Combined Effects of N-3 POLYUNSATURATED FATTY ACIDS and 1ALPHA, 25-DIHYDROXYVITAMIN D on BREAST CANCER Cell Growth

by

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ABSTRACT

COMBINED EFFECTS OF N-3 POLYUNSATURATED FATTY ACIDS AND 1ALPHA,25-DIHYDROXYVITAMIN D ON BREAST CANCER CELL GROWTH

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Omega-3 polyunsaturated fatty acids (PUFA) and vitamin D both have anti-cancer effects through common and unique pathways. The hypothesis of this thesis is that the combination of n-3 PUFA with 1,25(OH)2D3 will inhibit breast cancer cell growth in an additive or synergistic manner. A 3X3 factorial design was used to test the combinations of five PUFA treatments (α-linoleic acid (ALA, 18:3n3), eicosapentaenoic acid (EPA, 20:5n3) and docosahexaenoic acid (DHA, 22:6n3), γ-linolenic acid (GLA, 18:3n6) and arachidonic acid (AA, 20:4n6)) with 1,25(OH)2D3 on MCF-7, MDA-MB-231, and MCF-10A cell growth, and determine any potential synergism in combination treatments. MCF-7 and MCF-10A cells responded to PUFA and 1,25(OH)2D3 treatments, but combinations provided no potential synergism. MDA-MB-231 growth was not affected by 1,25(OH)2D3, while combinations treatments involving ALA, EPA, GLA, and AA caused potentially synergistic growth inhibition. This thesis presents the novel observation that PUFA are sensitizing MDA-MB-231 cells to 1,25(OH)2D3 treatment.
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<tr>
<td>α-MEM</td>
<td>α-modified Eagle’s medium</td>
</tr>
<tr>
<td>AA</td>
<td>Arachidonic acid</td>
</tr>
<tr>
<td>ALA</td>
<td>Alpha-linolenic acid</td>
</tr>
<tr>
<td>AUC</td>
<td>Area under the curve</td>
</tr>
<tr>
<td>BF$_3$-MeOH</td>
<td>Boron trifluoride-methanol</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>Cdk</td>
<td>Cyclin-dependent kinase</td>
</tr>
<tr>
<td>COX</td>
<td>Cyclooxygenase</td>
</tr>
<tr>
<td>DHA</td>
<td>Docosahexaenoic acid</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>EPA</td>
<td>Eicosapentaenoic acid</td>
</tr>
<tr>
<td>ERα</td>
<td>Estrogen Receptor α</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>GC</td>
<td>Gas chromatography</td>
</tr>
<tr>
<td>GLA</td>
<td>Gamma-linolenic acid</td>
</tr>
<tr>
<td>HDL</td>
<td>High-density lipoprotein</td>
</tr>
<tr>
<td>HER2</td>
<td>Human epidermal growth factor receptor 2</td>
</tr>
<tr>
<td>IU</td>
<td>International units</td>
</tr>
<tr>
<td>KCl</td>
<td>Potassium chloride</td>
</tr>
<tr>
<td>KOH</td>
<td>Potassium hydroxide</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>LDL</td>
<td>Low-density lipoprotein</td>
</tr>
<tr>
<td>LOX</td>
<td>Lipooxygenase</td>
</tr>
<tr>
<td>LT</td>
<td>Leukotriene</td>
</tr>
<tr>
<td>LTB₅</td>
<td>Leukotriene B₅</td>
</tr>
<tr>
<td>LTE₄</td>
<td>Leukotriene E₄</td>
</tr>
<tr>
<td>LTE₅</td>
<td>Leukotriene E₅</td>
</tr>
<tr>
<td>MARRS</td>
<td>Membrane-associated rapid-response steroid-binding receptor</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>NSAID</td>
<td>Non-steroid anti-inflammatory drug</td>
</tr>
<tr>
<td>PARP</td>
<td>Poly(ADP-ribose) polymerase</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PG</td>
<td>Prostaglandin</td>
</tr>
<tr>
<td>PGE₂</td>
<td>Prostaglandin E₂</td>
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<tr>
<td>PPARγ</td>
<td>Peroxisome proliferator-activated receptor γ</td>
</tr>
<tr>
<td>pRb</td>
<td>Retinoblastoma protein</td>
</tr>
<tr>
<td>PR</td>
<td>Progestin receptor</td>
</tr>
<tr>
<td>PS</td>
<td>Phosphatidyl serine</td>
</tr>
<tr>
<td>PUFA</td>
<td>Polyunsaturated fatty acids</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RXR</td>
<td>Retinoid X receptor</td>
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<tr>
<td>SDC-1</td>
<td>Syndecan-1</td>
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<tr>
<td>SRB</td>
<td>Sulforhodamine-B</td>
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<tr>
<td>TBST</td>
<td>Tris-buffered saline with Tween</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>TCA</td>
<td>Trichloroacetic acid</td>
</tr>
<tr>
<td>TEB</td>
<td>Terminal end bud</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>TX</td>
<td>Thromboxane</td>
</tr>
<tr>
<td>VDR</td>
<td>Vitamin D receptor</td>
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<tr>
<td>VDRE</td>
<td>Vitamin D response Element</td>
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<tr>
<td>VITAL</td>
<td>VITamin D and OmegA-3 TriaL</td>
</tr>
<tr>
<td>z-VAD.fmk</td>
<td>z-Val-Ala-Asp fluoromethyl ketone</td>
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Chapter 1: Literature Review

1.1 Introduction

Breast cancer is the malignant growth of breast tissues, and is a major cause of death in women worldwide. Within Canada, an estimated 22,700 new breast cancer diagnoses were made in 2012 (1). Globally in 2004, the World Health Organization estimated that 519,000 deaths were due to breast cancer (2). Although breast cancer is thought to be a disease of developed countries, rates around the world are increasing (2). As time progresses, breast cancer is becoming a global health issue.

The role of diet and nutrition in the prevention, treatment and development of breast cancer is an area of great interest. As the relationship between diet and environment and the development of cancer continues to be studied, several nutrients of interest have been highlighted. Omega-3 polyunsaturated fatty acids (n-3 PUFA) and vitamin D are among these nutrients. Despite the vast differences in their chemical structures, both n-3 PUFA and vitamin D have been observed to have anti-cancer effects by mediating cell proliferation, cell cycle progression, and apoptosis stimulation (3,4). This review discusses the current research on these molecules, their proposed mechanisms involved in anti-cancer effects.
1.2 Vitamin D and Breast Cancer

1.2.1 Structures and Metabolism

Vitamin D is a secosteroid hormone that is classically known for its roles in bone health and homeostasis. It is consumable in two forms, D3, from animal sources, and D2, from plant sources. Vitamin D2 metabolism is similar to D3, though less is known of its regulation. Vitamin D can be endogenously synthesized and its metabolism in the human body is regulated by several feedback loops, involving calcium, phosphate, and the active form of vitamin D, 1,25(OH)2D3. Vitamin D biosynthesis occurs in the epidermal cells of the skin. With exposure to ultraviolet radiation, the precursor molecule, 7-dehydrocholesterol, is rearranged to create pre-vitamin D (5). Pre-vitamin D is further altered to created 25(OH)D, or calcidiol.

Three cytochrome-P450 enzymes control vitamin D metabolism. CYP27A1 (25-hydroxylase) is found primarily in the liver, and hydroxylates pre-vitamin D at the 25C position, creating 25(OH)D, or calcidiol (6). Pre-vitamin D3 can also be converted to 25(OH)D by isomerization via heat and infrared irradiation. 25(OH)D is inactive, and is the primary circulating form of vitamin D. 25(OH)D travels through plasma bound to the vitamin D binding protein to arrive at target tissues (7). Blood serum levels of 25(OH)D are used to approximate vitamin D status, and is known to increase with supplementation (8). A second hydroxylation occurs at the C-1α position to activate 25(OH)D. CYP27B1 (1α-hydroxylase) is the enzyme responsible for this hydroxylation, and is primarily found
in the kidney (6). This reaction creates the active form of vitamin D, 1,25(OH)$_2$D$_3$, or calcitriol. CYP24A1 (24-hydroxylase) is the catabolic enzyme, deactivates 1,25(OH)$_2$D$_3$ hydroxylating at the 24C position, and creates 1α,24,25(OH)$_3$D$_3$ (6). **Figure 1.1** summarizes these activating hydroxylations. Hepatic and renal regulation of vitamin D metabolites allows for vitamin D to act in an endocrine fashion, although CYP27B1 and CYP24A1 are expressed in other tissues (9). This suggests tissue-localized metabolism of vitamin D can result in autocrine and paracrine functions.

![Vitamin D structure and hydroxylations](image)

**Figure 1.1** Vitamin D structure of and the hydroxylations involved in its metabolism (6). Ultraviolet light converts 7-dehydrocholesterol to pre-vitamin D$_3$, and heat and infrared radiation convert pre-vitamin D$_3$ to 25(OH)D (not shown in diagram). Additional hydroxylations at the 1α-C and the 24-C position by specific CYP (P450) enzymes can activate and deactivate vitamin D.

### 1.2.2 Vitamin D Receptors and Genomic Actions

Currently, there are two known vitamin D receptors. The vitamin D receptor (VDR) is a nuclear receptor, and is involved with multiple coactivators for its actions.
Alternatively, a membrane-associated rapid response, steroid binding receptor (MARRS) was more recently identified, and is activated by 1,25(OH)$_2$D$_3$ (10,11). MARRS has potential roles in mediating non-genomic actions of vitamin D, such as stimulating calcium and phosphate uptake in intestinal cells (11). Recent focus has also been placed on MARRS and its role in cancer growth (12).

1,25(OH)$_2$D$_3$ activates VDR by binding to its hormone binding domain, decreasing the amounts of VDR homodimer complexes, and favouring VDR heterodimerization with retinoid X receptors (RXR) (13). The VDR-RXR heterodimer has a high affinity for the vitamin D response elements (VDRE) present in genes regulated by vitamin D (see Figure 1.2) (14). The genomic actions of VDR-RXR when bound to VDRE are highly regulated by a multitude of co-activators and co-repressors, which allow for activation or suppression of a wide variety of genes (15). Additionally, the number and location of VDRE may impact the genomic effects that vitamin D has. VDRE may be present in both proximal and distal locations within gene promoters, and the number of VDRE may also vary, altering the overall impacts vitamin D has on target genes (16).

Comparisons of VDR expression in benign and malignant breast lesions have shown that malignant specimens have lower VDR levels; one study found 56% of breast tumors to have measurable VDR expression (17). Additionally, the estrogen receptor status of breast cancer tumors were found to have a positive association with VDR expression (17). In breast cancer cell lines, early work by Buras et al. (18) determined
Figure 1.2  Basic schematic of 1,25(OH)$_2$D$_3$ genomic action (14). 1,25(OH)$_2$D$_3$ enters the cell, and binds VDR-RXR, activating the complex to bind to VDRE in target genes. That cell lines had varied expression of VDR. MCF-7 cells express VDR, whereas MDA-MB-231 cells have limited VDR expression (17). Breast cancer cells also have variations in VDRE present in promoter regions of the vitamin D metabolism enzymes. MCF-7 cells were found to have more VDRE in the CYP24A1 promoter region when compared to non-malignant MCF-10A breast epithelial cells (18). Additionally, MCF-7 cells express CYP24A1 mRNA that is more stable, with a half life three times that of MCF-10A CYP24A1 mRNA (16). These findings suggest that MCF-7 cells have an enhanced ability to deactivate 1,25(OH)$_2$D$_3$. 
1.2.3 Vitamin D and Breast Cancer in Human Research

The discovery of an association between sunlight exposure, vitamin D and breast cancer risk dates as far back as the late 1980s, after similar observations were made with colon cancer risk. Garland and colleagues (19) observed an increased risk for both breast and colon cancer in populations in Northeast urban areas within the United States.

Additional observational studies looking at the relationship between vitamin D status, vitamin D supplementation, UV radiation and sun exposure, and relative breast cancer risk have reported conflicting evidence. In a 2005 study nested within the Nurses’ Health Study, plasma 25(OH)D concentrations were suggested to have a modest association with decreased breast cancer risk in post-menopausal women (20). Additionally, the inverse relationship between serum 25(OH)D was only seen with estrogen and progesterone receptor (PR) negative tumors (ER\(^-\)/PR\(^-\)), and not ER\(^+\)/PR\(^-\) or ER\(^+\)/PR\(^+\) tumors (20).

Conversely, a 2008 study found evidence of a strong inverse association in post-menopausal women with low serum 25(OH)D (<30nM) (21). Recently, vitamin D status in addition to UV exposure was suggested to be the necessary combination required to decrease breast cancer risk (22). This led to the theory that a specific threshold of vitamin D must be met in order to show positive results (22). Increasing the controversy, these studies are difficult to compare due to variances in geographic location, demographic sub-groups (such as menopausal versus post-menopausal), dietary habits, baseline serum 25(OH)D levels, and control for voluntary supplementation.
Vitamin D supplementation trials have provided evidence on how vitamin D status in breast cancer patient populations can be improved. In a study with premenopausal women receiving adjuvant chemotherapy, a supplement of 400IU/day failed to bring serum 25(OH)D levels to sufficient levels (≥30ng/mL) (23). Amir and colleagues (24) supplemented a breast cancer patient population with bone metastases with 10,000IU/day, an amount proposed to be biosynthesized within the epidermis with UV radiation. This high-dose of oral vitamin D was well tolerated by most participants, and was found to significantly increase serum 25(OH)D levels to more than sufficient levels after 4 months of supplementation (24). When comparing a daily low dose (1000IU/day) versus a weekly high dose (50,000IU/week), large weekly dose was found to be more effective at increasing serum 25(OH)D, while remaining well tolerated within the study population (25). These studies suggest that a higher dose of vitamin D supplementation is required to achieve sufficient levels of serum 25(OH)D in breast cancer patient populations, and that current recommendations (800ID/day for adults in Canada) are not reflective of this knowledge.

1.2.4 Vitamin D Effects on Apoptosis of Breast Cancer Cells

While it is generally accepted that 1,25(OH)₂D₃ can cause apoptosis in cancer cells, an interesting debate on the mechanisms that are involved has developed. Early work treating breast cancer cells with 1,25(OH)₂D₃ found that the treatments stimulated apoptosis, as characterized by observations of classic changes in cell morphology indicative of apoptosis (26). These morphological changes include cell shrinkage,
condensed cytoplasm and chromatin, reorganization of the nuclear matrix and fragmented DNA (26,27). Mechanistically, studies have found Bcl-2 to be involved in 1,25(OH)_2D_3-mediated apoptosis in breast cancer cells (26, 27). Bcl-2 is an important anti-apoptotic protein. Treatment with 1,25(OH)_2D_3 and its analogues caused Bcl-2 down regulation, suggesting vitamin D is working against this negative signaling pathway (26-29).

Interestingly, when Bcl-2 was overexpressed in MCF-7 cells, it caused almost complete protection from vitamin D-induced apoptosis, suggesting excess Bcl-2 may provide a mechanism for vitamin D resistance (29).

Another unique characteristic of vitamin D-induced apoptosis is that it appears to occur without the involvement of caspases (29-31). Caspases are a family of cysteine proteases that are mediators of apoptosis execution. Caspases were once thought to be necessary for apoptosis, however several have shown caspase-independent apoptosis is possible (32). The ability of 1,25(OH)_2D_3 to stimulate caspase-independent apoptosis has been shown through the use of caspase inhibitors, such as the broad-spectrum caspase inhibitor z-VAD.fmk. When caspase inhibitors are applied with 1,25(OH)_2D_3 treatment, they fail to inhibit breast cancer cell apoptosis (29,33). The absence of caspase activation in 1,25(OH)_2D_3-stimulated apoptosis may explain why apoptosis kinetics are slower with this treatment, as several studies found it took a minimum of 72h for apoptosis stimulation (26,28,29). One form of caspase-independent apoptosis that has been described is termed “parthanatos”. Parthanatos involves translocation of the apoptosis-inducing factor (AIF) from the mitochondria to the nucleus, causing large-scale DNA
fragmentation via unknown mechanisms (32). Further study is needed to assess if 1,25(OH)$_2$D$_3$-medicated apoptosis is another example of parthanatos.

The impact of 1,25(OH)$_2$D$_3$ on cytochrome c release is an area with conflicting evidence. Cytochrome c is a protein that is released from the mitochondria in response to pro-apoptotic stimuli (34). Some studies observed cytochrome c release in response to vitamin D treatment, whereas another did not (29,30). In the 2001 study by Narvaez and Welsh (30), 1,25(OH)$_2$D$_3$ treatment with zVAD.fmk caused Bax (a pro-apoptotic protein) translocation to mitochondria and subsequent cytochrome c release from the mitochondria, but some late-stage apoptosis characteristics were no longer present(30). These late-stage apoptosis characteristics included poly (ADP-ribose) polymerase (PARP) cleavage, phosphatidyserine (PS) externalization, and DNA fragmentation.

1.2.5 *Vitamin D Effects on Cell Cycle and Growth of Breast Cancer Cells*

The cell cycle is a complex system and delicate balance of cyclins (such as Cyclin A, Cyclin D, Cyclin E), and their associations with cyclin-dependent kinases (Cdk; such as Cdk2, Cdk4) and Cdk inhibitors (such as p21 and p27). Several studies have identified key areas of the cell cycle in which 1,25(OH)$_2$D$_3$ and its analogues appear to have growth inhibitory effects. Cell cycle halting at G1 phase is characteristic of 1,25(OH)$_2$D$_3$ treatment, and is achieved through unknown mechanisms that prevent progression to the S-phase (3,26,35,36).
The retinoblastoma protein (pRb) is involved in cell cycle progression to the S-phase when it becomes hyperphosphorylated, and may be involved in 1,25(OH)\textsubscript{2}D\textsubscript{3}-mediated G1 phase halting. 1,25(OH)\textsubscript{2}D\textsubscript{3} has been found to increase hypophosphorylated pRb levels, preventing pRb from binding to the E2F transcription factor family (27,36-38). When pRb is hyperphosphorylated, it can bind to the E2F transcription factors and induce transcription of the cyclins required for G1/S-phase transition (39). The culmination of events leading to G1/S-phase transition involves Cyclin E activation Cdk2, causing E2F transcription factors to induce transcription of DNA replication proteins, allowing cells to begin the S-phase (39). 1,25(OH)\textsubscript{2}D\textsubscript{3}-mediated increases in hypophosphorylated pRb occurs alongside changes in of Cyclin, Cdk, and Cdk-inhibitor expression and activation in breast cancer cells. MCF-7 cells treated with 1,25(OH)\textsubscript{2}D\textsubscript{3} experienced decreases in Cyclin D1 mRNA and protein expression as well as increases in p21 and p27, two universal inhibitors that act upon a wide range of Cyclins and Cdk's (35-38).

In 2001, Jensen et al. (38) conducted a thorough look at the cell cycle machinery involved in vitamin D-stimulated cell cycle arrest, and proposed 3 potential mechanisms to explain the observations. The proposed mechanisms included 1) a cascade of events leading to decreased Cdk4/Cyclin D1, causing a decrease in phosphorylation of pRb and preventing E2F transcription factors to promote Cyclin A/E expression and Cdk2 activation; 2) induction of p21 to inhibit Cdk2 activation; 3) down regulation of c-Myc (product of the protooncogene c-myc) which may induce Cdk2 activity (See Figure 1.3).
When Cdk2 and Cyclin A/E are prevented from interacting, cell cycle progression from G1 is inhibited. Despite the current understanding in cell cycle machinery, the manner in which 1,25(OH)2D3 mediates these actions remains unknown.

**Figure 1.3** Proposed mechanisms 1,25(OH)2D3 may use to halt cell cycle at G1-phase (38). 1,25(OH)2D3 acts through unknown mechanisms to disturb three proposed pathways that ultimately inhibit cell Cdk2 interaction with Cyclin A/E, preventing cell cycle progression to S-phase.
1.3 n-3 Polyunsaturated Fatty Acids

1.3.1 Structures and Metabolism

The n-3 PUFA are long-chain fatty acids with several carbon double bonds that are naturally available in the diet from the oil and fats in flaxseed, canola, soy, and fish. Unlike saturated or monounsaturated fatty acids, the multiple double bonds in PUFA create an increasingly irregular and bent structure (Figure 1.4). n-3 PUFA have had positive implications in multiple diseases, including cardiovascular disease, cancer, depression, neurological diseases, and childhood development (40,41). It is well known that unlike lower-order species, mammals are unable to create double bonds at both n-3 and n-6 carbon locations on fatty acid chains. As a result, the parent molecules of these fatty acids are essential for human consumption.

α-linolenic acid (18:3n3; ALA) is the parent molecule for n-3 PUFA. It can be metabolized to EPA (20:4n3) and DHA (22:6n3) through a series of desaturation and elongation reactions. The parent n-6 PUFA, linoleic acid (18:2n6; LA), can also be converted to longer chain n-6 PUFA such as γ-linoleic acid (18:3n6; GLA) and arachidonic acid (20:4n6; AA). n-3 and n-6 PUFA conversion to longer and more unsaturated fatty acids occurs through the actions of two types of enzymes: desaturases and elongases. Since both PUFA classes use the same enzymes, it is suggested that there is a competition between the two classes for the use of the desaturases and elongases (See Figure 1.5) (42,43). Although PUFA metabolism pathways have been known in some
detail for over 40 years, understanding the final conversion process between EPA and DHA has more recently been discovered (44,45). This conversion process takes place in both the endoplasmic reticulum, where PUFA are metabolized and can be removed for integration into the plasma membrane, and the peroxisome, where a final single β-oxidation cycle creates DHA (Figure 1.6) (44,45). This final step is not limited to EPA and DHA alone, but is necessary for any PUFA to increase chain length from 22 to 24 carbons (44,45).

**Figure 1.4:**  Fatty acid structures. A) Stearic acid (18:0), a saturated fatty acid. B) Oleic acid (18:1), a monounsaturated fatty acid with one carbon-carbon double bond. C) EPA (20:5n3), a polyunsaturated fatty acid with multiple carbon-carbon double bonds.
Figure 1.5: Summary of the n-3 and n-6 PUFA synthesis (42). Each chain utilizes the same enzymes, which has led to the theory that there is competition between n-3 and n-6 PUFA for these enzymes.

In humans, the rate of conversion from ALA to EPA and DHA is suggested to be extremely low. Several studies and reviews have shown that supplementation with ALA may result in higher EPA, but not DHA (43,46). Additionally, while most human studies use blood fractions as samples to determine n-3 PUFA status after ALA supplementation, it has been hypothesized that conversion rates may be tissue-specific. For example, the human brain is likely to have higher rates of conversion from shorter chain n-3 fatty acids to DHA (46). In order to increase EPA and DHA in humans, it is often recommended that
this be achieved through consumption of these fatty acids directly, and not to rely on ALA conversion.

1.3.2 n-3 PUFA and Breast Cancer Epidemiology and Human Trials

Ecological data has suggested that variations in dietary fat consumption may mediate breast cancer risk. This has supported the theory that n-3 PUFA may play a role in breast cancer prevention (47). Several studies have since attempted to clarify the relationship between fatty acid intake and breast cancer risk. However, several epidemiological reviews are controversial, and fail to determine if there is a positive or negative relationship (48-50). In several case-control studies, high n-3 PUFA intake from fatty fish sources and high n-3 PUFA levels in breast adipose tissue were both associated with decreased BC risk, supporting the hypothesis that n-3 PUFA are protective (51,52). Several groups have suggested that it is not the total amount of n-3 PUFA, but rather the ratio of n-6:n-3 PUFA that may play a larger role in mediating BC risk (51,53). Additionally, DHA has been found to impact tumor response to chemotherapy, and is suggested to have chemosensitizing effects in breast cancer patients (54,55). When comparing DHA levels in tumors and adjacent tissues, higher DHA levels in both tissues were correlated with an increased response to chemotherapy treatments (55). Furthermore, in a single-arm phase II trial, 1.8g/day DHA was found to improve chemotherapy outcomes, supporting claims that DHA can cause chemosensitization (54).
Figure 1.6: Schematic of EPA to DHA conversion, as summarized from (44,45). The conversion between EPA and DHA is more complicated than conversions earlier in the n-3 PUFA chain; once fatty acid chains reach a length of 22 carbons, a degradation step is required to create a double bond at the fourth carbon on 24-carbon long chains. One β-oxidation cycle, which takes place in peroxisomes, is required to shorten the fatty acid chain, creating 22:6n3 (DHA). This process is also required for other fatty acid chains longer than 22 carbons.

1.3.3 Proposed Mechanisms of n-3 PUFA Anti-Cancer Effects

1.3.3.1 Genomic Actions of n-3 PUFA via PPARγ

Peroxisome proliferator-activated receptor gamma (PPARγ) is one of three PPAR receptors, and controls expression of genes that encode enzymes involved in lipid metabolism pathways (56,57). PPARs are ligand-activated transcription factors that mediate their effects by binding to RXR, and to DNA sequences upstream of genes
responsive to PPAR activation or suppression (57). PPARγ has been found in human breast cancer cell lines, and its activation may stimulate breast cancer cell growth (58,59). n-3 and n-6 PUFA are both capable of binding to PPARγ, however they have different effects on its activation and downstream effects (60,61). In human breast cancer cell lines, n-3 PUFA are PPARγ antagonists and can reduce cell proliferation; conversely, n-6 PUFA activate PPARγ and stimulate cell growth (61). This suggests that n-3 PUFA in breast cancer cells may have growth inhibition effects via PPARγ antagonism, and inhibition of its downstream actions.

1.3.3.2 Eicosanoids

Eicosanoids are signaling molecules created by oxygenated 20-carbon fatty acids, and have a wide range of functions (62). Some of the best-described functions of eicosanoids include mediation of inflammation, platelet aggregations, and vasoconstriction and vasodilation (62). They are classified into multiple families, including prostaglandins (PG), leukotrienes (LT), and thromboxanes (TX) (62,63). Eicosanoids are created by the enzymes cyclooxygenase (COX) and lipoxygenase (LOX). The role of eicosanoids in breast cancer has been one of interest, as inflammation has been identified as a hallmark of cancer development, and eicosanoids are under local regulation within individual cells (63). n-3 and n-6 PUFA can both be oxygenated to produce eicosanoids, however the molecules they produce are suggested to have opposing effects. n-6 PUFA create pro-inflammatory eicosanoids whereas n-3 PUFA create anti-inflammatory eicosanoids (64). n-6 PUFA eicosanoids are produced from AA,
and include PGE$_2$ and LTE$_4$ (62). EPA is the substrate for n-3 PUFA eicosanoids, such as LTB$_5$ and LTE$_5$ (62,64). The anti-inflammatory effects of n-3 PUFA eicosanoids is likely to occur through two mechanisms: 1) production of less potent inflammatory cytokines (including LTB$_5$ and LTE$_5$) that are structurally similar to n-6 PUFA eicosanoids, and 2) physical displacement of AA from the plasma membrane, reducing the AA pool available for pro-inflammatory eicosanoid production (64,65). n-3 PUFA eicosanoids have been suggested to play a role in growth inhibition in breast cancer cells. Rose and Connolly (65) treated MDA-MB-231 cells with EPA or DHA in the presence of eicosanoid synthesis inhibitors, and observed that growth inhibition was dependent on LT synthesis, and not PG synthesis (65). Although eicosanoid production is a common point of discussion in breast cancer cell-culture studies, few articles have been published to clearly demonstrate any further relationships and roles eicosanoids may play in breast cancer cell growth. In humans, observations have been made to suggest that non-steroid anti-inflammatory drugs (NSAIDs), which act upon inhibiting COX and LOX activity, may cause a small decrease in breast cancer risk (66).

1.3.3.3 Lipid Peroxidation

In addition to PUFA oxygenation and eicosanoid production, several early studies suggested lipid peroxidation could negatively impact breast cancer cell growth (67,68). Lipid peroxidation is the oxidative degradation of lipids, and causes the production of reactive oxygen species (ROS) through various mechanisms (69). ROS are capable of creating a cytotoxic environment by increasing oxidative stress, which can affect several
cellular processes and promote DNA damage (69). Subsequently, lipid peroxidation has been considered to be a factor involved in mammary gland tumorigenesis and breast cancer development (70). In studies on breast cancer cells, EPA and DHA treatment increase oxidative stress and ROS production, while causing to decreased cell proliferation and increased apoptosis (71-73). For example, DHA was found to increase ROS accumulation, resulting in caspase-8 activation and subsequent apoptosis in MDA-MB-231 cells (71). In MCF-7 cells, EPA and DHA were found to generate more peroxidation products while ALA and GLA were less effective, suggesting the longer chain n-3 PUFA are more readily able to create peroxidation products in breast cancer cells (72). However, ROS generation is also a potential factor in tumorigenesis, as they can cause DNA modifications that may favour cancer development (74,75). Despite this possibility, several chemotherapies act through ROS accumulation to kill cancer cells, and DHA was found to enhance these effects (76,77).

1.3.3.4 Lipid Rafts

Lipid rafts are microdomains within the cell plasma membrane that are rich in cholesterol and sphingolipids, and have complex interactions with multiple receptors, proteins and signaling molecules (78). Changes and alterations in lipid raft composition has been implicated in health and disease, including cancer (79). Several in vitro studies have studied the effect of n-3 PUFA (specifically EPA and DHA) on lipid raft composition, and have confirmed they can alter lipid raft composition (80-82). EPA was found to displace AA from lipid rafts, while DHA has opposing interactions with
cholesterol, creating DHA-rich/cholesterol-poor domains (80,82-85). MCF-7 and MDA-MB-231 cells treated with EPA and DHA have been found to incorporate n-3 PUFA into their lipid rafts, causing changes in biochemical and biophysical features of lipid rafts (85). These changes included the amount and types of phospholipids present and physical height of the rafts (85). Additionally, DHA treatment of MDA-MB-231 cells was found to interfere with EGFR, with decreases in phosphorylated and lipid raft-localized EGFR (81). This evidence suggests altering receptor populations in lipid rafts may be a potential mechanism in which DHA has antiproliferative and pro-apoptotic effects (81).

Additionally, the DHA-mediated changes in lipid rafts were associated with an increase in phosphatidylserine (PS) externalization and plasma membrane “blebbing”, both characteristics of apoptosis (80). n-3 PUFA modulation of lipid rafts and the protein and receptor content within them is a possible mechanism in which their anti-cancer effects are mediated.

1.3.4 n-3 PUFA and Apoptosis

n-3 PUFA have been found to induce apoptosis in breast cancer cells through several possible mechanisms, many of which have previously been discussed in the current review: lipid raft alteration, PPARγ activation, and ROS production (71,84,86). Breast cancer cells treated with EPA and DHA were found to have decreased levels of the pro-apoptotic protein Bcl-2 and increased caspase-8 activation; these changes in apoptosis signaling pathways occurred alongside changes in lipid raft composition (85). Lipid rafts are also known to contain Fas/tumor necrosis factor (TNF) receptor families,
which can interact with apoptosis pathways (78). n-3 PUFA-mediated changes in lipid raft architecture may alter the amount and activation of these “death receptors”. DHA-mediated PPARγ activation in human breast cancer cells has been found to upregulate syndecan-1 (SDC-1), a transmembrane heparin sulfate proteoglycan that is known to inhibit cell growth and stimulate apoptosis (86-88). The pro-apoptotic effects of SDC-1 in breast cancer cells have been shown to involve inhibition of the MAPK/Erk/Bad signaling pathway (86). Additionally, the gene encoding the SDC-1 protein (Sdc-1) is a target of PPARγ; DHA activation of PPARγ induces SDC-1 expression, and is associated with subsequent increases in apoptosis (88). DHA treatment has also been found to cause a short-term (24h) activation of caspase-3, stimulating an early apoptotic response (89). The activation of caspase-3 is likely a downstream effect of one or many of these apoptotic mechanisms.

1.3.5  n-3 PUFA and Cell Cycle and Growth Regulation of Breast Cancer Cells

Studies assessing the impact of individual n-3 and n-6 PUFA on breast cancer cell growth have produced conflicting results. Among the first studies, Bégin and colleagues (67) were among the first to discover the potential of n-3 and n-6 to have anti-proliferative effects on breast cancer cells. The anti-proliferative effects of n-3 PUFA were found to be selective to cancer cell lines only, causing two non-malignant cell lines to experience a reduced growth rate while remaining growing and viable. However, these early studies found that DHA was less effective than ALA and EPA at inhibiting cell growth, and that its effects were not limited to malignant cells alone (67,68). In later
work, DHA was found to be more potent, with its efficacy varying depending on the cell lines used (65,72,90).

With evidence that n-3 PUFA have negative growth effects on breast cancer cells, studying their involvement with cell cycle regulation is an area of interest. However, the effect of n-3 PUFA on breast cancer cell cycle machinery has not been studied to the same extent as the overall anti-proliferative effects. An article by Chamras et al. (91) looked at the effects of multiple n-3 (ALA, EPA, DHA) and some n-6 PUFA (LA, AA) on cell cycle in MCF-7 cells, and saw no observable changes in molecules mediating cell cycle with PUFA treatments. Conversely, Lu and colleagues found that cyclin D1 was downregulated with DHA treatment (92). There is additional evidence in other types of malignancies that n-3 PUFA, specifically DHA, may significantly slow down the S-phase, or inhibit G1/S transition (93-95). Molecules mediating G1/S transition, such as Cyclin A/E and Cdk2, may be involved in the observed changes in cell cycle progression with DHA treatment. Additionally, n-3 PUFA alterations within the plasma membrane may have direct or indirect actions in altering membrane receptor signaling pathways involved in cell growth signals (94,95). Further study is needed to understand the mechanisms involved with n-3 PUFA, specifically DHA, and its role in inhibiting breast cancer cell growth.
1.4 \textit{n-3 PUFA and Vitamin D as a Combined Treatment}

1.4.1 \textit{Changes in Vitamin D and n-3 PUFA Metabolism in Cancer}

In the process of cancer development and progression, many changes are made within the cell to promote cancer growth. Among these changes, enzymatic regulation of n-3 PUFA and vitamin D metabolism in breast cancer is possible. Several studies have identified that breast cancer tumors express vitamin D enzymes, though their levels may be varied \((3,17,96)\). More aggressive phenotypes were found to have increased CYP24A1 and decreased CYP27B1, suggesting that as malignancy progresses, there is a higher rate of deactivating \(1,25(\text{OH})_2\text{D}_3\) \((17,96)\). This remains true when comparing breast cancer cell lines to non-malignant cell lines \((96,97)\). Additionally, the discovery of different splice variants of CYP27B1 found in MCF-7 cells has suggested that breast cancer cells may express a non-functional form of the enzyme \((98)\). Fatty acid profiles of breast cancer cells and tissues are abnormal compared to non-malignant tissues, with the possibility that fatty acid metabolism in breast cancer is also altered \((90,99,100)\). A recent study has shown that n-6 fatty acid conversion is up regulated in human breast cancer tissue, increasing the pro-tumorigenic prostaglandin PGE\(_2\) \((99)\). This information suggests cancer cells may preferentially metabolize n-6 PUFA to create factors that could potentiate their development \((99)\). Conversely, higher DHA was recorded in a small sample of tumor and adjacent tissues when compared to normal tissues \((100)\). Internal changes in vitamin D and n-3 PUFA metabolism to produce a favourable environment for
tumorigenesis and cancer growth speaks strongly to how sophisticated cancer may act to promote its development.

1.4.2 Interactions of Vitamin D and n-3 PUFA Genomic Mediators

In addition to their effects on cell proliferation and apoptosis, genomic mechanisms mediating n-3 PUFA and vitamin D effects may interact in several ways. Over time, complex interactions and cross-talk involving VDR, ERα and PPARγ have come to light. Figure 1.7 is a summary of some current relationships that have been discovered through in vitro work. VDR has been found to negatively regulate ERα via two VDRE present in the ERα gene promoter, suggesting that vitamin D can down regulate ERα expression (101,102). Decreased ERα expression subsequently decreases the amount of the receptor available for promoting growth stimulation effects. ERα is capable of physically associating and inhibiting PPARγ and its effects, including growth inhibition and apoptosis via SDC-1 (103). n-3 PUFA are involved with both ERα and PPARγ; DHA was found to cause proteasome-independent ERα degradation, and can bind to PPARγ to mediate cell growth inhibition (88,92). Conversely, n-6 PUFA have been suggested to bind and activate PPARγ, leading to growth stimulation signals (61). PPARγ and VDR negatively interact with one another through two potential mechanisms: 1) both receptors can physically associate and block activation by their respective ligands, and 2) both require RXR for binding to their response elements in DNA, and provide competition for RXR binding (102). The level of cross-talk between these nuclear receptors and the physiological impact they may have has yet to be elucidated.
Figure 1.7: Schematic of potential cross-talk between nuclear receptors affected by vitamin D metabolites, n-3 and n-6 PUFA in breast cancer cells. Dashed lines indicate inhibition and negative interaction as, while solid lines indicate stimulation and positive interactions.

1.4.3 Current n-3 PUFA and Vitamin D Research

As highlighted in this review, n-3 PUFA and vitamin D compounds both interact with mechanisms mediating cell proliferation and death. However, the specific molecular targets for each may vary. With unique targets that have similar results on cancer cell fate, the combination of these two molecule groups are thought to have additional benefits than each treatment alone. Despite the numerous possibilities of how vitamin D and n-3 PUFA may independently affect cancer growth, studies looking at the combined
effects of these molecules are few. Reviews have hypothesized that additive or synergistic effects of combining the two types of molecules is possible (104,105). Often, the rationale for studying this combination is to find if n-3 PUFA may sensitize cancer to vitamin D treatment, in order to reduce the effective dose, and potential toxicity, of vitamin D. Toxic levels of vitamin D, albeit quite high, have been known to cause hypercalcemia and hypercalciurea (24).

In vitro work studying liver and prostate cancers have been studied in response to combined n-3 PUFA and vitamin D treatment. Two studies found that the combination caused enhancement and potential synergism in growth inhibition (106,107). In androgen-dependent prostate cancer cells, the combination treatment caused synergistic growth inhibition and an increase of cells in the G1/S phase transition, which is consistent with 1,25(OH)\(_2\)D\(_3\) treatment effects on cell cycle halting (107). Liver cancer cells responded to combination treatments with dose-dependent and significant decreases in ['H]-thymidine incorporation into DNA, indicating decreased cell growth (106). However, both studies are limited by their experimental design, and neither indicates the specific amounts of EPA and DHA in their fish oil treatments.

Although laboratory-based evidence is minimal, a large clinical trial (the VITAL trial) in the USA is underway to observe the effects that vitamin D and n-3 PUFA supplements have on cancer and cardiovascular disease (108). The VITAL trial is testing the effects of 2000IU/day vitamin D and marine omega-3 fatty acid supplements (EPA +
DHA, 1g/day) both alone and in combination in men and women over 50 and 55, respectively. Results from this study are expected within the next 5 years.
Chapter 2: Rationale, Hypothesis and Objectives

2.1 Overall Study Rationale

As highlighted in the literature review (Chapter 1), n-3 PUFA and vitamin D compounds are both capable of inhibiting breast cancer cell growth. Although there is debate on the characteristics and mechanisms involved in growth inhibition, there is potential for the combination of n-3 PUFA and vitamin D to have an additional impact on breast cancer cell growth. Currently, only two studies have tested the combination of n-3 PUFA and vitamin D on prostate and liver cancer cell lines, and found that the combination resulted in a synergistic effect on inhibiting cell growth (106,107). It remains unknown whether breast cancer cells will respond to combination n-3 PUFA and vitamin D treatment in an additive or synergistic manner. The current study aims to explore the effects of combining 1,25(OH)2D3 with n-3 or n-6 PUFA on the growth of breast cancer cells.

2.2 Overall Study Hypothesis

The hypothesis of this thesis is that the combination of n-3 PUFA with 1,25(OH)2D3 will inhibit breast cancer cell growth in an additive or synergistic manner. Two studies were conducted in order to test this hypothesis. This hypothesis will be tested using cell culture techniques, and assessing breast cancer cell growth in response to combination treatments.
A pilot study was initially conducted to characterize growth inhibition by n-3 and n-6 PUFA in MCF-7, MDA-MB-231, and MCF-10A cells. This pilot study was conducted to determine three n-3 and n-6 PUFA concentrations, which are combined with three 1,25(OH)$_2$D$_3$ concentrations in the second study. To create these concentrations, MCF-7, MDA-MB-231, and MCF-10A responses to n-3 and n-6 PUFA treatment were averaged. Fatty acid concentrations causing 25, 50, and 75% of the averaged growth inhibition were calculated to create low, medium, and high treatment concentrations, respectively. Additionally, analysis was done to provide evidence that n-3 and n-6 PUFA treatment was effective for cellular incorporation of PUFA. Specific objectives were as follows:

1. To generate toxicity curves of the average growth inhibition of MCF-7, MDA-MB-231, and MCF-10A cells in response to ALA, EPA, DHA, GLA, or AA treatment using the Sulforhodamine B (SRB) dye assay; three levels of fatty acid treatment concentrations (low, medium, high) will be determined from the toxicity curves.

2. To confirm fetal bovine serum (FBS) is a successful vehicle for PUFA delivery to MCF-7, MDA-MB-231 and MCF-10A cells, and its ability to alter the fatty acid composition of cellular lipids through the use of gas chromatography.
2.4 Study Rationale and Objectives

The present study was conducted to test combinations of 1,25(OH)$_2$D$_3$ with ALA, EPA, DHA, GLA or AA and describe how they affect the growth of MCF-7, MDA-MB-231, and MCF-10A cells. A 3x3 design was used, combining three concentrations of PUFA treatment (low, medium, high; determined in the pilot study) with three 1,25(OH)$_2$D$_3$ concentrations. Specific objectives were as follows:

1. To measure growth response in MCF-7, MDA-MB-231, and MCF-10A cells after treatment with various combinations of 1,25(OH)$_2$D$_3$ and PUFA (ALA, EPA, DHA, GLA, or AA) through the SRB dye assay.

2. To determine the presence of possible synergism between n-3 or n-6 PUFA and 1,25(OH)$_2$D$_3$ on the growth of MCF-7, MDA-MB-231, and MCF-10A cells.
Chapter 3: PUFA Toxicity Curves and Determination of Inhibition Levels

3.1 Introduction

n-3 PUFA consumption has been associated with a decreased risk of several cancers, including breast cancer. However, when reviewing the epidemiological data available, discrepancies are present (48,109). The hypothesis that n-3 PUFA may affect cancer risk, progression, and treatment in humans is based on work in animal studies (110,111) and in vitro (65,67,68,72,90,91). Conversely, n-6 fatty acids are widely considered to be pro-inflammatory, and may mediate cancer risk in favour of development (110-112). Many studies have been done to understand the mechanisms involved in n-3 PUFA anti-cancer effects. There are several proposed mechanisms, including ROS formation, alterations in lipid raft architecture, and eicosanoid production (65,72,83,86,89,113).

Much breast cancer in vitro work utilizes cell lines that are derived from primary and metastatic breast cancer tumors. The MCF-7 and MDA-MB-231 cell lines were established in the early 1970s (114,115). Both cell lines were isolated and cultivated from pleural effusions from two different breast cancer patients, and are metastatic breast cancer cells. However, these two cell lines have varying characteristics. MCF-7 cells express ERα, and were recently determined to represent the luminal A breast cancer subtype (114,116,117). They are also HER2− and EGFR− (116). MDA-MB-231 cells are ER−, progesterone receptor negative (PR−) and HER2− (116). They are considered to represent
triple negative breast cancer, as they do not express any of the clinically relevant hormone receptors. MDA-MB-231 cells are also more aggressive and less differentiated that MCF-7 cells (115,116). Non-malignant breast epithelial cell lines have also been established, such as the MCF-10A cell line established over two decades ago (118). The MCF-10A cell line was cultivated and established from a sub-cutaneous mastectomy of breast tissue that was determined to be extensive fibrocystic disease (118). It is a spontaneously immortalized cell line and is used as a non-cancerous comparison in in vitro studies (118).

In the current study, MCF-7, MDA-MB-231, and MCF-10A are assessed for growth inhibition in response to n-3 and n-6 PUFA treatments. Three n-3 PUFA were tested; the parent n-3 PUFA ALA (18:3n3), as well as two long-chain n-3 PUFA, EPA (20:5n3) and DHA (22:6n3). Two n-6 PUFA were also tested, GLA (18:3n6) and AA (20:4n6). Serial dilutions of PUFA concentrations were used to treat the cells, and to create average growth inhibition curves. These curves represented the average growth response all three cell lines experienced with PUFA treatment. Using these curves, PUFA concentrations causing 25, 50 and 75% of the averaged cell response were calculated. The concentrations were defined as low, medium, and high treatment concentrations. This study was conducted to confirm the methods used, and to establish three levels of fatty acid treatment concentrations for future work.
3.2 Materials and Methods

3.2.1 Cell Culture

MCF-7, MDA-MB-231, and MCF-10A cells were from ATCC. MCF-7 cells were grown initially in α-modified Eagle’s medium (α-MEM; Lonza) supplemented with 1 mM sodium pyruvate (Lonza), 10% fetal bovine serum (FBS; PAA, Lot A70110-7001), 50U/mL penicillin and streptomycin (Gibco), and 10 µg/mL of insulin (Sigma-Aldrich). Dulbecco’s modified Eagle’s medium (DMEM; Lonza) media was later used when α-MEM became unavailable. MDA-MB-231 cells were grown in DMEM (Lonza) with 10% FBS (PAA, Lot A70110-7001), and 50 U/mL penicillin and streptomycin (Gibco). MCF-10A cells were grown in DMEM-F12 (1:1) media (Lonza) with 5% FBS (PAA, Lot A70110-7001), 10 µg/mL insulin (Sigma-Aldrich), 0.5 µg/mL hydrocortisone (Calbiochem), 20 ng/mL epidermal growth factor (Calbiochem), 100 ng/mL cholera toxin (Enzo Life Sciences), and 50 U/mL penicillin and streptomycin (Gibco). All cells were housed in an incubator at 37°C with 5% CO₂. Cells were maintained in 25cm² flasks, and were split prior to reaching full confluence. MCF-7 and MDA-MB-231 cells were trypsinized with 1x trypsin (10X trypsin-EDTA (Gibco) diluted in phosphate buffer solution (PBS)) for 2 to 5 min or until cells were no longer adherent. MCF-10A cells were trypsinized with 10x trypsin-EDTA (Gibco) for approximately 10 min, or until cells were no longer adherent.
3.2.3 Western Blotting for ERα and EGFR

Western blotting for EGFR and ERα for MCF-7, MDA-MB-231, and MCF-10A cell lines was completed to validate the cell lines used. Cells were washed twice with cold PBS and lysed with RIPA lysis buffer (150mM NaCl, 1% NP-40, 50mM Tris pH 7.5) with 0.2% SDS and a protease inhibitor tablet (Complete Mini protease inhibitor cocktail tablets, Roche) and agitated for 15 min at 4°C. Cells were then scraped while on ice, and lysate samples transferred to a microcentrifuge tube and spun at 15,000xg for 5 min at 4°C, after which the lysate was removed and stored at -20°C for future use. Prefabricated gradient gels (BioRad MiniPROTEAN TGX) with a 4-15% gradient were used. 10-25µg of whole cell lysates were loaded, and run at 50V for 15min followed by 100V for 1.5h. Proteins were transferred to nitrocellulose membrane (BioRad) via wet transfer in transfer buffer (10x buffer, 30g Tris base and 144g glycine in 1L, diluted to 1x buffer containing 100mL 10x stock, 200mL methanol, and 700mL ddH₂O) and run at 100V for 1h with cold packs. Fast green stain was used to detect protein presence and loading evenness on membranes. Membranes were soaked for 5 min in fast green stain solution (0.1% w/v Fast Green Stain (Fisher), 20% methanol, and 5% acetic acid), with agitation. After discarding the fast green stain solution, membranes were washed with fast green de-stain (20% methanol, 5% acetic acid, 75% ddH₂O) 5 X 5 min each and allowed to air dry. Pictures of the membranes were taken using the FluorChem HD2 system (Cell Biosciences) on the white light setting with 8ms exposure to confirm equal loading of samples prior to antibody probing. For antibody probing, membranes were blocked for 3 h in 1% milk powder, and washed in 0.05% tris-buffered saline with
Tween-20 (TBST). Membranes were probed with primary antibodies for EGFR (Santa Cruz, SC-03) and ERα (Santa Cruz, SC-7201) both at 1:500 dilution in 1% milk powder for 1 h, followed by TBST washing 5 X 5 min. Secondary goat-anti-rabbit antibody (Aviva Systems Biotech) at 1:50,000 dilution in 1% milk powder probed for 1 h, and washed with TBST 5 X 5 min. Membranes were exposed to a 1:1 ratio of chemiluminescence solution (Immuno-Star WesternC Kit, BioRad) for 1-2 min, and detected on the FluorChem HD2 system (Cell Biosciences) using the Alpha View Software (Alpha Innotech, v. 3.1.1.0).

3.2.3 PUFA Treatments

ALA, EPA, DHA, AA and GLA (Nu-Chek-Prep Inc.) were obtained as >99% pure fatty acids. Fatty acids were aliquotted into microcentrifuge tubes and stored under nitrogen at -20°C. ALA, EPA, DHA, GLA, or AA was added to 1mL of FBS in a microcentrifuge tube, placed in an incubator at 37°C, and vortexed every 5 min for 60 min to create stock concentrations of 40mM. Stock FBS-PUFA solutions were stored at -20°C.

3.2.4 Cell Growth Assay

Serial dilutions with final concentrations of 200, 100, 50, 25, 12.5, 6.25, 3.13, 1.57, 0.781, 0.390, and 0.019µM of PUFA treatments were plated in 96-well plates. Cells were plated simultaneously with PUFA treatments at 2000 cells/well. The addition of
FBS-PUFA treatment increased FBS concentration by a maximum of 0.5%. One column was left blank, containing only untreated media to use for a background reading for the SRB assay. Cells were grown in treated media for 48h.

3.2.5 Sulforhodamine-B Dye Assay, Absorbance Readings and PUFA Concentration Calculations

After 48h incubation, cell growth was determined using the SRB dye assay. Cells were fixed with 50µL/well of cold 80% TCA (Fisher Scientific), and placed at 4°C for 1 h. TCA and media were removed by washing the plates by immersing them in deionized water and flicking the fluid off five times. Plates dried overnight, and were stained by adding 50µL/well of 0.4% w/v SRB dye (Sigma-Aldrich) in 1% acetic acid, and sitting for approximately 30 min at room temperature. SRB dye was washed off the plates by immersion in 1% acetic acid, and flicking the fluid off a minimum of 3 times or until residual dye was gone. After being allowed to dry, the SRB dye was solubilized by adding 100µL of 10mM unbuffered Tris (Promega), and placed on a rotary shaker for approximately 15 min at room temperature. Absorbance readings were made initially on SpectraMax M2 (Molecular Devices) and later FlexStation3 (Molecular Devices) at 570nm using the Softmax Pro Software (Versions 5.2 and 5.4.1, respectively). Readings were repeated at 490nm if measurements were greater than 1.2 units (119). MCF-7, MDA-MB-231, and MCF-10A absorbance readings were combined and averaged for each PUFA treatment. The average absorbance measurement for each PUFA was plotted on a semi-log graph in Microsoft Excel. Growth inhibition curves were estimated by
hand. PUFA concentrations causing 25, 50, and 75% growth inhibition of the averaged cell response were calculated. These concentrations were defined as being the low, medium, and high PUFA concentrations. Averaged absorbance measurements were also calculated to express the percent of untreated cells.

3.2.6 Gas Chromatography

To confirm PUFA incorporation into treated cells, total lipid analysis via gas chromatography (GC) was performed on all three cell lines treated with the low concentration of all five PUFA (Table 3.1). Cells were plated with $4 \times 10^5$ to $6 \times 10^5$ cells per well, based on individual cell doubling times and growth inhibition caused by PUFA. Cells grew in treated media for 96h, with media changes at 48h and 72h. At 96h, cells were trypsinized with 0.25mL of 1x Trypsin-EDTA (Gibco) in PBS (MCF-7 and MDA-MB-231 cells) or 0.25mL of 10x Trypsin-EDTA (Gibco) (MCF-10A cells), resuspended, and spun into a pellet for 5 min at 800rpm. Cell pellets were frozen at -80°C until analysis. For lipid extraction, pellets were thawed, washed once with 5mL cold PBS, and spun down for 5 min at 800rpm. Excess PBS was removed, and samples remained on ice for the lipid extraction process. Prior to sample preparation, 5µL of 1µM 17:0 (heptadecanoic acid) standard was added to acid-washed GC vials. 750µL of 0.1M KCl was used to resuspend each sample, and for transfer to the glass GC vials. 2mL of freshly made 2:1 chloroform:methanol (CHCl$_3$:MeOH; Fisher Scientific) solution was added. Vials were capped and vortexed. 250µL KCl was added to the vials, vortexed again, and flushed with nitrogen gas for storage overnight at 4°C. The following day, samples were
spun at 1000rpm at 21°C for 10 min. Long Pasteur pipettes were used to remove the chloroform layer for transfer to new acid-washed GC vials, which were dried under nitrogen. Tubes were weighed pre- and post- sample addition and chloroform evaporation to determine the gram amount of fatty acids present in the sample. Dried samples were reconstituted with 100µL chloroform (Fisher). 2mL KOH in methanol (Fisher) was added to the tubes, vortexed, and flushed with nitrogen. Samples were saponified for 1h at 100°C, and allowed to cool for 10 min. 2mL of both hexane (EMD Millipore) and 14% BF$_3$-MeOH (Sigma Aldrich) were added to the vials, flushed with nitrogen and samples methylated for 1h at 100°C. After a 10-min cooling period, 2mL ddH$_2$O was added, and samples were centrifuged at 1000rpm for 10 min. Short Pasteur pipettes were used to extract the hexane layer of the samples, and transferred to acid-washed GC vials to be dried down. 100µL of hexane was used to reconstitute the samples for insertion to GC inserts. Samples were run on the Agilent Technologies 7890A GC system. Results were calculated using the integrated area under the curve (AUC) in reference to the 17:0 standard AUC, and are expressed as molar percent of total fatty acids.

3.3 Results

3.3.1 Presence of ERα and EGFR in MCF-7, MDA-MB-231, and MCF-10A cells

To confirm differential ERα and EGFR expression, Western blotting was used to determine the presence of these receptors in MCF-7, MDA-MB-231, and MCF-10A cells.
Figure 3.1 shows a representative blot, confirming MDA-MB-231 cells are ERα+/EGFR⁺, and MCF-7 cells are ERα⁺/EGFR⁻. MCF-10A cells were also found to be ERα⁺/EGFR⁻.

3.3.2 Cell Responses to ALA Treatment

ALA concentrations (≤ 50μM) resulted in minimal or no growth inhibition (Figure 3.2). 100μM ALA resulted in an average growth decrease of 78% relative to control cells. 200μM ALA caused decreased average cell growth to 32%, suggesting an ALA concentration that can inhibit average cell growth by 50% occurs between 100-200μM of ALA. The low, medium, and high concentrations were estimated to be 100μM, 160μM, and 220μM, respectively (Table 3.1).

<table>
<thead>
<tr>
<th>PUFA Level (μM)</th>
<th>ALA</th>
<th>EPA</th>
<th>DHA</th>
<th>GLA</th>
<th>AA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low</td>
<td>100</td>
<td>100</td>
<td>50</td>
<td>150</td>
<td>120</td>
</tr>
<tr>
<td>Medium</td>
<td>160</td>
<td>170</td>
<td>120</td>
<td>180</td>
<td>170</td>
</tr>
<tr>
<td>High</td>
<td>220</td>
<td>270</td>
<td>170</td>
<td>250</td>
<td>240</td>
</tr>
</tbody>
</table>

Table 3.1 The low, medium, and high concentrations of all PUFA treatments, as calculated from the toxicity curves. MCF-7, MDA-MB-231, and MCF-10A cells were treated with a serial dilution of 200-0.02μM for 48h. The SRB dye assay was used and absorbance measurements for all three cell lines were averaged to create average growth inhibition curves. 25, 50, and 75% average growth inhibition was calculated to create low, medium, and high n-3 and n-6 PUFA concentrations.
**Figure 3.1:** (A) Fast green staining of nitrocellulose membrane. MCF-7, MDA-MB-231 and MCF-10A cells were loaded into a gradient gel, and transferred to nitrocellulose membrane via wet transfer methods. Membranes were stained with fast green stain to visualize loading evenness. (B) Representative Western blot of MCF-7, MDA-MB-231 and MCF-10A cells to confirm cell type based on differential EGFR and ERα expression. EGFR was confirmed in MDA-MB-231 cells only. ERα was confirmed in MCF-7 and MCF-10A cells.
3.3.3 Cell Responses to EPA Treatment

All levels of EPA treatment caused some growth inhibition when compared to non-treated cells (Figure 3.3). Concentrations ≤ 50μM caused averaged cell growth decrease to ~80% of control. Above 100μM EPA, the surviving fraction of cells sharply declined. 100μM EPA caused an average of 79% of control growth, while 200μM EPA reduced average cell growth to 37% of control growth. Low, medium, and high levels of EPA causing an average of 25, 50, and 75% growth inhibition were determined to be 100μM, 170μM, and 270μM, respectively (Table 3.1).

3.3.4 Cell Responses to DHA Treatment

DHA treatment at 200μM caused the greatest decrease in growth when compared to control, substantially decreasing the remaining fraction of cells to an average of 10% (Figure 3.4). 100μM DHA reduced average cell growth to 58% of control, and 50μM DHA to 76% of control. Concentrations below 12.5μM caused minimal growth inhibition. The low, medium, and high levels of DHA treatment causing an average of 25, 50, and 75% growth inhibition were determined to be 50, 120, and 170μM, respectively (Table 3.1).
3.3.5 *Cell Responses to GLA Treatment*

200µM GLA caused a decrease in average cell growth to 45% of control (Figure 3.5). Concentrations ≤100µM had no impact on average cell growth. The low, medium, and high concentrations causing an average of 25, 50, and 75% growth inhibition of GLA were determined to be 150, 180, and 250µM, respectively (Table 3.1).

3.3.6 *Cell Responses to AA Treatment*

AA concentrations below 50µM caused a minimal decrease in averaged cell growth compared to control (Figure 3.6). Average cell growth was never less than 87% of control cell growth. At 100µM AA, the surviving fraction of cells was an average of 85% of control, and 200µM caused an average decrease in cell growth to 39% of control. Low, medium, and high concentrations causing an average of 25, 50, and 75% growth inhibition of AA were determined to be 120, 170, and 240µM, respectively (Table 3.1).
Figure 3.2 Absorbance readings of the averaged cell response to a serial dilution of ALA. Cells were plated in 96-well plates and treated with 200-0.019µM ALA. At 48h, cells were fixed with 50% TCA and stained with SRB dye. Absorbance readings from MCF-7, MDA-MB-231 and MCF-10A cells were averaged, and plotted in Microsoft Excel. The plot was used to estimate concentrations causing average growth inhibitions of 25, 50, and 75%. These concentrations were defined as the low, medium, and high ALA concentrations for later use; n=1.
Figure 3.3  Absorbance readings of the averaged cell response to a serial dilution of EPA. Cells were plated in 96-well plates and treated with 200-0.019µM EPA. At 48h, cells were fixed with 50% TCA and stained with SRB dye. Absorbance readings from MCF-7, MDA-MB-231 and MCF-10A cells were averaged, and plotted in Microsoft Excel. The plot was used to estimate concentrations causing average growth inhibitions of 25, 50, and 75%. These concentrations were defined as the low, medium, and high EPA concentrations for later use; n=1.
Figure 3.4  Absorbance readings of the averaged cell response to a serial dilution of DHA. Cells were plated in 96-well plates and treated with 200-0.019µM DHA. At 48h, cells were fixed with 50% TCA and stained with SRB dye. Absorbance readings from MCF-7, MDA-MB-231 and MCF-10A cells were averaged, and plotted in Microsoft Excel. The plot was used to estimate concentrations causing average growth inhibitions of 25, 50, and 75%. These concentrations were defined as the low, medium, and high DHA concentrations for later use, n=1.
Figure 3.5  Absorbance readings of the averaged cell response to a serial dilution of GLA. Cells were plated in 96-well plates and treated with 200-0.019µM GLA. At 48h, cells were fixed with 50% TCA and stained with SRB dye. Absorbance readings from MCF-7, MDA-MB-231 and MCF-10A cells were averaged, and plotted in Microsoft Excel. The plot was used to estimate concentrations causing average growth inhibitions of 25, 50, and 75%. These concentrations were defined as the low, medium, and high GLA concentrations for later use, n=1.
Figure 3.6  Absorbance readings of the averaged cell response to a serial dilution of AA. Cells were plated in 96-well plates and treated with 200-0.019µM AA. At 48h, cells were fixed with 50% TCA and stained with SRB dye. Absorbance readings from MCF-7, MDA-MB-231 and MCF-10A cells were averaged, and plotted in Microsoft Excel. The plot was used to estimate concentrations causing average growth inhibitions of 25, 50, and 75%. These concentrations were defined as the low, medium, and high AA concentrations for later use, n=1.
3.3.7 Gas Chromatography and PUFA Uptake into MCF-7, MDA-MB-231, and MCF-10A Cells

Gas chromatography was completed to measure PUFA amounts in cultured cells after 96h of treatment. This was used to confirm PUFA were successfully delivered to the treated cells. GC data in Tables 3.3-3.5 indicate PUFA incorporation into cells was successful, as treated cells exhibited an enrichment of the treated PUFA. GC analysis of FBS-PUFA stock solutions show that the PUFA added to FBS accounted for >90% of the fatty acid profile (Table 3.2). When comparing differences in cell line-specific uptake of PUFA treatments, MCF-10A cells exhibited the highest levels of PUFA incorporation (Table 3.5). Molar percentages of treatment PUFA in MCF-10A cells were 25-63%. MCF-7 uptake of treatment PUFA ranged from 13-28% (Table 3.3). MDA-MB-231 cells experienced the lowest range of treatment PUFA integration, achieving a molar percentage range of 9-21% (Table 3.4).
Table 3.2  Percent molar concentration (molar %) of fatty acid presence in FBS solutions. GC analysis was used to measure percent molar concentrations in FBS with or without PUFA emulsified. Percent molar concentrations were calculated using the integrated AUC of all fatty acids present in each sample.
Percent molar concentration (molar %) of fatty acid presence in MCF-7 cells after 96h treatment with low levels ALA, EPA, DHA, GLA, or AA, and a non-treated control. GC analysis was used to measure percent molar concentrations in whole cell samples. Cells were frozen in media, thawed, and washed once with cold PBS prior to GC analysis. Percent molar concentrations were calculated using the integrated AUC of all fatty acids present in each sample.

<table>
<thead>
<tr>
<th></th>
<th>ALA (molar %)</th>
<th>EPA (molar %)</th>
<th>DHA (molar %)</th>
<th>GLA (molar %)</th>
<th>AA (molar %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCF-7 + 100μM ALA</td>
<td>13.10</td>
<td>0.22</td>
<td>0.60</td>
<td>0.00</td>
<td>1.81</td>
</tr>
<tr>
<td>MCF-7 + 100μM EPA</td>
<td>0.39</td>
<td>14.22</td>
<td>0.74</td>
<td>0.00</td>
<td>1.20</td>
</tr>
<tr>
<td>MCF-7 + 50μM DHA</td>
<td>0.27</td>
<td>2.50</td>
<td>17.93</td>
<td>0.00</td>
<td>1.47</td>
</tr>
<tr>
<td>MCF-7 + 150μM GLA</td>
<td>0.20</td>
<td>0.21</td>
<td>0.43</td>
<td>16.18</td>
<td>9.18</td>
</tr>
<tr>
<td>MCF-7 + 120μM AA</td>
<td>0.22</td>
<td>0.00</td>
<td>0.47</td>
<td>0.50</td>
<td>28.69</td>
</tr>
<tr>
<td>MCF-7 - Control</td>
<td>0.32</td>
<td>0.50</td>
<td>0.98</td>
<td>0.00</td>
<td>3.87</td>
</tr>
</tbody>
</table>
Table 3.4  Percent molar concentration (molar %) of fatty acid presence in MDA-MB-231 (231) cells after 96h treatment with low levels of ALA, EPA, DHA, GLA, or AA, and a non-treated control. GC analysis was used to measure percent molar concentrations in whole cell samples. Cells were frozen in media, thawed, and washed once with cold PBS prior to GC analysis. Percent molar concentrations were calculated using the integrated AUC of all fatty acids present in each sample.

<table>
<thead>
<tr>
<th>Condition</th>
<th>ALA (molar %)</th>
<th>EPA (molar %)</th>
<th>DHA (molar %)</th>
<th>GLA (molar %)</th>
<th>AA (molar %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA-MB-231 + 100µM ALA</td>
<td>11.08</td>
<td>6.85</td>
<td>1.42</td>
<td>0.06</td>
<td>2.89</td>
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<tr>
<td>MDA-MB-231 + 100µM EPA</td>
<td>0.35</td>
<td>14.53</td>
<td>1.65</td>
<td>0.13</td>
<td>2.41</td>
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<tr>
<td>MDA-MB-231 + 50µM DHA</td>
<td>0.47</td>
<td>3.16</td>
<td>21.26</td>
<td>0.10</td>
<td>2.99</td>
</tr>
<tr>
<td>MDA-MB-231 + 150µM GLA</td>
<td>0.21</td>
<td>0.21</td>
<td>0.65</td>
<td>9.36</td>
<td>5.36</td>
</tr>
<tr>
<td>MDA-MB-231 + 120µM AA</td>
<td>0.68</td>
<td>1.11</td>
<td>0.52</td>
<td>0.50</td>
<td>19.33</td>
</tr>
<tr>
<td>MDA-MB-231 - Control</td>
<td>0.15</td>
<td>0.89</td>
<td>1.98</td>
<td>0.18</td>
<td>4.69</td>
</tr>
</tbody>
</table>
Table 3.5 Percent molar concentration of fatty acid presence in MCF-10A cells after 96h treatment with low levels of ALA, EPA, DHA, GLA, or AA and a non-treated control. Gas chromatography analysis was used to measure percent molar concentrations in whole cell samples. Cells were frozen in media, thawed, and washed once with cold PBS prior to GC analysis. Percent molar concentrations were calculated using the integrated AUC of all fatty acids present in each sample.

<table>
<thead>
<tr>
<th></th>
<th>ALA (molar %)</th>
<th>EPA (molar %)</th>
<th>DHA (molar %)</th>
<th>GLA (molar %)</th>
<th>AA (molar %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCF-10A + 100μM ALA</td>
<td>25.56</td>
<td>0.58</td>
<td>0.91</td>
<td>0.00</td>
<td>2.13</td>
</tr>
<tr>
<td>MCF-10A + 100μM EPA</td>
<td>0.50</td>
<td>29.73</td>
<td>0.59</td>
<td>0.10</td>
<td>1.40</td>
</tr>
<tr>
<td>MCF-10A + 50μM DHA</td>
<td>0.63</td>
<td>6.82</td>
<td>28.80</td>
<td>0.00</td>
<td>2.87</td>
</tr>
<tr>
<td>MCF-10A + 150μM GLA</td>
<td>0.17</td>
<td>0.19</td>
<td>0.53</td>
<td>30.63</td>
<td>1.38</td>
</tr>
<tr>
<td>MCF-10A + 120μM AA</td>
<td>0.21</td>
<td>0.22</td>
<td>0.53</td>
<td>2.33</td>
<td>36.47</td>
</tr>
<tr>
<td>MCF-10A - Control</td>
<td>0.42</td>
<td>0.80</td>
<td>1.71</td>
<td>0.09</td>
<td>3.82</td>
</tr>
</tbody>
</table>
Discussion

In this study, the growth inhibition effects of multiple n-3 and n-6 fatty acids were tested on MCF-7 and MDA-MB-231 cancer cell lines, and the spontaneously immortalized MCF-10A breast epithelial cell line. In order to establish PUFA concentrations for future work, the responses of all three cell lines to each PUFA were averaged. This allowed for the generation of average PUFA toxicity curves. Three levels of PUFA concentrations were calculated from the curves; concentrations causing 25, 50, and 75% of average growth inhibition were defined as being low, medium, and high PUFA concentrations. This pilot study was done to confirm methods used, and to show FBS is an acceptable vehicle for PUFA delivery in cultured cells. This was accomplished by using gas chromatography, and FBS-PUFA treatment resulted in increased PUFA incorporation into the cells.

Results from the current study show that MCF-7, MDA-MB-231, and MCF-10A cells experience growth inhibition when exposed to n-3 and n-6 PUFA. Growth inhibition generally occurred in a dose dependent manner. However, the dose response trend was only seen when PUFA concentrations were ≥50µM, with the exception of GLA. When concentrations causing an average of 50% growth inhibition were calculated, DHA was found to require the lowest concentration (120µM), and GLA required the highest concentration (180µM). PUFA concentrations calculated to cause 25% and 75% of average growth inhibition were calculated to be 50-150µM and 170-270µM, respectively.
Multiple studies have studied the impact that various fatty acids have on the growth of breast cancer cell lines. However, comparison across the literature provides several challenges, as the fatty acids used for treatment varied in the concentrations used, the molecular form used for treatment (acid form, esterified, etc.), and the vehicle used to treat the cells. For example, in early work by Grammatikos and colleagues (90), MCF-7 and MCF-10A cells were treated with a range of 6-30µM of ALA, EPA, DHA, and AA. This work showed that MDA-MB-231 cells were extremely sensitive to the lower levels of PUFA treatment, while MCF-7 cells responded to EPA and DHA in a dose-response manner. MCF-10A cells exhibited a higher resistance to PUFA treatment; concentrations of PUFA <24µM caused no changes in MCF-10A growth (90). In comparison, concentrations in the current study are considerably higher; PUFA concentrations 8-9x higher than those used by Grammatikos et al. (90).

In more recent work, higher concentrations of fatty acids have been used to treat breast cancer cells, as well as combinations of PUFA rather than monotreatments (81,86,91). Two of these studies used more comparable PUFA concentrations, treating MCF-7 and MDA-MB-231 cells with 100µM of treatment fatty acids (81,91). In these studies, 100µM DHA caused 65% and 54% of control cell growth in MDA-MB-231 and MCF-7 cells, respectively (81,91). The current study found 120µM DHA to cause an average growth inhibition of 50% across all three cell lines studied. However, cell responses to EPA in the present study are higher compared to other work. Studies testing breast cancer cell response to EPA found 100µM EPA to reduce MDA-MB-231 cell growth to 42% of control, and MCF-7 cell growth to 30% of control (81,91). The current
data shows that 170\(\mu\)M EPA can cause an average of 50% growth inhibition in MCF-7, MDA-MB-231 and MCF-10A cells. Since these three cell lines have been shown to have varied responses to PUFA treatments, the concentrations of EPA and DHA used in the present study may be comparable to more recent research on PUFA ability to inhibit breast cancer cell growth.

The vehicles used for in vitro fatty acid delivery to cells is varied, and may complicate comparison between studies and observed effects. While the current study used FBS as the vehicle, previous studies have used ethanol, bovine serum albumin (BSA), and growth medium supplemented with FBS (72,81,86,90,91). In studies using BSA and ethanol as the treatment vehicle, treatment concentrations of EPA and DHA were lower than studies utilizing serums or media (30\(\mu\)M vs 100\(\mu\)M). However, these vehicles and concentrations resulted in similar effects on breast cancer cell growth. For example, MCF-7 cells treated with 30\(\mu\)M of DHA in BSA cause 50% growth inhibition, whereas 100\(\mu\)M of DHA in growth media with 5% FBS caused 54% growth inhibition (90,91). In MDA-MB-231 cells, studies using BSA and ethanol as PUFA vehicles resulted in very high levels of growth inhibition; one study suggested DHA concentrations >10\(\mu\)M to be cytotoxic (72,90). Conversely, when MDA-MB-231 cells were treated with PUFA using fetal calf serum (FCS) as the vehicle, 100\(\mu\)M DHA caused a 65% decrease in MDA-MB-231 growth (91).

Animal serums in growth media are used to mimic the serum environment in the physiological setting, and contain components involved in normal fatty acid metabolism
are present. While albumin is present in FBS/FCS and is known to bind fatty acids, low- and high-density lipoproteins (HDL and LDL) are also present, and could play a role in altering the effective concentrations required to inhibit breast cancer cell growth (120,121). Additionally, using BSA and ethanol as a vehicle, while effective in treating cells in vitro, they may not be as strong of a representation of physiological fatty acid delivery. These differences in vehicles may explain some of the variations in concentrations required for breast cancer cell growth inhibition. Some have gone further, and have isolated LDL and HDL from in vivo systems, such as monkeys, and used these isolates as treatment in in vitro systems (86). This presents a unique method to try and achieve in vivo levels of n-3 and n-6 PUFA, as the concentrations of these fatty acids in the LDL and HDL will vary based on the study diet provided in the in vivo system employed. GC data from this study show that FBS as a vehicle for treating cells with fatty acids is successful, as shown by the increased presence of treatment PUFA in the cell membranes of all three cell lines studied.

In conclusion, the current study has confirmed the growth inhibitory effects of n-3 and n-6 PUFA in breast cancer cells. PUFA incorporation into treated cells was confirmed using GC analysis, and is strength of the current work. However, expressing growth inhibition responses as an average response of MCF-7, MDA-MB-231, and MCF-10A cells may be a weakness. With previous work showing the varied responses across MCF-7, MDA-MB-231, and MCF-10A cells, averaging the results fails to demonstrate how various sub-types of breast cancer may respond to PUFA treatments. However, this approach was used with the goal to generate three common treatment concentrations for
future work, and assessing variations in cell line responses was not a goal of the present study
Chapter 4: Combination of n-3 PUFA and 1,25(OH)₂D₃ on Breast Cancer Cell Growth

4.1 Introduction

Independent of one another, the negative impact n-3 PUFA and vitamin D molecules have on breast cancer cell growth has been an area of interest. However, very little has been done to assess the effects of combining n-3 PUFA and vitamin D on cancer cell growth. To date, only two studies have looked at the combination of n-3 PUFA and vitamin D on cancer cells, with one showing potential synergism (106,107). Chiang et al. (106) measured cell proliferation of liver cancer cells in response to various combinations of fish oil and 1,25(OH)₂D₃. This study found that a concentration of fish oil (18µg/mL), which failed to cause significant decreases in cell proliferation alone, was able to cause significantly lower cell proliferation when 10⁻⁷ and 10⁻⁶ M of 1,25(OH)₂D₃ were added (106). However, this study was not designed to observe any additive or synergistic responses with combination treatments, and changes in proliferation were compared to control cells, and not to other treatments. In a combination study on prostate cells, fish oil and 1,25(OH)₂D₃ was found to inhibit cell cycle progression past the G1/S phase, in what was described as a synergistic response (107). However, this study failed to define the criteria used to determine synergy, and failed to test if the response is possibly additive; any additional inhibition in cell proliferation beyond fish oil or 1,25(OH)₂D₃ treatment alone was considered to be a synergistic response (107). Additionally, neither of these
studies offered details on the fatty acid content of their fish oil treatments. Any affect that could be attributed to a specific combination of n-3 or n-6 PUFA cannot be described.

In this study, the combination of n-3 PUFA and 1,25(OH)_2D_3 and its effects on breast cancer cell growth is tested. It is hypothesized that the combination of treatments will cause an additive, if not synergistic effect on inhibiting breast cancer cell growth. A 3x3 factorial design was used in order to assess if breast cancer cells respond to treatment in a dose-dependent manner, or if there is an optimal combination of treatment concentrations. For the purpose of testing for potential synergy between n-3 or n-6 PUFA and 1,25(OH)_2D_3, a theoretical additive (TA) value was calculated. The TA value was created by adding the percent difference in growth caused by a given PUFA concentration to the percent difference in growth caused by a given 1,25(OH)_2D_3 concentration. This created a theoretical additive growth inhibition value that would be the result if both treatments acted upon cell growth in a 1+1=2 manner. This value was used to compare the actual observed growth inhibition measured after combination treatments were tested. This comparison was used to suggest if cell growth inhibition with combination treatment is beyond an additive effect, and possibly a synergistic response.
4.2 Materials and Methods

4.2.1 Cell Culture

Refer to section 4.2.1.

4.2.2 Combination n-3 PUFA and 1,25(OH)D₃ Treatment

40mM stock concentrations of ALA, EPA, DHA, GLA, and AA were made as previously described (Section 4.2.3). Two sources of 1,25(OH)₂D₃ were used during this study, a 4mM solution (donated by LEO Pharmaceuticals), and later a 1mM solution (Enzo Life Sciences). Both 1,25(OH)₂D₃ stock solutions were in ethanol. Treatment media was made fresh for each experiment, and brown tubes were used to limit light exposure to the treated media. A 3x3 factorial design was used, creating 9 different combination treatments and 6 control monotreatments (Figure 4.1). The 3x3 design utilized the low, medium, and high concentrations of all 5 fatty acids that were generated in the pilot study (Chapter 3; Figure 3.1), for combination with 100, 300, and 500nM 1,25(OH)₂D₃. The 1,25(OH)₂D₃ concentrations were determined based on previous work completed in the current lab (12). Treatment media was plated into 2 96 well plates, each with one column of cells with non-treated media for control Plate 1 (combination plate) contained a control cell column, the 9 combination treatments, and blank columns (growth media only for background readings after SRB staining). Plate 2 (control plate) contained a control column, and the 6 monotreatments, and blank columns. Appendix I
details the plating design. Each plate contained 8 technical replications. MCF-7, MDA-MB-231, and MCF-10A cells were trypsinized as previously described (Section 3.2.1), and plated at 2000 cells/well. All media was changed at 48h time points. At 96h, plates were fixed with 50% TCA, and stained with SRB dye for absorbance readings as previously described (Section 4.2.5). At minimum of 3 experimental replications were completed for each PUFA-1,25(OH)_{2}D_{3} combination for all 3 cell types. Cell responses to the 1,25(OH)_{2}D_{3} vehicle (ethanol) treatment corresponding to 500nM 1,25(OH)_{2}D_{3} treatment were separately assessed. The ethanol concentrations in growth media were ≤1%.

**Figure 4.1** Schematic of combination treatment design, creating 9 combinations. Each treatment was tested on its own, creating 15 total treatments. Low, medium, and high PUFA values were determined in study 1 of this thesis. Values for the PUFA levels can be found in Table 3.1.
4.2.3 Microscopy

To observe the morphological changes in response to treatments, phase-contrast imaging was employed. Cells were plated in 12 well plates at a density of 4000 cells/well for MCF-7 and MDA-MB-231, and 2000 cells/well for MCF-10A. Cells were treated with low, medium, and high levels of EPA (100, 170, and 270µM, respectively) and AA (120, 170, and 240µM, respectively) alone and in combination with 100nM of 1,25(OH)₂D₃. EPA and AA were used as representative treatments of all 5 PUFA tested. EPA and AA were chose due to early results showing their effectiveness and potential interaction with 1,25(OH)₂D₃ treatments to increase growth inhibition in the cell lines. 100nM 1,25(OH)₂D₃ was used due to results in previous work showing morphological changes in breast cancer cells at this concentration (26,29,37). Images were taken after 96h of treatment using the Olympus FSX1000 and the FSX-BSW software. Media for all plates were changed at 48h.

4.2.5 Statistical Analysis

Combination and monotreatments were assessed using factorial analysis of variance (ANOVA) with post-hoc Tukey’s test to compare means of all treated groups. The Tukey’s test produced lists of homologous subsets identifying treatments reaching significance. Independent samples t-tests were used to compare the observed growth in response to combination treatments to their associated TA values, similar to previous work on combination treatments (107). One-sample t-tests were used to test for
significance with vehicle (ethanol) treatment to untreated cells. SPSS Statistics (IBM, version 20) was used for all tests, and significance was determined at \( p \leq 0.05 \).

4.3 Results

4.3.1 Response of MCF-7, MDA-MB-231, and MCF-10A Cells to \( 1,25(OH)_2D_3 \)

Treatment

All three cell lines were characterized for their response to 96h of 100, 300, or 500nM \( 1,25(OH)_2D_3 \) treatment as a control for each combination treatment trial. As summarized in Table 4.1, each cell line had the different responses each to \( 1,25(OH)_2D_3 \) treatment. None of the cell lines responded to \( 1,25(OH)_2D_3 \) treatment in a dose-response manner. MCF-10A cells were the most sensitive to \( 1,25(OH)_2D_3 \) treatment, with the surviving fraction of cells ranging from 27-31% (Table 4.1). MCF-7 cells had a moderate response to \( 1,25(OH)_2D_3 \) treatment, which caused a cell response of 60-64% of untreated cell growth (Table 4.1). Our results confirmed MDA-MB-231 resistance to \( 1,25(OH)_2D_3 \); MDA-MB-231 cells experienced 11-15% growth stimulation as compared to control cells. Vehicle control treatments corresponding to the 500nM \( 1,25(OH)_2D_3 \) treatment did not significantly change cell growth compared to untreated cells.
4.3.2 Cell Responses to PUFA Treatments

Individual cell line responses to each PUFA treatment were measured, unlike the averaged cell responses determined in the pilot study (Chapter 3). MCF-7, MDA-MB-231 and MCF-10A cells responded to low, medium, and high concentrations of all five treatment PUFA, predominantly in a dose-response manner (Appendix II). MCF-7 cells experienced the greatest growth inhibition in response to EPA and GLA treatments; medium levels (120µM EPA and 180µM GLA) reduced growth to 32% and 36%, respectively, and high levels (170µM EPA and 250µM GLA) reduced growth to 9% and 11%, respectively. MDA-MB-231 cells experienced the lowest responses to PUFA treatment, however treatments were still effective. DHA caused the greatest levels of MDA-MB-231 inhibition, with the high DHA concentration (170µM) decreasing cell growth to 4% of control cell growth. AA treatment also inhibited MDA-MB-231 cells, with a maximum inhibition of 10% of control cell growth in response to the high AA concentration (240µM AA). ALA, EPA, and GLA inhibited MDA-MB-231 cells to a maximum inhibition of 72%, 26%, and 25%, respectively. MCF-10A cells were the most sensitive cells, with both n-3 and n-6 PUFA causing large decreases in cell growth. EPA and DHA were the least effective in inhibiting MCF-10A cell growth, but low concentrations (100µM EPA and 50µM DHA) were able to decrease cell growth to 12% and 22% of control cell growth, respectively. AA was the most effective at inhibiting MCF-10A cell growth, reducing growth to 1% with medium and high concentrations (170µM and 240µM, respectively).
Table 4.1: The average growth response of MCF-7, MDA-MB-231, and MCF-10A to 100, 300, and 500nM 1,25(OH)₂D₃ treatment for 96h, expressed as the percent of untreated control cells. Cells were plated in 96 well plates and were stained with SRB dye for absorbance readings. Results were generated from averaged responses from all 1,25(OH)₂D₃ monotreatments plated for comparison with combination treatments.

<table>
<thead>
<tr>
<th>96h 1,25(OH)₂D₃ Treatment</th>
<th>MCF-7 (% of control)</th>
<th>MDA-MB-231 (% of control)</th>
<th>MCF-10A (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100nM</td>
<td>64.6</td>
<td>115</td>
<td>30.6</td>
</tr>
<tr>
<td>300nM</td>
<td>60.0</td>
<td>112</td>
<td>29.4</td>
</tr>
<tr>
<td>500nM</td>
<td>60.4</td>
<td>111</td>
<td>27.0</td>
</tr>
</tbody>
</table>
MCF-7 Cells Response to Combination Treatments

MCF-7 cells experienced significantly lower cell growth when treated with combination treatments involving EPA and AA. 100μM EPA + 300nM 1,25(OH)₂D₃ caused 38% of control cell growth, which was significantly lower than 100μM EPA alone (p=0.008) and 300nM 1,25(OH)₂D₃ alone (p=0.002) (Figure 4.3). 100μM EPA + 500nM 1,25(OH)₂D₃ was also significantly lower than 100μM EPA alone (p=0.04) and 500nM 1,25(OH)₂D₃ alone (p=0.04) (Figure 4.3). MCF-7 cells treated with the low AA concentration (120μM AA) in combination with 300 and 500nM 1,25(OH)₂D₃ showed significant growth inhibition as compared to 120μM AA alone (p=0.02 and p=0.035, respectively) (Figure 4.6). Combination treatments with ALA, DHA, and GLA did not exhibit any significant changes in growth inhibition (Figure 4.2, 4.4, 4.5)
Figure 4.2: MCF-7 response to ALA + 1,25(OH)$_2$D$_3$ treatments at 96h. L: 100µM ALA; M: 160µM ALA; H: 220µM ALA; l: 100nM 1,25(OH)$_2$D$_3$; m: 300nM 1,25(OH)$_2$D$_3$; h: 500nM 1,25(OH)$_2$D$_3$. Cells were plated with treatment media and incubated for 96h, followed by SRB staining and absorbance readings to determine percent growth compared to control. Factorial ANOVA with post-hoc Tukey’s test was performed to determine significance, with n=3. Bars that do not share the same letter are significantly different, with p ≤ 0.05. Error bars are +/- SEM.
**Figure 4.3:** MCF-7 response to EPA + 1,25(OH)$_2$D$_3$ treatments. L: 100µM EPA; M: 170µM EPA; H: 270µM EPA; l: 100nM 1,25(OH)$_2$D$_3$; m: 300nM 1,25(OH)$_2$D$_3$; h: 500nM 1,25(OH)$_2$D$_3$. Cells were plated with treatment media and incubated for 96h, followed by SRB staining and absorbance readings to determine percent growth compared to control. Factorial ANOVA with post-hoc Tukey’s test was performed to determine significance, with n=5. Bars that do not share the same letter are significantly different, with p ≤ 0.05. Error bars are +/- SEM.
**Figure 4.4:** MCF-7 response to DHA + 1,25(OH)$_2$D$_3$ treatments. L: 50µM DHA; M: 120µM DHA; H: 170µM DHA; l: 100nM 1,25(OH)$_2$D$_3$; m: 300nM 1,25(OH)$_2$D$_3$; h: 500nM 1,25(OH)$_2$D$_3$. Cells were plated with treatment media and incubated for 96h, followed by SRB staining and absorbance readings to determine percent growth compared to control. Factorial ANOVA with post-hoc Tukey’s test was performed to determine significance, with n=5. Bars that do not share the same letter are significantly different, with p ≤ 0.05. Error bars are +/- SEM.
Figure 4.5: MCF-7 response to GLA + 1,25(OH)$_2$D$_3$ treatments. L: 150µM GLA; M: 180µM GLA; H: 250µM GLA; l: 100nM 1,25(OH)$_2$D$_3$; m: 300nM 1,25(OH)$_2$D$_3$; h: 500nM 1,25(OH)$_2$D$_3$. Cells were plated with treatment media and incubated for 96h, followed by SRB staining and absorbance readings to determine percent growth compared to control. Factorial ANOVA with post-hoc Tukey’s test was performed to determine significance, with n=3. Bars that do not share the same letter are significantly different, with $p \leq 0.05$. Error bars are +/- SEM.
Figure 4.6: MCF-7 response to AA + 1,25(OH)₂D₃ treatments. L: 120µM AA; M: 170µM AA; H: 240µM AA; l: 100nM 1,25(OH)₂D₃; m: 300nM 1,25(OH)₂D₃; h: 500nM 1,25(OH)₂D₃. Cells were plated with treatment media and incubated for 96h, followed by SRB staining and absorbance readings to determine percent growth compared to control. Factorial ANOVA with post-hoc Tukey’s test was performed to determine significance, with n=5. Bars that do not share the same letter are significantly different, with p ≤ 0.05. Error bars are +/- SEM.
In MDA-MB-231 cells, combinations involving 1,25(OH)$_2$D$_3$ with ALA, EPA, GLA and AA appear to sensitize cells to 1,25(OH)$_2$D$_3$ inhibitory effects (Figures 4.7, 4.8, 4.10, 4.11). As previously discussed, MDA-MB-231 cells do not exhibit growth inhibition when treated with 1,25(OH)$_2$D$_3$. ALA and EPA combinations with 1,25(OH)$_2$D$_3$ cause the greatest decreases in MDA-MB-231 growth inhibition as compared to control cells. In ALA combinations with 1,25(OH)$_2$D$_3$, where 1,25(OH)$_2$D$_3$ alone was found to stimulate MDA-MB-231 cell growth to 31-40% higher than control cells, combination treatment achieved growth as low as 38% of control cells. Combination treatments with ALA and 1,25(OH)$_2$D$_3$ decreased with a dose-response to both ALA and 1,25(OH)$_2$D$_3$ concentrations. The addition of 1,25(OH)$_2$D$_3$ to low, medium, and high ALA concentrations (100, 160, and 220µM ALA, respectively) enhanced growth inhibition by up to 14%, 28%, and 34%, respectively. In combinations involving the high EPA concentration (270µM EPA), the addition of 1,25(OH)$_2$D$_3$ caused the greatest amounts of growth inhibition; 270µM EPA alone inhibited MDA-MB-231 growth to 26% of control, which was decreased to 6-8% of control when 1,25(OH)$_2$D$_3$ was added. This response was not reflective of a dose-response to 1,25(OH)$_2$D$_3$. GLA and AA also experienced additional growth inhibition in the presence of 1,25(OH)$_2$D$_3$, though less drastically than ALA and EPA. GLA and 1,25(OH)$_2$D$_3$ combinations caused up to 19% additional growth inhibition compared to control and AA caused up to 12% added growth inhibition compared to control. These responses did not occur in a dose-response manner with regard to 1,25(OH)$_2$D$_3$ concentrations. Despite the differences in
growth when 1,25(OH)_2D_3 is treated in combination with ALA, EPA, GLA and AA, statistical significance in a factorial ANOVA was not reached. Combinations of 1,25(OH)_2D_3 with DHA did not enhance growth inhibition in MDA-MB-231 cells (Figure 4.9).
Figure 4.7: MDA-MB-231 response to ALA + 1,25(OH)$_2$D$_3$ treatments. L: 100µM ALA; M: 160µM ALA; H: 220µM ALA; l: 100nM 1,25(OH)$_2$D$_3$; m: 300nM 1,25(OH)$_2$D$_3$; h: 500nM 1,25(OH)$_2$D$_3$. Cells were plated with treatment media and incubated for 96h, followed by SRB staining and absorbance readings to determine percent growth compared to control. Factorial ANOVA with post-hoc Tukey’s test was performed to determine significance, with n=3. Bars that do not share the same letter are significantly different, with p ≤ 0.05. Error bars are +/- SEM.
**Figure 4.8:** MDA-MB-231 response to EPA + 1,25(OH)\(_2\)D\(_3\) treatments. L: 100µM EPA; M: 170µM EPA; H: 270µM EPA; l: 100nM 1,25(OH)\(_2\)D\(_3\); m: 300nM 1,25(OH)\(_2\)D\(_3\); h: 500nM 1,25(OH)\(_2\)D\(_3\). Cells were plated with treatment media and incubated for 96h, followed by SRB staining and absorbance readings to determine percent growth compared to control. Factorial ANOVA with post-hoc Tukey’s test was performed to determine significance, with n=5. Bars that do not share the same letter are significantly different, with p ≤ 0.05. Error bars are +/- SEM.
Figure 4.9: MDA-MB-231 response to DHA + 1,25(OH)₂D₃ treatments. L: 50µM DHA; M: 120µM DHA; H: 170µM DHA; l: 100nM 1,25(OH)₂D₃; m: 300nM 1,25(OH)₂D₃; h: 500nM 1,25(OH)₂D₃. Cells were plated with treatment media and incubated for 96h, followed by SRB staining and absorbance readings to determine percent growth compared to control. Factorial ANOVA with post-hoc Tukey’s test was performed to determine significance, with n=5. Bars that do not share the same letter are significantly different, with p ≤ 0.05. Error bars are +/- SEM.
**Figure 4.10:** MDA-MB-231 response to GLA + 1,25(OH)$_2$D$_3$ treatments. L: 150µM GLA; M: 180µM GLA; H: 250µM GLA; l: 100nM 1,25(OH)$_2$D$_3$; m: 300nM 1,25(OH)$_2$D$_3$; h: 500nM 1,25(OH)$_2$D$_3$. Cells were plated with treatment media and incubated for 96h, followed by SRB staining and absorbance readings to determine percent growth compared to control. Factorial ANOVA with post-hoc Tukey’s test was performed to determine significance, with n=3. Bars that do not share the same letter are significantly different, with p ≤ 0.05. Error bars are +/- SEM.
**Figure 4.11:** MDA-MB-231 response to AA + 1,25(OH)\(_2\)D\(_3\) treatments. L: 120µM AA; M: 170µM AA; H: 240µM AA; l: 100nM 1,25(OH)\(_2\)D\(_3\); m: 300nM 1,25(OH)\(_2\)D\(_3\); h: 500nM 1,25(OH)\(_2\)D\(_3\). Cells were plated with treatment media and incubated for 96h, followed by SRB staining and absorbance readings to determine percent growth compared to control. Factorial ANOVA with post-hoc Tukey’s test was performed to determine significance, with n=5. Bars that do not share the same letter are significantly different, with p ≤ 0.05. Error bars are +/- SEM.
With the robust growth inhibition caused by PUFA treatments alone (as discussed in section 4.3.2), the addition of 1,25(OH)$_2$D$_3$ provided little additional growth inhibition in the MCF-10A cell line (Figures 4.12-4.16). When n-3 and n-6 PUFA treatments were combined with 1,25(OH)$_2$D$_3$ treatments, any changes in MCF-10A growth were non-significant. In the combinations involving 100μM EPA and 50μM DHA with 1,25(OH)$_2$D$_3$, some additional growth inhibition was observed with 1,25(OH)$_2$D$_3$ addition. However, since 100μM and 50μM DHA caused 12% and 22% of control cell growth independent of 1,25(OH)$_2$D$_3$, and 1,25(OH)$_2$D$_3$ treatments alone inhibited MCF-10A cell growth as low as 27% of control cell growth, combination treatments did not cause any enhanced growth inhibition. No combination treatments were statistically significant from both respective PUFA and 1,25(OH)$_2$D$_3$ treatments.
Figure 4.12: MCF-10A response to ALA + 1,25(OH)\(_2\)D\(_3\) treatments. L: 100µM ALA; M: 160µM ALA; H: 220µM ALA; l: 100nM 1,25(OH)\(_2\)D\(_3\); m: 300nM 1,25(OH)\(_2\)D\(_3\); h: 500nM 1,25(OH)\(_2\)D\(_3\). Cells were plated with treatment media and incubated for 96h, followed by SRB staining and absorbance readings to determine percent growth compared to control. Factorial ANOVA with post-hoc Tukey’s test was performed to determine significance, with n=3. Bars that do not share the same letter are significantly different, with p ≤ 0.05. Error bars are +/- SEM.
Figure 4.13: MCF-10A response to EPA + 1,25(OH)₂D₃ treatments. L: 100µM EPA; M: 170µM EPA; H: 270µM EPA; l: 100nM 1,25(OH)₂D₃; m: 300nM 1,25(OH)₂D₃; h: 500nM 1,25(OH)₂D₃. Cells were plated with treatment media and incubated for 96h, followed by SRB staining and absorbance readings to determine percent growth compared to control. Factorial ANOVA with post-hoc Tukey’s test was performed to determine significance, with n=5. Bars that do not share the same letter are significantly different, with p ≤ 0.05. Error bars are +/- SEM.
Figure 4.14: MCF-10A response to DHA + 1,25(OH)$_2$D$_3$ treatments. L: 50µM DHA; M: 120µM DHA; H: 170µM DHA; l: 100nM 1,25(OH)$_2$D$_3$; m: 300nM 1,25(OH)$_2$D$_3$; h: 500nM 1,25(OH)$_2$D$_3$. Cells were plated with treatment media and incubated for 96h, followed by SRB staining and absorbance readings to determine percent growth compared to control. Factorial ANOVA with post-hoc Tukey’s test was performed to determine significance, with n=5. Bars that do not share the same letter are significantly different, with p ≤ 0.05. Error bars are +/- SEM.
Figure 4.15: MCF-10A response to GLA + 1,25(OH)_2D₃ treatments. L: 150µM GLA; M: 180µM GLA; H: 250µM GLA; l: 100nM 1,25(OH)_2D₃; m: 300nM 1,25(OH)_2D₃; h: 500nM 1,25(OH)_2D₃. Cells were plated with treatment media and incubated for 96h, followed by SRB staining and absorbance readings to determine percent growth compared to control. Factorial ANOVA with post-hoc Tukey’s test was performed to determine significance, with n=3. Bars that do not share the same letter are significantly different, with p ≤ 0.05. Error bars are +/- SEM.
Figure 4.16: MCF-10A response to AA + 1,25(OH)₂D₃ treatments. L: 120µM AA; M: 170µM AA; H: 240µM AA; l: 100nM 1,25(OH)₂D₃; m: 300nM 1,25(OH)₂D₃; h: 500nM 1,25(OH)₂D₃. Cells were plated with treatment media and incubated for 96h, followed by SRB staining and absorbance readings to determine percent growth compared to control. Factorial ANOVA with post-hoc Tukey’s test was performed to determine significance, with n=5. Bars that do not share the same letter are significantly different, with p ≤ 0.05. Error bars are +/- SEM.
4.3.6 *Comparison of Observed Cell Responses to Combination Treatment to Theoretical Additive Inhibition Levels*

To further characterize the effects combination treatments had on MDA-MB-231 cells, the measured (observed) changes in cell growth was compared to the corresponding TA value. The TA value is a round calculation to provide a theoretical growth response, with the assumption that PUFA and $1,25(\text{OH})_2\text{D}_3$ additivity occurs in a $1+1=2$ manner. Any growth inhibition beyond the TA value for a given combination could be considered to be a synergistic response of PUFA and $1,25(\text{OH})_2\text{D}_3$ treatments. To calculate the TA value for each combination of low, medium, or high PUFA with 100, 300 or 500nM $1,25(\text{OH})_2\text{D}_3$, the following formulas were used:

\[
\% \text{ TA growth inhibition} = \frac{(100\% - \% \text{ of control growth inhibited by PUFA treatment}) + (100\% - \% \text{ of control growth inhibited by } 1,25(\text{OH})_2\text{D}_3 \text{ treatment})}{100\%}
\]

**Formula 4.1:** % Theoretical additive growth inhibition formula

\[
100\% - \% \text{ TA growth inhibition} = \% \text{ TA growth}
\]

**Formula 4.2:** % Theoretical additive growth formula
The % TA growth value was plotted against observed values for MDA-MB-231 combinations with ALA, EPA, GLA, and AA. MCF-10A cells did not show any additional benefits with PUFA and 1,25(OH)$_2$D$_3$ combinations, and MCF-7 cells showed only minor benefits in certain combinations of PUFA and 1,25(OH)$_2$D$_3$, none of which were below that of the TA value. The low ALA concentration (100µM ALA) and high ALA concentration (200µM ALA) combined with 1,25(OH)$_2$D$_3$ were found to be significantly lower to the corresponding TA values (Figure 4.17). Low ALA and 500nM 1,25(OH)$_2$D$_3$ was the only exception. The observed growth of combinations with the high ALA concentration (220µM ALA) with all three 1,25(OH)$_2$D$_3$ concentrations were also significantly lower than the respective TA value (Figure 4.17). Although not statistically significant, combinations with the medium concentration of ALA (160µM) exhibited a trend (p-values with 100, 300, and 500nM 1,25(OH)$_2$D$_3$ calculated to be 0.07, 0.09, and 0.07, respectively). When 1,25(OH)$_2$D$_3$ was combined with EPA concentrations, high EPA (270µM) combinations with 1,25(OH)$_2$D$_3$ caused significantly less growth than the respective TA value growth, when a possible trend with low (100µM EPA) and medium (170µM EPA) levels are combined with 1,25(OH)$_2$D$_3$ as well (Figure 4.18). GLA combinations did not exhibit statistical significance when compared to their respective TA values, but appear to also have a trend (Figure 4.19). AA combinations exhibited a significant trend with all AA concentrations (120, 170, and 240µM AA) combined with 500nM 1,25(OH)$_2$D$_3$ (Figure 4.20). There was an additional benefit seen with AA and 100 and 300nM 1,25(OH)$_2$D$_3$, but they did not reach statistical significance.
Figure 4.17: MDA-MB-231 ALA + 1,25(OH)₂D₃ combination treatments (n=3) compared to the theoretical additive (TA) value. L: 100µM ALA; M: 160µM ALA; H: 220µM ALA; l: 100nM 1,25(OH)₂D₃; m: 300nM 1,25(OH)₂D₃; h: 500nM 1,25(OH)₂D₃. TA values were calculated by adding the growth response of PUFA and 1,25(OH)₂D₃ individually, for comparison to the observed growth response. * indicates TA values that are significantly different from their respective observed value, as determined by independent samples t-test with significance at p≤0.05. Error bars are +/- SEM.
Figure 4.18: MDA-MB-231 EPA + 1,25(OH)_2D_3 combination treatments (n=5) compared to the theoretical additive (TA) value. L: 100µM EPA; M: 170µM EPA; H: 270µM EPA; l: 100nM 1,25(OH)_2D_3; m: 300nM 1,25(OH)_2D_3; h: 500nM 1,25(OH)_2D_3. TA values were calculated by adding the growth response of PUFA and 1,25(OH)_2D_3 individually, for comparison to the observed growth response. * indicates TA values that are significantly different from their respective observed value, as determined by independent samples t-test with significance at p≤0.05. Error bars are +/- SEM.
Figure 4.19: MDA-MB-231 GLA + 1,25(OH)_{2}D_{3} combination treatments (n=3) compared to the theoretical additive (TA) value. L: 150μM GLA; M: 180μM GLA; H: 250μM GLA; l: 100nM 1,25(OH)_{2}D_{3}; m: 300nM 1,25(OH)_{2}D_{3}; h: 500nM 1,25(OH)_{2}D_{3}. TA values were calculated by adding the growth response of PUFA and 1,25(OH)_{2}D_{3} individually, for comparison to the observed growth response. * indicates TA values that are significantly different from their respective observed value, as determined by independent samples t-test with significance at p≤0.05. Error bars are +/- SEM.
Figure 4.20: MDA-MB-231 AA + 1,25(OH)$_2$D$_3$ combination treatments compared (n=5) to the theoretical additive (TA) value. L: 120µM AA; M: 170µM AA; H: 240µM AA; l: 100nM 1,25(OH)$_2$D$_3$; m: 300nM 1,25(OH)$_2$D$_3$; h: 500nM 1,25(OH)$_2$D$_3$. TA values were calculated by adding the growth response of PUFA and 1,25(OH)$_2$D$_3$ individually, for comparison to the observed growth response. * indicates TA values that are significantly different from their respective observed value, as determined by independent samples t-test with significance at $p \leq 0.05$. Error bars are +/- SEM.
4.3.7 Morphological Changes in Cells after Combination Treatments

To assess the physical and morphological changes to MCF-7 (Appendix III-IV), MDA-MB-231 (Appendix V-VI) and MCF-10A cells (Appendix VII-VIII), cells were treated with low, medium, and high concentrations alone, and combinations with 100nM 1,25(OH)_2D_3, and phase contrast images were taken. As EPA or AA concentrations increased, decreases in the cell population were observed. Cells treated with medium and high concentrations of EPA or AA alone and in combination appeared rounded with visible granules inside and decreased adherence to the plate surface. Small, round structures were also visible with EPA or AA treatment, with the possibility that they were apoptotic bodies resulting from treatments.

4.4 Discussion

The purpose of this thesis was to assess the effects of combined n-3 or n-6 PUFA and 1,25(OH)_2D_3 treatment on the growth of breast cancer cells. The effects of low, medium, and high concentrations of ALA, EPA, DHA, GLA, and AA (as determined in the pilot study, Chapter 2) and 100, 300, and 500nM 1,25(OH)_2D_3 were assessed alone and in combination. The present data has shown that treatments of ALA, EPA, GLA, and AA can sensitize a vitamin D-resistant cell line (MDA-MB-231) to 1,25(OH)_2D_3 treatment. This response was found to be beyond an additive effect of combination treatments, and is likely the result of synergistic interactions between these four PUFA and 1,25(OH)_2D_3. This is the only known study to test the combination of n-3 or n-6
PUFA and 1,25(OH)$_2$D$_3$ treatments on breast cancer cells, and these results are thought to be novel findings. Meanwhile, cell lines that are vitamin D-responsive (MCF-7 and MCF-10A) did not exhibit any additive or synergistic growth inhibition with PUFA and 1,25(OH)$_2$D$_3$ combination treatments.

Unlike the pilot study determining the average cell responses to PUFA treatments, the current study assessed individual cell line responses to n-3 or n-6 PUFA treatments. Previous work has produced conflicting evidence when characterizing MCF-7, MDA-MB-231 and MCF-10A responses to PUFA treatments. Some studies have suggested that the MDA-MB-231 cell line is most responsive to PUFA treatment, and MCF-10A cells are least responsive, while the current data suggests the opposite (90). It is possible that expression patterns within the cell lines have changed over time, and that their responses to PUFA treatments have been affected. For example, early work on MCF-7 cells found that cells from different labs had variable expression of ER and PR, along with alterations in chromosome structures (122). Additionally, when MCF-7 cells used in research laboratories were compared to the American Type Culture Collection (ATCC), from which most cells are purchased, cells grown and passaged in research labs had different expression patterns compared to ATCC cells (122). MCF-10A, typically ER$\alpha$, have been found to spontaneously express ER$\alpha$ after $>$20 cell passages; this may partially explain the presence of ER$\alpha$ in MCF-10A samples used in the current research (See section 3.3.1) (123). The differences in MDA-MB-231 response to n-3 and n-6 PUFA treatments may be explained in part by the vehicle used for PUFA treatments. In studies using BSA and ethanol as the vehicle for PUFA delivery to MDA-MB-231 cells, extensive growth
inhibition was recorded (72,90). One study found that PUFA concentrations >10µM in BSA were so effective at inhibiting MDA-MB-231 cell growth, that it was suggested these concentrations were cytotoxic to the cells (90). In another study, 20µg/mL of DHA in ethanol caused 95% growth inhibition in MDA-MB-231 cells (72). The present work, among others using serums and growth media as PUFA vehicles, have shown more moderate growth inhibition in MDA-MB-231 cells with higher treatment concentrations. For example, the current study found that 50µM DHA in FBS caused MDA-MB-231 cells to decrease growth to ~60% of control, and previous work testing 100µM DHA in FBS caused a 65% decrease in cell growth compared to control (81).

Responses to 1,25(OH)₂D₃ alone were comparable to previous work. In MCF-7 cells, 100nM 1,25(OH)₂D₃ has been reported to decrease cell growth to 56% of control after 72h treatment, compared to current data showing a 60% reduction at 96h (26). Another study found 500nM 1,25(OH)₂D₃ reduced MCF-7 growth to 65% of control after 72h of treatment, compared to 60% of control growth after 96h treatment in the current study (36). MDA-MB-231 cells did not exhibit any growth inhibition in the present work, and appeared to have slight stimulation with 1,25(OH)₂D₃ treatment. This is consistent with previous reports that this cell line is vitamin D resistant and exhibits very low VDR levels (18). MCF-10A cells were shown here to experience the greatest decrease in the remaining fraction of cells in response to 1,25(OH)₂D₃ treatment, which is also consistent with previous work (12). Additionally, the current work shows that there is no evidence of a dose-response to 1,25(OH)₂D₃ treatments across all cell lines.
The cell responses to combined treatments have uncovered a novel observation. While MCF-7 and MCF-10A cells did not appear to have much added benefit of combination treatments at 96h, MDA-MB-231 cells responded greater to combination treatments with ALA, EPA, GLA and AA compared to PUFA treatment alone. Given that the MDA-MB-231 cell line does not respond to vitamin D treatment, this suggests that these PUFA may sensitize MDA-MB-231 cells to 1,25(OH)\textsubscript{2}D\textsubscript{3} treatment. In order to suggest if synergism between the PUFA and 1,25(OH)\textsubscript{2}D\textsubscript{3} treatment is occurring, a basic formula to determine the “theoretical additive” value was used. This formula added the growth inhibition caused by each PUFA to growth inhibition caused by 1,25(OH)\textsubscript{2}D\textsubscript{3} treatments, in a 1+1=2 manner. The observed results were compared to their relative TA values to characterize the results, and suggest if there was a greater than additive effect occurring. In MDA-MB-231 cells treated with ALA, EPA, GLA, or AA and 1,25(OH)\textsubscript{2}D\textsubscript{3}, growth inhibition was found to be beyond the respective TA values, and was possibly a synergistic response. While a trend may exist across all combinations of ALA, EPA, GLA and AA with all three concentrations of 1,25(OH)\textsubscript{2}D\textsubscript{3}, significance was only found within certain combinations. However, treatment combinations with DHA, the longest PUFA tested, did not share this response; it is unknown if the length of PUFA is a factor in sensitizing MDA-MB-231 cells to 1,25(OH)\textsubscript{2}D\textsubscript{3} treatment.

Effects of combination treatment compared to PUFA-only treatment on cell morphology did not appear to be different. PUFA treatments alone and in combination with 100nM 1,25(OH)\textsubscript{2}D\textsubscript{3} caused a vast change in cell morphology, when medium and high EPA and AA concentrations were applied. As EPA and AA concentrations
increased, cells were less adherent, smaller in size, and spherical in shape. Excess amounts of cellular debris and much smaller sphere-shaped structures were visible, suggesting cells may have undergone apoptosis and apoptotic bodies remained. In physiological settings, apoptotic bodies would normally be cleared away by macrophages. Previous work has found that morphological changes consistent with apoptosis, such as cell shrinkage, cell membrane “blebbing”, and chromatin condensation, were visible in breast cancer cells after n-3 PUFA or 1,25(OH)_{2}D_{3} treatment, confirming the current observations (29,30,89). In order to show if combination treatments can cause cell changes indicative of apoptosis, a positive control treatment should be used in future work for further comparison.

Previous work on lung and prostate cancer has reported, where combinations of fish oil and 1,25(OH)_{2}D_{3} were found to cause synergistic growth inhibition (106,107). While neither study provides further information on the potential mechanisms at hand, potential interactions between n-3 PUFA and 1,25(OH)_{2}D_{3} have been discussed elsewhere (104). Mechanisms involved in the potential synergistic response to combination treatments in the MDA-MB-231 cell line remains unknown. The possibility that 1,25(OH)_{2}D_{3} may be acting in non-VDR-mediated pathways is a potential explanation. MDA-MB-231 cells have been found to express MARRS, a membrane-bound receptor that responds to 1,25(OH)_{2}D_{3} treatment (12). However, any potential roles MARRS may have in inhibiting breast cancer cell growth remain undiscovered. Much work in this area is needed to better understand how 1,25(OH)_{2}D_{3} interacts in
vitamin D resistant cells, and how PUFA may enhance the action of vitamin D in these cells.

In conclusion, the current study has assessed combinations of n-3 and n-6 PUFA with 1,25(OH)$_2$D$_3$, and has discovered potential 1,25(OH)$_2$D$_3$ sensitization in the MDA-MB-231 cell line with PUFA treatment. Further analysis is required to characterize mechanisms that may be involved or altered with these combination treatments, and if they are affecting rates of apoptosis and/or cell cycle progression. Understanding how PUFA may sensitize MDA-MB-231 cells to 1,25(OH)$_2$D$_3$ treatment is of great interest, and warrants future research.
Chapter 5: General Discussion and Future Directions

5.1 General Discussion

The two studies outlined in this thesis have shown that n-3 and n-6 PUFA are able to inhibit breast cancer cell growth, and are able to sensitize a vitamin D-resistant cell line to 1,25(OH)\textsubscript{2}D\textsubscript{3} treatment. When comparing theoretical additive values to the observed response, MDA-MB-231 cells appear to respond synergistically to PUFA and 1,25(OH)\textsubscript{2}D\textsubscript{3} treatment. The underlying mechanisms mediating the anti-cancer effects of n-3 PUFA and vitamin D have yet to be fully elucidated, though much work on both types of molecules has been done. Previous research has identified that both n-3 PUFA and 1,25(OH)\textsubscript{2}D\textsubscript{3} are able to cause apoptosis, growth inhibition, and changes in cell cycle machinery in breast cancer cells (26,28,37,72,87,89,91). The current work suggests that when PUFA and 1,25(OH)\textsubscript{2}D\textsubscript{3} are combined, there is an interaction that may be enhancing 1,25(OH)\textsubscript{2}D\textsubscript{3} effects within breast cancer cells. As breast cancer is growing to become a global health issue, interest in nutritional interventions to mediate risk, development, and treatments increases. This research provides evidence that n-3 PUFA and vitamin D may be beneficial as a nutritional component to breast cancer treatments regimens.

Three cell lines were used to study growth effects n-3 or n-6 PUFA and 1,25(OH)\textsubscript{2}D\textsubscript{3} on breast cancer cell growth. MCF-7 cells modeled a hormone responsive breast cancer, and MDA-MB-231 cells modeled a hormone-resistant form of breast
These cell lines were compared to a non-malignant breast epithelial cell line (MCF-10A). MCF-7 and MDA-MB-231 cell lines are commonly used to model cancer in \textit{in vitro} settings, though many breast cancer cell lines have now been established and characterized (116). Gene expression profiling of several cell lines has allowed them to be classified in comparison to clinical classifications that have been proposed, based on gene profiles (116,124,125). Neither MCF-7 nor MDA-MB-231 express HER2, and fail to model a large population of breast cancer subtypes present in patient populations (125). Cell lines that are HER2 positive, such as the BT474 and ZR-75 lines, could be used to model triple positive breast cancers, to account for the breast cancer population that has this hormone receptor profile.

The experimental design of the current studies involved plating cells simultaneously with treatment-supplemented media. If PUFA or 1,25(OH)$_2$D$_3$ treatments have early effects that could inhibit cellular attachment, this would be missed in the current study design. Treatment duration in the present research was assessed at 96h, and media was changed at 48h. Any cells that were prevented from adhering to the plate surface would have been removed with the media change at 48h. Previous cell culture work often seeded cells into plates for 24-48h to allow for cell adhesion and some growth prior to treating the cells (36,37,65,67,72,101). The current model could be used to examine the ability of PUFA and 1,25(OH)$_2$D$_3$ to inhibit normal breast cancer cell adhesion. To test this, media removed at 48h could be stained with trypan blue dye, and numbers of viable and non-viable cells could be counted. This would provide information on if the treatments are inhibiting cell adhesion and not cell viability, or if the cells are
dying. This should be taken into consideration in future work that chooses to plate cells and apply treatments simultaneously. This model could be changed in the future to allow 24-48h for cell attachment and potential cell growth prior to treatment. However, plating efficiency trials would need to be completed prior to initiating the longer time treatment experiments, to ensure cells do not grow past confluency or use all of the nutrients available in the media.

Vitamin D and n-3 PUFA have also been implicated in mammary gland development and subsequent breast cancer risk (30,48,126). In studies looking at mammary gland development through observations of terminal end buds (TEB), decreased vitamin D was found to decrease TEB differentiation, and n-3 PUFA are suggested to decrease TEB amounts (37,127). TEB are present during mammary gland development, are highly proliferative, and have been identified as sites of tumorigenesis in breast cancer tissues (127,128).

Several studies have looked at the benefit of fatty acids and vitamin D in conjunction with chemotherapy treatment. At the cellular level, DHA and GLA are able to enhance chemosensitivity and effectiveness of chemotherapeutic agents (76,77,129,130). Mechanisms that are proposed to be involved with these findings include augmenting uptake and efflux of chemotherapeutic drugs and enhancing the cytotoxicity of ROS-inducing chemotherapies (76,77,130). Studies using DHA and GLA in combination with ROS-inducing chemotherapies have suggested that they enhance the drug response by increasing lipid presence for peroxidation by chemotherapy and overall
lipid peroxidation products, allowing for enhanced breast cancer cell death (76,129). PUFA-mediated alterations of the plasma membrane and lipid rafts may play a role in altering membrane channels and receptors associated with drug resistance. For example, evidence shows that ATP-binding cassette (ABC) transporters, which are involved in multi-drug resistance in cancer, are localized to lipid rafts, whose composition may affect localization and function (131). In human clinical trials, DHA was tested in conjunction with various chemotherapies (54,55). These studies found that DHA supplementation enhanced chemosensitivity and caused a faster response to treatments (54,55). Vitamin D and its impact on breast cancer chemotherapy has been studied less than PUFA, however some evidence suggests vitamin D pre-treatment of cancer cells can enhance chemotherapy effectiveness (132,133). The chemotherapies used in these studies act upon breast cancer cells using mechanisms unique from 1,25(OH)_{2}D_{3}; inhibition of mitotic spindle formation, inhibition of topoisomerase II progression and ROS generation are the mechanisms involved in the tested chemotherapies (132,133). Ravid et al. (133) found that 1,25(OH)_{2}D_{3} treatment enhanced ROS production and ROS-inducing chemotherapies in MCF-7 cells, which is similar to previously discussed responses when DHA and GLA were combined with chemotherapy treatments in in vitro settings. This suggests the combination of vitamin D and PUFA with chemotherapies that induce oxidative stress to kill breast cancer cells may result in even further enhancement of treatment effectiveness.

Supplementation of n-3 PUFA and vitamin D to breast cancer patients is likely to be both safe and efficacious. Relatively high doses of vitamin D and fish oil supplementation have been found to be well tolerated and effective in raising plasma
25(OH)D, EPA and DHA levels (24,25,134). Additionally, clinical trials have found that women receiving adjuvant chemotherapy for breast cancer reduces their circulating vitamin D to insufficient levels, despite supplementation (23,135). With the positive effects that vitamin D may have on breast cancer treatment and overall health, higher supplementation doses may be necessary to maintain adequate serum 25(OH)D levels. However, vitamin D toxicity is a concern. Further work on high-dose vitamin D supplementation in this deficient population is needed, as well as examining whether PUFA supplementation may have any impact on serum 25(OH)D levels or toxicity.

5.2  Future Directions

To date, combination treatments with n-3 PUFA and vitamin D on breast cancer have not been investigated. There are many areas in which studying interactions between PUFA and vitamin D on breast cancer can continue. Within in vitro work, research needs to be done to identify and understand the mechanisms of both n-3 and n-6 PUFA and vitamin D in their involvement with cell proliferation, cell cycle regulation, and apoptosis stimulation in breast cancer cells. Based on the multiple potential mechanisms and genomic targets that PUFA and vitamin D act upon, it is likely that results presented in this research are a combination of several mechanisms.

There are multiple pathways that can be activated to stimulate apoptosis. These pathways and the molecules involved in signal transduction to stimulate apoptosis can be modulated by n-3 PUFA and vitamin D. Some of the mechanisms affected by n-3 PUFA
and vitamin D are common, with while others are unique to each treatment. One common mechanism is the down-regulation of Bcl-2 and the Bcl-2:Bax ratio (26,28,29). A unique characteristic of vitamin D-mediated apoptosis is that it is caspase-independent (29,31,37). However, vitamin D-mediated apoptosis retains several common late-stage apoptosis characteristics, including PARP-cleavage, PS externalization, and DNA fragmentation (37). Conversely, n-3 PUFA-mediated apoptosis has been found to involve caspase-3 activation (52,89). Additionally, research has shown that caspase-3 can inactivate VDR via three potential cleavage sites (136). n-3 PUFA, through caspase-3 activation, may cause downstream VDR cleavage, limiting the ability of 1,25(OH)₂D₃ to stimulate its genomic actions. A unique mechanism of n-3 PUFA stimulated apoptosis that has been found is through the upregulation of SDC-1 (86-88). Future studies on the combination of PUFA and 1,25(OH)₂D₃ should measure the impacts on caspase-3 activation, Bcl-2 and Bax levels, and VDR activity to understand the interactions these treatments may have on apoptosis mechanisms in breast cancer cells.

Changes in cell cycle have been observed more with vitamin D treatments, though some evidence suggests n-3 PUFA affect it as well, and warrants further research with combination treatments (35,36,87,93,95). Vitamin D and its analogues are known to directly impact several regulators of the cell cycle pathway, including pRb phosphorylation, p21 and p27. Overall, 1,25(OH)₂D₃ causes cell cycle halting at the G1/S phase (26,35-37). n-3 PUFA have less clear interactions with cell cycle machinery. There is some evidence that they do not alter the cell cycle profile at all (91). However, receptors that are typically involved in stimulating breast cancer cell growth, ERα and
EGFR, are both impacted by n-3 PUFA and vitamin D treatments. ERα was found to be degraded by DHA, while negative VDRE in the promoter region of ERα have been identified, allowing vitamin D to decrease ERα mRNA transcription (92,101,137). EGFR is affected by changes in lipid raft profiles with n-3 PUFA treatment, preventing its dimerization and activation of growth signaling pathways that it mediates. Moreover, EGFR contains VDRE in its promoter region, to which 1,25(OH)\(_2\)D\(_3\) has been found to cause negative regulation of EGFR expression (83,138). Future work testing the combination of PUFA with 1,25(OH)\(_2\)D\(_3\) on cell growth and cell cycle machinery should measure changes in cell cycle staging, the molecules mediating cell cycle phase changes, as well as levels of ERα and EGFR.

MDA-MB-231 cells were found to have increased growth inhibition after combination PUFA and 1,25(OH)\(_2\)D\(_3\) treatment, despite the lack of VDR in the cell line. To elucidate how PUFA can sensitize these cells to 1,25(OH)\(_2\)D\(_3\), studies should be done to assess any changes in VDR, or other vitamin D receptors. Additionally, expression and downstream effects of other nuclear receptors, such as ERα and PPARγ could prove interesting, with their negative interactions highlighted in the literature review (see Figure 1.7). If changes in these nuclear receptors are found, it may help to explain how vitamin D and PUFA molecules may interact at the cellular and genomic level when combined.

This research would translate well to in vivo research. Animal models could be used to test breast cancer xenograft growth in response to diets supplemented with n-3
PUFA and vitamin D. Use of transgenic models, such as the *Fat-1* mouse that can endogenously create n-3 PUFA from conception, and VDR knockout mice are potential models to look at n-3 PUFA and vitamin D interactions (139-142). If feasible, combining these two models may provide a unique model to observe mammary gland development and tumorigenesis risk with lifelong exposure to n-3 PUFA without functional VDR, as well as any changes in tumor growth and development. Lastly, human clinical trials assessing the impacts of supplementing both n-3 PUFA and vitamin D on breast cancer risk and treatments would be of great interest. With the VITAL trial currently underway, and previous clinical trials showing additional benefits these molecules may have with chemotherapy treatments, it is clear that interest in this combination exists within clinical research (108,130).
References


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Appendix I: 96-well plate organization for combination PUFA and 1,25(OH)\(_2\)D\(_3\) (1,25D) treatments. Two plates are used for each experiment; one combination plate for all 9 combination treatments, and a control plate with the 6 monotreatments. Each plate has one column of untreated control cells, used as an internal control for each plate. Similarly, blank columns containing untreated media and no cells were used as a background reading after cells were stained with SRB dye for absorbance measurements. Cell responses to treatments were expressed as the percent fraction of their respective control cells. 8 technical replications were in each experiment, and were averaged prior to percent of control calculations. Low, medium, and high PUFA concentrations are listed in Table 3.1.
Appendix II: Summary table of MCF-7, MDA-MB-231, and MCF-10A growth response to n-3 and n-6 PUFA treatment. Cells were plated in 96-well plates with treatment media, and incubated for 96h. SRB staining and absorbance reading was performed, and data was averaged and expressed as percent of control cell growth. Low, medium, and high PUFA concentrations are as follows: Low ALA: 100µM; Medium ALA: 160µM; High ALA: 220µM; Low EPA: 100µM; Medium EPA: 170µM; High EPA: 270µM; Low DHA: 50µM; Medium DHA: 120µM; High DHA: 170µM; Low GLA: 150µM; Medium GLA: 180µM; High GLA: 250µM; Low AA: 120M; Medium AA: 170µM; High AA: 240µM.

<table>
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<th>PUFA Concentration</th>
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<th>AA</th>
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<td>62</td>
<td>71</td>
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<td></td>
<td>High</td>
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<td>9</td>
<td>26</td>
<td>11</td>
<td>30</td>
</tr>
<tr>
<td>MDA-MB-231</td>
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<td>75</td>
<td>61</td>
<td>53</td>
<td>37</td>
</tr>
<tr>
<td></td>
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<td>46</td>
<td>8</td>
<td>45</td>
<td>21</td>
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<td></td>
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**Appendix III:** Phase contrast images of MCF-7 cells treated with 100, 170, and 270µM EPA alone and in combination with 100nM 1,25(OH)₂D₃ for 96h. Images were captured on Olympus FSX1000 and the FSX-BSW program, with 100x magnification. EPA treatment appears to be causing morphological changes similar to apoptosis; cells treated with 170µM and 270µM EPA with and without 100nM 1,25(OH)₂D₃ are shrunken, spherical, and have less adherence with smaller structures that appear to be apoptotic bodies (indicated by arrows).
Appendix IV: Phase contrast images of MCF-7 cells treated with 120, 170, and 240µM AA alone and in combination with 100nM 1,25(OH)₂D₃ for 96h. Images were captured on Olympus FSX1000 and the FSX-BSW program, with 100x magnification. AA treatment appears to be causing morphological changes similar to apoptosis; cells treated with 170µM and 240µM AA with and without 100nM 1,25(OH)₂D₃ are shrunken, spherical, and have less adherence with smaller structures that may be apoptotic bodies (indicated by arrows).
Appendix V: Phase contrast images of MDA-MB-231 cells treated with 100, 170, and 270µM EPA alone and in combination with 100nM 1,25(OH)₂D₃ for 96h. Images were captured on Olympus FSX1000 and the FSX-BSW program, with 100x magnification. EPA treatment appears to be causing morphological changes similar to apoptosis; cells treated with 170µM and 270µM EPA with and without 100nM 1,25(OH)₂D₃ are shrunken, spherical, and have less adherence with smaller structures that appear to be apoptotic bodies (indicated by arrows), and excess cell debris.
Appendix VI: Phase contrast images of MDA-MB-231 cells treated with 120, 170, and 240µM AA alone and in combination with 100nM 1,25(OH)₂D₃ for 96h. Images were captured on Olympus FSX1000 and the FSX-BSW program, with 100x magnification. AA treatment appears to be causing morphological changes similar to apoptosis; cells treated with 170µM and 240µM AA with and without 100nM 1,25(OH)₂D₃ are shrunken, spherical, and have less adherence with smaller structures that appear to be apoptotic bodies (indicated by arrows), and excess cell debris.
Appendix VII: Phase contrast images of MCF-10A cells treated with 100, 170, and 270µM EPA alone and in combination with 100nM 1,25(OH)₂D₃ for 96h. Images were captured on Olympus FSX1000 and the FSX-BSW program, with 100x magnification. EPA treatment appears to be causing morphological changes similar to apoptosis; cells treated with 170µM and 270µM EPA with and without 100nM 1,25(OH)₂D₃ are shrunken, spherical, and have less adherence with smaller structures that appear to be apoptotic bodies (indicated by arrows), and excess cell debris.
Appendix VIII: Phase contrast images of MCF-10A cells treated with 120, 170, and 240µM AA alone and in combination with 100nM 1,25(OH)\textsubscript{2}D\textsubscript{3} for 96h. Images were captured on Olympus FSX1000 and the FSX-BSW program, with 100x magnification. AA treatment appears to be causing morphological changes similar to apoptosis; cells treated with 170µM and 240µM AA with and without 100nM 1,25(OH)\textsubscript{2}D\textsubscript{3} are shrunken, spherical, and have less adherence with smaller structures that appear to be apoptotic bodies (indicated by arrows), and excess cell debris.