied). When examining the effect of dose on relative efficacy, some researchers have found that DHA decreases cell viability to a greater extent than EPA at lower concentrations in triple negative and ER+ human breast cancer cells, suggesting that DHA is more potent (see Chapter 3). There was limited data on the relative efficacy of DHA:EPA mixtures *in vitro*; therefore, the initial hypothesis that more DHA in mixture will exert greater anti-cancer effect could not be confirmed through the critical review presented in Chapter 3. Of note, in the published literature, the relative efficacy of EPA and DHA on relative incorporation into lipid rafts and on cell growth *in vitro* differed between breast cancer cell lines representing distinct breast cancer subtypes. One *in vivo* study compared DHA and EPA enriched diets to that of a 1:1 DHA:EPA diet in rats induced with mammary carcinogenesis [92]. In this study, DHA had a greater effect than EPA at the same dose and the 1:1 DHA:EPA mixture. This suggests that DHA exerts a greater anti-effect and that the presence of EPA blunts the effect of DHA. This observation provided preliminary evidence that there is a difference in anti-cancer effect between DHA, EPA, and DHA:EPA mixtures.

Table 6.1.1: Summary of literature review evaluating the relative efficacy of EPA and DHA for anti-cancer effects in human breast cancer models

	***in vitro:* ER-, PR-, Her2- cell lines**	***in vitro:* ER+ cell lines**	***in vivo:* animal feeding models**
Membrane incorporation*	Whole cell EPA>DHA Lipid raft DHA>EPA	Whole cell & lipid raft EPA>DHA	DHA>EPA>1:1 DHA:EPA
↑Cell Death	DHA>EPA	DHA>EPA	
↓Cell Growth	DHA>EPA	DHA=EPA	DHA>EPA & 1:1 DHA:EPA**

"↑"=significant increase ($p<0.05$); "↓"=significant decrease ($p<0.05$); "triple negative breast cancer"=Triple Negative breast cancer; "ER"=estrogen receptor; "PR"=progesterone receptor, "Her2=human epidermal growth factor receptor; "EPA"=eicosapentaenoic acid; "DHA"=docosahexaenoic acid. *=Relative efficacy of membrane incorporation determined using fold changes in EPA and/or DHA from control condition. **=Tumour multiplicity assessed in animal feeding model (not cell growth).

Objective 2: Determine if differences in cell viability exist between the effect of DHA, EPA, and DHA:EPA mixtures when provided at the same total concentration on MDA-MB-231 and SK-BR-3 human breast cancer cell lines and determine if changes in tumour fatty acid composition explains these differences.

Differential effects of n-3 LCPUFA treatments

In MDA-MB-231 breast cancer cells, all treatments (EPA, DHA, 1:1 DHA:EPA, and 2:1 DHA:EPA) were cytotoxic to the cells to same extent except DHA at 200 μM (see Table 6.1.2 for decreases in viability at each dose tested). The ratio of n-3 LCPUFA in mixtures did not change the cytotoxic effect of treatment, as more DHA in mixture did not enhance cytotoxicity (see Table 6.1.2 for decreases in viability). This is in contrast to what was predicted from the literature review (Chapter 3) that showed that in MDA-MB-231 breast cancer cells when DHA and EPA were compared directly, DHA appeared to have a greater anti-cancer effect. This higher DHA effect was only observed at the higher fatty acid concentration of 200 μM.

Table 6.1.2: Summary of results from the present thesis, by objective and breast cancer cell line

Objective	Summary of Results	
	MDA-MB-231	**SK-BR-3**
Objective 2: Determine if differences in cell viability exist with DHA, EPA, & DHA:EPA mixtures	Decrease in cell viability: • At 100 μM: DHA=EPA=1:1=2:1 ↓25-29% • At 150 μM: DHA=EPA=1:1=2:1 ↓19-26% • At 200 μM: DHA: ↓ 59% EPA=1:1=2:1: ↓36-44%	Decrease in cell viability: • At 100 μM: EPA↓35% DHA & 2:1 ↓22-24% 1:1 ↓17% • At 150 μM: EPA: ↓44 % DHA=1:1=2:1: ↓31-37% • At 200 μM: EPA: ↓47 % DHA=1:1=2:1: ↓33-39%
Objective 2: Determine if changes in tumour fatty acid composition can explain differences in cell viability	In general, across doses and lipid fractions: • When provided EPA, DHA or 2:1: EPA+DPA≈DHA content (%w/w) • When provide 1:1: EPA+DPA> DHA content (%w/w) • ↓AA with ↑ doses of EPA	In general, across doses and lipid fractions: • When provide EPA, DHA or 1:1 EPA+DPA≈DHA content (%w/w) • When provide 2:1: DHA>EPA+DPA[2] content (%w/w) • ↓AA with ↑ doses of EPA
Objective 3: Identify potential membrane-associated receptors to explain the effects of n-3 LCPUFAs on cell death & growth	<u>Effect on proteins related to cell death:</u> • EPA, 1:1 & 2:1 ↑ whole cell CD95 (EPA =1:1 and EPA>2:1) • DHA & 2:1 ↑ cell surface CD95 (DHA=2:1) • All treatments ↓whole cell RIPK1 & FADD (EPA=DHA=1:1=2:1) <u>Effect on proteins related to cell growth:</u>	<u>Effect on proteins related to cell death:</u> • EPA ↑ whole cell CD95 and ↓ FADD • DHA & 2:1 ↓ whole cell CD95 and ↑ cell surface CD95 (DHA=2:1) • 1:1 & 2:1 ↑RIPK1 (1:1=2:1) <u>Effect on proteins related to cell growth:</u>

[2] This only approached significance for total PL (100 and 200 μM) and PI at 200 μM.

	• 1:1 ↑ whole cell EGFR • EPA & 2:1 ↓ cell surface EGFR (EPA=2:1) • All treatments ↑ whole cell pEGFR (EPA=DHA=1:1=2:1)	• DHA ↑ whole cell Her2
Objective 4: Determine if the effects of DHA and/or EPA are consistent in 2D are consistent in 3D	Compared to effect of n-3 LCPUFA treatment in whole cell FA in 2D culture: • EPA ↑ number of spheroids • Whole cell content of DHA and EPA+DPA (%w/w) not different • Similar relative incorporation of n-3 LCPUFA to 2D model when provide EPA or DHA: EPA+DPA≈DHA content (%w/w) • Distinct relative incorporation of n-3 LCPUFA to 2D model when provide 1:1: EPA+DPA>DHA content (%w/w)	Compared to effect of n-3 LCPUFA treatment in whole cell FA in 2D culture: • EPA & DHA ↑ number of AG (EPA=DHA) • EPA & 1:1 ↓ number of spheroids/AG (EPA=1:1) • Whole cell content of DHA and EPA+DPA (%w/w) slightly higher • Distinct relative incorporation of n-3 LCPUFA to 2D model when provide 100 μM EPA or DHA: EPA+DPA>DHA[3] content (%w/w) • Distinct relative incorporation of n-3 LCPUFA to 2D model when provide 1:1: EPA+DPA≈DHA content (% w/w)

"↑ "=significant increase; "↓"=significant decrease; "≈"=similar to; "="=equal to; "DHA"=docosahexaenoic acid; "EPA"=eicosapentaenoic acid; 1:1= 1:1 DHA:EPA mixture; 2:1= 2:1 DHA:EPA mixture; "DPA"=docosapentaenoic acid; "AA"=arachidonic acid; CD95= cluster of differentiation 95; "EGFR"= Epidermal Growth Factor Receptor; "pEGFR"= phosphorylated EGFR; "RIPK1"= Receptor-interacting serine/threonine-protein kinase 1; "FADD"= Fas-associated protein with death domain; "Her2"=Human Epidermal Growth Factor Receptor; "SC"=spheroids; "AG"=aggregates.

[3] When provide 150 μM EPA or DHA relative incorporation same in 2D as 3D (EPA+DPA≈DHA content)

In SK-BR-3 breast cancer cells, EPA was more cytotoxic than DHA and DHA:EPA mixtures at all doses tested (Table 6.1.2). Ratios predicted cytotoxicity at 100 μM (more DHA in treatment decreased cell viability) and incorporation into total PL and PL classes. At 200 μM ratios did not predict the degree of cytotoxicity as both mixtures decreased viability to the same extent but incorporation was predictable based on the ratio.

Interpretation of results: changes in tumour fatty acid composition

In the present study, whole cell FA, total PL, and PL classes were examined. It is important to note that the methods used in this study did not involve the extraction of the plasma membrane from breast cancer cells for fatty acid analysis (see Chapter 3). It is likely that incorporation into whole cell FA, total PL, and PL classes is representative of the incorporation of n-3 LCPUFA into the plasma membrane, as the plasma membrane is the largest membrane constituent of total cellular membranes [142]. There are several other subcellular membranes that are associated with organelles including the endoplasmic reticulum, mitochondria, golgi apparatus, and endosomes (reviewed in [143]). The lipid compositions of these subcellular membranes differ; however, when provided to cells, DHA and/or EPA are incorporated into these subcellular membranes (reviewed in [7, 144]).

As previously stated, de novo synthesis of PL is regulated by the Kennedy pathway and the fatty acid composition of PL is regulated by the Lands cycle [17]. In the present study, n-3 LCPUFA were provided to breast cancer cells bound to BSA. Since PL were not provided to breast cancer cells, alterations in total PL are most likely attributed to changes in de novo lipogenesis. This is consistent with the current literature, as researchers have shown that DHA containing PL are generated via the Kennedy pathway [25].

Predictability of ratios

If DHA and EPA were incorporated as predicted by the ratio in DHA:EPA mixtures into whole cell fatty acids, total PL and PL classes in tumour cells, it would be expected that with a 1:1 mixture EPA+DPA and DHA content (%w/w) would be similar and with a 2:1 DHA:EPA mixture there would be approximately double the amount of DHA compared to EPA+DPA. In MDA-MB-231 breast cancer cells the ratios in DHA:EPA mixtures did not predict EPA+DPA and DHA content (see Table 6.1.2 for relative incorporation of EPA and/or DHA). More EPA+DPA was incorporated when a 1:1 mixture was provided and there were similar amounts of EPA+DPA and DHA content with a 2:1 mixture. In MDA-MB-231 breast cancer cells there was predictability of incorporation at 150 μM into total PL with 1:1 DHA:EPA and into PE at 200 μM with 1:1 and 2:1 DHA:EPA treatments. This suggests that measuring total PL is not necessarily predictive of the cytotoxic effect on the cells and suggests that one has to measure specific PL to understand how these fatty acids changes might predict the anti-cancer effects.

Incorporation into whole cell fatty acids, total PL and PL classes was more predictable in SK-BR-3 breast cancer cells. The differences in the predictability between breast cancer cell lines may be due to differences in growth rates. The population doubling time for MDA-MB-231 breast cancer cells is shorter than that of SK-BR-3 breast cancer cells (31 vs. 45 hours)[145]. The slower doubling time in SK-BR-3 cells may allow the ratio of the DHA:EPA mixtures to be incorporated. The cellular morphology may also play a role. SK-BR-3 breast cancer cells are grape-like, while MDA-MB-231 breast cancer cells are stellate shaped with disorganized nuclei and non-uniform size and shape. These elongated cell bodies overlap and cluster when grown in 2D [146], so it is unlikely that each of these cells is exposed to the exact dose of EPA and/or DHA when provided alone or in a mixture. SK-BR-3 breast cancer cells are rounder and more symmetrical; therefore, it is more plausible that the n-3 LCPUFA mixture is incorporated with less impediments [146].

Effect of n-3 LCPUFA treatments on AA

In both breast cancer cell lines, AA concentration in the cell decreased with increasing doses of EPA in total PL. There was a more potent decrease in AA with EPA in SK-BR-3 breast cancer than MDA-MB-231 breast cancer cells (larger % decrease in AA with same EPA containing treatments, see Table 6.1.3). The decrease in cell viability with 100 μM EPA was larger in SK-BR-3 than MDA-MB-231 breast cancer cells (35% vs. 25% decrease). It is likely that EPA was better able to compete with AA for incorporation at a lower dose in this cell line and had subsequent effects on breast cancer cell viability. AA release from the membrane by phospholipase A_2 has been shown to induce intrinsic apoptosis in liver hepatoma cells [147]. Blockage of AA incorporation into PL in promonocytic cells has also been shown to induce apoptosis [148]. In breast cancer, inhibition of AA acid metabolite hydroxy-eicosatetraenoic acid (20-HETE), decreased breast cancer cell invasion and metastases (reviewed in [149]). Together, this suggests that the decrease observed in total PL AA may have contributed to increased cell death.

Table 6.1.3: Decrease in total PL AA in MDA-MB-231 and SK-BR-3 human breast cancer cells with 100 μM n-3 LCPUFA treatments

	%w/w Total PL AA with 80 μM OA/LA	**%w/w Total PL AA with EPA-Containing Treatments (100 μM EPA, 1:1 DHA:EPA, 2:1 DHA:EPA)**	**% Decrease in Total PL AA**
MDA-MB-231	2.85±0.31	1.07-1.77	38-62
SK-BR-3	5.59±0.38	2.00-2.58	54-64

"PL"=phospholipid; "AA"=arachidonic acid; "EPA"=eicosapentaenoic acid

There was a plateau in the decrease of AA into PL at doses >75 μM in both cell lines suggesting a threshold value of AA (Figure 5.2.2C & 5.2.4E). However, there was a dose-dependent decrease in AA in PC, PE, and PI lipid fractions (Figure 6.1.1 and 6.1.2). In both

MDA-MB-231 and SK-BR-3 breast cancer cells, AA content was lowest (%w/w) in the PC fraction. In MDA-MB-231 breast cancer cells the largest decreases in AA with n-3 LCPUFA were in the PE fraction and in SK-BR-3 breast cancer cells the greatest decrease was observed in the PC fraction (Table 6.1.4).

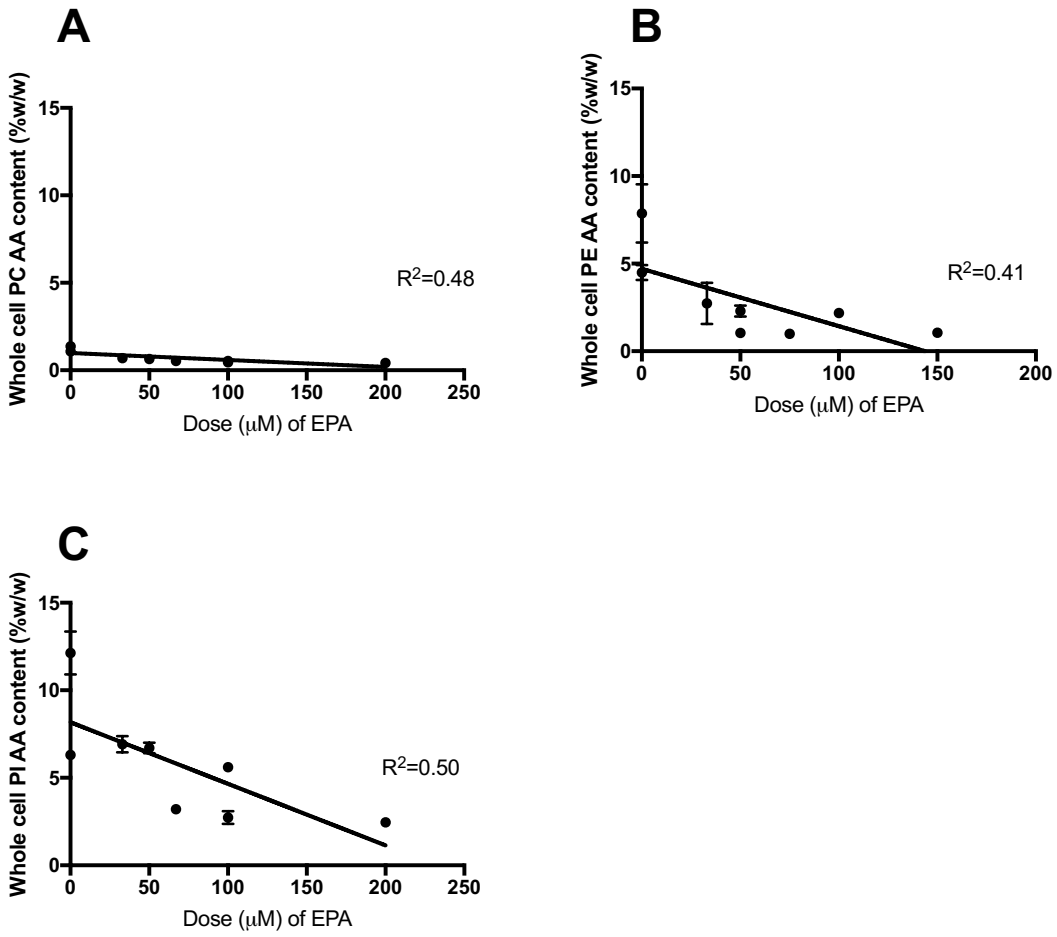

Figure 6.1.1: Dose-effect of n-3 LCPUFA treatments in MDA-MB-231 breast cancer cells on total PL AA content (%w/w) in: (A) PC; (B) PE; (C) PI.

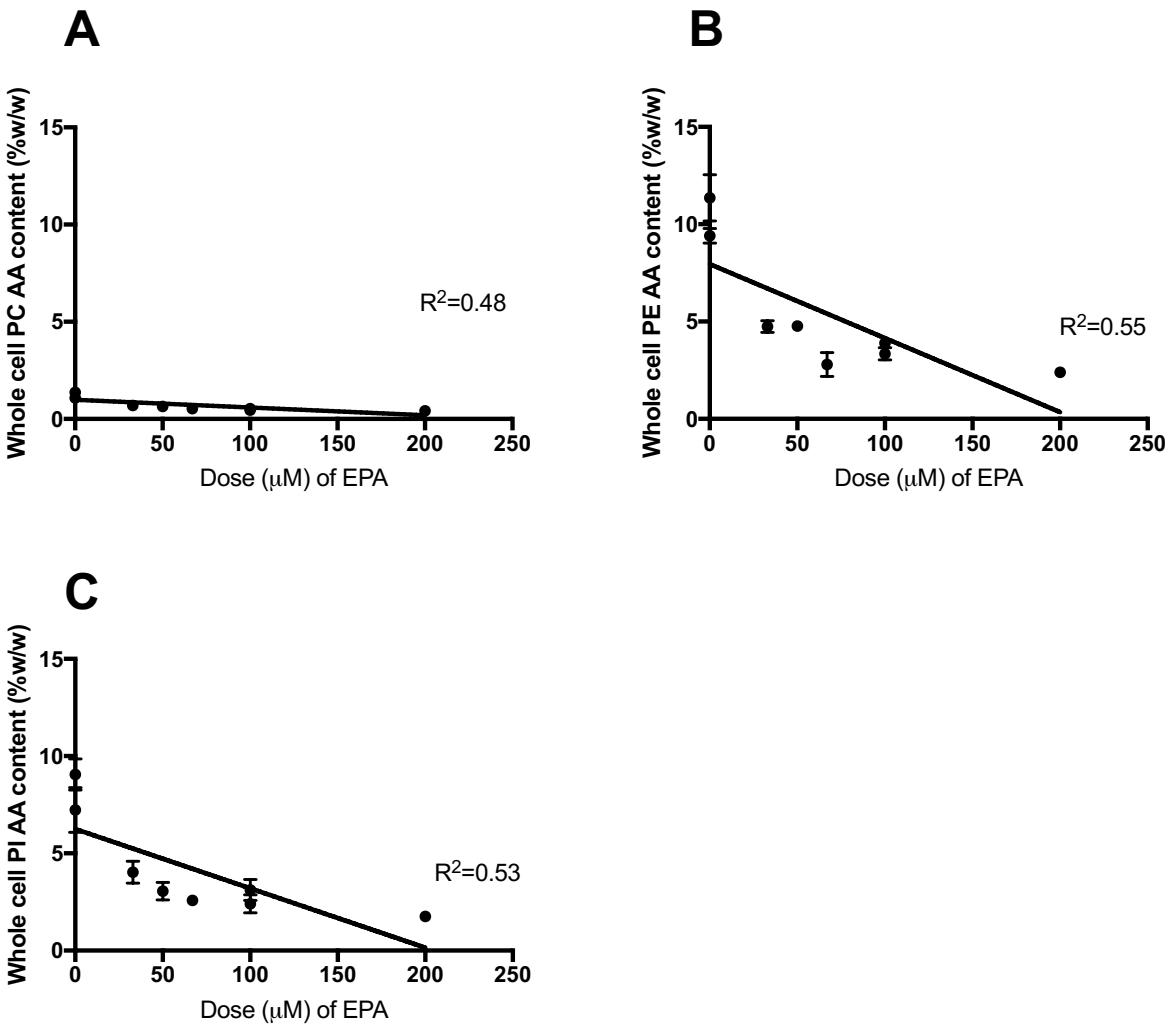

Figure 6.1.2: Dose-effect of n-3 LCPUFA treatments in SK-BR-3 breast cancer cells on total PL AA content (%w/w) in: (A) PC; (B) PE; (C) PI.

Table 6.1.4: Decrease in AA in whole cell PL classes of MDA-MB-231 and SK-BR-3 human breast cancer cells with n-3 LCPUFA treatments

Breast Cancer Cell Line	PL Fraction	Range in % Decrease AA with 100 and 200 μM EPA Containing Treatments
MDA-MB-231	PC	49-61
	PE	65-78
	PI	43-61
SK-BR-3	PC	53-80
	PE	49-79
	PI	44-81

"PL"=phospholipid; "PC"=phosphatidylcholine; "PE"=phosphatidylethanolamine; "PI"=phosphatidylinositol; "AA"=arachidonic acid; "EPA"=eicosapentaenoic acid.

Incorporation of EPA+DPA and DHA into whole cell fatty acids, total PL, and PL classes did not explain cell viability in MDA-MB-231 breast cancer cells. The relative incorporation of EPA+DPA and DHA differed between DHA, EPA, 2:1 treatments with that of the 1:1 treatment, yet all treatments caused the same cytotoxic effect. In SK-BR-3 breast cancer cells, EPA consistently decreased cell viability better but DHA was incorporated either to the same extent or greater extent than EPA+DPA. The effect of increasing dose of EPA on AA in specific PL fractions explained above may explain the decreases in viability.

Objective 3: To identify potential membrane-associated receptors that could explain the effects of n-3 LCPUFAs on cell death and growth pathways in MDA-MB-231 and SK-BR-3 human breast cancer cells.

Cell growth

In MDA-MB-231 breast cancer cells, 1:1 DHA:EPA increased whole cell EGFR, while EPA and 2:1 DHA:EPA decreased cell surface EGFR. These changes coincided with preferential incorporation of EPA+DPA into whole cell total PI with EPA and 1:1 DHA:EPA

treatments. PI is found exclusively on the inner leaflet of the plasma membrane and is responsible for signal transduction of many pathways [142]. A literature gap is the investigation of the role of the lipid composition of PI on EGFR activation and translocation in human breast cancer cells, presenting an opportunity for future research. Phosphatidylinositol 4,5-bisphosphate (PIP_2), the major PI in the plasma membrane [150] has been shown to modulate EGFR activation [151]. PIP_2 is phosphorylated to phosphatidylinositol (3,4,5)-trisphosphate (PIP_3) by PI 3-kinase (PI3K)[150]. PI3K activation is increased in cancer, which leads to increased membrane PIP_3,which activates Akt and as a result proliferative signaling [150]. In Chapter 3, DHA:EPA mixtures decreased pAkt [37] and DHA alone decreased Akt [32] in MDA-MB-231 breast cancer cells, suggesting that n-3 LCPUFA can decrease proliferative signaling in breast cancer. Phosphatase and tensin homolog (PTEN) is a tumour suppressor that dephosphorylates PIP_3 to PIP_2. However, PTEN is frequently mutated and inactivated in cancer [150]. A fish oil diet decreased PIP_3 and increased PTEN in a rodent model of breast cancer [152]. This shows that there is a relationship between the PI3K/PTEN/Akt signaling axis and n-3 LCPUFA. It is plausible that altering the lipid composition of PI with EPA and DPA may impact the PI3K/PTEN/Akt signaling axis and, as a result, EGFR activation. However, further studies are needed to determine the role of n-3 LCPUFA on PI and its role in EGFR activation and translocation.

A previous study from our lab group used a 1:1.5 DHA:EPA mixture in MDA-MB-231 breast cancer cells and did not see significant changes in whole cell EGFR with or without LA in the media [28]. In the present study EPA and DHA when provided at a ratio of 1:1 increased whole cell EGFR, while 2:1 DHA:EPA, DHA , and EPA treatments had no effect. Other studies have showed that DHA, not EPA, decreases whole cell EGFR [26, 32]. Of note, these studies were different in experimental design as one study dissolved EPA and DHA in ethanol [26] rather than conjugating to BSA, while the other study used smaller doses of EPA and DHA (30 and 50 μM) with a much shorter exposure period [32]. We previously saw in MDA-MB-231

breast cancer cells that a DHA:EPA mixture increased whole cell pEGFR [28]. This was associated with increased cell death and increased p38 MAPK, suggesting that the increased pEGFR is promoting death, not growth. In this thesis, MDA-MB-231 breast cancer cells all n-3 LCPUFA treatments significantly increased pEGFR. In contrast, Corsetto et al. [26], found that whole cell pEGFR decreased when EPA or DHA was provided. This difference may be accounted for by dose, as Corsetto et al. [26] used a much higher dose of 230 μM.

In SK-BR-3 breast cancer cells the effect of treatment was examined on Her2, a common therapeutic agent for Her2+ breast cancer [153]. DHA at 100 μM significantly increased Her2, which has potential clinical implications as this would increase the number/concentration of the Her2 receptors in tumours and potentially increase the efficacy of the drug. This is also of importance as there is a high frequency of developing drug resistance with Her2+ breast cancer (reviewed in [154-157]). With EPA there was a trend towards decreased Her2, although this did not reach statistical significance ($p=0.09$). This may have clinical implications as EPA at a higher dose could significantly decrease Her2 as a therapeutic target. In Zou et al. [158], DHA in SK-BR-3 breast cancer cells increased Her2 after 24 hours with heregulin, a Her3 specific ligand. However, from 48-72 hours, Her2 was significantly decreased. These differences may also be due to differences in experimental design, as Zou et al. [158] only allowed cells to adhere over night before treating with DHA or differences in n-3 LCPUFA treatment as no background fatty acids were used.

Cell death

In MDA-MB-231 breast cancer cells, DHA at 100 μM or 66 μM (2:1 DHA:EPA) increased cell surface CD95, while EPA and both DHA:EPA mixtures increased whole cell CD95. Previous work conducted by our lab group has shown that DHA simultaneously increases cell surface and plasma membrane lipid raft content of CD95 in MDA-MB-231 breast cancer cells [40]. Here, DHA's effect on increasing cell surface CD95 (likely by increasing

translocation to the lipid rafts) was blunted when EPA was present, so a higher dose of DHA was needed. This can be explained by lipid incorporation data, as the presence of EPA in DHA:EPA mixtures capped total PL DHA at ~5%. It is likely that cell surface CD95 increased with increasing dose of DHA in MDA-MB-231 breast cancer cells and this might account for the enhanced killing with 200 μM DHA compared to other treatments.

In SK-BR-3 breast cancer cells, DHA and 2:1 DHA:EPA treatments increased cell surface CD95, while decreasing whole cell CD95. DHA alone also increased whole cell Her2. These changes were accompanied by more DHA into PC with DHA and 2:1 DHA:EPA treatments, suggesting that changes in CD95 and Her2 could be predicted by changes in this PL fraction. PC is the most abundant PL in the plasma membrane [143]. The relationship between PC fatty acid composition and CD95 or Her2 has not been explored. However, researchers have examined PC specific phospholipase C (PC-PLC) and these membrane associated receptors. Her2 has been shown to co-localize with PC-PLC and inhibition of this enzyme decreases membrane Her2 in human breast cancer cells [159]. CD95 signal transduction has also been linked to PC-PLC activation [160]. It is possible that changes in the FA composition of PC with n-3 LCPUFA may have implications on growth and death pathways as PC-PLC acts on this PL. Further studies are needed to determine if n-3 LCPUFA directly affect these membrane-mediated mechanisms.

This thesis presented novel insight into the FA composition of PL classes with differing doses and ratios of n-3 LCPUFA. However, a large body of research has investigated the importance of the relative quantity of PL classes on cellular proliferation. For example, increases in membrane 20:4-PC in fibroblasts has been shown to inhibit proliferation by preventing translocation of Akt to the plasma membrane [17]. There was no effect of saturated and monounsaturated PC, suggesting that PUFA PC are more important in regulating proliferation. Cellular PE and PC content has also been associated with proliferation and inversely associated with apoptosis [161]. The ratio of PL may also be important in cancer

progression. A higher PC:PE ratio has been associated with metastases in colorectal cancer cells [162]. Future studies could investigate the effect of DHA and/or EPA on the relative amounts of membrane PE, PC, PI, sphingomyelin, and phosphatidylserine to further elucidate the effects of these treatments on membrane dynamics and how this relates to changes in cellular proliferation.

Effects of treatments on cell death and growth receptors explaining cell viability

In MDA-MB-231 the effect of EPA and DHA:EPA mixtures on cell surface and whole cell CD95 and whole cell EGFR do not explain why all treatments killed to same extent at 100 and 150 μM (see Table 6.1.2 for relative efficacy of these treatments). However, all treatments decreased whole cell RIPK1 and FADD to the same extent while also increasing whole cell pEGFR to the same extent. This suggests that similar activation of receptors related to cell growth and similar effect on downstream apoptotic signaling proteins may explain similarities in cell death.

In addition to EPA's more potent effect on decreasing PL AA in SK-BR-3 breast cancer cells, EPA's greater anti-cancer effect in this cell line may be explained by EPA increased whole cell CD95 and decreased FADD. At 100 μM we saw that DHA and 2:1 increase cell surface CD95, while 1:1 DHA:EPA did not. This is probably why the 1:1 treatment was less effective at decreasing cell viability at 100 μM compared to DHA and 2:1 treatments at 100 μM. At 200 μM, the DHA-containing treatments (DHA, 1:1, and 2:1) had the same effect on decreasing cell viability (33-39%). It is possible that at this dose, cell surface CD95 expression is more similar between DHA-containing treatments. A dose-effect study would need to be conducted to determine changes in CD95 with DHA containing treatments to confirm this. It is also possible that since DHA is implicated in many other signaling pathways (reviewed in [7, 8]) the degree of cell death is activated in a similar manner at this dose. The observed increase in cell surface CD95 and whole cell Her2 with 100 μM DHA in SK-BR-3 breast cancer

cells did not translate to enhanced cytotoxicity when compared to the 100 μM EPA. This suggests that the decrease in FADD and increase in whole cell CD95 with 100 μM EPA had a greater effect on cell viability.

Objective 4: Determine if the effects of DHA and/or EPA, on cell viability and lipids in 2D culture are consistent when MDA-MB-231 and SK-BR-3 breast cancer cells are grown in a 3D on Top cell culture model.

Growth and incorporation

It was hypothesized that the same effects and differences between n-3 LCPUFA treatments seen in 2D cell culture would be seen in 3D cell culture. In both breast cancer subtypes, the effects and differences between n-3 LCPUFA in 2D on tumour cell death were not seen in 3D cell culture (Table 6.1.2). In MDA-MB-231 breast cancer cells in 2D cell culture, 150 μM n-3 LCPUFA treatments caused mean decreases in cell viability of 19-26%. In 3D cell culture, EPA enhanced growth of MDA-MB-231 spheroids.

Cell viability of SK-BR-3 breast cancer grown in 2D cell culture decreased 31-44% with 150 μM n-3 LCPUFA treatments. DHA and EPA increased the number of SK-BR-3 breast cancer AG grown in 3D cell culture while EPA and 1:1 DHA:EPA decreased the number of spheroids/AG. These changes occurred without changing pixel area, suggesting that EPA with or without DHA affect AG formation. The changes in AG and spheroids/AG may be explained by whole cell content n-3 LCPUFA as whole cell EPA+DPA content with EPA was similar to increases in DHA and EPA+DPA content with the 1:1 DHA:EPA treatment (16.15% EPA+DPA and 16.46% EPA+DPA+DHA, respectively), while DHA content with DHA was lower (12.73% DHA)(see Table 5.2.26). These differences in Σ n-3 LCPUFA between treatments did not reach significance in whole cell fatty acids; however, it is possible that there are differences between treatments in EPA+DPA and DHA content in total PL and PL classes

not investigated in the present thesis that may explain the effect of n-3 LCPUFA on cell growth parameters.

In MDA-MB-231 cells, whole cell DHA and EPA+DPA content with DHA, EPA, or 1:1 DHA:EPA were similar between 2D (at either dose provided) and 3D cell culture models. In SK-BR-3 cells there was a significant, but small increase in DHA and EPA+DPA content with DHA, EPA, or 1:1 DHA:EPA in 3D compared to 2D. Collectively, this indicates that incorporation is similar in both models, but results in cell growth in 3D and significant cell death in 2D. The same relative incorporation of EPA+DPA and DHA into whole cell FA was seen in MDA-MB-231 cells in 2D and 3D cell culture models when EPA or DHA was provided (Table 6.1.2). This suggests that the same trends in the relative incorporation of EPA+DPA and DHA with EPA or DHA treatments in MDA-MB-231 total PL and PL classes observed in 2D cell culture at this dose are occurring in 3D cell culture. The relative incorporation of EPA+DPA and DHA with DHA:EPA mixtures were not the same in 2D cell culture as 3D cell culture for either breast cancer cell line.

2. Discussion and future directions

N-3 LCPUFA incorporation in MDA-MB-231 and SK-BR-3 breast cancer cells grown in 2D cell culture model of breast cancer

Consistent with the results of the critical literature review (Chapter 3), there were differences between the breast cancer subtypes studied on the relative incorporation of EPA+DPA and/or DHA in whole cell FA, PL, and PL classes with EPA, DHA, or DHA:EPA treatments. In MDA-MB-231 breast cancer cells at 100 μM, the presence of EPA in DHA:EPA mixtures limited total PL DHA to ~5%, showing competition for PL incorporation (Table 6.2.1). This was not seen in SK-BR-3 breast cancer cells with the same DHA:EPA mixtures. In addition, there were also differences in the amount of n-3 LCPUFA incorporated. For each

dose tested, MDA-MB-231 breast cancer cells incorporated more n-3 LCPUFA %w/w than SK-BR-3 breast cancer cells into whole cell FA and PL (Table 6.2.1).

When comparing breast cancer subtypes, MDA-MB-231 breast cancer cells had more whole cell, PL, and PL class DPA with EPA containing treatments relative to control than SK-BR-3 breast cancer cells. Triple negative breast cancer has been shown to have a 4.6-fold increase in ELVOL5, an elongase that facilitates the elongation of EPA to DPA, compared to normal breast tissue [163], which may explain the high amount of DPA seen in this breast cancer cell line.

In both breast cancer cell lines, the magnitude of increase in total PL DHA was lower with treatments at 200 μM compared to 100 and/or 150 μM. There was a large decrease in cell viability with DHA at this dose (59% and 33%, in MDA-MB-231 and SK-BR-3, respectively). It is possible that PL DHA was so low at this dose because a small proportion of live cells remain and would be slower to incorporate DHA. This data also suggests that there is a maximum amount of DHA that can be incorporated into human breast cancer cells with increasing dose of n-3 LCPUFA. The phenomenon of a maximum membrane DHA content has been observed in the PC and PE fractions of human neuroblastoma (SH-SY5Y & IGR-N-91) and retinoblastoma (Y79) cells [164] as well as plasma PL in metastatic breast cancer patients receiving chemotherapy and DHA supplements [165, 166](Table 6.2.2). In metastatic breast cancer patients, maximum DHA incorporation was associated with improvements in the efficacy of chemotherapy [165].

In MDA-MB-231 breast cancer cells, 200 μM DHA decreased cell viability to the greatest extent, even though PL DHA was higher at 100 and 150 μM. This suggests that the effect of DHA may be attributed to other defined anti-cancer effects of DHA not associated directly to the membrane including inhibition of Wnt/β-Catenin, PI3k/Akt/mTOR, JAK-STAT, or NFκB pathways (reviewed in [7, 8]).

Table 6.2.1: Change in EPA+DPA and DHA in the PL fraction of MDA-MB-231 and SK-BR-3 breast cancer cells incubated with 100 μM of control or n-3 LCPUFA treatments

n-3 LCPUFA	Breast Cancer Cell Line	80 μM OA/LA	DHA	EPA	1:1 DHA:EPA	2:1 DHA:EPA
EPA+DPA	MDA-MB-231	0.4 ± 0.08^{d}		12.3 ± 0.50^{a}	10.1 ± 0.45^{b}	5.0 ± 0.31^{c}
	SK-BR-3	0.5 ± 0.03^{c}		5.6 ± 0.65^{a}	4.0 ± 0.43^{ab}	3.1 ± 0.17^{b}
DHA	MDA-MB-231	0.2 ± 0.07^{c}	13.0 ± 0.05^{a}		5.9 ± 0.36^{b}	5.1 ± 0.44^{b}
	SK-BR-3	0.2 ± 0.01^{d}	7.0 ± 0.28^{a}		3.2 ± 0.05^{c}	4.7 ± 0.37^{b}

“”EPA”=eicosapentaenoic acid; “DPA”=docosapentaenoic acid; “DHA”=docosapentaenoic acid.

Table 6.2.2: Maximum amount of DHA (%w/w) incorporated into total PL, PC, and PE *in vitro* and *in vivo* models of cancer

Breast Cancer Model		Dose (μM)	DHA Max Total PL (%w/w)	DHA Max PC (%w/w)	DHA Max PE (%w/w)
Human Cancer Cell Lines	MDA-MB-231	200	16.0	8.1	10.5
	SK-BR-3	200	11.3	12.1	18.3
	IGR-N-91 [164]	70		12.6	31.8
	SH-SY5Y [164]	70		10.6	32.7
	Y79 [164]	70		30.1	39.2
Human Intervention Study		1.8 g DHA daily [165]	8.3*		

“”%w/w”=percent weight/weight of identified FA;“DHA”=docosahexaenoic acid; “PL”=phospholipid; “PC”=phosphatidylcholine; “PE”=phosphatidylethanolamine. “*” =DHA max in total plasma PL.

Regulation of RIPK1 and FADD in MDA-MB-231 and SK-BR-3 breast cancer cells grown in 2D cell culture model of breast cancer

RIPK1 and FADD are important apoptotic proteins that may interact in one of 3 ways to promote apoptosis: 1) downstream of TNF-R1 in complex 2 (RIPK1/FADD/pro-capsase 8); 2) downstream of DISC complexes formed at the plasma membrane (FADD, pro-caspase-8, Death domain of CD95 or TRAIL receptors); and 3) the ripoptosome (RIPK1, FADD, pro-capsase 8), which forms independently of the death receptor and mitochondrial mediated apoptotic pathways (reviewed in [167]).

It was hypothesized that breast cancer cells with DHA and/or EPA will promote increases in the amount of proteins associated with cell death and that this would be consistent with changes in viability. All 100 μM n-3 LCPUFA treatments caused significant decreases in whole cell FADD in MDA-MB-231 and with 100 μM EPA decreased whole cell FAAD in SK-BR-3. A previous study by our lab showed that 60 μM DHA when provided with an 80 μM OA/LA background decreased lipid raft FADD content [40]. FADD levels are regulated by ubiquitination and subsequent degradation by the proteasome [168]. It is possible that cell death through DHA and/or EPA decreased FADD by promoting ubiquitination and degradation during the 72-hour exposure period. At 100 μM all MDA-MB-231 all treatments decreased cell viability and FADD to the same extent. At 100 μM EPA cause the greatest decrease in breast cancer cell viability and was the only treatment to significantly decrease whole cell FADD. Therefore, the changes in FADD were consistent with changes in viability in both cell lines at 100 μM.

There were differences between cell lines on the effect of n-3 LCPUFA treatments on whole cell RIPK1. In MDA-MB-231 breast cancer cells all 100 μM n-3 LCPUFA treatments caused significant decreases in whole cell RIPK1, while 1:1 and 2:1 treatment in SK-BR-3 breast cancer cells increased RIPK1. This could be due to either differences in growth rate or differences in apoptotic signal transduction between cell lines. As previously stated, SK-BR-3

breast cancer cells have a longer doubling time compared to MDA-MB-231 breast cancer cells, so they may incorporate EPA and/or DHA at a slower rate and, as a result, there may be temporal differences in the activation of RIPK1. RIPK1 is not essential for extrinsic apoptosis, so this marker may not be indicative of how EPA and/or DHA promote cell death in this cell line. It is possible that other signaling pathways are being altered including Wnt/β-Catenin, PI3k/Akt/mTOR, JAK-STAT, or NFκB pathways (reviewed in [7]). Lastly, RIPK1 is also subject to degradation by the proteasome [169]. This could be more tightly regulated in MDA-MB-231 breast cancer cells in response to n-3 LCPUFA treatments than SK-BR-3. Future studies are needed to elucidate these mechanisms.

3D cell culture methods: Suitability, limitations, and future directions

To determine the suitability of the present 3D cell culture models for mimicking n-3 LCPUFA incorporation *in vivo*, the relative trends in DHA and/or EPA were compared and contrasted to that of Yuri et al. who [92] studied the effect of diets enriched in DHA, EPA, and a 1:1 DHA:EPA mixture in rats with MNU induced mammary carcinogenesis (Table 6.2.3). In both breast cancer cell lines grown in 3D cell culture, when EPA or DHA were provided at 150 μM, the relative amount of whole cell of EPA+DPA and DHA content (%w/w) were approximately equal. *In vivo*, whether looking at mammary tissue or serum fatty acids in rats, when an EPA supplemented diet was provided there was more whole cell EPA+DPA than whole cell DHA with a DHA supplemented diet. This suggests that the present 3D models do not predict whole cell FA incorporation of EPA and DHA when provided alone *in vivo.* When a 1:1 DHA:EPA mixture was provided to SK-BR-3 breast cancer cells, whole cell EPA+DPA and DHA content were approximately equal. This trend of relative incorporation was also seen *in vivo* in serum of rats fed DHA:EPA diets. This suggests that in SK-BR-3 breast cancer cell line the 3D cell culture model may be predictive of whole cell EPA+DPA and DHA content in serum with DHA:EPA mixtures. The matrigel used in the present thesis contained laminin,

collagen IV, entactin/nidogen, and heparan sulfate proteoglycans [170]. Naturally derived matrigels may also contain small amounts of undefined compounds containing PL [68]; however, it is unlikely that the small amount of these compounds would significantly induce changes in whole cell fatty acid composition of breast cancer cells extracted from the gel for lipid analysis.

Table 6.2.3: Comparison of the relative % w/w of EPA+DPA and DHA in whole cell FA with n-3 LCPUFA treatments in in MDA-MB-231 and SK-BR-3 breast cancer cells grown in 3D cell culture with whole cell FA in mammary tissue or serum

Breast Cancer Model	**Cell Line or Tissue**	**% w/w EPA+DPA with EPA vs. % w/w DHA with DHA**	**Ratio**	**% w/w EPA+DPA vs. % w/w DHA with 1:1 DHA:EPA**	**Ratio**	**Predict-able Based on 1:1 Ratio?**
3D Cell Culture	MDA-MB-231	EPA+DPA≈DHA	1	EPA+DPA>DHA	2	NO
	SK-BR-3	EPA+DPA≈DHA	1	EPA+DPA≈DHA	1	YES
Rats induced with MNU	Mammary Tissue	EPA+DPA>DHA	2	DHA>EPA+DPA	2	NO
	Serum FA	EPA+DPA>DHA	3	EPA+DPA≈DHA	1	YES

"MNU"= N-Nitroso-N-methylurea; "EPA"=eicosapentaenoic acid; "DPA"=docosapentaenoic acid; "DHA"=docosahexaenoic acid; "n-3 LCPUFA"=n-3 long chain polyunsaturated fatty acid; "FA"= fatty acid.

There was significant growth with n-3 LCPUFA treatments in both breast cancer cell lines when grown in 3D culture in the present study. This is in contrast to a convincing body of evidence showing *in vivo* that EPA and/or DHA decreases tumour growth in rodent models of mammary carcinogenesis [152, 171-174]. Hongisto et al. [175] found that JIMT-1 breast cancer cells grew 1.9 fold faster in Matrigel than in a 2D cell culture model and 7.2 fold slower in a poly-2-hydroxyethyl methacrylate induced anchorage-independent 3D cell culture model. Hongisto et al. [175] used the same Matrigel used in the present study (Basement Membrane

Matrix Growth Factor Reduced), suggesting that the scaffolding provided by this hydrogel may promote tumour cell growth. Synthetic hydrogels or other 3D models should be explored as alternative 3D models that might be better to determine the effect of n-3 LCPUFA on breast tumour growth.

There are several potential reasons why n-3 LCPUFA treatments did not appear to decrease growth in the 3D on-top Cell culture model pertaining to the experimental design including the length of n-3 LCPUFA exposure period, the dose of n-3 LCPUFA, and the amount of Matrigel used. If a longer exposure period, higher n-3 LCPUFA dose, or less Matrigel was used to coat the cell culture plates, it is plausible that there may have been more of an effect of treatment on tumour growth. In addition, analysis of phase-contrast images may not be capturing all of the effects of n-3 LCPUFA. It is consistent in both *in vitro* and *in vivo* studies in the literature that DHA and/or EPA decrease breast cancer cell viability and tumour growth. However, there were increases in the morphological growth parameters assessed, which are commonly used endpoints to assess the effect of potential therapeutics in 3D culture models. Collectively, this suggests that these indices are not appropriate to assess effects of n-3 LCPUFA. Nuclear staining breast cancer cells in 3D with DAPI would increase the accuracy of spheroid counts and 3D specific colorimetric assays would determine whether or not the cells captured with phase-contrast images were undergoing metabolic changes. These could be explored in future studies to further elucidate the effects of n-3 LCPUFA on breast cancer cells grown in 3D cell culture.

This research has contributed to the current state of knowledge surrounding n-3 LCPUFA and breast cancer by: 1) directly comparing and contrasting the effect of DHA and EPA to dietary relevant DHA:EPA mixtures in the presence of physiologically relevant fatty acids (OA and LA) and 2) developing a novel 3D on-top cell culture model and determining that this model incorporates n-3 LCPUFA similarly to 2D cell culture models but current indices used to measure the efficacy of treatment in 3D cell culture (morphological changes)

are not appropriate to study the anti-cancer effects of n-3 LCPUFA in MDA-MB-231 and SK-BR-3 breast cancer cells. Overall, this thesis has shown that in 2D cell culture DHA, EPA, and DHA:EPA (1:1 and 2:1) mixtures do not exert the same anti-cancer effect as determined by incorporation of n-3 LCPUFA into breast cancer cells and effect on proteins related to cell growth and death pathways. Incorporation of the different n-3 LCPUFA treatments into whole cell or PL did not always predict the effects on tumour cell viability or explain the effects on cell death and growth pathways in either cell line. This work has also identified several opportunities for future research to further elucidate the beneficial, pleiotropic anti-cancer effects of DHA and/or EPA. In 2D cell culture this may include the establishment of maximum membrane n-3 LCPUFA content in distinct breast cancer subtypes, studying the effect of DHA and/or EPA on cell growth and death pathways not directly related to the plasma membrane (ex. Wnt/β-Catenin, PI3k/Akt/mTOR, JAK-STAT, or NFκB pathways), examining the relationship of specific PL classes (ex. PC and PI) and receptors related to cell death and growth (ex. EGFR or CD95), and quantifying changes in n-3 LCPUFA in PL classes to determine if DHA and/or EPA affect the PE:PC ratio. Changes in membrane fluidity with DHA and/or EPA treatments could also be assessed using fluorescent probes to determine if this may be a mechanism by which n-3 LCPUFA exert an anti-cancer effect [176]. Collectively, this research is important for future work using n-3 LCPUFA mixtures to target breast cancer tumours.

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Appendices

Appendix 1: The effect of n-3 LCPUFA treatments on SK-BR-3 paclitaxel resistant breast cancer cell lines

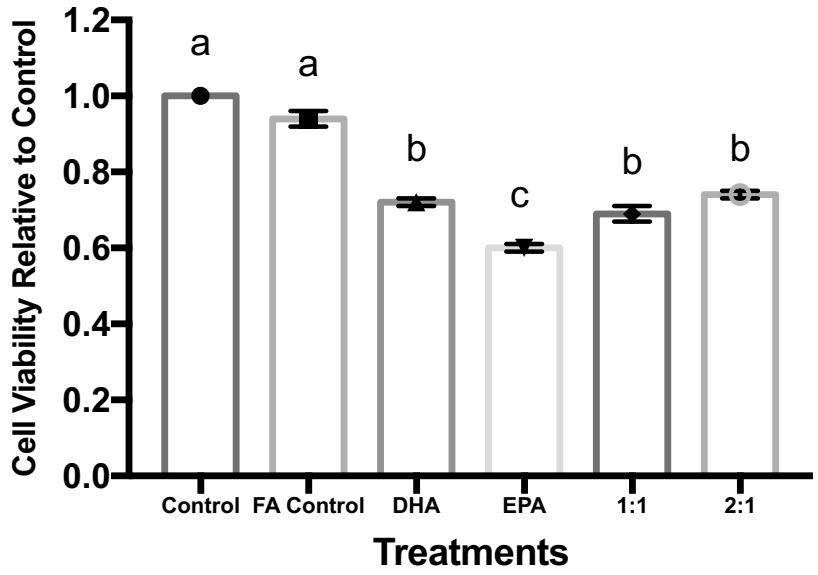

Figure 1: Cell Viability of paclitaxel resistant SK-BR-3 human breast cancer cells incubated with n-3 LCPUFA treatments (measured by counting cells) at 100 μM (n=3 separate passages). Bars that do not share a common letter are significantly different ($p<0.05$).

The effect of n-3 LCPUFA treatments were investigated at 100 μM in paclitaxel resistant SK-BR-3 human breast cancer cells to determine if drug resistance affects how breast cancer cells respond to treatment with EPA and/or DHA as described in chapter four. EPA when provided at 100 μM decreased cell viability by 40% ($p<0.05$), while DHA and DHA:EPA mixtures decreased to a lesser extent (26-31%, $p<0.05$) (Figure 1). Of note, the observed decreases with EPA, DHA, and DHA:EPA mixtures are larger in magnitude than those observed with parental SK-BR-3 breast cancer cells (35% decrease with EPA and 17-24% with DHA containing treatments, Figure 5.1.3). This suggests that n-3 LCPUFA treatments may be more potent in resistant breast cancer cell lines of this subtype.

Appendix 2: Justification for control fatty acid treatment

To increase the translatability of the present *in vitro* work, a control fatty acid background was used in the cell culture media which better represents fatty acids found abundantly *in vivo*. In healthy adults, LA is the most abundant PUFA in plasma (28-32%) [177, 178] and serum PL (19%) [179], while OA is the most abundant monounsaturated fatty acid (MUFA) in plasma (18-24%)[177, 178] and serum PL (10%) [179]. Most studies evaluating the anti-cancer effects of EPA and DHA have not evaluated these n-3 LCPUFA in the presence of other fatty acids. Our lab has previously shown that the presence of LA in the media with DHA:EPA mixtures blunts the beneficial anti-cancer effects of these n-3 LCPUFA on decreasing cell viability [28]. LA is also needed for mammary carcinogenesis models *in vivo* [180]. Collectively, this suggests that physiologically relevant fatty acids should also be considered to determine the potential efficacy of n-3 LCPUFA treatments. The control fatty acid treatment used for in the present study was an 80μM OA/LA mixture (40μM OA and 40μM LA). This treatment has been used previously by our lab in MDA-MB-231 breast cancer cells to optimize growth [27, 40]. This control treatment did not exert cytotoxic effects in these studies or the present research.

Appendix 3: Flow cytometry median fluorescence intensity analysis

MDA-MB-231 breast cancer cells have high basal levels of EGFR [181-184] and SK-BR-3 breast cancer cells highly express Her2 [2, 183]. Due to these high basal levels of these membrane-associated proteins, median fluorescence intensity was used to determine the effects of EPA and/or DHA on the intensity of staining among positive cells. Median fluorescence intensity is able to detect subtle differences in fluorescence intensity that are missed when using other analyses such as the percentage of positively expressing cells [185]. There was no significant difference in either cell line between treatments on the percentage of positive and negative cells with staining (Tables 1 and 2).

Table 1: Percentage of positive and negative MDA-MB-231 breast cancer cells with staining for CD95 (APC) or EGFR (FITC) with 100 μM n-3 LCPUFA treatments

Treatment	**Protein of interest (fluorophore for staining)**			
	CD95 (APC)		**EGFR (FITC)**	
	% APC+ cells	**% APC- cells**	**% FITC+ cells**	**% FITC- cells**
180 μM OA/LA	99.0±0.75[a]	0.96±0.76[a]	99.6±0.18[a]	0.44±0.17[a]
DHA	99.9±0.04[a]	0.07±0.03[a]	99.4±0.25[a]	0.59±0.25[a]
EPA	99.8±0.08[a]	0.18±0.08[a]	99.8±0.10[a]	0.20±0.11[a]
1:1 DHA:EPA	99.9±0.05[a]	0.12±0.05[a]	99.3±0.25[a]	0.66±0.25[a]
2:1 DHA:EPA	99.9±0.04[a]	0.10±0.04[a]	99.5±0.26[a]	0.51±0.26[a]

Data presented as mean±SEM. Within a column, treatments that share the same letter are not significantly different from one another ($p>0.05$). "APC"= Allophycocyanin; "FITC"= Fluorescein."CD95"= cluster of differentiation and "EGFR"=epidermal growth factor.

Table 2: Percentage of positive and negative SK-BR-3 breast cancer cells with staining for CD95 (APC) or EGFR (FITC) with 100 µM n-3 LCPUFA treatments

Treatment	Protein of interest (fluorophore for staining)			
	CD95 (APC)		EGFR (FITC)	
	% APC+ cells	% APC+ cells	% FITC+ cells	% FITC- cells
180 µM OA/LA	86.9±1.45 [a]	13.1±1.45 [a]	99.9±0.03 [a]	0.03±0.03 [a]
DHA	90.8±0.82 [a]	9.2±0.82 [a]	99.8±0.03 [a]	0.17±0.03 [a]
EPA	89.8±0.69 [a]	10.2±0.70 [a]	99.9±0.03 [a]	0.13±0.03 [a]
1:1 DHA:EPA	90.2±1.05 [a]	9.8±0.60 [a]	99.9±0.03 [a]	0.13±0.03 [a]
2:1 DHA:EPA	90.3±0.41 [a]	9.7±0.39 [a]	99.9±0.00 [a]	0.10±0.00 [a]

Data presented as mean±SEM. Within a column, treatments that share the same letter are not significantly different from one another ($p>0.05$). "APC"= Allophycocyanin; "FITC"= Fluorescein. "CD95"= cluster of differentiation and "EGFR"=epidermal growth factor.

The median fluorescence intensity (relative to 180 µM OA/LA), was used to determine the effect of n-3 LCPUFA treatments on EGFR or Her2 staining intensity. Three different types of gates were used to ensure that all positive cells were captured in data analysis (Figure 1). Gate 3 (positive) encompassed all positive cells (excluded debris in bottom left corner), gate 2 (blue) was more stringent than gate 3, and gate 1 (green) was the most stringent looking at the positive cells that stained with the most intensity. Gate 1 data was presented in Chapter 5 to represent the positive cells that stained with the most intensity for EGFR(FITC) and/or CD95 (APC).

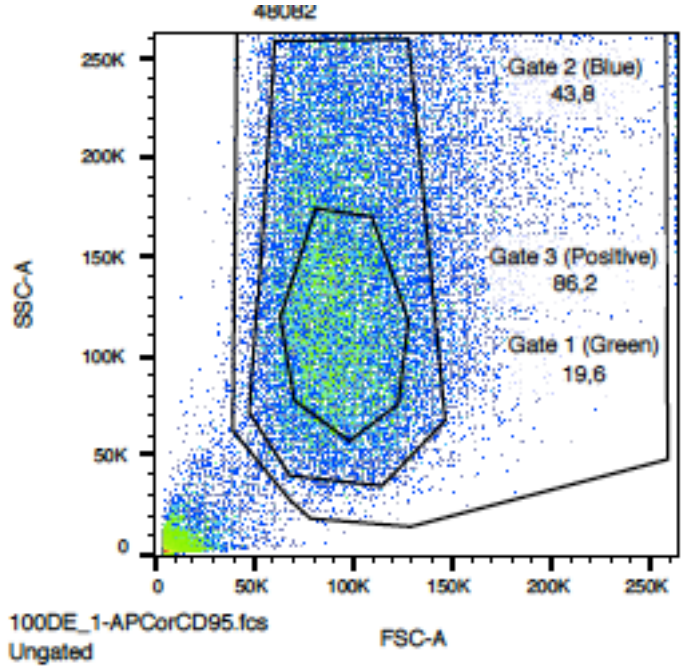

Figure 1: Gates used on a forward (FSC-A) versus side scatter area (SSC-A) density plot to capture positive cells stained for CD95 in MDA-MB-231 breast cancer cells treated with 100 µM of a 1:1 DHA:EPA treatment +80 µM OA/LA. Subsequent analyses were done to compare the median fluorescence intensity of positive cells relative to control.

An isotype control (BD Pharmigen, FITC Mouse IgG2a, K isotype control) was used as a negative control in both cell lines in order to distinguish between non-specific background signal and specific antibody signal [186, 187]. In both cell lines, cells stained with isotype control had a significantly lower median fluorescence intensity of positive cells than cells stained for EGFR or CD95. The median fluorescence intensity of the isotype control was not different from unstained cells (see scatter area density plot for SK-BR-3 breast cancer cells in Figure 2). Collectively, this indicates that there was minimal non-specific binding.

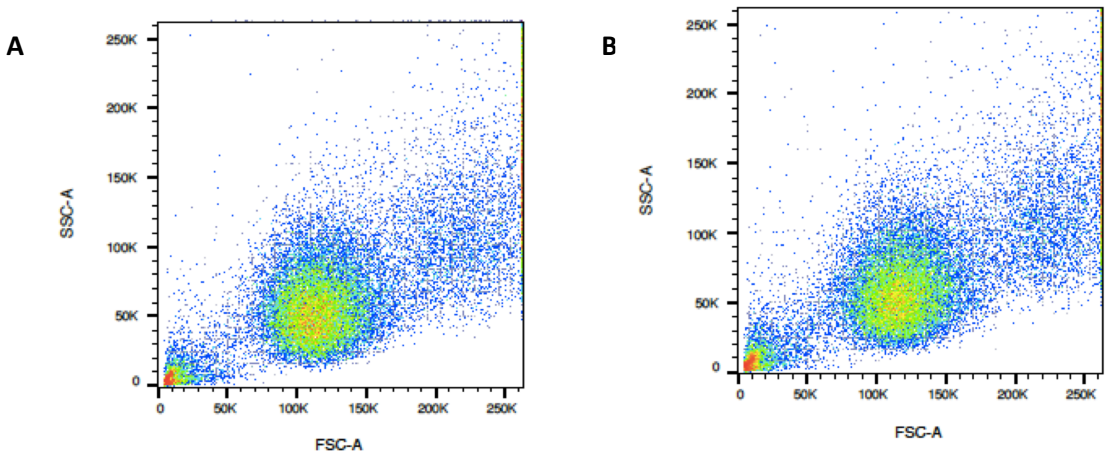

Figure 2: FSC-A vs. SSC-A density plot comparing median fluorescence intensity for (A) SK-BR-3 breast cancer cells treated with 180 μM OA/LA and stained with isotype control and (B) SK-BR-3 breast cancer cells treated with 180 μM OA/LA without staining.

Appendix 4: Changes in complete fatty profiles in whole cell fatty acids, total PL, and PL classes of MDA-MB-231 and SK-BR-3 breast cancer cells incubated with n-3 LCPUFA treatments

List of Tables for Complete Fatty Acid Profiles

Table 1: Change in whole cell fatty acids of MDA-MB-231 cells incubated with control or 100 μM n-3 LCPUFA treatments

Fatty Acid	80 μM OA/LA	DHA	EPA	1:1 DHA:EPA	2:1 DHA:EPA	180 μM OA/LA
14:0	1.24±0.20^{a}	1.02±0.06^{a}	0.98±0.15^{a}	1.01±0.02^{a}	0.76±0.06^{ab}	0.40±0.01^{b}
16:0	14.09±2.12^{a}	10.69±0.54^{ab}	11.23±3.61^{ab}	9.97±0.69^{ab}	6.71±0.12^{ab}	4.13±0.03^{b}
16:1n-9	0.80±0.04^{a}	0.84±0.06^{a}	0.74±0.09^{a}	0.89±0.08^{a}	0.65±0.04^{ab}	0.40±0.01^{b}
17:0	0.30±0.02^{a}	0.25±0.02^{a}	0.30±0.10^{a}	0.24±0.01^{a}	0.18±0.02^{a}	0.13±0.00^{a}
18:0	11.68±1.19^{a}	7.22±0.40^{a}	9.36±4.11^{a}	6.35±0.45^{a}	4.52±0.16^{a}	3.29±0.02^{a}
18:1n-9	10.36±0.83^{c}	8.86±0.13^{c}	7.43±0.05^{c}	8.86±0.46^{c}	28.96±0.23^{b}	37.54±0.22^{a}
18:2n-6	44.45±2.37^{a}	39.72±0.58^{a}	31.37±2.59^{b}	28.99±0.71^{b}	20.82±0.17^{c}	45.54±0.27^{a}
18:3n-6	0.30±0.02^{a}	0.26±0.03^{ab}	0.16±0.02^{bc}	0.14±0.01^{bc}	0.11±0.00^{c}	0.20±0.00^{b}
18:3n-3	1.12±0.36^{a}	0.29±0.03^{b}	0.19±0.02^{b}	0.24±0.03^{b}	0.54±0.01^{a}	1.26±0.01^{a}
20:2n-6	5.94±0.27^{a}	2.64±0.04^{c}	1.37±0.02^{d}	1.22±0.04^{d}	0.95±0.01^{d}	3.78±0.04^{b}
20:3n-6	3.54±0.45^{a}	1.93±0.04^{b}	0.74±0.09^{c}	0.89±0.01^{c}	0.81±0.01^{c}	1.41±0.04^{bc}
20:4n-6	2.29±0.11^{a}	1.17±0.06^{b}	0.69±0.00^{c}	0.84±0.07^{c}	0.80±0.02^{c}	0.78±0.04^{c}
20:5n-3	0.50±0.09^{c}	0.41±0.01^{c}	17.27±2.55^{a}	11.00±0.53^{b}	6.81±0.04^{b}	0.05±0.00^{c}
24:0	0.39±0.10^{bc}	0.06±0.02^{c}	1.10±0.13^{a}	0.70±0.15^{ab}	0.41±0.04^{bc}	0.02±0.00^{c}
24:1n-9	0.41±0.04^{a}	0.27±0.02^{ab}	0.24±0.08^{ab}	0.20±0.03^{b}	0.34±0.02^{ab}	0.36±0.01^{ab}
22:4n-6	1.70±0.04^{a}	0.59±0.04^{b}	0.35±0.03^{c}	0.38±0.01^{bc}	0.36±0.02^{c}	0.44±0.02^{bc}
22:5n-6	0.53±0.18^{a}	0.42±0.04^{a}	0.61±0.15^{a}	0.27±0.03^{a}	0.20±0.04^{a}	0.13±0.04^{a}
22:5n-3	0.23±0.01^{b}	0.81±0.05^{b}	15.71±2.89^{a}	11.66±0.54^{ab}	6.79±0.01^{b}	0.10±0.00^{b}
22:6n-3	0.12±0.02^{c}	22.56±1.03^{a}	0.17±0.02^{c}	16.15±1.17^{b}	19.28±0.17^{ab}	0.05±0.00^{c}
ΣEPA+DPA	0.73±0.08^{c}	1.22±0.04^{c}	32.97±5.44^{a}	22.66±1.06^{ab}	13.60±0.05^{b}	0.14±0.00^{c}
ΣSFA	27.71±3.11^{a}	19.24±1.00^{a}	22.97±7.85^{a}	18.28±1.28^{a}	12.59±0.32^{a}	7.96±0.23^{b}
ΣMUFA	11.57±0.81^{c}	9.96±0.16^{c}	8.42±0.15^{a}	9.94±0.53^{c}	29.95±0.21^{a}	38.30±0.04^{b}
ΣPUFA	60.73±2.30^{a}	70.80±1.11^{a}	68.62±7.99^{a}	71.78±1.74^{a}	57.46±0.31^{a}	53.74±0.25^{a}
Σn-3	1.98±0.33^{c}	24.07±1.02^{b}	33.33±5.44^{ab}	39.05±2.24^{a}	33.42±0.16^{ab}	1.45±0.01^{c}
Σn-6	58.75±2.38^{a}	46.73±0.49^{b}	35.29±2.55^{c}	32.73±0.70^{c}	24.04±0.15^{d}	52.29±0.25^{ab}

Values are mean percent composition ± SEM (n=3). "OA/LA"=oleic acid (18:1n-9)/linoleic acid (18:2n-6);"EPA=eicosapentaenoic acid (20:5n-3); "DHA"=docosahexaenoic acid (22:6n-3); "Σ EPA+DPA"=summation of EPA and docosapentaenoic acid (22:5n-3); "SFA"=saturated fatty acids; "MUFA"=monounsaturated fatty acids; "PUFA"=polyunsaturated fatty acids. For each fatty acid identified, values that do not share a common letter are significantly different ($p<0.05$).

Table 2: Change in whole cell fatty acids of MDA-MB-231 cells incubated with control or 150 μM n-3 LCPUFA treatments

Fatty Acid	80 μM OA/LA	DHA	EPA	1:1 DHA:EPA	2:1 DHA:EPA	230 μM OA/LA
14:0	0.58±0.09^{a}	0.55±0.06^{a}	0.48±0.01^{a}	0.56±0.01^{a}	0.54±0.04^{a}	0.47±0.06^{a}
16:0	6.42±0.95^{a}	5.63±0.37^{a}	5.05±0.23^{a}	5.46±0.10^{a}	5.33±0.33^{a}	5.23±1.27^{a}
16:1n-9	0.55±0.10^{a}	0.52±0.05^{a}	0.50±0.03^{a}	0.53±0.02^{a}	0.51±0.04^{a}	0.47±0.05^{a}
17:0	0.17±0.02^{a}	0.14±0.01^{a}	0.13±0.01^{a}	0.14±0.00^{a}	0.13±0.01^{a}	0.14±0.02^{a}
18:0	4.85±0.72^{a}	3.74±0.38^{a}	3.65±0.31^{a}	3.65±0.07^{a}	3.42±0.20^{a}	4.60±1.60^{a}
18:1n-9	35.23±2.85^{a}	25.18±0.94^{b}	23.88±0.66^{b}	24.73±0.31^{b}	24.32±0.98^{a}	32.31±2.83^{b}
18:2n-6	40.17±6.02^{a}	22.32±0.49^{b}	22.43±0.09^{b}	24.04±0.32^{b}	22.86±0.40^{b}	46.32±6.67^{a}
18:3n-6	0.25±0.00^{a}	0.13±0.01^{b}	0.10±0.00^{b}	0.10±0.00^{b}	0.13±0.02^{b}	0.23±0.03^{a}
18:3n-3	1.54±0.23^{a}	0.44±0.02^{b}	0.33±0.01^{b}	0.40±0.00^{b}	0.44±0.05^{b}	1.31±0.23^{a}
20:2n-6	4.11±0.04^{a}	0.89±0.05^{b}	0.64±0.02^{b}	0.87±0.01^{b}	0.87±0.01^{b}	4.22±0.12^{a}
20:3n-6	2.07±0.29^{a}	0.79±0.03^{b}	0.47±0.00^{b}	0.66±0.01^{b}	0.76±0.01^{b}	1.76±0.23^{a}
20:4n-6	1.85±0.66^{a}	0.74±0.18^{a}	0.52±0.01^{a}	0.56±0.01^{a}	0.66±0.01^{a}	1.02±0.17^{a}
20:5n-3	0.21±0.02^{d}	0.50±0.02^{d}	24.75±0.93^{a}	11.37±0.01^{b}	8.79±0.42^{c}	0.30±0.06^{d}
24:0	0.14±0.03^{a}	0.04±0.01^{a}	0.71±0.45^{a}	0.67±0.11^{a}	0.31±0.06^{a}	0.15±0.04^{a}
24:1n-9	0.53±0.10^{a}	0.24±0.02^{ab}	0.15±0.01^{b}	0.19±0.00^{b}	0.25±0.03^{ab}	0.40±0.11^{ab}
22:4n-6	0.84±0.20^{a}	0.28±0.01^{ab}	0.23±0.00^{b}	0.27±0.00^{b}	0.32±0.02^{ab}	0.64±0.18^{ab}
22:5n-6	0.24±0.01^{b}	0.09±0.02^{c}	0.43±0.04^{a}	0.26±0.05^{b}	0.15±0.02^{bc}	0.24±0.01^{bc}
22:5n-3	0.15±0.03^{d}	0.63±0.01^{d}	15.49±0.48^{a}	9.51±0.15^{b}	7.82±0.32^{c}	0.12±0.02^{d}
22:6n-3	0.08±0.01^{d}	37.15±2.10^{a}	0.07±0.01^{d}	16.02±0.34^{c}	22.39±1.27^{b}	0.08±0.01^{d}
ΣEPA+DPA	0.36±0.05^{d}	1.14±0.01^{d}	40.24±1.33^{a}	20.88±0.16^{b}	16.61±0.74^{c}	0.42±0.08^{d}
ΣSFA	12.16±1.80^{a}	10.10±0.81^{a}	10.01±1.00^{a}	10.48±0.28^{a}	9.74±0.63^{a}	10.59±2.99^{a}
ΣMUFA	36.31±3.04^{a}	25.93±0.99^{b}	24.53±0.64^{b}	25.46±0.31^{b}	25.09±1.00^{b}	33.18±2.97^{ab}
ΣPUFA	51.53±4.74^{a}	63.97±1.74^{a}	65.46±1.39^{a}	64.06±0.58^{a}	65.18±1.55^{a}	56.23±5.95^{a}
Σn-3	1.98±0.29^{b}	38.73±2.09^{a}	40.64±1.32^{a}	37.30±0.48^{a}	39.44±1.96^{a}	1.81±0.30^{b}
Σn-6	49.54±5.01^{a}	25.24±0.41^{b}	24.81±0.08^{b}	26.76±0.28^{b}	25.73±0.41^{b}	54.42±6.25^{a}

Values are mean percent composition ± SEM (n=3). "OA/LA"=oleic acid (18:1n-9)/linoleic acid (18:2n-6);"EPA=eicosapentaenoic acid (20:5n-3); "DHA"=docosahexaenoic acid (22:6n-3); "Σ EPA+DPA"=summation of EPA and docosapentaenoic acid (22:5n-3); "SFA"=saturated fatty acids; "MUFA"=monounsaturated fatty acids; "PUFA"=polyunsaturated fatty acids. For each fatty acid identified, values that do not share a common letter are significantly different ($p<0.05$).

Table 3: Change in whole cell fatty acids of SK-BR-3 cells incubated with control or 100 μM n-3 LCPUFA treatments

Fatty Acid	80 μM OA/LA	DHA	EPA	1:1 DHA:EPA	2:1 DHA:EPA	180 μM OA/LA
14:0	1.37±0.04^{ab}	1.46±0.17^{a}	1.15±0.30^{ab}	1.18±0.22^{ab}	1.15±0.19^{ab}	0.45±0.09^{b}
16:0	17.62±1.20^{a}	21.21±0.33^{a}	17.50±0.49^{a}	19.40±0.43^{a}	18.11±0.96^{a}	9.53±0.34^{b}
16:1n-9	2.46±0.25^{a}	1.94±0.04^{a}	2.45±0.04^{a}	2.26±-0.12^{a}	2.15±0.09^{a}	1.19±0.04^{b}
17:0	0.31±0.01^{b}	0.42±0.02^{a}	0.37±0.02^{ab}	0.37±0.01^{ab}	0.35±0.01^{ab}	0.21±0.02^{c}
18:0	15.18±2.39^{a}	12.38±0.20^{a}	10.91±0.19^{ab}	11.67±0.09^{a}	10.54±0.32^{ab}	6.23±0.47^{b}
18:1n-9	37.48±3.43^{a}	36.54±1.40^{a}	40.08±1.02^{a}	38.56±1.13^{a}	34.65±0.30^{a}	41.34±0.72^{b}
18:2n-6	13.04±3.45^{b}	11.78±0.09^{b}	13.69±0.04^{b}	12.30±0.17^{b}	18.50±0.21^{b}	31.04±0.68^{a}
18:3n-6	0.54±0.00^{a}	0.36±0.01^{a}	0.41±0.04^{c}	0.42±0.02^{bc}	0.69±0.05^{ab}	1.68±0.05^{a}
18:3n-3	1.82±0.38^{a}	0.94±0.06^{b}	0.71±0.01^{b}	0.76±0.06^{b}	0.71±0.00^{b}	0.55±0.01^{b}
20:2n-6	1.28±0.46^{a}	1.10±0.03^{a}	0.57±0.02^{a}	0.70±0.03^{a}	1.00±0.04^{a}	1.06±0.05^{a}
20:3n-6	2.68±0.33^{a}	2.79±0.04^{a}	1.02±0.03^{c}	1.54±0.07^{bc}	2.16±0.17^{ab}	2.64±0.15^{a}
20:4n-6	3.39±1.10^{a}	1.48±0.09^{ab}	0.83±0.11^{b}	0.93±0.04^{ab}	1.48±0.18^{ab}	2.42±0.25^{ab}
20:5n-3	0.42±0.13^{c}	0.24±0.05^{c}	5.62±0.40^{a}	3.28±0.18^{b}	2.70±0.27^{b}	0.17±0.04^{c}
24:0	0.22±0.09^{a}	0.14±0.03^{a}	0.35±0.04^{a}	0.24±0.05^{a}	0.28±0.06^{a}	0.13±0.03^{a}
24:1n-9	0.54±0.09^{ab}	0.76±0.05^{a}	0.44±0.02^{b}	0.46±0.03^{b}	0.46±0.01^{b}	0.16±0.01^{c}
22:4n-6	1.27±0.04^{a}	0.76±0.03^{b}	0.30±0.02^{e}	0.37±0.02^{de}	0.54±0.04^{cd}	0.67±0.06^{bc}
22:5n-6	0.13±0.01^{a}	0.23±0.03^{a}	0.22±0.02^{a}	0.27±0.02^{a}	0.26±0.04^{a}	0.27±0.08^{a}
22:5n-3	0.20±0.09^{c}	0.26±0.06^{c}	3.25±0.31^{a}	2.66±0.21^{ab}	2.20±0.21^{b}	0.14±0.03^{c}
22:6n-3	0.07±0.01^{c}	5.23±0.72^{a}	0.13±0.01^{c}	2.63±0.23^{b}	2.05±0.16^{b}	0.12±0.04^{c}
ΣEPA+DPA	0.62±0.21^{c}	0.50±0.10^{c}	8.87±0.66^{a}	5.94±0.38^{b}	4.91±0.46^{b}	0.31±0.05^{c}
ΣSFA	34.70±2.96^{a}	35.60±0.64^{a}	30.28±0.77^{a}	32.86±0.56^{a}	30.43±1.39^{a}	16.54±0.80^{b}
ΣMUFA	40.47±3.75^{a}	39.23±1.44^{a}	42.98±1.00^{a}	41.29±1.03^{a}	37.27±0.29^{a}	42.69±0.76^{a}
ΣPUFA	24.83±3.93^{b}	25.17±0.87^{b}	26.74±0.81^{b}	25.85±0.66^{b}	32.30±1.23^{a}	40.77±0.87^{a}
Σn-3	2.51±0.28^{c}	6.67±0.64^{b}	9.71±0.66^{a}	9.33±0.56^{ab}	7.67±0.62^{ab}	0.99±0.06^{c}
Σn-6	22.32±3.88^{b}	18.49±0.23^{b}	17.03±0.16^{b}	16.52±0.10^{b}	24.63±0.61^{b}	39.78±0.89^{a}

Values are mean percent composition ± SEM (n=3). "OA/LA"=oleic acid (18:1n-9)/linoleic acid (18:2n-6);"EPA=eicosapentaenoic acid (20:5n-3); "DHA"=docosahexaenoic acid (22:6n-3); "Σ EPA+DPA"=summation of EPA and docosapentaenoic acid (22:5n-3); "SFA"=saturated fatty acids; "MUFA"=monounsaturated fatty acids; "PUFA"=polyunsaturated fatty acids. For each fatty acid identified, values that do not share a common letter are significantly different ($p<0.05$).

Table 4: Change in whole cell fatty acids of SK-BR-3 cells incubated with control or 150 μM n-3 LCPUFA treatments

Fatty Acid	80 μM OA/LA	DHA	EPA	1:1 DHA:EPA	2:1 DHA:EPA	280 μM OA/LA
14:0	1.54±0.22^{a}	1.20±0.14^{ab}	1.12±0.15^{ab}	1.00±0.06^{ab}	1.23±0.23^{ab}	0.45±0.10^{b}
16:0	18.60±0.50^{a}	19.14±2.27^{a}	16.84±1.86^{a}	17.09±2.22^{a}	17.98±3.62^{a}	10.37±1.37^{a}
16:1n-9	2.32±0.09^{a}	1.29±0.19^{a}	2.30±0.25^{a}	2.05±0.25^{a}	1.97±0.41^{a}	1.01±0.20^{a}
17:0	0.37±0.01^{a}	0.40±0.06^{a}	0.38±0.04^{a}	0.40±0.07^{a}	0.37±0.08^{a}	0.23±0.08^{a}
18:0	11.46±0.67^{a}	11.16±1.49^{a}	10.83±1.38^{a}	10.47±1.54^{a}	10.24±2.19^{a}	10.05±3.76^{a}
18:1n-9	32.60±1.35^{a}	26.58±2.74^{a}	34.72±3.75^{a}	32.17±4.60^{a}	30.85±6.47^{a}	31.73±5.55^{a}
18:2n-6	19.67±0.26^{ab}	17.13±1.47^{b}	10.00±1.00^{b}	11.34±1.21^{b}	9.19±1.77^{b}	28.05±4.14^{a}
18:3n-6	0.86±0.07^{ab}	0.48±0.07^{ab}	0.39±0.09^{ab}	0.40±0.07^{ab}	0.38±0.09^{b}	0.90±0.17^{a}
18:3n-3	0.86±0.06^{a}	0.57±0.06^{a}	0.55±0.10^{a}	0.57±0.09^{a}	0.59±0.14^{a}	0.72±0.35^{a}
20:2n-6	1.86±0.10^{a}	1.20±0.02^{b}	0.41±0.05^{c}	0.72±0.11^{c}	0.49±0.09^{c}	0.58±0.09^{c}
20:3n-6	3.90±0.08^{a}	2.51±0.25^{b}	0.73±0.08^{c}	1.19±0.07^{c}	1.23±0.26^{c}	1.51±0.32^{bc}
20:4n-6	2.73±0.10^{a}	0.94±0.16^{bc}	0.59±0.11^{c}	0.93±0.07^{bc}	0.81±0.19^{c}	1.75±0.25^{b}
20:5n-3	0.42±0.09^{b}	0.21±0.05^{b}	6.39±0.81^{a}	4.56±0.43^{a}	2.35±0.48^{b}	0.10±0.02^{b}
24:0	0.34±0.03^{ab}	0.11±0.03^{b}	0.62±0.14^{a}	0.38±0.10^{ab}	0.24±0.06^{ab}	0.08±0.02^{b}
24:1n-9	0.46±0.04^{a}	0.47±0.02^{a}	0.35±0.03^{ab}	0.38±0.03^{a}	0.36±0.05^{ab}	0.16±0.05^{b}
22:4n-6	1.56±0.11^{a}	0.40±0.03^{b}	0.21±0.03^{b}	0.32±0.02^{b}	0.31±0.05^{b}	0.39±0.07^{b}
22:5n-6	0.21±0.01^{a}	0.32±0.04^{a}	0.42±0.11^{a}	0.35±0.06^{a}	0.31±0.06^{a}	0.28±0.08^{a}
22:5n-3	0.18±0.02^{b}	0.25±0.04^{b}	3.00±0.35^{a}	2.97±0.30^{a}	1.87±0.33^{a}	0.27±0.13^{b}
22:6n-3	0.06±0.00^{c}	6.85±0.47^{a}	0.17±0.02^{c}	2.02±0.27^{b}	2.61±0.45^{b}	0.10±0.02^{c}
ΣEPA+DPA	0.60±0.06^{c}	0.47±0.09^{c}	9.39±1.14^{a}	7.53±0.71^{ab}	4.22±0.81^{b}	0.37±0.12^{c}
ΣSFA	32.31±1.25^{a}	32.02±3.96^{a}	29.80±3.44^{a}	29.34±3.87^{a}	30.06±6.17^{a}	21.20±5.28^{a}
ΣMUFA	35.38±1.38^{a}	28.34±2.95^{a}	37.37±4.00^{a}	34.60±4.87^{a}	33.18±6.92^{a}	32.90±5.52^{a}
ΣPUFA	32.31±0.39^{a}	30.87±2.28^{a}	22.87±2.66^{a}	25.36±2.44^{a}	20.15±3.80^{a}	34.66±4.49^{a}
Σn-3	1.52±0.13^{b}	7.89±0.47^{a}	10.11±1.26^{a}	10.12±0.97^{a}	7.42±1.38^{a}	1.19±0.45^{b}
Σn-6	30.80±0.40^{a}	22.98±1.90^{ab}	12.76±1.41^{b}	15.25±1.57^{b}	12.72±2.47^{b}	33.47±4.21^{a}

Values are mean percent composition ± SEM (n=3). "OA/LA"=oleic acid (18:1n-9)/linoleic acid (18:2n-6);"EPA=eicosapentaenoic acid (20:5n-3); "DHA"=docosahexaenoic acid (22:6n-3); "Σ EPA+DPA"=summation of EPA and docosapentaenoic acid (22:5n-3); "SFA"=saturated fatty acids; "MUFA"=monounsaturated fatty acids; "PUFA"=polyunsaturated fatty acids. For each fatty acid identified, values that do not share a common letter are significantly different ($p<0.05$).

Table 5: Change in fatty acids in total PL of MDA-MB-231 cells incubated with control or 100 μM n-3 LCPUFA treatments

Fatty Acid	80 μM OA/LA	DHA	EPA	1:1 DHA:EPA	2:1 DHA:EPA	180 μM OA/LA
14:0	0.99±0.08^{ab}	1.36±0.12^{a}	1.02±0.06^{ab}	1.17±0.07^{ab}	1.08±0.08^{ab}	0.90±0.05^{b}
16:0	15.88±0.54^{bc}	18.49±0.14^{a}	15.44±0.41^{bc}	17.57±0.49^{ab}	16.86±0.35^{abc}	14.84±0.58^{c}
16:1n-9	0.39±0.00^{d}	0.70±0.01^{a}	0.53±0.05^{c}	0.69±0.01^{ab}	0.58±0.01^{bc}	0.38±0.01^{d}
17:0	0.46±0.02^{a}	0.51±0.02^{a}	0.54±0.03^{a}	0.56±0.04^{a}	0.56±0.01^{a}	0.45±0.02^{a}
18:0	19.66±0.14^{bc}	21.58±0.03^{a}	19.29±0.12^{c}	20.14±0.41^{abc}	21.20±0.61^{ab}	19.50±0.34^{bc}
18:1n-9	8.64±0.26^{bc}	10.34±0.24^{a}	7.96±0.05^{c}	9.62±0.15^{ab}	9.04±0.12^{abc}	8.17±0.55^{bc}
18:2n-6	35.36±2.13^{ab}	23.78±0.27^{c}	34.97±0.33^{ab}	25.99±0.24^{c}	30.71±0.47^{b}	37.65±2.12^{a}
18:3n-6	0.14±0.01^{a}	0.17±0.01^{a}	0.13±0.01^{a}	0.14±0.01^{a}	0.14±0.02^{a}	0.14±0.01^{a}
18:3n-3	0.31±0.04^{a}	0.22±0.04^{a}	0.10±0.00^{a}	0.15±0.02^{a}	0.20±0.05^{a}	0.24±0.02^{a}
20:2n-6	6.24±0.15^{a}	2.06±0.05^{c}	2.63±0.01^{b}	2.17±0.02^{c}	3.01±0.05^{bc}	6.49±0.09^{a}
20:3n-6	1.87±0.17^{a}	1.48±0.01^{abc}	0.74±0.01^{c}	0.96±0.03^{c}	1.19±0.08^{bc}	1.61±0.20^{ab}
20:4n-6	2.85±0.31^{a}	2.04±0.07^{abc}	1.07±0.02^{c}	1.42±0.03^{c}	1.77±0.03^{ab}	2.46±0.33^{ab}
20:5n-3	0.08±0.03^{c}	0.48±0.02^{c}	5.77±0.23^{a}	5.03±0.22^{a}	2.40±0.14^{b}	0.05±0.02^{c}
24:0	1.01±0.14^{a}	0.92±0.12^{a}	0.63±0.05^{a}	0.81±0.07^{a}	0.77±0.04^{a}	0.95±0.07^{a}
24:1n-9	2.32±0.17^{a}	0.65±0.02^{b}	0.57±0.01^{b}	0.56±0.06^{b}	0.65±0.02^{b}	2.07±0.17^{a}
22:4n-6	1.11±0.16^{a}	0.90±0.12^{a}	0.78±0.05^{a}	0.95±0.08^{a}	0.83±0.05^{a}	1.03±0.08^{a}
22:5n-6	2.17±0.23^{a}	0.87±0.03^{b}	1.07±0.06^{b}	1.12±0.11^{b}	1.26±0.04^{b}	2.10±0.24^{a}
22:5n-3	0.29±0.06^{d}	0.42±0.55^{d}	6.56±0.26^{a}	5.03±0.23^{b}	2.62±0.17^{c}	0.26±0.07^{d}
22:6n-3	0.23±0.07^{c}	13.04±0.05^{a}	0.19±0.06^{c}	5.92±0.36^{b}	5.13±0.44^{b}	0.19±0.05^{c}
ΣEPA+DPA	0.37±0.08^{d}	0.90±0.05^{d}	12.33±0.50^{a}	10.06±0.45^{b}	5.02±0.31^{c}	0.31±0.09^{d}
ΣSFA	38.00±0.77^{b}	42.86±0.31^{a}	36.92±0.56^{b}	40.24±0.97^{ab}	40.46±1.04^{ab}	36.65±0.83^{b}
ΣMUFA	11.35±0.42^{a}	11.69±0.27^{a}	9.06±0.10^{b}	10.87±0.23^{ab}	10.27±0.14^{ab}	10.62±0.73^{ab}
ΣPUFA	50.64±1.19^{ab}	45.45±0.45^{b}	54.02±0.68^{a}	48.88±0.95^{ab}	49.26±1.13^{ab}	52.22±2.07^{a}
Σn-3	0.90±0.19^{c}	14.15±0.57^{a}	12.62±0.45^{b}	16.13±0.80^{a}	10.36±0.71^{b}	0.74±0.15^{c}
Σn-6	49.74±1.38^{a}	31.30±0.12^{c}	41.40±0.23^{b}	32.76±0.18^{c}	38.91±0.43^{b}	51.48±2.00^{a}

Values are mean percent composition ± SEM (n=3). "OA/LA"=oleic acid (18:1n-9)/linoleic acid (18:2n-6);"EPA=eicosapentaenoic acid (20:5n-3); "DHA"=docosahexaenoic acid (22:6n-3); "Σ EPA+DPA"=summation of EPA and docosapentaenoic acid (22:5n-3); "SFA"=saturated fatty acids; "MUFA"=monounsaturated fatty acids; "PUFA"=polyunsaturated fatty acids. For each fatty acid identified, values that do not share a common letter are significantly different ($p<0.05$).

Table 6: Change in fatty acids in total PL of MDA-MB-231 cells incubated with control or 150 μM n-3 LCPUFA treatments

Fatty Acid	80 μM OA/LA	DHA	EPA	1:1 DHA:EPA	2:1 DHA:EPA	230 μM OA/LA
14:0	1.21±0.09^{a}	1.52±0.31^{a}	1.48±0.21^{a}	1.45±0.15^{a}	1.74±0.09^{a}	1.10±0.18^{a}
16:0	17.03±0.29^{bc}	20.29±0.81^{a}	18.46±0.56^{abc}	19.23±0.64^{ab}	20.25±0.07^{a}	16.45±0.69^{c}
16:1n-9	0.47±0.01^{b}	0.81±0.03^{a}	0.78±0.02^{a}	0.77±0.03^{a}	0.80±0.02^{a}	0.47±0.01^{b}
17:0	0.46±0.03^{b}	0.53±0.01^{ab}	0.58±0.01^{a}	0.59±0.03^{a}	0.54±0.01^{ab}	0.46±0.02^{b}
18:0	18.90±0.21^{b}	20.27±0.17^{a}	19.24±0.09^{b}	19.60±0.19^{ab}	19.51±0.03^{ab}	18.94±0.18^{b}
18:1n-9	11.79±0.10^{a}	10.53±0.10^{b}	10.16±0.09^{bc}	9.50±0.18^{c}	9.55±0.04^{c}	11.70±0.14^{a}
18:2n-6	30.83±1.11^{a}	19.50±0.12^{c}	23.78±0.21^{b}	22.56±0.56^{bc}	21.27±0.27^{bc}	32.59±1.28^{a}
18:3n-6	0.14±0.01^{a}	0.17±0.02^{a}	0.14±0.00^{a}	0.15±0.00^{a}	0.14±0.01^{a}	0.13±0.01^{a}
18:3n-3	0.45±0.07^{b}	0.17±0.02^{a}	0.13±0.01^{b}	0.14±0.00^{ab}	0.13±0.00^{ab}	0.38±0.03^{b}
20:2n-6	5.35±0.05^{a}	1.41±0.04^{d}	1.92±0.02^{b}	1.91±0.02^{b}	1.62±0.05^{c}	5.46±0.03^{a}
20:3n-6	2.34±0.22^{a}	1.06±0.01^{b}	0.68±0.01^{b}	0.78±0.02^{b}	0.82±0.01^{b}	2.07±0.18^{a}
20:4n-6	3.80±0.45^{a}	1.49±0.01^{b}	1.07±0.01^{ab}	1.13±0.01^{b}	1.20±0.00^{b}	3.30±0.38^{a}
20:5n-3	0.06±0.01^{c}	0.57±0.05^{d}	8.73±0.14^{a}	5.87±0.03^{b}	4.15±0.11^{c}	0.03±0.01^{c}
24:0	1.10±0.07^{a}	0.77±0.07^{ab}	0.67±0.09^{b}	0.71±0.04^{ab}	0.76±0.11^{ab}	1.08±0.07^{ab}
24:1n-9	2.69±0.20^{a}	0.63±0.01^{b}	0.63±0.02^{b}	0.58±0.03^{b}	0.58±0.02^{b}	2.43±0.22^{a}
22:4n-6	1.29±0.07^{a}	0.91±0.09^{a}	0.91±0.14^{a}	0.95±0.06^{a}	0.97±0.13^{a}	1.27±0.13^{a}
22:5n-6	1.53±0.07^{a}	0.54±0.04^{b}	0.85±0.11^{b}	0.94±0.05^{b}	0.76±0.08^{b}	1.51±0.14^{b}
22:5n-3	0.32±0.02^{d}	0.54±0.07^{d}	9.34±0.12^{a}	5.42±0.05^{b}	3.65±0.11^{c}	0.29±0.01^{d}
22:6n-3	0.23±0.02^{d}	18.29±0.67^{a}	0.46±0.09^{d}	7.73±0.28^{b}	11.55±0.17^{c}	0.34±0.07^{d}
ΣEPA+DPA	0.38±0.02^{d}	1.11±0.12^{d}	18.07±0.26^{a}	11.29±0.07^{a}	7.80±0.23^{c}	0.33±0.01^{d}
ΣSFA	38.69±0.19^{b}	43.37±0.92^{a}	40.43±0.62^{ab}	41.58±0.78^{ab}	42.81±0.12^{a}	38.03±0.97^{b}
ΣMUFA	14.96±0.30^{a}	11.96±0.08^{b}	11.56±0.10^{bc}	10.85±0.22^{c}	10.93±0.06^{c}	14.60±0.16^{a}
ΣPUFA	46.35±0.44^{a}	44.67±0.86^{a}	48.00±0.54^{a}	47.57±0.59^{a}	46.27±0.18^{a}	47.37±1.07^{a}
Σn-3	1.06±0.08^{b}	19.58±0.77^{a}	18.66±0.26^{a}	19.16±0.34^{a}	19.48±0.37^{a}	1.04±0.08^{b}
Σn-6	45.29±0.52^{a}	25.09±0.19^{d}	29.34±0.42^{b}	28.42±0.64^{c}	26.78±0.21^{bcd}	46.33±1.01^{a}

Values are mean percent composition ± SEM (n=3). "OA/LA"=oleic acid (18:1n-9)/linoleic acid (18:2n-6);"EPA=eicosapentaenoic acid (20:5n-3); "DHA"=docosahexaenoic acid (22:6n-3); "*Σ* EPA+DPA"=summation of EPA and docosapentaenoic acid (22:5n-3); "SFA"=saturated fatty acids; "MUFA"=monounsaturated fatty acids; "PUFA"=polyunsaturated fatty acids. For each fatty acid identified, values that do not share a common letter are significantly different ($p<0.05$).

Table 7: Change in fatty acids in total PL of MDA-MB-231 cells incubated with control or 200 μM n-3 LCPUFA treatments

Fatty Acid	80 μM OA/LA	DHA	EPA	1:1 DHA:EPA	2:1 DHA:EPA	280 μM OA/LA
14:0	1.44±0.06^{bc}	2.13±0.26^{a}	1.77±0.08^{a}	1.85±0.04^{a}	1.59±0.02^{ab}	1.17±0.02^{c}
16:0	18.44±0.57^{b}	22.63±0.63^{a}	19.54±0.22^{a}	21.01±0.19^{a}	19.49±0.62^{a}	15.84±0.01^{b}
16:1n-9	0.55±0.01^{a}	0.76±0.19^{a}	0.90±0.02^{a}	0.91±0.02^{a}	0.86±0.02^{a}	0.51±0.01^{a}
17:0	0.45±0.02^{b}	0.70±0.05^{a}	0.76±0.07^{a}	0.66±0.02^{a}	0.61±0.01^{ab}	0.44±0.01^{b}
18:0	20.44±0.42^{a}	19.72±0.46^{a}	20.31±0.10^{a}	20.56±0.35^{a}	19.67±0.46^{a}	18.86±0.32^{a}
18:1n-9	14.32±0.13^{a}	11.10±0.32^{bc}	10.65±0.21^{c}	10.52±0.29^{c}	9.94±0.18^{c}	11.94±0.10^{b}
18:2n-6	24.81±0.18^{b}	19.46±0.57^{d}	17.81±0.27^{e}	16.13±0.18^{f}	21.10±0.26^{c}	34.90±0.05^{a}
18:3n-6	0.21±0.01^{a}	0.21±0.03^{a}	0.19±0.01^{a}	0.20±0.01^{a}	0.19±0.01^{a}	0.15±0.01^{a}
18:3n-3	0.56±0.01^{a}	0.18±0.03^{b}	0.19±0.01^{b}	0.19±0.00^{b}	0.18±0.01^{b}	0.42±0.01^{a}
20:2n-6	3.91±0.08^{b}	1.51±0.23^{c}	1.20±0.04^{c}	1.04±0.06^{c}	1.33±0.02^{c}	5.30±0.06^{a}
20:3n-6	2.63±0.03^{a}	1.06±0.07^{c}	0.56±0.02^{d}	0.62±0.01^{d}	0.71±0.03^{d}	1.65±0.05^{b}
20:4n-6	4.62±0.06^{a}	1.00±0.07^{c}	0.83±0.02^{c}	0.91±0.04^{c}	0.95±0.04^{c}	2.32±0.07^{b}
20:5n-3	0.12±0.08^{d}	0.45±0.09^{d}	11.34±0.38^{a}	7.15±0.37^{b}	5.26±0.29^{c}	0.47±0.01^{d}
24:0	1.25±0.14^{a}	0.68±0.03^{b}	0.61±0.05^{b}	0.62±0.07^{b}	0.58±0.11^{b}	0.84±0.11^{a}
24:1n-9	2.81±0.06^{a}	0.58±0.09^{c}	0.55±0.00^{c}	0.55±0.01^{c}	0.53±0.02^{c}	1.82±0.05^{b}
22:4n-6	1.57±0.18^{a}	0.98±0.10^{ab}	0.97±0.07^{ab}	0.96±0.11^{ab}	0.88±0.14^{b}	1.36±0.15^{ab}
22:5n-6	1.16±0.15^{a}	0.52±0.08^{b}	0.56±0.04^{b}	0.45±0.07^{b}	0.57±0.08^{b}	1.61±0.19^{a}
22:5n-3	0.37±0.06^{d}	0.35±0.06^{d}	11.05±0.42^{a}	6.60±0.28^{b}	4.63±0.27^{c}	0.24±0.01^{d}
22:6n-3	0.32±0.13^{c}	15.98±1.22^{a}	0.22±0.05^{c}	9.06±0.64^{b}	10.90±0.84^{b}	0.15±0.02^{c}
ΣEPA+DPA	0.49±0.14^{d}	0.80±0.12^{d}	22.38±0.80^{a}	13.76±0.66^{b}	9.90±0.56^{c}	0.71±0.01^{d}
ΣSFA	42.04±0.70^{a}	45.85±1.16^{a}	42.98±0.43^{a}	44.71±062^{a}	41.95±1.18^{a}	37.15±0.24^{b}
ΣMUFA	17.68±0.14^{a}	12.43±0.55^{c}	12.10±0.23^{c}	11.97±0.31^{c}	11.33±0.18^{c}	14.27±0.16^{b}
ΣPUFA	40.28±0.56^{c}	41.71±1.16^{c}	44.92±0.57^{abc}	43.32±0.92^{bc}	46.72±1.35^{ab}	48.58±0.26^{a}
Σn-3	1.37±0.28^{c}	16.96±1.28^{b}	22.79±0.75^{a}	23.01±1.30^{a}	20.98±1.39^{ab}	1.28±0.03^{c}
Σn-6	38.91±0.50^{b}	24.75±0.82^{c}	22.13±0.32^{d}	20.31±0.38^{d}	25.74±0.09^{c}	47.30±0.28^{a}

Values are mean percent composition ± SEM (n=3). "OA/LA"=oleic acid (18:1n-9)/linoleic acid (18:2n-6);"EPA=eicosapentaenoic acid (20:5n-3); "DHA"=docosahexaenoic acid (22:6n-3); "*Σ* EPA+DPA"=summation of EPA and docosapentaenoic acid (22:5n-3); "SFA"=saturated fatty acids; "MUFA"=monounsaturated fatty acids; "PUFA"=polyunsaturated fatty acids. For each fatty acid identified, values that do not share a common letter are significantly different ($p<0.05$).

Table 8: Change in fatty acids in total PL of SK-BR-3 cells incubated with control or 100 μM n-3 LCPUFA treatments

Fatty Acid	80 μM OA/LA	DHA	EPA	1:1 DHA:EPA	2:1 DHA:EPA	180 μM OA/LA
14:0	1.49±0.06^{a}	1.39±0.23^{a}	1.55±0.26^{a}	1.34±0.03^{a}	1.06±0.11^{a}	1.02±0.03^{a}
16:0	21.49±0.03^{ab}	24.66±0.77^{a}	23.55±0.90^{ab}	24.19±0.43^{a}	24.11±0.46^{a}	20.67±0.66^{ab}
16:1n-9	3.25±0.36^{a}	1.89±0.13^{a}	2.90±0.05^{a}	2.83±0.18^{a}	2.52±0.13^{a}	2.33±0.28^{a}
17:0	0.55±0.02^{a}	0.63±0.01^{a}	0.67±0.04^{a}	0.66±0.04^{a}	0.60±0.02^{a}	0.55±0.02^{a}
18:0	19.01±0.65^{a}	18.75±0.47^{a}	19.29±0.42^{a}	18.35±0.47^{a}	17.48±0.29^{a}	15.97±0.23^{a}
18:1n-9	26.81±0.84^{ab}	23.00±0.81^{b}	25.63±0.20^{b}	24.26±0.07^{b}	25.40±0.69^{b}	30.50±1.18^{a}
18:2n-6	13.73±0.64^{b}	14.87±0.34^{ab}	15.05±0.51^{ab}	15.25±0.52^{ab}	14.59±0.57^{ab}	16.92±0.83^{a}
18:3n-6	0.21±0.04^{a}	0.18±0.03^{a}	0.17±0.02^{a}	0.24±0.07^{a}	0.17±0.01^{a}	0.15±0.01^{a}
18:3n-3	0.64±0.16^{a}	0.36±0.05^{a}	0.41±0.06^{a}	0.32±0.11^{a}	0.45±0.06^{a}	0.83±0.19^{a}
20:2n-6	1.43±0.14^{ab}	1.15±0.06^{b}	0.97±0.03^{b}	0.91±0.19^{b}	0.96±0.04^{b}	1.78±0.02^{a}
20:3n-6	2.43±0.16^{a}	1.86±0.03^{ab}	0.80±0.07^{c}	1.14±0.06^{c}	1.29±0.06^{bc}	1.91±0.19^{a}
20:4n-6	5.59±0.38^{a}	2.74±0.09^{c}	2.00±0.10^{c}	2.14±0.06^{c}	2.58±0.12^{c}	4.31±0.27^{b}
20:5n-3	0.06±0.01^{c}	0.11±0.02^{c}	3.04±0.27^{a}	2.25±0.18^{b}	1.71±0.02^{b}	0.12±0.05^{c}
24:0	1.83±0.08^{a}	0.27±0.05^{b}	0.20±0.03^{b}	0.19±0.03^{b}	0.17±0.01^{b}	1.56±0.09^{a}
24:1n-9	0.15±0.01^{a}	0.11±0.05^{a}	0.13±0.01^{a}	0.23±0.16^{a}	0.08±0.04^{a}	0.09±0.02^{a}
22:4n-6	0.24±0.10^{a}	0.10±0.02^{a}	0.46±0.12^{a}	0.39±0.21^{a}	0.23±0.14^{a}	0.22±0.13^{a}
22:5n-6	0.47±0.22^{a}	0.64±0.02^{a}	0.38±0.12^{a}	0.42±0.14^{a}	0.51±0.18^{a}	0.53±0.23^{a}
22:5n-3	0.42±0.02^{cd}	0.33±0.04^{d}	2.59±0.37^{a}	1.72±0.25^{ab}	1.37±0.16^{bc}	0.36±0.02^{cd}
22:6n-3	0.19±0.01^{d}	6.97±0.28^{a}	0.21±0.04^{d}	3.17±0.05^{c}	4.73±0.37^{b}	0.18±0.01^{d}
ΣEPA+DPA	0.49±0.03^{c}	0.44±0.06^{c}	5.63±0.65^{a}	3.97±0.43^{ab}	3.07±0.17^{b}	0.48±0.03^{c}
ΣSFA	44.37±0.78^{ab}	45.70±0.99^{a}	45.25±1.24^{a}	44.73±0.86^{a}	43.42±0.77^{ab}	39.77±0.93^{b}
ΣMUFA	30.22±1.19^{ab}	25.00±0.99^{c}	28.66±0.23^{abc}	27.32±0.25^{bc}	28.00±0.70^{bc}	32.92±1.36^{a}
ΣPUFA	25.42±0.48^{a}	29.30±0.49^{a}	26.09±1.05^{a}	27.95±1.11^{a}	28.58±1.07^{a}	27.31±0.61^{a}
Σn-3	1.31±0.14^{b}	7.77±0.25^{a}	6.25±0.62^{a}	7.46±0.38^{a}	8.25±0.49^{a}	1.49±0.22^{b}
Σn-6	21.67±0.47^{ab}	19.67±0.29^{b}	19.03±0.37^{b}	19.35±0.70^{b}	19.05±0.59^{b}	23.91±0.64^{a}

Values are mean percent composition ± SEM (n=3). "OA/LA"=oleic acid (18:1n-9)/linoleic acid (18:2n-6);"EPA=eicosapentaenoic acid (20:5n-3); "DHA"=docosahexaenoic acid (22:6n-3); "*Σ* EPA+DPA"=summation of EPA and docosapentaenoic acid (22:5n-3); "SFA"=saturated fatty acids; "MUFA"=monounsaturated fatty acids; "PUFA"=polyunsaturated fatty acids. For each fatty acid identified, values that do not share a common letter are significantly different ($p<0.05$).

Table 9: Change in fatty acids in total PL of SK-BR-3 cells incubated with control or 200 μM n-3 LCPUFA treatments

Fatty Acid	80 μM OA/LA	DHA	EPA	1:1 DHA:EPA	2:1 DHA:EPA	280 μM OA/LA
14:0	1.81±0.09^{a}	1.30±0.01^{b}	1.36±0.07^{b}	1.12±0.04^{b}	1.27±0.05^{b}	1.19±0.06^{b}
16:0	20.99±0.60^{c}	26.84±0.52^{a}	23.25±0.29^{b}	23.21±0.25^{b}	24.70±0.30^{b}	20.69±0.12^{c}
16:1n-9	2.18±0.44^{ab}	1.52±0.10^{ab}	2.74±0.19^{a}	2.26±0.19^{ab}	2.02±0.14^{ab}	1.36±0.30^{b}
17:0	0.50±0.02^{ab}	0.57±0.03^{a}	0.55±0.02^{a}	0.55±0.02^{a}	0.55±0.02^{a}	0.41±0.03^{b}
18:0	17.86±0.28^{a}	15.99±0.47^{a}	16.94±0.57^{a}	16.12±0.31^{a}	16.33±0.12^{a}	12.80±0.03^{b}
18:1n-9	24.93±0.97^{b}	19.84±0.62^{c}	23.37±0.36^{b}	23.29±0.40^{b}	22.63±0.09^{bc}	29.63±0.33^{a}
18:2n-6	14.65±0.72^{b}	15.39±0.32^{b}	16.94±0.68^{b}	16.05±0.37^{b}	14.94±0.14^{b}	19.62±0.27^{a}
18:3n-6	0.40±0.05^{ab}	0.23±0.02^{b}	0.31±0.07^{b}	0.36±0.07^{ab}	0.31±0.06^{b}	0.67±0.08^{a}
18:3n-3	0.63±0.04^{a}	0.31±0.02^{b}	0.29±0.01^{b}	0.35±0.02^{b}	0.30±0.02^{b}	0.53±0.04^{a}
20:2n-6	1.53±0.17^{ab}	1.02±0.07^{bc}	0.78±0.06^{c}	0.85±0.10^{c}	0.85±0.08^{c}	2.03±0.18^{a}
20:3n-6	3.47±0.43^{a}	1.70±0.07^{bc}	0.79±0.04^{c}	1.08±0.06^{c}	1.22±0.08^{c}	2.41±0.28^{b}
20:4n-6	5.72±0.25^{a}	1.69±0.17^{c}	1.25±0.13^{c}	1.60±0.09^{c}	1.75±0.08^{c}	3.97±0.18^{b}
20:5n-3	0.06±0.03^{d}	0.16±0.03^{d}	4.99±0.30^{a}	3.42±0.12^{b}	2.36±0.11^{c}	0.10±0.02^{d}
24:0	1.41±0.17^{a}	1.16±0.06^{a}	0.98±0.07^{a}	1.06±0.05^{a}	1.05±0.08^{a}	1.14±0.26^{a}
24:1n-9	2.53±0.18^{a}	0.34±0.00^{b}	0.35±0.03^{b}	0.34±0.02^{b}	0.33±0.01^{b}	2.24±0.16^{a}
22:4n-6	0.05±0.01^{a}	0.10±0.05^{a}	0.20±0.10^{a}	0.13±0.04^{a}	0.26±0.16^{a}	0.12±0.04^{a}
22:5n-6	0.45±0.07^{a}	0.38±0.03^{a}	0.45±0.09^{a}	0.43±0.03^{a}	0.39±0.01^{a}	0.45±0.06^{a}
22:5n-3	0.54±0.04^{c}	0.20±0.05^{c}	4.20±0.41^{a}	2.73±0.19^{b}	1.84±0.13^{b}	0.45±0.04^{c}
22:6n-3	0.29±0.00^{d}	11.25±0.64^{a}	0.26±0.04^{d}	5.04±0.11^{c}	6.90±0.02^{b}	0.20±0.02^{d}
ΣEPA+DPA	0.59±0.07^{d}	0.36±0.08^{d}	9.20±0.61^{a}	6.15±0.06^{b}	4.21±0.07^{c}	0.55±0.02^{d}
ΣSFA	42.57±0.74^{b}	45.86±1.04^{a}	43.08±0.67^{ab}	42.07±0.23^{b}	43.90±0.11^{ab}	36.23±0.35^{c}
ΣMUFA	29.64±1.18^{b}	21.70±0.72^{d}	26.45±0.56^{bc}	25.89±0.59^{c}	24.98±0.14^{cd}	33.23±0.34^{a}
ΣPUFA	27.78±1.20^{b}	32.44±0.91^{a}	30.48±1.02^{ab}	32.04±0.49^{ab}	31.12±0.23^{ab}	30.54±0.43^{ab}
Σn-3	1.51±0.04^{c}	11.93±0.66^{a}	9.75±0.62^{b}	11.54±0.13^{ab}	11.40±0.06^{ab}	1.29±0.04^{c}
Σn-6	26.28±1.24^{a}	20.51±0.32^{b}	20.72±0.50^{b}	20.50±0.40^{b}	19.72±0.23^{b}	29.26±0.39^{a}

Values are mean percent composition ± SEM (n=3). "OA/LA"=oleic acid (18:1n-9)/linoleic acid (18:2n-6);"EPA=eicosapentaenoic acid (20:5n-3); "DHA"=docosahexaenoic acid (22:6n-3); "*Σ* EPA+DPA"=summation of EPA and docosapentaenoic acid (22:5n-3); "SFA"=saturated fatty acids; "MUFA"=monounsaturated fatty acids; "PUFA"=polyunsaturated fatty acids. For each fatty acid identified, values that do not share a common letter are significantly different ($p<0.05$).

Table 10: Change in fatty acids in PC of MDA-MB-231 cells incubated with control or 100 μM n-3 LCPUFA treatments

Fatty Acid	80 μM OA/LA	DHA	EPA	1:1 DHA:EPA	2:1 DHA:EPA	180 μM OA/LA
14:0	0.46±0.26^{a}	0.45±0.24^{a}	0.26±0.02^{a}	0.29±0.02^{a}	0.24±0.03^{a}	0.29±0.05^{a}
16:0	26.37±1.20^{a}	25.83±1.79^{a}	27.88±0.81^{a}	26.53±1.42^{a}	25.69±0.40^{a}	23.43±0.93^{a}
16:1n-9	0.13±0.01^{a}	0.21±0.08^{a}	0.15±0.01^{a}	0.14±0.02^{a}	0.13±0.01^{a}	0.13±0.02^{a}
17:0	0.51±0.03^{a}	0.40±0.14^{a}	0.30±0.01^{a}	0.26±0.03^{a}	0.25±0.03^{a}	0.36±0.07^{a}
18:0	9.42±0.25^{bc}	11.36±0.17^{a}	9.37±0.10^{bc}	9.64±0.05^{b}	9.52±0.22^{bc}	8.27±0.45^{c}
18:1n-9	27.62±0.44^{a}	24.70±0.72^{b}	27.91±0.64^{a}	27.73±0.20^{a}	28.44±0.22^{a}	23.93±0.48^{b}
18:2n-6	27.49±0.29^{b}	25.64±0.62^{bc}	22.98±0.28^{d}	24.30±0.23^{cd}	25.59±0.16^{bc}	36.14±0.44^{a}
18:3n-6	0.10±0.05^{a}	0.08±0.03^{a}	0.06±0.00^{a}	0.06±0.01^{a}	0.05±0.01^{a}	0.06±0.02^{a}
18:3n-3	0.95±0.06^{a}	0.45±0.02^{b}	0.32±0.01^{b}	0.38±0.02^{b}	0.45±0.01^{b}	0.80±0.06^{a}
20:2n-6	2.90±0.19^{b}	1.43±0.05^{c}	0.95±0.05^{c}	1.18±0.05^{c}	1.20±0.08^{c}	3.74±0.12^{a}
20:3n-6	1.35±0.17^{a}	0.77±0.01^{bc}	0.34±0.01^{c}	0.47±0.05^{c}	0.59±0.02^{bc}	0.91±0.10^{b}
20:4n-6	1.37±0.25^{a}	1.06±0.09^{ab}	0.53±0.07^{b}	0.64±0.05^{b}	0.70±0.01^{b}	0.83±0.06^{ab}
20:5n-3	0.23±0.04^{d}	0.44±0.02^{d}	4.73±0.50^{a}	2.72±0.24^{b}	1.91±0.03^{c}	0.23±0.02^{d}
24:0	0.07±0.02^{a}	0.14±0.06^{a}	0.08±0.01^{a}	0.11±0.02^{a}	0.16±0.01^{a}	0.07±0.01^{a}
24:1n9	0.47±0.08^{a}	0.25±0.11^{a}	0.22±0.02^{a}	0.19±0.02^{a}	0.22±0.05^{a}	0.33±0.04^{a}
22:4n-6	0.18±0.04^{a}	0.29±0.13^{a}	0.16±0.03^{a}	0.17±0.01^{a}	0.22±0.03^{a}	0.12±0.05^{a}
22:5n-6	0.07±0.01^{a}	0.18±0.07^{a}	0.13±0.02^{a}	0.08±0.02^{a}	0.13±0.03^{a}	0.12±0.06^{a}
22:5n-3	0.18±0.03^{d}	0.41±0.05^{d}	4.53±0.56^{a}	2.50±0.35^{b}	1.54±0.13^{c}	0.14±0.06^{d}
22:6n-3	0.14±0.03^{c}	6.76±0.84^{a}	0.13±0.04^{c}	2.61±0.40^{b}	2.95±0.26^{b}	0.08±0.03^{c}
ΣEPA+DPA	0.40±0.00^{d}	0.84±0.07^{d}	9.27±1.06^{a}	5.22±0.59^{b}	3.46±0.14^{c}	0.37±0.06^{d}
ΣSFA	36.82±1.34^{a}	38.19±2.04^{a}	37.89±0.71^{a}	36.83±1.48^{a}	35.86±0.42^{a}	32.43±1.28^{a}
ΣMUFA	28.22±0.48^{a}	25.15±0.74^{b}	28.28±0.63^{a}	28.06±0.20^{a}	28.79±0.18^{a}	24.39±0.49^{b}
ΣPUFA	34.96±0.86^{ab}	35.24±1.29^{ab}	31.78±1.29^{b}	35.11±1.28^{ab}	35.34±0.61^{ab}	43.18±0.82^{a}
Σn-3	1.49±0.09^{b}	8.00±0.80^{a}	6.63±1.03^{a}	8.21±0.99^{a}	6.86±0.41^{a}	1.25±0.12^{b}
Σn-6	33.47±0.78^{b}	29.45±0.73^{c}	25.15±0.36^{d}	26.90±0.34^{cd}	28.49±0.20^{c}	41.93±0.70^{a}

Values are mean percent composition ± SEM (n=2-3). "OA/LA"=oleic acid (18:1n-9)/linoleic acid (18:2n-6);"EPA=eicosapentaenoic acid (20:5n-3); "DHA"=docosahexaenoic acid (22:6n-3); "*Σ* EPA+DPA"=summation of EPA and docosapentaenoic acid (22:5n-3); "SFA"=saturated fatty acids; "MUFA"=monounsaturated fatty acids; "PUFA"=polyunsaturated fatty acids. For each fatty acid identified, values that do not share a common letter are significantly different ($p<0.05$).

Table 11: Change in fatty acids in PE of MDA-MB-231 cells incubated with control or 100 μM n-3 LCPUFA treatments

Fatty Acid	80 μM OA/LA	DHA	EPA	1:1 DHA:EPA	2:1 DHA:EPA	180 μM OA/LA
14:0	0.71±0.09^{a}	0.73±0.17^{a}	0.68±0.19^{a}	4.93±3.06^{a}	0.80±0.32^{a}	0.86±0.10^{a}
16:0	8.80±0.17^{a}	8.88±0.66^{a}	8.64±0.28^{a}	9.90±1.68^{a}	8.23±0.80^{a}	9.37±0.57^{a}
16:1n-9	0.45±0.05^{a}	0.46±0.07^{a}	0.37±0.03^{a}	0.66±0.15^{a}	0.37±0.06^{a}	0.32±0.03^{a}
17:0	0.54±0.05^{a}	0.47±0.03^{a}	0.52±0.04^{a}	0.60±0.03^{a}	0.47±0.03^{a}	0.47±0.02^{a}
18:0	24.82±0.54^{a}	27.23±2.50^{a}	27.54±0.50^{a}	27.80±0.62^{a}	25.58±0.99^{a}	24.66±0.77^{a}
18:1n-9	28.05±0.21^{a}	24.00±1.21^{a}	25.12±0.72^{a}	22.26±3.09^{a}	27.75±0.74^{a}	25.77±0.53^{a}
18:2n-6	14.44±1.15^{b}	13.44±1.28^{b}	11.05±0.28^{b}	9.78±1.48^{b}	11.88±1.16^{b}	20.57±0.58^{a}
18:3n-6	0.16±0.02^{a}	0.11±0.00^{a}	0.12±0.01^{a}	0.48±0.25^{a}	0.19±0.02^{a}	0.17±0.03^{a}
18:3n-3	0.98±0.23^{ab}	0.53±0.05^{ab}	0.38±0.02^{b}	0.66±0.20^{ab}	0.43±0.29^{ab}	1.11±0.06^{a}
20:2n-6	2.24±0.44^{b}	1.19±0.14^{b}	1.24±0.06^{b}	1.14±0.08^{b}	1.29±0.34^{b}	3.73±0.20^{a}
20:3n-6	1.67±0.39^{a}	1.17±0.04^{a}	0.78±0.04^{a}	0.81±0.10^{a}	0.96±0.37^{a}	1.70±0.04^{a}
20:4n-6	7.87±1.66^{a}	3.10±0.50^{bc}	2.18±0.13^{c}	2.29±0.32^{c}	2.73±1.17^{c}	4.80±0.32^{b}
20:5n-3	0.30±1.14^{c}	0.70±0.04^{c}	9.45±0.68^{a}	5.75±0.86^{b}	4.33±1.30^{b}	0.40±0.08^{c}
24:0	0.29±0.01^{a}	0.19±0.01^{a}	0.12±0.02^{a}	0.41±0.27^{a}	0.15±0.06^{a}	0.32±0.06^{a}
24:1n9	5.21±1.44^{a}	0.98±0.12^{c}	0.98±0.09^{c}	0.93±0.02^{c}	0.90±0.92^{c}	3.68±0.04^{b}
22:4n-6	0.24±0.04^{a}	0.23±0.02^{a}	0.15±0.02^{a}	0.58±0.39^{a}	0.21±0.05^{a}	0.37±0.05^{a}
22:5n-6	0.39±0.05^{a}	0.35±0.08^{a}	0.40±0.05^{a}	0.88±0.54^{a}	0.41±0.26^{a}	0.38±0.05^{a}
22:5n-3	0.61±0.75^{c}	0.34±0.10^{c}	9.91±0.78^{a}	4.70±0.65^{b}	3.28±0.91^{b}	0.54±0.01^{c}
22:6n-3	0.45±0.06^{c}	15.58±3.02^{a}	0.36±0.05^{c}	6.89±0.70^{b}	10.05±1.20^{b}	0.46±0.30^{c}
ΣEPA+DPA	0.92±1.88^{c}	1.04±0.09^{c}	19.36±1.45^{a}	10.45±1.51^{b}	7.61±2.20^{b}	0.95±0.06^{c}
ΣSFA	34.37±0.79^{a}	28.42±2.01^{a}	37.52±0.67^{a}	43.63±5.49^{a}	35.22±1.99^{a}	35.68±1.44^{a}
ΣMUFA	33.96±1.69^{a}	25.45±1.29^{ab}	26.47±0.66^{ab}	23.85±2.93^{b}	29.02±0.27^{ab}	29.78±0.59^{a}
ΣPUFA	31.67±2.23^{a}	31.54±3.26^{a}	36.01±1.33^{a}	30.48±2.69^{a}	35.75±2.43^{a}	34.10±0.96^{a}
Σn-3	2.58±1.50^{b}	17.16±3.04^{a}	20.10±1.44^{a}	18.39±2.02^{a}	18.09±5.34^{a}	2.63±0.21^{b}
Σn-6	29.09±3.73^{a}	19.58±0.62^{b}	15.92±0.12^{c}	15.97±0.77^{c}	17.66±3.33^{c}	31.74±0.81^{a}

Values are mean percent composition ± SEM (n=2-3). "OA/LA"=oleic acid (18:1n-9)/linoleic acid (18:2n-6);"EPA=eicosapentaenoic acid (20:5n-3); "DHA"=docosahexaenoic acid (22:6n-3); "*Σ* EPA+DPA"=summation of EPA and docosapentaenoic acid (22:5n-3); "SFA"=saturated fatty acids; "MUFA"=monounsaturated fatty acids; "PUFA"=polyunsaturated fatty acids. For each fatty acid identified, values that do not share a common letter are significantly different ($p<0.05$).

Table 12: Change in fatty acids in PI of MDA-MB-231 cells incubated with control or 100 μM n-3 LCPUFA treatments

Fatty Acid	80 μM OA/LA	DHA	EPA	1:1 DHA:EPA	2:1 DHA:EPA	180 μM OA/LA
14:0	2.92±0.69^{a}	2.11±0.31^{a}	2.25±0.23^{a}	1.91±0.12^{a}	1.88±0.48^{a}	2.65±0.23^{a}
16:0	4.81±0.92^{a}	4.44±0.59^{a}	4.92±0.55^{a}	4.16±0.19^{a}	3.66±0.80^{a}	4.50±0.59^{a}
16:1n-9	0.35±0.13^{a}	0.17±0.03^{a}	0.18±0.02^{a}	0.18±0.03^{a}	0.75±0.54^{a}	0.22±0.03^{a}
17:0	0.19±0.06^{a}	0.11±0.02^{a}	0.15±0.02^{a}	0.11±0.01^{a}	0.13±0.03^{a}	0.15±0.02^{a}
18:0	40.27±0.57^{a}	37.83±3.83^{a}	42.03±1.84^{a}	41.98±1.22^{a}	41.78±1.61^{a}	41.45±0.95^{a}
18:1n-9	14.23±0.11^{a}	13.82±0.39^{a}	13.08±0.64^{a}	13.20±0.62^{a}	13.96±0.38^{a}	12.30±0.29^{a}
18:2n-6	11.79±0.23^{cd}	13.92±0.62^{b}	10.22±0.26^{d}	11.51±0.23^{cd}	12.53±0.48^{bc}	17.42±0.38^{a}
18:3n-6	0.25±0.10^{a}	0.12±0.02^{a}	0.14±0.01^{a}	0.11±0.01^{a}	0.13±0.03^{a}	0.15±0.02^{a}
18:3n-3	0.62±0.07^{a}	0.30±0.01^{b}	0.20±0.01^{b}	0.26±0.03^{b}	0.28±0.02^{b}	0.61±0.08^{a}
20:2n-6	2.35±0.10^{b}	1.08±0.03^{c}	0.65±0.04^{c}	0.81±0.02^{c}	0.95±0.02^{c}	3.13±0.15^{a}
20:3n-6	6.93±0.51^{a}	4.16±0.26^{ab}	2.03±0.03^{c}	2.89±0.12^{bc}	3.54±0.15^{b}	5.70±0.32^{a}
20:4n-6	12.13±1.23^{a}	6.67±0.58^{bc}	5.61±0.14^{c}	6.71±0.31^{bc}	6.92±0.46^{bc}	9.34±0.43^{ab}
20:5n-3	0.14±0.08^{d}	0.61±0.02^{d}	9.78±0.53^{a}	5.68±0.31^{b}	3.83±0.33^{c}	0.14±0.02^{d}
24:0	1.83±0.13^{a}	0.77±0.04^{c}	0.47±0.04^{c}	0.61±0.05^{c}	0.65±0.03^{c}	1.25±0.08^{b}
22:4n-6	0.52±0.08^{a}	0.57±0.15^{a}	0.97±0.47^{a}	1.08±0.35^{a}	0.64±0.02^{a}	0.66±0.15^{a}
22:5n-3	0.28±0.02^{c}	0.63±0.07^{c}	7.04±0.63^{a}	4.86±0.39^{b}	3.49±0.31^{b}	0.18±0.01^{c}
22:6n-3	0.30±0.05^{c}	9.43±3.35^{a}	0.35±0.07^{c}	3.93±0.53^{b}	4.90±0.49^{b}	0.15±0.01^{c}
ΣEPA+DPA	0.43±0.07^{c}	1.24±0.09^{c}	16.83±1.11^{a}	10.53±0.71^{b}	7.31±0.65^{b}	0.32±0.01^{c}
ΣSFA	50.03±1.72^{a}	45.25±3.67^{a}	49.82±1.63^{a}	48.77±1.07^{a}	48.10±2.68^{a}	50.00±1.11^{a}
ΣMUFA	14.58±0.05^{a}	13.99±0.36^{a}	13.27±0.62^{a}	13.39±0.61^{a}	14.71±0.67^{a}	12.52±0.26^{a}
ΣPUFA	35.26±1.72^{a}	34.34±3.33^{a}	36.91±1.02^{a}	37.85±1.32^{a}	37.19±2.21^{a}	37.47±1.29^{a}
Σn-3	1.30±0.18^{c}	7.83±3.42^{bc}	17.31±1.10^{a}	14.73±1.20^{ab}	12.49±1.12^{ab}	1.08±0.05^{c}
Σn-6	33.96±1.87^{a}	26.51±0.54^{b}	19.61±0.12^{c}	23.12±0.26^{bc}	24.70±1.10^{bc}	36.39±1.31^{a}

Values are mean percent composition ± SEM (n=2-3). "OA/LA"=oleic acid (18:1n-9)/linoleic acid (18:2n-6);"EPA=eicosapentaenoic acid (20:5n-3); "DHA"=docosahexaenoic acid (22:6n-3); "*Σ* EPA+DPA"=summation of EPA and docosapentaenoic acid (22:5n-3); "SFA"=saturated fatty acids; "MUFA"=monounsaturated fatty acids; "PUFA"=polyunsaturated fatty acids. For each fatty acid identified, values that do not share a common letter are significantly different ($p<0.05$).

Table 13: Change in fatty acids in PC of MDA-MB-231 cells incubated with control or 200 μM n-3 LCPUFA treatments

Fatty Acid	80 μM OA/LA	DHA	EPA	1:1 DHA:EPA	2:1 DHA:EPA	280 μM OA/LA
14:0	1.21±0.40^{a}	1.04±0.29^{a}	1.09±0.39^{a}	1.09±0.10^{a}	1.02±0.46^{a}	0.89±0.41^{a}
16:0	27.93±3.78^{a}	27.70±1.07^{a}	27.10±1.73^{a}	28.08±0.62^{a}	29.24±1.04^{a}	17.14±1.25^{b}
16:1n-9	1.06±0.11^{a}	1.38±0.09^{a}	1.70±0.17^{a}	1.56±0.10^{a}	1.50±0.12^{a}	1.37±0.54^{a}
17:0	0.62±0.08^{a}	0.60±0.01^{a}	0.63±0.01^{a}	0.64±0.00^{a}	0.64±0.01^{a}	0.40±0.05^{b}
18:0	10.71±0.46^{a}	11.40±0.84^{a}	9.34±0.47^{ab}	10.54±0.46^{a}	10.25±0.60^{ab}	7.49±0.40^{b}
18:1n-9	28.09±1.75^{b}	24.97±0.42^{b}	27.86±1.10^{b}	26.77±0.35^{b}	25.94±0.22^{b}	37.33±0.39^{a}
18:2n-6	21.99±2.39^{ab}	20.99±0.24^{b}	20.00±0.54^{b}	19.89±0.22^{b}	19.52±0.09^{b}	28.31±1.83^{a}
18:3n-6	0.26±0.07^{a}	0.11±0.00^{a}	0.09±0.01^{a}	0.09±0.03^{a}	0.08±0.00^{a}	0.44±0.28^{a}
18:3n-3	0.28±0.10^{a}	0.13±0.00^{a}	0.09±0.01^{a}	0.12±0.02^{a}	0.13±0.00^{a}	0.46±0.39^{a}
20:2n-6	2.78±0.20^{a}	1.02±0.03^{b}	0.75±0.04^{b}	0.85±0.03^{b}	0.90±0.04^{b}	2.71±0.15^{a}
20:3n-6	1.05±0.03^{a}	0.57±0.04^{b}	0.25±0.01^{c}	0.30±0.03^{c}	0.33±0.01^{c}	0.52±0.01^{b}
20:4n-6	1.08±0.17^{a}	0.81±0.02^{ab}	0.42±0.01^{b}	0.45±0.01^{b}	0.53±0.03^{b}	0.51±0.06^{b}
20:5n-3	0.44±0.10^{c}	0.32±0.02^{c}	5.59±0.21^{a}	3.63±0.11^{b}	2.92±0.20^{b}	0.52±0.36^{c}
24:0	0.58±0.03^{a}	0.18±0.04^{b}	0.16±0.04^{b}	0.13±0.01^{b}	0.21±0.09^{b}	0.32±0.06^{ab}
24:1n9	0.57±0.17^{a}	0.15±0.03^{a}	0.12±0.03^{a}	0.16±0.03^{a}	0.20±0.06^{a}	0.43±0.22^{a}
22:4n-6	0.32±0.12^{a}	0.11±0.02^{a}	0.09±0.03^{a}	0.10±0.04^{a}	0.14±0.04^{a}	0.31±0.13^{a}
22:5n-6	0.28±0.12^{a}	0.09±0.03^{a}	0.12±0.02^{a}	0.16±0.06^{a}	0.08±0.02^{a}	0.26±0.17^{a}
22:5n-3	0.42±0.07^{c}	0.36±0.03^{c}	4.32±0.06^{a}	2.74±0.11^{b}	2.27±0.20^{b}	0.29±0.19^{c}
22:6n-3	0.34±0.01^{d}	8.07±0.48^{a}	0.28±0.16^{d}	2.68±0.11^{c}	4.10±0.35^{b}	0.29±0.21^{d}
ΣEPA+DPA	0.79±0.09^{c}	0.68±0.04^{c}	9.91±0.26^{a}	6.37±0.19^{b}	5.19±0.37^{b}	0.81±0.54^{c}
ΣSFA	41.04±4.34^{a}	40.92±0.55^{a}	38.31±1.63^{a}	40.49±0.28^{a}	41.37±0.76^{a}	26.25±1.38^{b}
ΣMUFA	29.72±1.85^{b}	26.51±0.36^{b}	29.68±0.90^{b}	28.49±0.27^{b}	27.63±0.30^{b}	39.13±0.99^{a}
ΣPUFA	29.24±2.57^{a}	32.57±0.37^{a}	32.00±0.75^{a}	31.02±0.04^{a}	31.00±0.81^{a}	34.62±0.80^{a}
Σn-3	1.48±0.24^{b}	8.87±0.52^{a}	10.29±0.22^{a}	9.17±0.28^{a}	9.42±0.70^{a}	1.56±1.14^{b}
Σn-6	27.76±2.60^{ab}	23.70±0.26^{b}	21.72±0.54^{b}	21.85±0.27^{b}	21.58±0.12^{b}	33.06±1.30^{a}

Values are mean percent composition ± SEM (n=3). "OA/LA"=oleic acid (18:1n-9)/linoleic acid (18:2n-6);"EPA=eicosapentaenoic acid (20:5n-3); "DHA"=docosahexaenoic acid (22:6n-3); "*Σ* EPA+DPA"=summation of EPA and docosapentaenoic acid (22:5n-3); "SFA"=saturated fatty acids; "MUFA"=monounsaturated fatty acids; "PUFA"=polyunsaturated fatty acids. For each fatty acid identified, values that do not share a common letter are significantly different ($p<0.05$).

Table 14: Change in fatty acids in PE of MDA-MB-231 cells incubated with control or 200 μM n-3 LCPUFA treatments

Fatty Acid	80 μM OA/LA	DHA	EPA	1:1 DHA:EPA	2:1 DHA:EPA	280 μM OA/LA
14:0	1.47±0.98^{a}	2.65±1.84^{a}	1.50±1.03^{a}	2.80±2.04^{a}	0.54±0.11^{a}	0.87±0.32^{a}
16:0	13.07±1.69^{a}	15.31±0.75^{a}	13.21±0.35^{a}	15.63±1.79^{a}	16.11±1.49^{a}	11.64±0.32^{a}
16:1n-9	0.66±0.16^{a}	0.99±0.16^{a}	1.02±0.06^{a}	1.13±0.38^{a}	1.77±1.08^{a}	0.63±0.11^{a}
17:0	0.87±0.06^{ab}	1.40±0.22^{a}	0.90±0.03^{ab}	1.38±0.10^{a}	0.88±0.03^{ab}	0.79±0.09^{ab}
18:0	25.96±1.17^{bc}	30.69±1.21^{ab}	31.60±0.59^{a}	31.98±1.22^{a}	31.34±0.90^{a}	22.69±0.45^{c}
18:1n-9	28.53±0.88^{ab}	19.06±0.68^{c}	22.99±1.15^{bc}	19.90±1.38^{c}	20.25±1.65^{c}	34.32±0.52^{a}
18:2n-6	14.86±0.55^{b}	8.11±0.43^{c}	9.44±0.63^{c}	7.67±0.93^{c}	7.90±0.49^{c}	18.65±0.34^{a}
18:3n-6	0.21±0.09^{a}	0.60±0.33^{a}	0.26±0.15^{a}	0.95±0.77^{a}	0.66±0.54^{a}	0.16±0.07^{a}
18:3n-3	0.35±0.13^{a}	1.20±0.43^{a}	0.51±0.22^{a}	1.02±0.70^{a}	0.37±0.15^{a}	0.25±0.01^{a}
20:2n-6	2.78±0.19^{b}	1.03±0.09^{c}	1.15±0.05^{c}	1.09±0.14^{c}	1.23±0.16^{c}	3.50±0.08^{a}
20:3n-6	1.57±0.10^{a}	0.86±0.11^{ab}	0.49±0.10^{b}	0.80±0.28^{b}	0.47±0.09^{b}	0.88±0.04^{ab}
20:4n-6	4.49±0.43^{a}	1.37±0.11^{b}	1.06±0.04^{b}	0.99±0.06^{b}	1.04±0.18^{b}	2.11±0.22^{b}
20:5n-3	0.31±0.13^{c}	0.67±0.26^{c}	6.48±0.80^{a}	3.60±0.45^{b}	3.12±0.38^{b}	0.30±0.04^{c}
24:0	3.06±0.41^{a}	0.96±0.18^{bc}	0.65±0.10^{c}	0.82±0.21^{bc}	0.85±0.11^{bc}	1.80±0.12^{b}
24:1n9	0.27±0.12^{a}	1.13±0.60^{a}	1.68±1.42^{a}	1.27±0.97^{a}	0.36±0.12^{a}	0.36±0.25^{a}
22:4n-6	0.36±0.04^{a}	1.36±0.83^{a}	0.29±0.19^{a}	0.56±0.29^{a}	0.48±0.30^{a}	0.18±0.06^{a}
22:5n-6	0.34±0.09^{a}	0.97±0.36^{a}	0.46±0.18^{a}	0.57±0.25^{a}	0.37±0.10^{a}	0.22±0.05^{a}
22:5n-3	0.43±0.09^{bc}	1.11±0.54^{bc}	5.64±0.88^{a}	2.82±0.40^{b}	2.58±0.27^{bc}	0.35±0.04^{c}
22:6n-3	0.40±0.00^{c}	10.54±1.86^{a}	0.68±0.20^{c}	5.03±1.12^{bc}	9.67±1.22^{ab}	0.31±0.11^{c}
ΣEPA+DPA	0.74±0.17^{d}	1.79±0.79^{cd}	12.12±1.67^{a}	6.42±0.84^{b}	5.69±0.61^{bc}	0.65±0.07^{d}
ΣSFA	44.43±1.00^{b}	51.02±0.54^{a}	47.85±1.18^{ab}	52.60±1.31^{a}	49.73±1.92^{ab}	37.79±0.56^{c}
ΣMUFA	29.45±0.60^{b}	21.17±0.14^{d}	25.69±0.41^{c}	22.31±1.26^{d}	22.38±0.52^{cd}	35.32±0.28^{a}
ΣPUFA	26.11±1.26^{a}	27.81±0.43^{a}	26.45±1.45^{a}	25.09±0.13^{a}	27.89±1.91^{a}	26.90±0.32^{a}
Σn-3	1.50±0.30^{b}	13.52±0.77^{a}	13.30±1.51^{a}	12.47±1.30^{a}	15.74±1.76^{a}	1.20±0.05^{b}
Σn-6	24.62±0.97^{a}	14.29±0.85^{b}	13.15±0.31^{b}	12.61±1.27^{b}	12.15±0.53^{b}	25.70±0.32^{a}

Values are mean percent composition ± SEM (n=3). "OA/LA"=oleic acid (18:1n-9)/linoleic acid (18:2n-6);"EPA=eicosapentaenoic acid (20:5n-3); "DHA"=docosahexaenoic acid (22:6n-3); "*Σ* EPA+DPA"=summation of EPA and docosapentaenoic acid (22:5n-3); "SFA"=saturated fatty acids; "MUFA"=monounsaturated fatty acids; "PUFA"=polyunsaturated fatty acids. For each fatty acid identified, values that do not share a common letter are significantly different ($p<0.05$).

Table 15: Change in fatty acids in PI of MDA-MB-231 cells incubated with control or 200 μM n-3 LCPUFA treatments

Fatty Acid	80 μM OA/LA	DHA	EPA	1:1 DHA:EPA	2:1 DHA:EPA	280 μM OA/LA
14:0	1.37±0.60^{a}	0.74±0.22^{a}	3.05±2.48^{a}	2.02±1.58^{a}	0.62±0.05^{a}	0.83±0.16^{a}
16:0	8.40±1.11^{a}	11.03±1.73^{a}	7.96±1.18^{a}	8.23±0.84^{a}	8.26±0.63^{a}	6.67±0.67^{a}
16:1n-9	1.76±1.03^{a}	0.68±0.15^{a}	1.58±1.11^{a}	2.66±2.08^{a}	0.45±0.03^{a}	0.40±0.04^{a}
17:0	2.11±1.39^{a}	0.64±0.02^{a}	1.24±0.48^{a}	1.00±0.23^{a}	0.72±0.02^{a}	0.35±0.01^{a}
18:0	39.61±2.84^{a}	52.97±0.45^{a}	45.60±6.16^{a}	49.72±3.03^{a}	52.72±0.87^{a}	43.66±0.22^{a}
18:1n-9	14.34±1.46^{ab}	13.29±0.61^{ab}	10.85±1.76^{b}	11.01±1.05^{b}	12.70±0.17^{ab}	18.03±0.46^{a}
18:2n-6	11.57±0.97^{b}	12.28±0.45^{b}	7.49±1.07^{c}	9.20±1.06^{bc}	10.66±0.28^{bc}	17.10±0.08^{a}
18:3n-6	1.65±1.32^{a}	0.19±0.02^{a}	2.86±2.70^{a}	0.50±0.36^{a}	0.21±0.03^{a}	0.14±0.01^{a}
18:3n-3	0.60±0.20^{a}	0.40±0.10^{a}	1.76±1.38^{a}	0.67±0.34^{a}	0.34±0.02^{a}	0.24±0.05^{a}
20:2n-6	2.43±0.28^{a}	0.68±0.03^{b}	1.08±0.66^{ab}	0.69±0.16^{b}	0.61±0.02^{b}	2.17±0.12^{ab}
20:3n-6	5.20±0.52^{a}	2.18±0.09^{c}	1.37±0.27^{c}	1.15±0.10^{c}	1.66±0.11^{c}	3.67±0.14^{b}
20:4n-6	6.31±0.26^{a}	3.03±0.02^{bc}	2.45±0.26^{c}	2.73±0.36^{c}	3.20±0.23^{bc}	4.16±0.33^{b}
20:5n-3	0.41±0.13^{d}	0.53±0.08^{d}	8.85±2.45^{a}	7.21±0.88^{b}	5.55±0.33^{c}	0.18±0.03^{d}
24:0	1.51±0.20^{a}	0.29±0.07^{a}	0.86±0.72^{a}	1.03±0.80^{a}	0.49±0.09^{a}	0.80±0.19^{a}
24:1n9	1.43±0.45^{a}	0.33±0.06^{a}	1.01±0.82^{a}	0.74±0.41^{a}	0.66±0.23^{a}	0.65±0.08^{a}
22:4n-6	0.68±0.16^{a}	0.26±0.05^{a}	1.07±0.86^{a}	0.60±0.38^{a}	0.59±0.26^{a}	0.46±0.12^{a}
22:5n-6	0.62±0.02^{a}	0.51±0.21^{a}	0.93±0.60^{a}	0.85±0.24^{a}	0.56±0.09^{a}	0.48±0.18^{a}
22:5n-3	0.41±0.07^{b}	0.45±0.03^{b}	3.62±0.97^{a}	3.34±0.55^{a}	2.88±0.18^{a}	0.24±0.06^{b}
22:6n-3	0.32±0.08^{c}	7.73±0.76^{a}	1.51±1.05^{bc}	2.58±0.72^{bc}	4.60±0.37^{ab}	0.14±0.01^{c}
ΣEPA+DPA	0.81±0.18^{b}	0.97±0.10^{b}	12.47±3.42^{a}	10.55±1.43^{a}	8.42±0.51^{ab}	0.42±0.06^{b}
ΣSFA	53.00±0.79^{b}	65.67±1.08^{a}	58.71±3.10^{ab}	62.00±0.79^{a}	62.81±0.39^{a}	52.31±0.39^{b}
ΣMUFA	17.53±0.51^{ab}	14.30±0.79^{b}	13.44±0.55^{b}	14.41±1.51^{b}	13.81±0.09^{b}	19.09±0.51^{a}
ΣPUFA	30.20±0.53^{a}	28.21±1.00^{a}	32.98±3.01^{a}	29.51±2.16^{a}	30.86±0.91^{a}	28.99±0.47^{a}
Σn-3	1.74±0.46^{b}	9.10±0.86^{a}	15.74±1.19^{a}	13.80±1.81^{a}	13.37±0.87^{a}	0.80±0.10^{b}
Σn-6	28.46±0.14^{a}	19.11±0.38^{b}	17.24±3.88^{b}	15.72±0.36^{b}	17.49±0.22^{b}	28.19±0.40^{a}

Values are mean percent composition ± SEM (n=3). "OA/LA"=oleic acid (18:1n-9)/linoleic acid (18:2n-6);"EPA=eicosapentaenoic acid (20:5n-3); "DHA"=docosahexaenoic acid (22:6n-3); "*Σ* EPA+DPA"=summation of EPA and docosapentaenoic acid (22:5n-3); "SFA"=saturated fatty acids; "MUFA"=monounsaturated fatty acids; "PUFA"=polyunsaturated fatty acids. For each fatty acid identified, values that do not share a common letter are significantly different ($p<0.05$).

Table 16: Change in fatty acids in PC of SK-BR-3 cells incubated with control or 100 μM n-3 LCPUFA treatments

Fatty Acid	80 μM OA/LA	DHA	EPA	1:1 DHA:EPA	2:1 DHA:EPA	180 μM OA/LA
14:0	2.04±0.40^{a}	1.29±0.21^{a}	1.38±0.23^{a}	1.16±0.32^{a}	1.58±0.30^{a}	1.44±0.25^{a}
16:0	31.88±3.96^{a}	34.53±1.94^{a}	30.47±2.20^{a}	30.61±2.28^{a}	31.97±2.66^{a}	30.74±2.46^{a}
16:1n9	3.92±0.14^{a}	2.37±0.07^{c}	4.12±0.08^{a}	3.51±0.29^{ab}	3.31±0.03^{ab}	2.88±0.08^{bc}
17:0	0.49±0.06^{a}	0.53±0.01^{a}	0.52±0.02^{a}	0.62±0.06^{a}	0.49±0.00^{a}	0.47±0.01^{a}
18:0	11.11±0.55^{ab}	11.70±0.35^{ab}	12.00±0.25^{a}	11.29±0.38^{ab}	11.07±0.33^{ab}	10.10±0.40^{b}
18:1n9	27.03±1.51^{a}	21.66±0.73^{b}	26.37±0.56^{a}	24.97±0.41^{ab}	24.79±0.48^{ab}	28.11±0.54^{a}
18:2n-6	14.59±2.73^{a}	16.06±1.01^{a}	17.13±1.25^{a}	16.44±0.86^{a}	17.02±1.50^{a}	18.25±1.66^{a}
18:3n-6	0.62±0.10^{ab}	0.34±0.05^{b}	0.46±0.04^{ab}	0.57±0.10^{ab}	0.41±0.03^{ab}	0.73±0.11^{a}
18:3n-3	0.75±0.09^{a}	0.37±0.02^{b}	0.33±0.03^{b}	0.66±0.14^{ab}	0.34±0.01^{b}	0.54±0.02^{b}
20:2n-6	1.23±0.18^{ab}	0.87±0.03^{bc}	0.65±0.03^{c}	0.78±0.12^{c}	0.73±0.01^{c}	1.33±0.09^{a}
20:3n-6	2.20±0.23^{a}	1.54±0.10^{bc}	0.82±0.03^{d}	0.98±0.11^{cd}	1.08±0.14^{cd}	1.79±0.04^{ab}
20:4n-6	2.52±0.75^{a}	1.32±0.10^{ab}	0.96±0.07^{b}	1.11±0.11^{b}	1.18±0.15^{ab}	2.09±0.31^{ab}
20:5n-3	0.14±0.11^{c}	0.13±0.01^{c}	2.30±0.30^{a}	1.78±0.22^{ab}	1.20±0.09^{b}	0.12±0.03^{c}
24:0	0.29±0.08^{a}	0.21±0.07^{a}	0.18±0.01^{a}	0.58±0.24^{a}	0.17±0.02^{a}	0.27±0.07^{a}
24:1n9	0.38±0.14^{a}	0.19±0.05^{a}	0.21±0.05^{a}	0.53±0.17^{a}	0.18±0.04^{a}	0.33±0.04^{a}
22:4n-6	0.40±0.23^{a}	0.28±0.12^{a}	0.39±0.11^{a}	0.67±0.29^{a}	0.40±0.14^{a}	0.36±0.16^{a}
22:5n-6	0.15±0.07^{a}	0.18±0.01^{a}	0.23±0.08^{a}	0.49±0.13^{a}	0.22±0.09^{a}	0.19±0.01^{a}
22:5n-3	0.14±0.03^{c}	0.16±0.02^{c}	1.26±0.10^{a}	1.16±0.12^{a}	0.73±0.05^{b}	0.16±0.03^{c}
22:6n-3	0.14±0.01^{d}	6.29±0.73^{a}	0.23±0.03^{cd}	2.05±0.24^{bc}	3.13±0.39^{b}	0.11±0.03^{d}
ΣEPA+DPA	0.30±0.12^{c}	0.29±0.02^{c}	3.56±0.40^{a}	2.94±0.31^{ab}	1.93±0.14^{b}	0.28±0.05^{c}
ΣSFA	45.80±3.06^{a}	48.26±2.43^{a}	44.55±2.33^{a}	44.27±2.59^{a}	45.28±2.65^{a}	43.01±3.01^{a}
ΣMUFA	31.33±0.78^{a}	24.22±0.85^{b}	30.70±0.55^{a}	29.00±0.73^{a}	28.28±0.50^{ab}	31.32±0.57^{a}
ΣPUFA	22.87±2.29^{a}	27.52±1.78^{a}	24.75±1.78^{a}	26.71±2.03^{a}	26.44±2.15^{a}	25.67±2.47^{a}
Σn-3	1.16±0.07^{b}	6.94±0.73^{a}	4.12±0.47^{a}	5.66±0.68^{a}	5.40±0.55^{a}	0.93±0.10^{b}
Σn-6	21.70±2.27^{a}	20.58±1.24^{a}	20.63±1.35^{a}	21.05±1.36^{a}	21.05±1.60^{a}	24.74±2.37^{a}

Values are mean percent composition ± SEM (n=2-3). "OA/LA"=oleic acid (18:1n-9)/linoleic acid (18:2n-6);"EPA=eicosapentaenoic acid (20:5n-3); "DHA"=docosahexaenoic acid (22:6n-3); "*Σ* EPA+DPA"=summation of EPA and docosapentaenoic acid (22:5n-3); "SFA"=saturated fatty acids; "MUFA"=monounsaturated fatty acids; "PUFA"=polyunsaturated fatty acids. For each fatty acid identified, values that do not share a common letter are significantly different ($p<0.05$).

Table 17: Change in fatty acids in PE of SK-BR-3 cells incubated with control or 100 μM n-3 LCPUFA treatments

Fatty Acid	80 μM OA/LA	DHA	EPA	1:1 DHA:EPA	2:1 DHA:EPA	180 μM OA/LA
14:0	0.21±0.08^{a}	0.35±0.25^{a}	0.33±0.18^{a}	0.23±0.09^{a}	0.23±0.00^{a}	0.55±0.32^{a}
16:0	11.40±1.96^{a}	10.33±1.62^{a}	10.97±0.83^{a}	11.63±2.36^{a}	12.53±2.86^{a}	12.64±2.63^{a}
16:1n-9	1.28±0.13^{a}	1.04±0.38^{a}	1.61±0.30^{a}	1.17±0.08^{a}	1.32±0.06^{a}	1.38±0.43^{a}
17:0	0.58±0.07^{a}	0.68±0.18^{a}	0.81±0.14^{a}	0.67±0.06^{a}	0.71±0.12^{a}	0.70±0.15^{a}
18:0	26.08±0.31^{ab}	27.41±1.09^{a}	25.43±0.95^{ab}	25.93±0.76^{ab}	27.48±0.24^{ab}	22.69±0.65^{b}
18:1n-9	28.81±0.60^{a}	21.57±1.23^{b}	25.10±0.78^{ab}	23.15±2.06^{ab}	23.05±1.19^{ab}	28.72±0.97^{a}
18:2n-6	9.76±0.74^{a}	8.74±0.16^{a}	9.50±0.06^{a}	9.04±1.12^{a}	8.21±0.62^{a}	11.02±0.41^{a}
18:3n-6	0.36±0.03^{a}	0.41±0.21^{a}	0.33±0.18^{a}	0.31±0.07^{a}	0.22±0.06^{a}	0.56±0.18^{a}
18:3n-3	0.77±0.02^{a}	0.65±0.19^{a}	0.52±0.15^{a}	0.43±0.05^{a}	0.40±0.05^{a}	0.77±0.09^{a}
20:2n-6	1.11±0.01^{b}	1.05±0.08^{b}	0.83±0.04^{b}	0.82±0.01^{b}	0.79±0.03^{b}	1.88±0.08^{a}
20:3n-6	2.81±0.09^{a}	2.14±0.01^{b}	1.14±0.02^{c}	1.37±0.20^{c}	1.38±0.13^{c}	2.32±0.06^{ab}
20:4n-6	9.41±0.38^{a}	6.07±0.37^{b}	3.89±0.22^{c}	4.77±0.05^{bc}	4.74±0.31^{bc}	7.99±0.21^{a}
20:5n-3	0.13±0.01^{c}	0.43±0.14^{c}	6.96±0.98^{a}	4.87±0.39^{ab}	3.40±0.02^{b}	0.37±0.21^{c}
24:0	0.19±0.01^{a}	0.53±0.35^{a}	0.47±0.29^{a}	0.26±0.05^{a}	0.23±0.00^{a}	0.16±0.01^{a}
24:1n9	4.77±0.11^{a}	0.73±0.14^{b}	0.92±0.05^{b}	0.39±0.04^{b}	0.58±0.20^{b}	4.85±0.12^{a}
22:4n-6	0.26±0.01^{b}	0.25±0.03^{b}	0.77±0.37^{ab}	1.26±0.22^{a}	1.05±0.01^{ab}	0.44±0.05^{ab}
22:5n-6	0.22±0.07^{a}	0.58±0.34^{a}	0.41±0.11^{a}	0.42±0.01^{a}	0.32±0.02^{a}	0.37±0.02^{a}
22:5n-3	1.29±0.01^{c}	0.67±0.07^{c}	9.56±1.58^{a}	5.25±0.69^{b}	3.33±0.09^{bc}	1.48±0.05^{c}
22:6n-3	0.57±0.02^{c}	16.33±2.54^{a}	0.45±0.14^{c}	8.05±1.25^{b}	10.03±0.47^{b}	0.51±0.06^{c}
ΣEPA+DPA	1.42±0.01^{c}	1.11±0.20^{c}	16.52±2.56^{a}	10.12±1.08^{b}	6.74±0.07^{bc}	1.85±0.26^{bc}
ΣSFA	38.46±1.70^{a}	39.31±0.24^{a}	38.01±0.72^{a}	38.72±1.40^{a}	41.18±2.81^{a}	36.75±2.56^{a}
ΣMUFA	34.85±0.60^{a}	23.35±1.46^{b}	27.62±1.03^{b}	24.71±2.10^{b}	24.94±1.05^{b}	34.94±1.12^{a}
ΣPUFA	26.70±1.16^{b}	37.34±1.70^{a}	34.37±1.75^{ab}	36.60±0.72^{a}	33.88±1.76^{ab}	27.72±1.14^{b}
Σn-3	2.76±0.04^{b}	18.09±2.15^{a}	17.48±2.27^{ab}	18.61±2.27^{a}	17.17±0.59^{ab}	3.13±0.29^{b}
Σn-6	23.94±1.15^{a}	19.25±0.45^{a}	16.88±0.52^{a}	17.99±1.55^{a}	16.71±1.17^{a}	24.59±0.87^{a}

Values are mean percent composition ± SEM (n=2-3). "OA/LA"=oleic acid (18:1n-9)/linoleic acid (18:2n-6);"EPA=eicosapentaenoic acid (20:5n-3); "DHA"=docosahexaenoic acid (22:6n-3); "*Σ* EPA+DPA"=summation of EPA and docosapentaenoic acid (22:5n-3); "SFA"=saturated fatty acids; "MUFA"=monounsaturated fatty acids; "PUFA"=polyunsaturated fatty acids. For each fatty acid identified, values that do not share a common letter are significantly different ($p<0.05$).

Table 18: Change in fatty acids in PI of SK-BR-3 cells incubated with control or 100 μM n-3 LCPUFA treatments

Fatty Acid	80 μM OA/LA	DHA	EPA	1:1 DHA:EPA	2:1 DHA:EPA	180 μM OA/LA
14:0	0.31±0.06^{a}	0.47±0.11^{a}	0.52±0.02^{a}	0.66±0.07^{a}	0.58±0.22^{a}	0.53±0.18^{a}
16:0	13.41±2.82^{a}	17.29±2.65^{a}	18.24±1.50^{a}	18.81±2.45^{a}	14.06±1.65^{a}	17.46±2.68^{a}
16:1n-9	1.20±0.14^{a}	1.26±0.06^{a}	1.89±0.34^{a}	2.19±0.31^{a}	1.44±0.36^{a}	1.53±0.15^{a}
17:0	0.78±0.03^{a}	0.95±0.05^{a}	0.93±0.01^{a}	0.97±0.05^{a}	0.92±0.03^{a}	0.96±0.13^{a}
18:0	41.55±0.39^{a}	35.29±0.91^{b}	34.56±1.33^{b}	33.33±1.84^{b}	34.51±0.86^{b}	32.24±1.14^{b}
18:1n-9	16.74±0.85^{a}	18.61±1.16^{a}	18.86±1.48^{a}	17.90±0.81^{a}	20.30±0.78^{a}	20.90±1.51^{a}
18:2n-6	10.41±0.99^{a}	13.10±0.99^{a}	12.33±1.05^{a}	11.72±0.72^{a}	13.55±0.50^{a}	12.49±1.13^{a}
18:3n-6	0.32±0.08^{a}	0.39±0.05^{a}	0.51±0.18^{a}	0.67±0.13^{a}	0.56±0.15^{a}	0.53±0.11^{a}
18:3n-3	0.43±0.05^{a}	0.53±0.08^{a}	0.51±0.17^{a}	0.74±0.14^{a}	0.56±0.10^{a}	0.60±0.03^{a}
20:2n-6	0.58±0.06^{a}	0.67±0.07^{a}	0.49±0.09^{a}	0.63±0.08^{a}	0.71±0.06^{a}	0.73±0.06^{a}
20:3n-6	4.35±0.43^{a}	2.97±0.25^{ab}	1.52±0.19^{b}	1.83±0.15^{b}	2.34±0.20^{b}	2.55±0.23^{b}
20:4n-6	7.23±1.15^{a}	3.26±0.38^{b}	3.12±0.54^{ab}	3.06±0.46^{b}	4.03±0.56^{ab}	4.44±0.59^{ab}
20:5n-3	0.30±0.08^{d}	0.37±0.05^{cd}	2.48±0.25^{a}	1.41±0.10^{b}	1.19±0.16^{bc}	0.53±0.21^{d}
24:0	0.26±0.05^{b}	0.38±0.06^{ab}	0.54±0.01^{ab}	0.71±0.05^{a}	0.58±0.15^{ab}	0.49±0.11^{ab}
24:1n9	0.57±0.15^{a}	0.37±0.04^{a}	0.54±0.01^{a}	0.68±0.10^{a}	0.60±0.22^{a}	0.57±0.05^{a}
22:4n-6	0.45±0.14^{a}	1.50±0.96^{a}	0.73±0.22^{a}	0.78±0.02^{a}	0.96±0.22^{a}	1.64±0.74^{a}
22:5n-6	0.41±0.03^{b}	0.52±0.07^{b}	0.68±0.19^{ab}	1.03±0.09^{a}	0.87±0.15^{ab}	0.52±0.01^{b}
22:5n-3	0.35±0.05^{b}	0.38±0.04^{b}	1.11±0.18^{a}	1.08±0.13^{a}	0.89±0.23^{ab}	0.56±0.07^{ab}
22:6n-3	0.35±0.14^{b}	1.68±0.31^{a}	0.43±0.07^{b}	0.75±0.05^{b}	1.34±0.22^{ab}	0.35±0.01^{b}
ΣEPA+DPA	0.65±0.11^{c}	0.74±0.10^{c}	3.59±0.43^{a}	2.49±0.18^{b}	2.07±0.39^{bc}	1.09±0.26^{c}
ΣSFA	56.32±3.05^{a}	54.38±1.87^{a}	54.80±2.86^{a}	54.49±0.57^{a}	50.66±1.13^{a}	51.68±2.80^{a}
ΣMUFA	18.51±0.94^{a}	20.24±1.10^{a}	21.29±1.15^{a}	20.77±0.58^{a}	22.35±0.20^{a}	22.99±1.46^{a}
ΣPUFA	25.17±2.12^{a}	25.38±0.84^{a}	23.91±1.72^{a}	23.45±1.04^{a}	27.00±0.93^{a}	24.95±1.15^{a}
Σn-3	1.43±0.18^{d}	2.96±0.17^{bc}	4.53±0.18^{a}	3.99±0.22^{b}	3.97±0.51^{ab}	2.04±0.29^{cd}
Σn-6	23.74±2.17^{a}	22.42±0.74^{a}	19.38±1.53^{a}	19.46±1.18^{a}	23.03±0.42^{a}	22.90±1.11^{a}

Values are mean percent composition ± SEM (n=2-3). "OA/LA"=oleic acid (18:1n-9)/linoleic acid (18:2n-6);"EPA=eicosapentaenoic acid (20:5n-3); "DHA"=docosahexaenoic acid (22:6n-3); "*Σ* EPA+DPA"=summation of EPA and docosapentaenoic acid (22:5n-3); "SFA"=saturated fatty acids; "MUFA"=monounsaturated fatty acids; "PUFA"=polyunsaturated fatty acids. For each fatty acid identified, values that do not share a common letter are significantly different ($p<0.05$).

Table 19: Change in fatty acids in PC of SK-BR-3 cells incubated with control or 200 μM n-3 LCPUFA treatments

Fatty Acid	80 μM OA/LA	DHA	EPA	1:1 DHA:EPA	2:1 DHA:EPA	280 μM OA/LA
14:0	2.17±0.44^{a}	1.42±0.39^{a}	2.01±0.29^{a}	2.07±0.68^{a}	1.67±0.89^{a}	1.23±0.01^{a}
16:0	29.82±0.49^{a}	34.81±1.48^{a}	30.59±1.69^{a}	32.28±2.42^{a}	32.17±2.97^{a}	27.08±0.16^{a}
16:1n-9	2.70±0.21^{bc}	1.63±0.11^{c}	3.72±0.03^{a}	3.00±0.15^{ab}	2.50±0.25^{bc}	1.78±0.03^{c}
17:0	0.53±0.05^{a}	0.48±0.04^{a}	0.52±0.05^{a}	0.46±0.02^{a}	0.48±0.01^{a}	0.38±0.03^{a}
18:0	12.27±1.95^{a}	12.72±3.38^{a}	10.69±0.11^{a}	10.22±0.36^{a}	10.69±0.07^{a}	8.69±1.21^{a}
18:1n-9	21.87±1.47^{ab}	15.21±1.27^{b}	21.26±0.58^{ab}	20.66±1.08^{ab}	20.48±1.59^{ab}	26.39±0.21^{a}
18:2n-6	19.60±1.50^{a}	17.16±1.39^{a}	21.10±1.71^{a}	19.57±1.91^{a}	18.61±2.15^{a}	26.22±0.67^{a}
18:3n-6	0.40±0.06^{a}	0.30±0.14^{a}	0.34±0.09^{a}	0.30±0.02^{a}	0.30±0.03^{a}	0.48±0.06^{a}
18:3n-3	0.72±0.02^{a}	0.25±0.01^{c}	0.33±0.04^{c}	0.29±0.00^{c}	0.30±0.00^{c}	0.51±0.00^{b}
20:2n-6	2.15±0.20^{a}	0.81±0.08^{b}	0.72±0.04^{b}	0.76±0.10^{b}	0.78±0.12^{b}	2.26±0.01^{a}
20:3n-6	3.21±0.06^{a}	1.35±0.12^{c}	0.64±0.04^{d}	0.84±0.10^{cd}	1.06±0.22^{cd}	2.15±0.12^{b}
20:4n-6	2.87±0.11^{a}	0.78±0.07^{b}	0.57±0.03^{b}	0.70±0.14^{b}	0.79±0.18^{b}	1.56±0.03^{a}
20:5n-3	0.02±0.01^{c}	0.14±0.03^{bc}	4.20±1.02^{a}	2.68±0.72^{ab}	1.95±0.53^{abc}	0.02±0.01^{bc}
24:0	0.28±0.12^{a}	0.24±0.13^{a}	0.19±0.15^{a}	0.22±0.11^{a}	0.18±0.10^{a}	0.16±0.01^{a}
24:1n9	0.78±0.01^{a}	0.13±0.02^{b}	0.25±0.14^{b}	0.12±0.02^{b}	0.11±0.02^{b}	0.55±0.02^{a}
22:4n-6	0.12±0.05^{a}	0.20±0.07^{a}	0.30±0.03^{a}	0.13±0.02^{a}	0.18±0.13^{a}	0.25±0.04^{a}
22:5n-6	0.18±0.05^{a}	0.14±0.08^{a}	0.18±0.15^{a}	0.14±0.10^{a}	0.15±0.10^{a}	0.11±0.00^{a}
22:5n-3	0.15±0.01^{b}	0.12±0.02^{b}	2.10±0.57^{a}	1.64±0.48^{ab}	1.25±0.38^{ab}	0.10±0.01^{b}
22:6n-3	0.13±0.00^{b}	12.09±1.86^{a}	0.29±0.11^{b}	3.94±1.46^{ab}	6.34±2.11^{ab}	0.08±0.00^{b}
ΣEPA+DPA	0.17±0.01	0.26±0.05	6.30±1.58	4.32±1.20	3.20±0.91	0.12±0.01
ΣSFA	45.06±1.36^{a}	49.68±4.21^{a}	44.00±2.29^{a}	45.25±3.59^{a}	45.19±3.90^{a}	37.54±1.09^{a}
ΣMUFA	25.36±1.67^{a}	16.97±1.36^{b}	25.22±0.69^{ab}	23.77±1.21^{ab}	23.10±1.81^{ab}	28.72±0.22^{a}
ΣPUFA	29.51±1.66^{a}	33.36±3.46^{a}	30.77±2.98^{a}	30.97±4.80^{a}	31.72±5.71^{a}	33.74±0.87^{a}
Σn-3	0.98±0.03^{a}	12.60±1.91^{a}	6.92±1.43^{ab}	8.54±2.65^{ab}	9.84±3.03^{ab}	0.71±0.00^{b}
Σn-6	28.53±1.69^{a}	20.76±1.59^{a}	23.85±1.55^{a}	22.43±2.15^{a}	21.87±2.68^{a}	33.03±0.86^{a}

Values are mean percent composition ± SEM (n=2-3). "OA/LA"=oleic acid (18:1n-9)/linoleic acid (18:2n-6);"EPA=eicosapentaenoic acid (20:5n-3); "DHA"=docosahexaenoic acid (22:6n-3); "*Σ* EPA+DPA"=summation of EPA and docosapentaenoic acid (22:5n-3); "SFA"=saturated fatty acids; "MUFA"=monounsaturated fatty acids; "PUFA"=polyunsaturated fatty acids. For each fatty acid identified, values that do not share a common letter are significantly different ($p<0.05$).

Table 20: Change in fatty acids in PE of SK-BR-3 cells incubated with control or 200 μM n-3 LCPUFA treatments

Fatty Acid	80 μM OA/LA	DHA	EPA	1:1 DHA:EPA	2:1 DHA:EPA	280 μM OA/LA
14:0	0.15±0.05^{a}	0.31±0.16^{a}	0.16±0.09^{a}	0.51±0.47^{a}	0.28±0.21^{a}	0.22±0.07^{a}
16:0	9.49±1.88^{a}	16.07±2.63^{a}	14.71±2.82^{a}	12.69±1.24^{a}	13.47±0.87^{a}	15.06±1.08^{a}
16:1n-9	0.85±0.11^{a}	0.66±0.10^{a}	0.94±0.11^{a}	0.77±0.17^{a}	0.67±0.03^{a}	0.68±0.09^{a}
17:0	0.42±0.03^{a}	0.55±0.05^{a}	0.80±0.28^{a}	1.09±0.65^{a}	0.61±0.07^{a}	0.46±0.04^{a}
18:0	25.24±0.97^{b}	26.28±0.56^{ab}	25.57±0.55^{ab}	24.40±1.03^{b}	32.44±2.03^{a}	22.39±1.65^{b}
18:1n-9	22.93±0.79^{ab}	16.14±0.64^{b}	20.54±0.49^{ab}	18.99±1.21^{b}	15.82±3.09^{b}	27.20±1.26^{a}
18:2n-6	12.59±0.67^{a}	9.48±0.77^{a}	11.27±0.86^{a}	9.96±0.41^{a}	11.45±1.81^{a}	14.20±1.12^{a}
18:3n-6	0.26±0.04^{a}	0.15±0.03^{a}	0.14±0.03^{a}	0.13±0.01^{a}	0.18±0.02^{a}	0.23±0.02^{a}
18:3n-3	0.67±0.01^{a}	0.40±0.03^{b}	0.37±0.02^{b}	0.37±0.02^{b}	0.42±0.02^{b}	0.72±0.06^{a}
20:2n-6	1.64±0.17^{b}	0.95±0.10^{b}	0.84±0.10^{b}	0.91±0.09^{b}	0.62±0.29^{b}	2.95±0.13^{a}
20:3n-6	4.39±0.35^{a}	1.90±0.17^{bc}	0.92±0.06^{c}	1.27±0.12^{c}	1.54±0.11^{bc}	2.67±0.15^{b}
20:4n-6	11.36±1.19^{a}	3.97±0.38^{b}	2.40±0.21^{b}	3.34±0.31^{b}	2.80±0.62^{b}	5.94±0.32^{b}
20:5n-3	0.07±0.01^{c}	0.37±0.06^{c}	8.69±0.81^{a}	6.71±0.47^{a}	3.53±0.64^{b}	0.09±0.03^{c}
24:0	0.21±0.01^{a}	0.19±0.02^{a}	0.07±0.00^{a}	0.11±0.00^{a}	0.23±0.12^{a}	0.25±0.07^{a}
24:1n9	7.06±0.93^{a}	1.12±0.14^{b}	0.83±0.04^{b}	0.94±0.01^{b}	0.49±0.33^{b}	4.81±0.50^{a}
22:4n-6	0.26±0.07^{a}	0.43±0.10^{a}	0.33±0.23^{a}	0.27±0.17^{a}	0.79±0.26^{a}	0.56±0.05^{a}
22:5n-6	0.17±0.06^{a}	0.29±0.23^{a}	0.21±0.12^{a}	0.35±0.28^{a}	0.13±0.06^{a}	0.19±0.08^{a}
22:5n-3	1.44±0.19^{d}	0.54±0.08^{d}	10.71±1.11^{a}	7.12±0.60^{b}	3.64±0.48^{c}	0.95±0.06^{d}
22:6n-3	0.80±0.07^{c}	18.25±0.22^{a}	0.49±0.06^{c}	10.05±0.81^{b}	10.72±0.33^{b}	0.42±0.06^{c}
ΣEPA+DPA	1.52±0.18^{d}	0.91±0.12^{d}	19.40±1.92^{a}	13.84±1.06^{b}	7.17±1.12^{c}	1.04±0.04^{d}
ΣSFA	35.51±2.53^{a}	43.41±3.38^{a}	41.32±2.64^{a}	38.81±1.32^{a}	47.04±3.30^{a}	38.38±0.77^{a}
ΣMUFA	30.83±1.02^{a}	17.93±0.54^{b}	22.31±0.56^{b}	20.70±1.38^{b}	16.98±3.38^{b}	32.70±1.22^{a}
ΣPUFA	33.66±1.99^{a}	30.65±5.01^{a}	36.37±3.21^{a}	40.49±2.70^{a}	35.82±0.09^{a}	28.92±0.63^{b}
Σn-3	2.99±0.25^{b}	19.49±0.42^{a}	20.25±1.88^{a}	24.26±1.89^{a}	18.31±1.43^{a}	2.18±0.14^{b}
Σn-6	30.67±1.74^{a}	17.18±1.18^{b}	16.12±1.33^{b}	16.23±0.81^{b}	17.51±1.35^{b}	26.74±0.68^{a}

Values are mean percent composition ± SEM (n=2-3). "OA/LA"=oleic acid (18:1n-9)/linoleic acid (18:2n-6);"EPA=eicosapentaenoic acid (20:5n-3); "DHA"=docosahexaenoic acid (22:6n-3); "*Σ* EPA+DPA"=summation of EPA and docosapentaenoic acid (22:5n-3); "SFA"=saturated fatty acids; "MUFA"=monounsaturated fatty acids; "PUFA"=polyunsaturated fatty acids. For each fatty acid identified, values that do not share a common letter are significantly different ($p<0.05$).

Table 21: Change in fatty acids in PI of SK-BR-3 cells incubated with control or 200 μM n-3 LCPUFA treatments

Fatty Acid	80 μM OA/LA	DHA	EPA	1:1 DHA:EPA	2:1 DHA:EPA	280 μM OA/LA
14:0	0.20±0.10^{a}	0.17±0.07^{a}	0.42±0.30^{a}	0.63±0.39^{a}	0.47±0.33^{a}	0.24±0.12^{a}
16:0	12.43±0.90^{a}	20.22±2.98^{a}	24.14±1.76^{a}	22.17±6.53^{a}	26.71±1.79^{a}	19.08±0.82^{a}
16:1n-9	0.56±0.08^{a}	0.90±0.04^{a}	0.91±0.15^{a}	0.94±0.28^{a}	0.82±0.23^{a}	0.58±0.12^{a}
17:0	0.59±0.03^{a}	0.83±0.05^{a}	0.73±0.11^{a}	0.81±0.03^{a}	0.78±0.02^{a}	0.61±0.01^{a}
18:0	43.08±1.30^{a}	36.84±0.56^{ab}	31.69±0.52^{ab}	34.63±4.03^{ab}	34.61±3.00^{ab}	30.57±1.21^{b}
18:1n-9	12.03±1.02^{b}	13.82±0.88^{b}	18.45±2.56^{ab}	15.51±0.21^{ab}	16.51±1.43^{ab}	22.45±0.89^{a}
18:2n-6	13.03±0.62^{a}	16.41±1.04^{a}	12.75±0.23^{a}	13.48±1.88^{a}	13.70±1.09^{a}	16.04±0.41^{a}
18:3n-6	0.18±0.06^{a}	0.36±0.17^{a}	0.20±0.08^{a}	0.37±0.14^{a}	0.23±0.01^{a}	0.25±0.06^{a}
18:3n-3	0.25±0.01^{a}	0.30±0.02^{a}	0.41±0.12^{a}	0.36±0.03^{a}	0.28±0.05^{a}	0.44±0.02^{a}
20:2n-6	0.74±0.05^{ab}	0.48±0.06^{bc}	0.37±0.03^{c}	0.45±0.05^{c}	0.42±0.04^{c}	0.81±0.04^{a}
20:3n-6	5.54±0.23^{a}	2.99±0.31^{b}	1.07±0.18^{c}	1.54±0.19^{bc}	2.10±0.13^{bc}	2.67±0.33^{b}
20:4n-6	9.06±0.81^{a}	2.33±0.27^{b}	1.75±0.21^{b}	2.41±0.47^{b}	2.58±0.13^{b}	3.55±0.41^{b}
20:5n-3	0.14±0.04^{d}	0.12±0.02^{d}	3.43±0.07^{a}	2.28±0.13^{b}	1.61±0.15^{c}	0.12±0.00^{d}
24:0	0.16±0.07^{a}	0.16±0.01^{a}	0.22±0.08^{a}	0.36±0.12^{a}	0.17±0.03^{a}	0.12±0.01^{a}
24:1n9	0.69±0.06^{a}	0.14±0.03^{b}	0.29±0.04^{b}	0.48±0.08^{b}	0.23±0.04^{b}	0.45±0.04^{ab}
22:4n-6	0.67±0.30^{a}	1.56±0.05^{a}	0.92±0.52^{a}	1.49±0.09^{a}	1.05±0.70^{a}	1.10±0.50^{a}
22:5n-6	0.32±0.21^{a}	0.15±0.05^{a}	0.64±0.51^{a}	0.33±0.02^{a}	0.65±0.51^{a}	0.45±0.30^{a}
22:5n-3	0.19±0.01^{b}	0.14±0.01^{b}	1.36±0.07^{a}	1.02±0.19^{a}	0.90±0.12^{a}	0.20±0.04^{b}
22:6n-3	0.14±0.02^{c}	2.16±0.15^{a}	0.24±0.04^{c}	0.73±0.17^{bc}	1.07±0.19^{b}	0.13±0.01^{c}
ΣEPA+DPA	0.34±0.05^{c}	0.26±0.04^{c}	4.79±0.14^{a}	3.30±0.32^{b}	2.51±0.26^{b}	0.33±0.04^{c}
ΣSFA	56.45±1.18^{a}	58.22±2.55^{a}	57.20±1.74^{a}	58.61±3.03^{a}	62.73±4.40^{a}	50.63±1.10^{a}
ΣMUFA	13.28±1.12^{a}	14.86±0.81^{a}	19.65±2.46^{a}	16.93±0.01^{a}	17.56±1.62^{a}	23.48±1.01^{a}
ΣPUFA	30.27±1.53^{a}	27.01±1.83^{a}	23.15±0.72^{a}	24.46±3.02^{a}	24.58±2.09^{a}	25.73±0.43^{a}
Σn-3	0.73±0.08^{b}	2.73±0.09^{ab}	5.44±0.02^{a}	4.39±0.46^{ab}	3.86±0.50^{ab}	0.93±0.05^{ab}
Σn-6	29.54±1.56^{a}	24.28±1.74^{a}	17.71±0.73^{a}	20.07±2.56^{a}	20.71±1.59^{a}	24.88±0.37^{a}

Values are mean percent composition ± SEM (n=3-4). "OA/LA"=oleic acid (18:1n-9)/linoleic acid (18:2n-6);"EPA=eicosapentaenoic acid (20:5n-3); "DHA"=docosahexaenoic acid (22:6n-3); "*Σ* EPA+DPA"=summation of EPA and docosapentaenoic acid (22:5n-3); "SFA"=saturated fatty acids; "MUFA"=monounsaturated fatty acids; "PUFA"=polyunsaturated fatty acids. For each fatty acid identified, values that do not share a common letter are significantly different ($p<0.05$).

Table 22: Change in whole cell fatty acids of MDA-MB-231 Cells Grown in 3D with control or 150 μM n-3 LCPUFA treatments

Fatty Acid	80 μM OA/LA	DHA	EPA	1:1 DHA:EPA
14:0	1.22±0.25[a]	0.80±0.21[a]	0.89±0.23[a]	0.87±0.21[a]
16:0	18.88±1.84[a]	12.50±1.18[a]	12.96±2.04[a]	12.49±1.65[a]
16:1n-9	0.76±0.05[a]	0.42±0.08[a]	0.67±0.17[a]	0.54±0.08[a]
17:0	0.35±0.03[a]	0.38±0.07[a]	0.40±0.09[a]	0.40±0.08[a]
18:0	25.91±7.01[a]	12.01±0.26[a]	12.08±1.77[a]	11.40±1.47[a]
18:1n-9	19.77±3.93[a]	19.36±1.03[a]	17.03±0.53[a]	17.33±0.77[a]
18:2n-6	20.24±3.87[a]	18.23±1.28[a]	17.25±0.59[a]	18.26±0.62[a]
18:3n-6	0.31±0.05[a]	0.17±0.01[ab]	0.16±0.03[b]	0.13±0.01[b]
18:3n-3	1.20±0.71[a]	0.32±0.03[a]	0.19±0.02[a]	0.22±0.03[a]
20:2n-6	1.75±0.49[a]	0.80±0.19[a]	0.47±0.08[a]	0.57±0.08[a]
20:3n-6	1.83±0.38[a]	1.09±0.09[ab]	0.70±0.12[b]	0.87±0.14[ab]
20:4n-6	3.26±0.18[a]	1.35±0.30[b]	0.94±0.09[b]	1.14±0.06[b]
20:5n-3	0.15±0.03[c]	0.27±0.09[c]	20.71±0.89[a]	12.88±0.16[b]
24:0	0.39±0.11[a]	0.31±0.06[a]	0.91±0.60[a]	0.84±0.55[a]
24:1n-9	1.33±0.56[a]	0.55±0.07[a]	0.57±0.18[a]	0.50±0.07[a]
22:4n-6	0.98±0.44[a]	0.46±0.19[a]	1.01±0.67[a]	0.34±0.11[a]
22:5n-6	0.89±0.15[a]	0.40±0.22[a]	0.87±0.21[a]	0.57±0.17[a]
22:5n-3	0.43±0.12[b]	0.82±0.09[b]	11.55±2.31[a]	10.38±1.22[a]
22:6n-3	0.35±0.07[c]	29.72±4.17[a]	0.62±0.45[c]	10.23±2.53[b]
ΣEPA+DPA	0.58±0.09[c]	1.09±0.05[c]	32.25±2.20[a]	23.27±1.31[b]
ΣSFA	46.75±8.73[a]	25.99±1.34[a]	27.24±3.52[a]	26.00±2.77[a]
ΣMUFA	21.85±3.89[a]	20.33±0.90[a]	18.26±0.38[a]	18.37±0.76[a]
ΣPUFA	31.40±5.00[b]	53.64±2.13[a]	54.47±3.34[a]	55.60±2.36[a]
Σn-3	2.13±0.69[b]	31.13±4.19[a]	33.07±2.41[a]	33.72±2.04[a]
Σn-6	29.27±4.83[a]	22.51±2.13[a]	21.40±1.03[a]	21.88±0.81[a]

Values are mean percent composition ± SEM (n=3-5). "OA/LA"=oleic acid (18:1n-9)/linoleic acid (18:2n-6);"EPA=eicosapentaenoic acid (20:5n-3); "DHA"=docosahexaenoic acid (22:6n-3); "*Σ* EPA+DPA"=summation of EPA and docosapentaenoic acid (22:5n-3); "SFA"=saturated fatty acids; "MUFA"=monounsaturated fatty acids; "PUFA"=polyunsaturated fatty acids. For each fatty acid identified, values that do not share a common letter are significantly different ($p<0.05$).

Table 23: Change in whole cell fatty acids of SK-BR-3 cells grown in 3D with control or 150 μM n-3 LCPUFA treatments

Fatty Acid	80 μM OA/LA	DHA	EPA	1:1 DHA:EPA
14:0	1.97±0.46[a]	2.19±1.17[a]	1.83±0.68[a]	2.19±1.06[a]
16:0	24.15±1.28[a]	21.45±3.35[a]	20.50±2.42[a]	21.47±0.79[a]
16:1n-9	1.73±0.35[a]	1.23±0.35[a]	1.30±0.24[a]	1.40±0.27[a]
17:0	0.44±0.04[a]	0.50±0.05[a]	0.50±0.04[a]	0.50±0.04[a]
18:0	30.75±4.30[a]	19.30±2.96[a]	18.84±2.14[a]	18.55±0.65[a]
18:1n-9	16.43±2.38[a]	13.63±2.66[a]	16.03±1.58[a]	15.87±1.97[a]
18:2n-6	11.18±1.48[a]	13.47±2.68[a]	17.09±1.15[a]	16.93±0.56[a]
18:3n-6	0.91±0.19[a]	0.29±0.06[a]	0.36±0.04[a]	0.37±0.06[a]
18:3n-3	1.70±1.38[a]	0.26±0.07[a]	0.30±0.08[a]	0.31±0.08[a]
20:2n-6	1.47±0.85[a]	0.27±0.04[a]	0.23±0.02[a]	0.32±0.05[a]
20:3n-6	0.81±0.18[a]	5.26±4.40[a]	0.52±0.02[a]	0.72±0.11[a]
20:4n-6	4.11±0.79[a]	6.73±4.88[a]	3.90±1.79[a]	2.27±0.45[a]
20:5n-3	1.23±0.75[c]	0.20±0.07[c]	14.88±0.72[a]	7.23±1.08[b]
24:0	0.22±0.05[a]	0.30±0.07[a]	0.22±0.06[a]	0.24±0.07[a]
24:1n-9	0.93±0.30[a]	0.42±0.12[a]	0.45±0.07[a]	0.47±0.12[a]
22:4n-6	0.49±0.16[a]	0.41±0.20[a]	0.35±0.02[a]	0.47±0.18[a]
22:5n-6	1.00±0.25[a]	1.12±0.60[a]	1.19±0.45[a]	1.45±0.62[a]
22:5n-3	0.26±0.07[b]	0.25±0.07[b]	1.27±0.23[a]	0.91±0.12[a]
22:6n-3	0.21±0.08[b]	12.73±1.36[a]	0.25±0.06[b]	8.32±1.72[a]
ΣEPA+DPA	1.49±0.77[c]	0.45±0.14[c]	16.15±0.94[a]	8.14±1.02[b]
ΣSFA	57.54±4.50[a]	43.74±6.25[a]	41.90±4.92[a]	42.96±0.86[a]
ΣMUFA	19.09±2.95[a]	15.28±2.99[a]	17.78±1.69[a]	17.75±2.13[a]
ΣPUFA	23.38±2.04[b]	40.98±3.35[a]	40.32±3.23[a]	39.30±01.26[a]
Σn-3	3.40±1.30[b]	13.43±1.51[a]	16.69±0.96[a]	16.78±2.68[a]
Σn-6	19.98±2.50[a]	27.55±3.52[a]	23.63±2.89[a]	22.52±1.77[a]

Values are mean percent composition ± SEM (n=3). "OA/LA"=oleic acid (18:1n-9)/linoleic acid (18:2n-6);"EPA=eicosapentaenoic acid (20:5n-3); "DHA"=docosahexaenoic acid (22:6n-3); "*Σ* EPA+DPA"=summation of EPA and docosapentaenoic acid (22:5n-3); "SFA"=saturated fatty acids; "MUFA"=monounsaturated fatty acids; "PUFA"=polyunsaturated fatty acids. For each fatty acid identified, values that do not share a common letter are significantly different ($p<0.05$).