Docosahexaenoic Acid Attenuates Bioenergetic Function and Inhibits Mammary Carcinoma Survival and Progression

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biochemistry

by

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May, 2013
Breast cancer is the second leading cause of cancer-related mortality for women in the United States. Only 5-10 percent of diagnosed breast cancer cases are attributed to known hereditary factors, making breast cancer arguably an acquired disease. A major contributor to breast cancer incidence is diet. The glycolytic switch, known as the Warburg Effect, is observed in many malignant models and results in a unique metabolic shift in energy metabolism where tumor cells increase anaerobic glycolysis even in the presence of normal oxygen levels. The stabilization of the Warburg metabolic phenotype and malignant transformation is linked to the activities of two major contributing factors, hypoxia-inducible factor 1α (HIF-1α) and myelocytomatosis (Myc) oncogene. Together, HIF-1α and Myc induce a cascade of events that mediate changes in metabolism, cell signaling, proliferation, growth, and differentiation. The current study evaluated whether polyunsaturated fatty acids (PUFAs), specifically docosahexaenoic acid (DHA), could modify the malignant network coordinated by HIF-1α and Myc. Interestingly, DHA treatment in the breast cancer cell lines BT-474 and MDA-MB-231 decreased protein expression level and transcriptional activity of HIF-1α and suppressed cancer cell metabolism. Functional consequences of suppressing HIF-1α activity led to a significant inhibition of glucose metabolism by 50%. Glucose uptake, glucose usage through glycolysis, and total glucose oxidation were all depressed upon DHA supplementation. DHA decreased the oxygen consumption rates (OCR) and extracellular acidification rates (ECAR) as well as the bioenergetic profile of cancer cells. Moreover, cells treated with DHA had significant decreases in intracellular ATP levels, which induced
phosphorylation of the metabolic stress marker AMP-activated protein kinaseα (AMPKα) at Thr172. Increases in metabolic stress and oxidative stress also led to increased cellular apoptosis in cancer cells treated with DHA. Myc activity was found to be partially responsible for the induction of apoptosis in cancer cells, although the way in which apoptosis was activated was opposite between the two cancer cell lines, BT-474 and MDA-MB-231. Observations revealed that DHA supplementation in the BT-474 cell line stimulated total phosphorylation and transcriptional activity and induced apoptotic events involving Bcl-2-associated X protein (BAX), partially through a Myc-dependent mechanism. However, the MDA-MB-231 cells treated with DHA resulted in decreases in both Myc total phosphorylation and transcriptional activity. Similar to the BT-474 cell line, MDA-MB-231 cells treated with DHA resulted in decreases in proliferation and cell viability and increases in apoptosis, but Myc did not seem to be required. Taken together, the mechanisms by which DHA induces metabolic stress and apoptosis through targeting the function of HIF-1α and Myc activities contribute to the impaired growth and survival of malignant cell lines. These data provide rationale for clinical cancer intervention with DHA.
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UNR Molecular Biosciences Graduate Symposium Poster Presentation – 2009 Awarded 2nd Place – Omega-3-PUFA–Induced Downregulation of HIF-1alpha.


American Association for Cancer Research Annual Conference Poster Presentation – 2008 – Dietary omega-3 polyunsaturated fatty acid supplementation decreases tumor and serum cholesterol, but increases tumor cholesterol levels.
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Chapter 1 – Literature Review

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1.1 Introduction to Cancer

Cancer has been misconceived as a single disease of the aged-population, but indeed it is several diseases that can affect anyone at any age [21]. There is much debate on the origin of cancer because several contradictions have inundated the field with controversy [9], [22–24]. The oncogenic paradox hypothesis suggests the possibility that the process of malignant transformation may follow one of two routes, a specific order of events taking place or unspecific events that lead to a disordered system [25]. This hypothesis remains highly controversial, however several environmental and epigenetic factors have been shown to initiate cancer and prolonged exposure to these agents could possibly induce carcinogenesis [5], [25]. The term cancer is defined as the uncontrolled growth of cells with the ability to invade other tissues. Malignant transformation, shown in Figure 1, displays a simplified model of tumor initiation, promotion and progression. Epidemiological evidence indicates that tumor appearance after an exposure to a carcinogen may take as many as 20 years [5]. Illustrated in the model, several barriers to tumor progression exist, including DNA repair process, the availability of nutrition, the requirement of angiogenesis to allow the tumor to increase in size and responses to hypoxia. Once cells become genetically mutated, indicated by the color, tumor
microenvironmental selection may enhance mutagenesis and can drive tumor progression, resulting in a heterogeneous population of cells (Figure 1) [5].

![Image](image.png)

**Figure 1.** Malignant transformation adapted from Loeb et al., 2011 [5]. Several factors mediate malignant transformation, including environmental or endogenous sources. These genetic or epigenetic factors contribute to mutations that escape repair, leading to propagation of oncogenic mutation and stabilization. Other microenvironmental factors, like angiogenesis and hypoxia facilitate malignant transformation.

For cells to become malignant, they must undergo certain cellular transformations, which have been described as cancer hallmarks (Figure 2) [6]. This multistep process, whether specific or unspecific, enables normal cells to progress into a neoplastic and malignant state, which makes cancer cells distinct from normal cells and provides the basic understanding of cancer biology. Moreover, cancer cells gain genetic alterations in a multistep process that promotes heterogeneous populations of cells, making cancer difficult to therapeutically target (Figure 1) [26]. The initial 6 hallmarks of cancer were identified in the early 2000s, which included resisting cell death, sustaining proliferative signaling, evading growth suppressors, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis [24]. Over the past decade,
4 more cancer hallmarks have been added to the list, including deregulating cellular energetics, genome instability and mutation, tumor promoting inflammation and avoiding immune destruction. Each hallmark poses a potential niche for therapeutic intervention, which has been reviewed by Hanahan and Weinberg in 2011 [6].

Figure 2. Hallmarks of Cancer adapted from Hanahan et al., 2011 [6], An illustration of reported functions that cells acquire for malignant transformation. Described in text.
1.1.1 Breast Cancer

Breast cancer is the most frequently diagnosed cancer for women in the United States and is estimated to be the second highest cause of cancer-related death for women [27]. Breast cancer incidence before 25 years of age is about 10 cases per 100,000 women, but increases 100-fold by age 45 and 230,000 new cases are estimated to be diagnosed this year alone [27], [28]. Significant progress has been made in biomarker discovery and early diagnostic testing has provided therapeutic opportunities to decrease mortality rates. Additionally, depending on the stage of breast cancer, survival rates have improved as well [27].

Similar to other solid tumors, like colon and lung, breast cancer cells display similar genetic and metabolic profiles. What makes breast cancer unique is that fact that it is arguably an acquired disease, with only 5-10% of the diagnosed cases are estimated to be attributed to known heredity factors [1]. Several key epigenetic and environmental sources mediate mammary carcinogenesis, including geographic location, smoking, alcohol consumption, physical activity, obesity and diet [1], [29]. Risk factors that contribute to breast cancer are summarized in Table 1. However, there is still a clear need for the identification of phenotypic and genetic traits that further explain causes of breast cancer, which will provide means to accurately assess breast cancer risk, prevention and therapy. Breast cancer risk in relation to diet will be assessed in Section 1.6.
Table 1. Risk factors for breast cancer adapted from Dumitrescu et al., 2005 [1].

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<thead>
<tr>
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<th>Magnitude of risk</th>
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<td><strong>Breast Cancer Risk Factors</strong></td>
<td></td>
</tr>
<tr>
<td>Increasing age</td>
<td>++</td>
</tr>
<tr>
<td>Geographical region (USA and western countries)</td>
<td>++</td>
</tr>
<tr>
<td>Family history of breast cancer</td>
<td>++</td>
</tr>
<tr>
<td>Mutations in BRCA1 and BRCA2 genes</td>
<td>++</td>
</tr>
<tr>
<td>Mutations in other high-penetrance genes (p53, ATM, NBS1, LKB1)</td>
<td>++</td>
</tr>
<tr>
<td>Ionizing radiation exposure (in childhood)</td>
<td>++</td>
</tr>
<tr>
<td>History of benign breast disease</td>
<td>++</td>
</tr>
<tr>
<td>Late age of menopause (&gt;54)</td>
<td>++</td>
</tr>
<tr>
<td>Early age of menarche (&lt;12)</td>
<td>++</td>
</tr>
<tr>
<td>Nulliparity and older age at first birth</td>
<td>++</td>
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<tr>
<td>High mammographic breast density</td>
<td>++</td>
</tr>
<tr>
<td>Hormonal replacement therapy</td>
<td>+</td>
</tr>
<tr>
<td>Oral contraceptives recent use</td>
<td>+</td>
</tr>
<tr>
<td>Obesity in postmenopausal women</td>
<td>+</td>
</tr>
<tr>
<td>Tall stature</td>
<td>+</td>
</tr>
<tr>
<td>Alcohol consumption (~1 drink/day)</td>
<td>+</td>
</tr>
<tr>
<td>High insulin-like growth factor I (IGF-I) levels</td>
<td>++</td>
</tr>
<tr>
<td>High prolactin levels</td>
<td>+</td>
</tr>
<tr>
<td><strong>Probable factors</strong></td>
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<td>High saturated fat and well-done meat intake</td>
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<td>High socioeconomic status</td>
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<td><strong>Factors that decrease breast cancer risk</strong></td>
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</tr>
<tr>
<td>Fruit and vegetables consumption</td>
<td>-</td>
</tr>
<tr>
<td>Physical activity</td>
<td>-</td>
</tr>
<tr>
<td>Chemopreventive agents</td>
<td>-</td>
</tr>
<tr>
<td>Non-steroidal anti-inflammatory drugs</td>
<td>-</td>
</tr>
<tr>
<td>Polymorphisms in low-penetrance genes (see text)</td>
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++ (moderate to high increase in risk); -- (moderate to high decrease in risk); + (low to moderate increase in risk); - (low to moderate decrease in risk)
1.1.2 Breast Cancer Subtypes

Whole-genome analyses using microarray expression systems have led to the discovery of 5 distinct subtypes of breast carcinomas, which are, Luminal A, Luminal B, HER2+, Basal-like, and Normal-like. Each subtype has a unique phenotypic footprint with specific clinical outcomes [30–32].

Luminal tumors are the most common diagnosed breast cancer. They start in the inner (luminal) lining in the mammary ducts. Luminal A tumors usually are estrogen receptor-positive (ER+) and/or progesterone receptor-positive (PR+). They also are HER2/neu-negative (HER2−), are characterized as a low or moderate tumor grade, and only about 12-15% have mutations in p53. Luminal B tumors usually are ER+ and/or PR+. They are HER2/neu-positive (HER2+) and highly positive for Ki67, which is an indicator for cells actively dividing. Diagnoses of Luminal B tumors often happens at a younger age for women and tend to have a poorer prognosis, including poorer tumor grade, larger tumor size, lymph node-positive and about 30% positive for p53 mutation. HER2+ tumors are ER+/PR−, lymph node-positive, with 75% containing a mutant p53.

Approximately 10-15% of breast cancer diagnoses designate this molecular profile and have poor prognosis associated with it due to its frequent recurrence and metastasis. Basal-like/triple negative cancers are cells that are similar to cells of the outer (basal) lining the mammary ducts. They are ER+/PR−, HER2−, and most contain mutations in p53. These tumors are correlated with poor prognosis and are often highly aggressive. Normal-like breast cancer subtypes are less commonly diagnosed (6-10% of all breast cancers). These tumors are small and usually associated with good prognosis. Of all of
the subtypes, Luminal A and Normal-like tumors are correlated with the best prognosis with high survival rates and low recurrence rates. All subtypes, classifications and prevalence are summarized in Table 2 and Figure 3 [33–38].

**Table 2.** Subtype classification and prevalence for breast cancers.

<table>
<thead>
<tr>
<th>Subtype</th>
<th>Classification</th>
<th>Prevalence (approximate)</th>
</tr>
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<tbody>
<tr>
<td>Luminal A</td>
<td>ER+ and/or PR+, HER2-, low Ki67</td>
<td>40%</td>
</tr>
<tr>
<td>Luminal B</td>
<td>ER+ and/or PR+, HER2+ (or HER2- with high Ki67)</td>
<td>20%</td>
</tr>
<tr>
<td>HER2 type</td>
<td>ER-, PR-, HER2+</td>
<td>10-15%</td>
</tr>
<tr>
<td>Triple negative/basal-like</td>
<td>ER-, PR-, HER2-, cytokeratin 5/6 + and/or HER1+</td>
<td>15-20%</td>
</tr>
<tr>
<td>Normal-like</td>
<td></td>
<td>6-10%</td>
</tr>
</tbody>
</table>

*These are the most common profiles for each subtype and may or may not express each feature.

**Figure 3.** Main subtypes of breast cancer and prognosis adapted from Sims et al., 2007 [7].
The mammary epithelial ductal carcinoma lines specifically tested in this report are BT-474 and MDA-MB-231. Subtype classification of the BT-474 cell line fell to Luminal B and HER2\(^+\) and the MDA-MB-231 subtype classification fell to Basal-like, triple negative [2]. BT-474 cell line is demonstrated to have a mitochondrially active phenotype, based on high oxygen consumption rates, and the MDA-MB-231 cell line is shown to have a glycolytically active phenotype based on high glycolytic rates and lactate production. These two cell lines provide an opportunity to compare two metabolically distinct cancer cell lines. Additionally, a breast epithelial non-tumorigenic cell line, MCF-10A, was used as a comparative model. Although it is immortalized in cell culture and classified as a Basal-like subtype, it has been shown to not induce a malignant tumor in immunocompromised mice (Table 3) [2]. Images of the structural morphological phenotypes of each cell line are shown in Figure 4.

<table>
<thead>
<tr>
<th>Table 3.</th>
<th>IHC analysis of breast cancer cell lines adapted from Subik et al., 2010 [2].</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abbreviations: IHC (immunohistochemistry), ER (estrogen receptor), PR (progesterone receptor), HER2 (human epidermal growth factor receptor 2), CK5/6 (cytokeratin 5/6), EGFR (epidermal growth factor receptor), AR (androgen receptor).</td>
<td></td>
</tr>
<tr>
<td>ER</td>
<td>PR</td>
</tr>
<tr>
<td>------------------------------</td>
<td>------------------------------</td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td>0</td>
</tr>
<tr>
<td>SKBR-3</td>
<td>0</td>
</tr>
<tr>
<td>MDA-MB-231-UR</td>
<td>0</td>
</tr>
<tr>
<td>MCF-12A</td>
<td>0</td>
</tr>
<tr>
<td>HBL101</td>
<td>0</td>
</tr>
<tr>
<td>MDA-MD-435</td>
<td>0</td>
</tr>
<tr>
<td>MCF-7</td>
<td>6</td>
</tr>
<tr>
<td>HS578T</td>
<td>0</td>
</tr>
<tr>
<td>MCF-10A</td>
<td>0</td>
</tr>
<tr>
<td>BT-20</td>
<td>0</td>
</tr>
<tr>
<td>MCF-10F</td>
<td>0</td>
</tr>
<tr>
<td>468</td>
<td>0</td>
</tr>
<tr>
<td>AU 565</td>
<td>0</td>
</tr>
<tr>
<td>ZR-75-1</td>
<td>3</td>
</tr>
<tr>
<td>BT-483</td>
<td>0</td>
</tr>
<tr>
<td>BT-474</td>
<td>0</td>
</tr>
<tr>
<td>MDA-MB-453</td>
<td>0</td>
</tr>
</tbody>
</table>

Abbreviations: IHC (immunohistochemistry), ER (estrogen receptor), PR (progesterone receptor), HER2 (human epidermal growth factor receptor 2), CK5/6 (cytokeratin 5/6), EGFR (epidermal growth factor receptor), AR (androgen receptor).
Figure 4. **Cell line specific images.** (A) Image of MDA-MB-231 cell line. (B) Image of BT-474 cell line. (C) Image of MCF-10A non-transformed cell line.
1.2 Cancer Metabolism

Cancer cells display an altered metabolic phenotype when compared to normal cells [6], [9]. It has been almost a century since it was first discovered that the metabolic phenotype of cancer cells is different than that of normal cells [39]. These observations made by Warburg, now termed the Warburg effect, demonstrated that cancer cells utilize aerobic fermentation instead of oxidative metabolism to maintain the bioenergetic demands for survival and proliferation [39]. The process utilizes glucose to yield lactic acid in addition to acquiring 2 net ATP molecules ($\text{C}_6\text{H}_{12}\text{O}_6 \rightarrow 2\text{CH}_3\text{CH(OH)}\text{COOH} + 2\text{ ATP}$). However, the question remains as to what propagates the metabolic transformation of cancer cells. Darlington in 1948 provided evidence that the driver for carcinogenic transformation were “mutant particles in the cytoplasm” [40]. These arguments along with new technologies and expanded metabolomic research have brought the focus back to metabolism with the ultimate goal to target and exploit metabolic differences between normal and cancer cells making them a strong metabolic hallmark [41–43]. It is argued that the genesis of cancer is mediated by genetic mutations, however the origin of cancer remains unclear and several influences can initiate the disease, including cellular metabolism [9].

1.2.1 Warburg Effect

Warburg’s initial explanation as to why cancer cells display an altered phenotype has been controversial. His proposed theory was based on what was thought to be a
physiological impairment or damaged mitochondria and the increased glycolytic flux was regarded as a compensatory response mechanism to maintain energy function and viability in cancer cells [39]. The theory also maintained the notion that the cells had irreversible damage to respiration and that this was the primary cause of cancer [39]. However, it has been shown that increased glycolytic fermentation is only a symptom of cancer and not the cause of it [44].

1.2.2 A Metabolic Disease

Recent interest in cancer metabolism has readdressed Warburg’s findings and emerging evidence provides insight into the origin of cancer and argues that carcinogenesis is caused from deregulated cellular metabolism [9]. Cells must generate enough usable energy, commonly stored as ATP, to maintain viability and perform programmed functions. Despite suitable \( O_2 \) for mitochondrial oxidative phosphorylation, the resulting elevation in glycolytic metabolism provides sufficient levels of ATP to sustain cellular energetic demands [45]. In functionally respiring cells, about 88% of the energy is produced by oxidative phosphorylation and the other 12% is generated through substrate level phosphorylation, by glycolysis in the cytoplasm and by the TCA cycle in the mitochondria. The functionality of the mitochondria in cancer cells remains highly controversial. However, numerous reports show that cancer cells have a structurally unstable and non-functional mitochondria unable to produce sufficient levels of energy to maintain cellular energetic demands [46–50]. Recent studies provide evidence that mitochondrial dysfunction also creates a mutator phenotype, thereby creating
chromosomal instability and expression of gene mutations [51–54]. Furthermore, deregulation of the mitochondria has been shown to alter redox-sensitive regulators on DNA transcription and repair, providing the platform for aberrant mutant phenotypes in cancer [54], [55].

The metabolic reprogramming of cancer cells is very complex and the molecular mechanisms that trigger these changes have been proposed to be mainly driven by defects in oxidative phosphorylation [8]. The metabolic targets have been classified as either direct targets of the metabolic enzymes themselves or indirect targets, such as signaling pathways that are switched on or off resulting in disordered metabolism [3], [56]. A cell that survives damage to oxidative phosphorylation holds the potential to become malignant because of the changes that may occur in oncogenic expression, “Oncogenesis” (Figure 5) [8]. Additionally, several changes affecting HIF-1 stabilization can drive transformation into a malignant state, “Transformation” (Figure 5) [8]. Some of the factors that cause damage to oxidative phosphorylation include, inflammation, carcinogens, radiation, hypoxia, viral infections, and morphological tissue damage [9]. When mitochondrial damage persists, oncogenic gain-of-function mutations or loss of tumor suppressors may lead to the evolution of transformation and to metabolic reprogramming of cancer cells (Figure 5). The propagation of mitochondrial dysfunction and gene abnormalities simultaneously enhance the energy-generating pathways, like glycolysis to sustain the energy requirements of the cells and cellular dependence on substrate level phosphorylation has been associated with an enhanced degree of
malignancy. Taken together, the metabolic reprogramming paths to malignancy where all of the hallmarks of cancer are expressed are summarized in Figure 6.

Figure 5. Molecular mechanisms of the metabolic reprogramming of cancer cells adapted from Kroemer et al., 2008 [8]. Pink represents oncogenic gain-of-function and green represents loss of tumor suppressors affecting the PI3K/Akt/mTOR/HIF axis and/or deactivation of the p53 system. These alterations further propagate metabolic changes associated with malignant transformation. Note that arrows connecting different proteins do not necessarily indicate direct interactions. Abbreviations: ACL (ATP citrate lyase), AMPK (AMP-activated kinase), CA9 and CA12 (carbonic anhydrases 9 and 12), ChoK (choline kinase), CPT (carnitine palmitoyltransferase) FH (fumarate hydratase), GLUT (glucose transporter), HIF (hypoxia-inducible factor), HK (hexokinase), OXPHOS (oxidative phosphorylation), LAT1 (L-type amino acid transporter 1), LDHA (lactate dehydrogenase isofrom A), MCT (monocarboxylate transporter), mTOR (mammalian target of rapamycin) NF (neurofibromin), PDK (pyruvate dehydrogenase kinase), PFK (pyruvate dehydrogenase kinase), PFK (phosphofructokinase), PI3K (phosphatidylinositol 3-kinase), PIP3 (phosphatidylinositol trisphosphate), PGM (phosphoglycerate mutase), PHD (prolyl hydroxylase), PKM2 (pyruvate kinase isoform M2), SCO2 (synthesis of cytochrome c oxidase 2), SDH (succinate dehydrogenase), TSC (tuberous sclerosis complex), VDAC (voltage-dependent anion channel), VHL (von Hippel-Lindau ubiquitin ligase).
Figure 6. Link between altered-cancer cell metabolism and hallmarks of cancer adapted from Seyfried et al., 2010 [9]. Damage caused by exogenous sources in oxidative phosphorylation propagates a change in the functional state of the mitochondria. Mitochondrial deregulation and reactive oxygen species increase genomic instability that stabilizes oncogenes and inhibits tumor suppressors, driving the metabolic reprogramming to the Warburg phenotype. In this way, the cancer cells acquire all of the hallmarks of cancer. Abbreviations: RTG (retrograde), SLP (substrate level phosphorylation), OxPhos (oxidative phosphorylation), HIF-1α (hypoxia-inducible 1α), VEGF (vascular endothelial growth factor).

1.3 Tumor Microenvironment

Cancer has been regarded as a wound that never heals [57]. Certain growth factors and cytokines released by the surrounding tissue in fact support chronic inflammation and tumor progression [58]. The degradation of the extracellular matrix also helps to progress tumor invasion and metastasis, which enhances the angiogenic response [58]. Furthermore, anaerobic respiration increases lactate production decreasing the pH of the
surrounding tissue, which further breaks down the extracellular matrix, and enhances the ability for tumors to invade [59]. The microenvironmental mechanisms driving tumor aggressiveness and resistance to therapies have recently been brought to attention. Potential targeting of some of these mechanisms may help promote better prognosis for patients and provide new strategies for current therapies (Table 4) [3].

Table 4. Microenvironmental drivers of aggressive breast cancer adapted from Ward et al., 2013 [3].

<table>
<thead>
<tr>
<th>Mechanism</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vascular and lymphatic abnormalities</td>
<td>Increased interstitial pressure causing inhibition of drug delivery</td>
</tr>
<tr>
<td>Hypoxia</td>
<td>Resistance to drugs that target proliferation and radiation</td>
</tr>
<tr>
<td>Slows cell cycling</td>
<td>Decreased apoptosis</td>
</tr>
<tr>
<td>Decreases pro-apoptotic Bcl-2 family members</td>
<td>Decreased apoptosis and increased resistance to treatment</td>
</tr>
<tr>
<td>BID, BAD and BAX</td>
<td>Decreased apoptosis</td>
</tr>
<tr>
<td>Induces IAP-2 expression</td>
<td>Resistance to radiotherapy</td>
</tr>
<tr>
<td>Activates PI3K/Akt pathway</td>
<td>Increased genetic instability</td>
</tr>
<tr>
<td>Decreased O2</td>
<td>DNA damage</td>
</tr>
<tr>
<td>Inhibits DNA repair pathways</td>
<td>Loss of E-cadherin and increased invasive potential</td>
</tr>
<tr>
<td>Generates reactive oxygen species</td>
<td>Increased invasive and metastatic potential</td>
</tr>
<tr>
<td>Activation of bryx oxidase-Snail pathway</td>
<td>Loss of anti-tumour defences, accumulation of cytokines and growth factors</td>
</tr>
<tr>
<td>Increased expression of chemokine receptors</td>
<td></td>
</tr>
<tr>
<td>Disturbs immune cell function</td>
<td></td>
</tr>
<tr>
<td>ph changes</td>
<td></td>
</tr>
<tr>
<td>Alkaline pH</td>
<td></td>
</tr>
<tr>
<td>Preservation of ATP</td>
<td>Inhibits apoptosis</td>
</tr>
<tr>
<td>Bypass of cell cycle checkpoints</td>
<td>Genetic instability</td>
</tr>
<tr>
<td>Increased glycolysis</td>
<td>Metabolic adaptation</td>
</tr>
<tr>
<td>Acidic pile</td>
<td>Resistance to chemotherapy</td>
</tr>
<tr>
<td>Altered drug structure and uptake</td>
<td>ECM damage, increased invasion and metastasis</td>
</tr>
<tr>
<td>Increased protease expression</td>
<td>Increases angiogenesis</td>
</tr>
<tr>
<td>Increased expression of IL-8 and VEGF</td>
<td>Loss of anti-tumour activities</td>
</tr>
<tr>
<td>Decreased activation of effector immune cells</td>
<td></td>
</tr>
</tbody>
</table>

1.3.1 Hypoxia

Tumor hypoxia is frequently found in solid tumors and occurs when the tumor outgrows its blood supply (Figure 7). Approximately one third of breast tumors have hypoxic regions [60]. The hypoxic regions found in breast tumors have O2 concentrations as low as 0.3%, whereas normal tissue has 9% [60]. It has been estimated that 40-50% of
breast cancers have hypoxic regions where chemotherapy and radiation are less effective and bioreductive alkylating agents have been used to target hypoxic areas in tumors [60].

The main forces that drive cancer cell aggressiveness and invasiveness are hypoxia and acidification of the microenvironment [41], [61]. The diffusion limit of O₂ is about 100-200 μm from the blood capillary. It has been suggested that as the tumor volumes reach more than 1-2 mm³, the disorganized vasculature and leakiness of the blood vessels cause hypoxic regions to develop within the tumor [61]. As the tumor cells proliferate and O₂ and other nutrients become limited, cells can become necrotic.

Figure 7. Illustration of high and low oxygenated environments in relation to vasculature in solid tumors adapted from Trédan et al., 2007 [10].
1.4 Metabolic Reprogramming by HIF

The adaptive survival mechanism by tumor cells has been shown to be primarily through the stabilization and regulation of hypoxia-inducible factors (HIFs). HIF proteins are transcriptional factors that are responsible for the metabolic reprogramming of hypoxic cells to enable cell survival in severe microenvironments [12]. Although other HIF isoforms exists, this report will only focus on the involvement of hypoxia-inducible factor 1α (HIF-1α).

The involvement of HIF-1α in cancer metabolism contributes to the development of an invasive, metastatic, and lethal cancer phenotype. Several genes have been shown to be modulated by HIF-1α, including genes involved with glycolysis, cell cycle control, proliferation, erythropoiesis, angiogenesis, and metastasis (Figure 8) [62].

![Figure 8. Regulation of HIFs on genes encoding protein that promote tumor growth and metastasis adapted from Bertout et al., 2011 [11].](image)
The HIF-1α subunit contains a basic/helix-loop-helix/PAS (bHLH-PAS) domain [63] that mediates DNA binding. HIF-1α protein levels have also been reported to modify HIF-1 transcriptional activity [64]. Under normoxic conditions, a prolyl hydroxylase, PDH2, mediates binding of the von Hippel-Lindau (VHL) protein to HIF-1α, which recruits a ubiquitin ligase and targets HIF-1α for proteasomal degradation [65]. Since PDH2 uses O₂ as its substrate, hypoxic conditions inhibit its activity [66].

The principle function of HIF-1α regulation is to metabolic reprogram a cell under stressed conditions (Figure 9A). HIF-1α has been shown to activate the transcription of major glucose transporters 1 and 3 (GLUT 1/3), as well as every enzyme of glycolysis [67]. Hexokinase isoforms 1 and 2, lactate dehydrogenase A (LDHA) and monocarboxylate transporter 4 (MCT4) are also regulated by HIF-1α [67], [68]. Pyruvate is a control point for metabolism. Cells have the option to convert pyruvate to acetyl coenzyme A by pyruvate dehydrogenase (PDH) for entry into the tricarboxylic acid (TCA) cycle or be converted to lactate by LDHA. Interestingly, another point of HIF-1α control is pyruvate dehydrogenase kinase (PDK). PDK phosphorylates and inactivates the catalytic domain of PDH. Studies have shown that PDK1 is encoded by four genes and activated by HIF-1 [69], [70]. Through PDK activation, pyruvate is forced to lactate fermentation and shunted away from the mitochondria, which reduces metabolism through the TCA cycle, thus decreasing delivery of NADH and FADH₂ to the electron transport chain. This adaptive response is critical for hypoxia to allow for cell survival; however HIF-1α stabilization has been linked to cell immortality and may lead to tumorigenesis.
HIF-1α activates transcription of a gene encoding the BH3 domain protein (BNIP3), which induces mitochondrial selective autophagy [71]. This response has been shown to be an adaptive response to prolonged hypoxic conditions to maintain cell viability [71]. Additionally, HIF-1α was found to control the efficiency of respiration in response to changes in cellular O₂ concentrations by transcriptionally activating genes responsible for the regulation of the mitochondrial cytochrome c oxidase (Complex IV) [72]. Taken together, HIF-1α appears to be a major contributor to O₂ management and consumption to maintain the balance ATP and ROS production in cells (Figure 9B).
Figure 9. HIF-1α metabolic regulation adapted from Semenza et al., 2010 [12]. Low O₂ concentrations increase the stability of HIF-1α and increase expression of genes leading to anaerobic respiration and inhibition of oxidative phosphorylation. (A) Regulation of HIF-1α protein synthesis and stability and HIF-1-dependent metabolic reprogramming. The rate of translation of HIF-1α mRNA into protein in cancer cells is determined by the activity of upstream tumor suppressor proteins (green ovals), oncoproteins (orange rectangles) and mTOR activity. HIF-1α protein stability is regulated by O₂ and a prolyl hydroxylation catalyzed by PHD2. Hydroxylation is required for the binding of VHL, which recruits an ubiquitin ligase that targets HIF-1α for proteasomal degradation. Loss of function for any of the tumor suppressor genes encoding FH, IDH, or SDH inhibits PHD2 activity. HIF-1α then activates transcription of target genes encoding proteins (yellow rectangles) that are major contributors in the metabolic reprogramming of cancer cells. Abbreviations: PTKs (protein tyrosine kinases), PI3K (phosphatidylinositol-3-kinase), S6K (ribosomal protein S6 kinase), ALD (aldolase), PGK (phosphoglycerate kinase), ENO (enolase), and PKM (pyruvate kinase M). Other HIF-1α-regulated glycolytic enzymes that are not shown: GPI (glucosephosphate isomerase), PFKL (phosphofructokinase L), TPI (triosephosphate isomerase), GAPDH (glyceraldehyde-3-phosphate dehydrogenase), and PGM (phosphoglyceromutase). (B) HIF-1α regulated oxidative and glycolytic metabolism of glucose. HIF-1α-regulated genes (yellow ovals) convert extracellular glucose to extracellular lactate and block entry of pyruvate into the TCA cycle. Loss of function for each of the TCA cycle enzymes shown in green is associated with tumor formation and HIF-1α stabilization is increased by PHD2 inhibition by accumulation of the enzyme substrate. Arrows indicate the direction of the TCA cycle. Abbreviations: CS (citrate synthase), ACON (aconitase), αKGDH (α-ketoglutarate dehydrogenase), SCS (succinyl-CoA synthetase), and MDH (malate dehydrogenase).
1.5 Biological Function of Myc

The Myelocytomatosis (Myc) protooncogene is a bridge to many growth promoting signal transduction pathways and several ligand-membrane receptor complexes. Myc belongs to a family of transcription factors and have been characterized based on their tissue association, including cancer Myc (c-Myc), viral Myc (v-Myc), neuroblastoma Myc (NMyC), and lung Myc (LMyc). Initial studies of Myc revealed its potential to reprogram and transform normal embryonic fibroblasts [73]. Further analyses discovered that Myc can regulate up to 15% of human genes involved in cell growth, proliferation, differentiation, apoptosis, and metabolism through its basic/helix-loop-helix (bHLH) / leucine zipper domain [74]. Associations between Myc’s heteromeric partners, Myc associated factor X (Max) (Myc transcriptional activation) and Max associated dimerization protein (Mad) (Myc transcriptional repression), mediate Myc-complex nuclear translocation, which binds to E-boxes with the consensus core sequence CACGTG or with noncanonical sequences [75]. The associations between Max and Mad induce specific cellular responses and DNA binding initiates recruitment of several coactivators to E-box elements (Figure 10) [76], [77].

Although several groups have identified the Myc protein regulation on target gene transcription, Myc posttranslational modification regulation on these downstream targets remain unclear. Many serine (Ser) and threonine (Thr) residues have been identified along the Myc protein, however only two sites, Ser62 and Thr58 have shown to elicit either transformation or apoptosis [78–81]. Phosphorylation of Ser62 activates recruitment of binding partners, Max or Mad, and Thr58 phosphorylation activates Myc
proteasomal degradation. A comprehensive list of cellular target genes identified to be directly and/or indirectly regulated by Myc has now been made available (http://www.myccancergene.org/site/mycTargetDB.asp) [82].

A recent emergence of Myc regulation has been established that identifies functions of Myc in metabolic pathways, including amino acid and nucleotide synthesis, regulation of lipid metabolism, glycolysis, and mitochondrial homeostasis [83], [84]. New insights into Myc as a redox sensor has demonstrated that Myc phosphorylation not only regulates protein stability, but also determines the choice of target genes [85]. However, the relevance of posttranslational modification to Myc in the regulation of each gene will have to be further analyzed. The functional consequence of Myc phosphorylation, with no changes in protein level, in response to cellular stresses poses another hurdle for therapeutic intervention on Myc.
Figure 10. Myc regulation on cell fate adapted from Nilsson et al., 2004 [13]. (A) (Left) Traditional Myc-Max complex network. (Right) Alternatively, the Myc-Max complex can activate promoters by direct binding to E-boxes not occupied by other complexes. Mad and Mnt work as antagonists and repressors of Myc. Abbreviations: Mnt (Max-binding protein), Mad (Max dimerization protein), c-Myc (myelocytomatosis cancer-related oncogene), ODC (ornithine decarboxylase).
1.5.1 Myc Expression in Breast Cancer

A major player involved in oncogenic transformation is Myc oncogene and its activity has been reported to be modified in over 70% of cancers [86], [87]. Myc was first discovered as v-Myc avian myelocytomatosis viral-related oncogene homolog over 30 years ago in patients with Burkitt’s lymphoma. Initial findings suggested that Myc therapies could potentially reverse many cancerous states and cure over 40% of patients with overexpressed Myc. Unfortunately, the complexity of Myc activities other than gene amplification, such as transcriptional regulation and mRNA and protein stabilization, are involved in Myc modification, which makes Myc a complex target for inhibition. Gene amplification differences have been reported in about 15% of tumors, whereas overexpression in Myc mRNA has been found in 35% of tumors [88], [89]. Furthermore, enhanced Myc protein activity has been found in over 40% of breast cancer [90], [91].

Transcriptional regulation of Myc promoter is complex and remains unclear. However, several signaling pathways, transcription factors and cis regulatory elements have been associated with transcriptional regulation of Myc [76]. Myc amplification has shown promise as a biomarker for aggressiveness of breast cancer [92]. However, due to the heterogeneity of breast cancer, pathological correlation with Myc protein overexpression has been poor [92]. Microarray analyses have correlated Myc overexpression with distinct breast cancer subtypes [19], which are summarized in Table 5. High Myc overexpression in the Basal-like subtype was confirmed by DNA microarray analysis (N=148) compared to the other four breast cancer subtypes [19].
These data suggest that Myc overexpression is involved in subtype-specific pathways in breast cancer.

Table 5. Distribution of Myc overexpressing tumors.

<table>
<thead>
<tr>
<th>Subtype</th>
<th>Myc Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Luminal A and B</td>
<td>11%</td>
</tr>
<tr>
<td>HER2 type</td>
<td>9%</td>
</tr>
<tr>
<td>Triple negative/Basal-like</td>
<td>50%</td>
</tr>
<tr>
<td>Normal-like</td>
<td>38%</td>
</tr>
</tbody>
</table>

1.5.2 Targeting Myc

Targeting Myc therapeutically poses several problems. Normal cells require Myc for proper cellular function. Additionally, Myc induces paradoxical effects ("double-edged sword") promoting cell proliferation or causing apoptosis depending on the circumstance, making it hard to develop novel drug targets. Nevertheless, inhibition of pathways that cross-talk with Myc have great potential for therapeutic strategies. Studies examining downregulation of Myc and Myc-associated pathways have been shown to impair tumor growth with the induction of cell cycle arrest in cancer cells [82], [90], [93]. Conversely, although it is not completely clear, cells overexpressing Myc have been shown to have a high rate of apoptosis [94].
The regions of Myc required for transformation are also required for apoptosis and several Myc target genes, such as Bcl-2-associated X protein (Bax), have been implicated in Myc-induced apoptosis [95]. Several studies have shown that Myc-induced apoptosis has a specific requirement for Bax to induce mitochondrial apoptosis, which is summarized in Figure 11 [14], [96–98].

**Figure 11.** Model for Bax requirement for Myc-induced apoptosis adapted from Cao et al., 2008 [14]. Cytotoxic events mediate activation of caspase 2 releasing cytosolic-sequestered Bax to the mitochondrial membrane. Myc activation regulates Bax activator that facilitates cytochrome c release into the cytosol, which recruits Apaf-1 to initiate Caspase 3/9 activation that leads to apoptosis. Abbreviations: c-Myc (myelocytomatosis cancer-related oncogene), Bax (bcl-2-associated X protein), Apaf-1 (apoptotic protease factor 1).

### 1.5.3 HIF-Myc Complex

As previously stated, Myc plays a central role in cell cycle progression. However, the induction of HIF-1α by hypoxia has shown to counteract Myc and inhibit Myc
transcriptional activity [99]. HIF-1α disrupts Myc DNA binding complexes through the repressed target of p21 resulting in Myc displacement and decreased promoter interaction [15], [99]. These HIF-1α-mediated effects can be attenuated by Myc-overexpressing cells or deficiencies in VHL [15], [100]. The expression kinetics of HIF/Myc interplay are complex, but distinct, in solid tumors and change by sensing cellular O₂ levels (Figure 12) [101].

**Figure 12.** HIF-1α effects on Myc transcriptional activity adapted from Gordan et al., 2007 [15]. Increases in HIF-1α levels concomitantly disrupt Myc complexes. Induction of Mxi causes transcriptional repression of Myc target genes. Abbreviations: Max (Myc-associated factor X), HIF-1α (Hypoxia-inducible factor 1α), c-Myc (myelocytomatosis cancer-related oncogene), SP1 (specificity protein 1), ARNT (aryl hydrocarbon receptor nuclear translocator), Mxi (Max-interacting protein 1), CKIs (cyclin-dependent kinase inhibitor protein), ODC (ornithine decarboxylase).
1.6 Fatty Acid Nomenclature and Classification

It is important to understand that lipids are major bioactive compounds. A fatty acid is a carboxylic acid with a long aliphatic chain. Fatty acid chains differ by length and have been distributed into 4 categories, including short-chain fatty acid (SCFA – less than 6 carbons), medium-chain fatty acid (MCFA – 6-12 carbons), long-chain fatty acid (LCFA – 12-22 carbons), and very long-chain fatty acid (VLCFA – more than 22 carbons) [102]. Fatty acids can be saturated, containing no double bonds, monounsaturated, containing one double bond, or polyunsaturated, containing 2 or more double bonds (Figure 13). The double bonds in the fatty acids can exist in cis or trans configuration, however for the purpose of this report, the cis configuration will only be referred to. Some LCFA are characterized as essential fatty acids (EFA) because they cannot be made by the body and must be obtained from dietary sources. EFAs are important to several biological processes, like eicosanoid biosynthesis, phospholipid membrane reorganization (lipid raft), and DNA modification [16], [103]. Since EFAs cannot be synthesized, bodily EFA depletion would induce cachexia and eventual death.

A list of well-known fatty acids in Table 6 identifies the different types of fatty acids and common dietary sources. Interestingly, all of the polyunsaturated fatty acids (PUFAs) depicted are EFAs and need to be obtained from the diet.
Figure 13. Molecular structure of fatty acids adapted from White et al., 2009 [4]. Structural differences between increases in unsaturation.

Table 6. Well-known fatty acids and their dietary sources adapted from White et al., 2009 [4].

<table>
<thead>
<tr>
<th>Name</th>
<th>Number of carbon atoms</th>
<th>Type of fatty acid</th>
<th>Essential fatty acid</th>
<th>Common sources</th>
</tr>
</thead>
<tbody>
<tr>
<td>Palmitic acid</td>
<td>16</td>
<td>Saturated</td>
<td>No</td>
<td>Palm oil</td>
</tr>
<tr>
<td>Stearic acid</td>
<td>18</td>
<td>Saturated</td>
<td>No</td>
<td>Animal fat</td>
</tr>
<tr>
<td>Oleic acid</td>
<td>18</td>
<td>Monounsaturated</td>
<td>No</td>
<td>Olive oil</td>
</tr>
<tr>
<td>Linoleic acid</td>
<td>18</td>
<td>Polyunsaturated</td>
<td>Yes</td>
<td>Safflower oil</td>
</tr>
<tr>
<td>Linolenic acid</td>
<td>18</td>
<td>Polyunsaturated</td>
<td>Yes</td>
<td>Soybean oil</td>
</tr>
<tr>
<td>Arachidonic acid</td>
<td>20</td>
<td>Polyunsaturated</td>
<td>Yes</td>
<td>Meat, dairy</td>
</tr>
<tr>
<td>Eicosapentaenoic acid</td>
<td>20</td>
<td>Polyunsaturated</td>
<td>Yes</td>
<td>Fish oil</td>
</tr>
<tr>
<td>Docosahexaenoic acid</td>
<td>22</td>
<td>Polyunsaturated</td>
<td>Yes</td>
<td>Fish oil</td>
</tr>
</tbody>
</table>
The nomenclature of fatty acids, specifically PUFAs, is designated in short-hand notation, which describes the position of the double bond that is closest to the methyl terminus of the acyl chain and termed omega (n). They have been classified into groups, such as n-3, n-6, n-9, and conjugated fatty acids.

### 1.6.1 Biological Metabolism of n-3 PUFAs

The shortest n-3 PUFA is α-linoleic acid (ALA, C18:3) and can be synthesized from linoleic acid (LA, C18:2) by desaturation, catalyzed by Δ15-desaturase. Although humans do not possess the Δ15-desaturase enzyme and cannot synthesize ALA or LA, they can metabolize ALA into other longer chain n-3 fatty acids. This occurs by a series of desaturation and elongation reactions that mainly take place in the liver (Figure 14) [104]. n-3 and n-6 PUFAs compete for the Δ4 and Δ6-desaturation enzymes and it is thought that Δ6-desaturases are rate limiting in this pathway [105–107]. The activities of Δ6 and Δ5-desaturases are regulated by nutritional status, hormones, and negative feedback inhibition from the products in the pathway [16]. Furthermore, the conversion to EPA and DHA in humans is poor and conversion to DHA is especially limited. Humans cannot produce sufficient levels of DHA. therefore dietary supplementation is necessary [106], [107].
1.6.2 Incorporation of Dietary Lipids

Fatty acids are usually ingested as triacylglycerol (TAG) and broken down into free fatty acids and monoglycerides by lipase [108]. Emulsification of the fats allow for
better digestion. Once digested, the monoglycerides and fatty acids associate with bile salts and phospholipids to form micelles, which can contain fat soluble vitamins and cholesterol as well. Inside the enterocyte the monoglycerides and fatty acids are re-synthesized into TAG and along with cholesterol and fat soluble vitamins are incorporated into chylomicrons and released into the blood, where they can be taken up by other tissues in the body [108].

1.6.3 Lipids in Cancer

The health effect of dietary changes in the intake of fatty acids has gained attention over the last few decades. Pooling analysis and epidemiological studies have estimated that 35% of cancer cases might be related to related to diet [109]. Other contributing factors to the increases in cancer risk are excessive caloric intake, increased obesity, decreased physical exercise, and smoking, all of which have been directly correlated to increasing incidence in breast and colon cancer [110]. It has been well characterized that populations who consume western style diets, enriched in n-6 PUFAs, are at risk for many diseases, such as cardiovascular disease, cancer, diabetes, and inflammatory diseases [111–113].

Dietary lipids have been extensively studied in the association with breast cancer survival and recurrence [114]. The amount and source of dietary lipids play an important role in cancer risk because variation in the rates of cancer is seen among different geographic locations [109]. Epidemiological studies have shown that Japanese
immigrants who have migrated to the United States had increased cancer occurrences after a single generation. Interestingly, cancer incidence was reversed when the Japanese immigrants migrated back to Japan, which suggests that the changes in cancer incidence may be environmental and related to diet [115], [116]. Moreover, data on dietary intervention studies carried out in animals [117], [118] and humans [119–121] demonstrate that dietary intake of lipids directly modify lipid storage and membrane lipid composition. Furthermore, PUFAs have been shown to alter several biological systems, such as preferential replacement of arachidonic acid (AA, C20:4) in membrane phospholipids, changes in formation of lipid raft membrane signaling platforms, altered substrates for eicosanoids, transcriptional and post translational modification to proteins, and hormone signaling [16], [103], [112], [122–124].

n-3 PUFAs have been shown to reduce malignant transformation, angiogenesis, and tumor growth in cell culture [125] and in animal models [126–129]. The anti-cancer effects of EPA and DHA have also presented promise clinically [121], [130], showing enhancement to existing chemo and radiation therapies [117], [131], [132]. While there are limited clinical trials that have examined the effects of n-3 fatty acids in cancer, the existing studies show positive responses [133]. Moreover, n-3 PUFAs have been shown to counteract the negative effects of chemotherapy and recent studies suggest that DHA and EPA supplementation helped to reduce weight loss in cancer patients receiving chemotherapy [29], [129], [132].
1.7 Project Aims

Dietary lipids have long been studied in association with breast cancer survival and recurrence [121]. A recent large cohort study showed that women who were already treated for early breast cancer and reduced the amount of dietary fat intake to 22% of the total energy intake [134] led to a reduction of the rate of recurrences by 24%. This intervention study marked for the first time the fact that dietary intervention can modify breast cancer outcome to an extent close to what is achievable by the current adjuvant treatments [121]. Since the intervention study was solely based on a quantitative change in total dietary fat intake, the possibility of dietary intervention targeting specific individual lipids might produce even greater benefits than gross changes in the diet. Therefore, the aim of the project was to identify ways in which n-3 and n-6 PUFAs mediate changes in mammary carcinogenesis survival and progression. Analysis of the metabolic and programmed pathways regulated by cancer cells were assessed in response to n-3 and n-6 PUFA supplementation. This was investigated through:

1. Determining the effects of n-3 and n-6 PUFAs on HIF-1α expression levels and metabolic pathways regulated by HIF-1α in the breast cancer lines, BT-474, MDA-MB-231 and the non-transformed cell line, MCF-10A. (Chapter 2)

2. Comparing the metabolic profiles (acquired using Seahorse XF96 Analyzer) of BT-474, MDA-MB-231, MCF-10A cell lines to identify the impacts of n-3 and n-6 PUFAs on metabolism. (Chapter 2)
3. Determining programmed cellular responses upon treatment of n-3 and n-6 PUFAs in BT-474, MDA-MB-231, and the MCF-10A by oncogenic analysis of Myc activity. (Chapter 3)
Chapter 2 - Docosahexaenoic acid attenuates cancer cell metabolism and the Warburg phenotype by targeting bioenergetic function in breast cancer through HIF-1α

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2.1 Abstract

Docosahexaenoic acid (DHA, C22:6) depresses mammary carcinoma proliferation and growth in tissue culture and in animal models. The current study was designed to explore the role of interrupting bioenergetic pathways in BT-474 and MDA-MB-231 breast cancer cell lines representing respiratory and glycolytic phenotypes, respectively and comparing the impacts of DHA with the non-transformed cell line, MCF-10A. DHA enrichment resulted in decreases in HIF-1α total protein levels and transcriptional activity in the two tumor lines and not in the non-transformed MCF-10A cells. Downregulation of downstream targets of HIF-1α, including the glucose transporter 1 (GLUT 1) and lactate dehydrogenase (LDH), were observed in both cancer cell lines. Glucose uptake, total glucose oxidation, glycolytic metabolism, and lactate production were significantly decreased in response to DHA supplementation as well. Further metabolic investigation revealed that DHA supplementation significantly altered the bioenergetic profile of cancer cells in a dose-dependent manner; thereby enhancing metabolic injury and decreasing oxidative metabolism. DHA-induced metabolic changes
led to a marked decrease of intracellular ATP levels by 50% in both cancer cell lines which mediated activation of metabolic stress marker, 5’ AMP-activated protein kinase (AMPK) phosphorylation at Thr172. These findings show that DHA contributes to impaired cancer cell growth and survival by altering cancer cell metabolism and increasing metabolic stress by directly altering HIF-1α-associated metabolism, providing a rationale for enhancement of current cancer prevention models and current therapies by combining them with dietary sources, like DHA.

2.2 Introduction

Breast cancer is the second highest cause for cancer-related mortality in the United States, and over 230,000 women are estimated to be diagnosed in 2013 [27]. Analysis of the bioenergetic metabolism of cancer cells has recently come into focus [3], [135], although it has been almost a century since Warburg’s initial findings that cancer cells exhibit a glycolytic phenotype over oxidative metabolism [39]. The functional state of mitochondria plays a critical role in carcinogenic invasion and metastasis because it has been shown to help stabilize the Warburg phenotype [59].

The metabolic reprogramming of cancer cells is dependent on the activity of hypoxia-inducible factor (HIF) pathway, mainly HIF-1α, which mediates the adaptation of cells under hypoxic conditions [136], [137]. Once stabilized, HIF-1α transcriptionally regulates the expression of over 1000 target genes, including genes responsible for regulation of cancer cell metabolism [12], [138]. Breast cancer patient biopsies have demonstrated a correlation of poor prognosis [139], tumor aggressiveness [140], and
early relapse [141] with increased HIF-1α protein levels. Additionally, HIF-1α level is associated with increased metastatic potential [33], [38], [142], [143], and resistance to both chemotherapy [97], [144], [145] and radiation therapy [146].

With the focus back to cancer cell metabolism, dietary factors and inhibitors of glycolysis and molecular targets, such as HIF-1α, are being widely studied to treat or enhance the effectiveness of other therapeutics in the treatment of cancer [3], [147]. The effects of dietary n-3 polyunsaturated fatty acids (PUFAs) have been suggested to have anti-cancer properties, and its effects on cancer cell metabolism presents another possible mechanism for inhibiting cancer survival and progression [16]. Studies on cardiac tissue have shown n-3 PUFAs are capable of changing cellular metabolism after ischemia [148–150]. Another recent study showed that n-3 PUFAs can increase proton leak in colon cancer cells [151]. Additionally, changes in proliferation and cancer cell survival are suggested to be mediated through peroxisome proliferator-activated receptor (PPAR) isoforms α [152] and γ [153], and that n-3 PUFAs, specifically eicosapentaenoic acid (EPA; C20:5) and docosahexaenoic acid (DHA; C22:6), can alter activities of HIF-1α [126]. However, the changes in HIF-1α-associated metabolism by n-3 PUFAs have not been fully elucidated.

In the current study, we hypothesize that DHA will result induce changes to HIF-1α activity and alter HIF-1α regulation on downstream metabolism. In order to test this hypothesis and compare the impact of DHA supplementation on the bioenergetic signature of cancer cells, two metabolically distinct breast cancer cell lines were employed (1) BT-474, with a mitochondrially active phenotype, and (2) MDA-MB-231,
with a glycolytically active phenotype. A non-transformed breast epithelial MCF-10A cell line was also examined.

Together, the data presented demonstrate that n-3 PUFA supplementation deregulates cancer bioenergetic function through HIF-1α-associated metabolism, suggesting that supplementation with n-3 PUFAs could enhance current therapies and lead to new approaches in the treatment of cancer.

2.3 Materials & Methods

Cell Lines & Reagents – BT-474 mammary ductal carcinoma cells, MDA-MB-231 mammary adenocarcinoma cells and MCF-10A non-tumorigenic mammary epithelial cells were purchased from ATCC (Manassas, VA). Fatty acid methyl esters (FAME) (Sigma, St. Louis, MO) were dissolved in ethanol (EtOH), flushed with nitrogen gas, protected from light and stored at -20°C for no more than 60 days.

Cell Culture – BT-474 mammary ductal carcinoma cells were maintained in HybriCare (ATCC, Manassas, VA) supplemented with 10% FBS (Hyclone, Logan, UT). MDA-MB-231 mammary adenocarcinoma cells were maintained in RPMI-1640 (Thermo Scientific, Rochester, NY) supplemented with 10% FBS (Hyclone, Logan, UT). MCF-10A non-tumorigenic mammary epithelial cells were maintained in DMEM/F12 (Thermo Scientific, Rochester, NY) supplemented with 5% FBS (Hyclone, Logan, UT). Cells were grown as monolayers at 37°C in a humidified environment with 5% CO₂.
**Integrated Cellular Metabolism Assays** – As previously described previously [154], [155] basal oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were measured in the Seahorse XF-96 Flux Analyzer (Seahorse Bioscience Inc. Billerica, MA). 1.0 x 10^4 cells per well of BT-474, MDA-MB-231 or MCF-10A were seeded in 100 µl of cell culture medium at 37°C overnight prior to treatment with specified PUFAs or an equal volume of EtOH. The plates were then treated for 48 hours prior to the experiment. The conditioned media was aspirated and cells were washed twice with PBS. The cells were then equilibrated with assay media lacking sodium bicarbonate at 37°C for 30 minutes in an incubator lacking CO₂. Basal OCR was measured followed by four treatments of glycolytic and mitochondrial inhibitors: 2-deoxy-D-glucose, oligomycin, carbonyl cyanide 4-trifluoromethoxy-phenylhydrazone (FCCP), and antimycin A. Measurements were standardized to protein concentration in each well. A minimum of four wells were utilized per treatment group and data represent at least n=3 independent experiments ± SD.

**XTT Assay** – The Cell Proliferation Kit II from Roche (Indianapolis, IN) was used following the manufacture’s instruction. Briefly, an equal number of cells were seeded in 96-well plates before specific treatments. At the end of the incubation times, XTT reagents (XTT labeling reagent and electron coupling reagent) were mixed and added to each well for 4-24 hours before being read at 450 nm (with a 650 nm reference wavelength) on a SpectraMax M5 machine (Molecular Devices, Sunnyvale, CA).
Cell Viability Assays – Cells were trypsinized and counted using trypan blue staining and a hemocytometer. Unstained cells were counted as viable.

Luciferase Assays – Cells were transfected with 100 ng of HIF Cignal™ reporter construct along with a negative and positive control (Qiagen, Valencia, CA). After 6 hours of transfection, medium was changed with the addition of either 100 µM of DHA, LA or an equal volume of EtOH and cells were incubated for 24 hours. Cells were then treated with 50 µM CoCl₂ for 24 hours and then harvested for reporter gene assays. Dual Luciferase assay (Promega, Madison, WI) was performed following manufacturer protocol, and promoter activity values are expressed as relative units standardized to the XTT assay (Roche, Indianapolis, IN).

Immunoblotting – As described previously [127], cells were washed with ice-cold PBS and lysed using GTP-lysis buffer [50 mM HEPES (pH 7.5), 15 mM NaCl, 6 mM sodium deoxycholate, 1% NP-40, 10% glycerol, 10 mM MgCl₂,1 mM EDTA] containing freshly added protease and phosphatase inhibitors. Tumors were homogenized in the same buffer. Samples were centrifuged at 16,000 × g for 10 min at 4°C. Supernatants were analyzed for protein concentration using BIO RAD’s DC assay (Hercules, CA). Samples were resolved by SDS-PAGE and transferred to either nitrocellulose or (Polyvinylidene fluoride) PVDF membranes (BIO RAD, Hercules, CA) and probed with specific antibodies. Detection was performed using HRP-conjugated secondary antibodies and visualized with ECL (GE Healthcare, Buckinghamshire, UK). Specific antibodies were purchased for pAMPK (1:1000), AMPK (1:1000), LDH
(1:2000) (Cell Signaling Technologies, Danvers, MA), GLUT-1 (1:1000) (EMD Millipore Corporation, Billerica, MA), HIF-1α (1:500) (Santa Cruz Biotechnology, Inc., Dallas, TX), and β-actin (1:5000) (Abcam, Cambridge, MA).

**Glucose Uptake** – Measurements of glucose uptake was done as previously described [156] with modification. Briefly, an equal number of cells were seeded in 10 cm plates and allowed to settle for 24 hours before specific treatments. At the end of specified treatment times, the cells were standardized using viable cell counts. 5 x 10^5 cells were washed once with warmed (37°C) PBS prior to resuspension in 500 μL KRBH with 0.5% BSA and 0.5 mM glucose and incubated for 30 min at 37°C. Resuspension media was removed and 250 μL of KRBH/0.5%BSA/0.5mM glucose containing 1 μCi of 2-deoxy-D-\(^3\)H-glucose (PerkinElmer, Waltham, MA) was added each sample and incubated for 5 min at 37°C. Following incubation, media was removed and samples were placed on ice before washing 3 times with ice-cold PBS containing 5 mM glucose (Figure 15). Cells were collected by centrifugation and the cell pellet was solubilized in 0.1 M NaOH. The samples were placed into 4 mL scintillation vials with 2 mL of Ecolite(+)™ liquid scintillation cocktail (MP Biochemicals, Santa Ana, CA) and radioactivity was measured by a Tri-Carb 2900TR (Packard Instrument Company, Meriden, CN) liquid scintillation analyzer.
Figure 15. $^3$H-2-deoxy-glucose structure and cellular action adapted from Aft et al., 2002 [17]. (A) Comparison of glucose and 2-deoxy-glucose. (B) 2-DG is taken up into the cell by GLUT 1 and metabolized by Hexokinase. 2-DG-PO$_4$ cannot be further metabolized and is trapped in the cell. Using $^3$H-2-DG allows for quantitation of glucose uptake.

**Glycolysis Measurements** – Measurements of glucose usage through glycolysis was done as previously described [157], [158] with slight modification. Briefly, an equal number of cells were seeded in 10 cm plates and allowed to settle for 24 hours before specific treatments. At the end of specified treatment times, the cells were standardized using viable cell counts. $1 \times 10^6$ cells were washed once with warmed (37°C) PBS prior to resuspension in 250 μL HBSS and incubated for 1 hour at 37°C. 250 μL of HBSS
containing 8 mM glucose and 5 μCi of 5-^3^H-glucose (PerkinElmer, Waltham, MA) were added to each sample and incubated in a shaking H_2O bath at 90 rpm for 1 hour at 37°C. Following incubation, samples were taken in triplicate and 100 μL were transferred to uncapped 0.25 mL PCR tubes containing an equal volume of 0.2 N HCl. 0.5 mL of H_2O was added to the outside of the PCR tubes not allowing the H_2O and the contents of the PCR tubes to mix. The vials were sealed and diffusion was allowed to occur for a minimum of 24 hours. The samples were then placed into 4 mL scintillation vials with 2 mL of Ecolite(+)™ liquid scintillation cocktail (MP Biochemicals, Santa Ana, CA). The amounts of diffused and undiffused ^3^H were determined by scintillation counting on a Tri-Carb 2900TR (Packard Instrument Company, Meriden, CN). Appropriate ^3^H-glucose-only and ^3^H_2O-only controls were included, enabling the calculation of ^3^H_2O in each sample to calculate the rate of glycolysis, as described previously [159].
3H-5-Glucose metabolism through glycolysis adapted from Kahn Academy 2013 [18]. 3H-5-Glucose is metabolized through glycolysis and the 3H is released at 3H2O, allowing for quantitation of glucose usage through glycolysis.

**Total Glucose Oxidation Measurements** – Glucose oxidation measurements were done as previously described [160] with slight modification. Briefly, an equal number of cells were seeded in 10 cm plates and allowed to settle for 24 hours before specific treatments. At the end of specified treatment times, the cells were standardized using viable cell counts. Fully oxygenated incubation media (KRB-HEPES with 0.12 M sodium bicarbonate) with O2:CO2 (95:5) and adjusted to pH 7.4 was prepared prior to the addition of 0.1 μCi per flask U-14C-glucose (33.3 μCi per millimole of substrate) (PerkinElmer, Waltham, MA) and 1 mM D-glucose. The reaction was initiated by the addition of 0.5 mL of cell suspension (1 x 10^7 viable cells) in flasks with sealed caps fitted with a suspended center well containing filter paper and incubated for 90 min at
37°C. The reactions were acidified by the addition of 0.2 mL of concentrated perchloric acid (HClO₄) (Sigma, St. Louis, MO). 0-min metabolic production rates for each sample were prepared by the addition of cell suspension followed immediate acidification with HClO₄. After incubations are terminated, center wells were filled with 0.3 mL of benzethonium hydroxide (C₂₇H₄₃NO₃) (Sigma, St. Louis, MO) to capture CO₂ and were allowed to sit at room temperature for 1 hour. Center wells were placed in scintillation vials and filled with 4 mL of Ecolite(+)™ liquid scintillation cocktail (MP Biochemicals, Santa Ana, CA) before scintillation counting on a Tri-Carb 2900TR (Packard Instrument Company, Meriden, CN). Matched set of experiments were used for analysis of lactate production.

**Glucose / Lactate Assay Kits** – Absorbance based Glucose and Lactate Assay Kit II (Biovision, Milpitas, CA) were used following the manufacturer protocol. Briefly, an equal number of cells were seeded in 6-well plates and allowed to settle for 24 hours before specific treatments. At the end of the specified treatment times, the cells were standardized using viable cell counts. The cells were then mixed with the respective kit reaction mixtures and were allowed to sit at room temperature for 30 min before being read at OD₄₅₀ nm on a SpectraMax M5 machine (Molecular Devices, Sunnyvale, CA). The kits allow for the quantification of the amount of glucose or L(+)-lactate or in the samples by generating a product which interacts with a probe to produce color (λₘₐₓ=450 nm).
**Quantitative RT-PCR** – Cells were treated with either 100 μM of LA, DHA or an equal volume of EtOH for 48 hours. Total RNA was isolated and purified using RNeasy mini kit (Qiagen, Valencia, CA) following the manufacturer’s protocol. cDNA was then synthesized using cDNA Synthesis VILO kit (Invitrogen, Grand Island, NY). Quantities of cDNA were measured by quantitative real-time PCR on a CFX96 Real-Time PCR System (Bio-Rad, Hercules, CA). The reaction used SYBR green FAST master mix and conditions followed manufacture’s recommendations (Applied Biosystems, Carlsbad, CA). GLUT 1 primer set was designed using Primer3 Input [161] (GLUT 1: Sense: GCCTGGATCTCCCCACTCTAG; Anti-sense: CTCCCAACTGGTCTCAGGTAAGA). Published primer sets were used for HIF-1α [162] (Sense: GTCGGACAGCCTCACCAAACAGAGC; Anti-sense: GTTAACCTTGATCCAAAGCTCTTGAG), LDHA [163] (Sense: ACCCAGTTTCCACCATGTATT; Anti-sense: CCCAAAATGCAAGGAACACT) and β-actin [164] (Sense: CGTCTTCCCCTCCATCG; Anti-sense: CTCCTTAATGTCACGCAC). The relative abundance of mRNA was determined by comparative Ct method [165]. Data represents at least n=3 independent experiments ± SD.

**Statistical Analysis** – All experimental results were independently repeated at least three times. All quantitative data shown represent the compiled data as percentages versus control treatments with error bars representing standard deviation and statistical analyses were performed using the Student’s t test and/or ANOVA with the Tukey
method for pairwise comparison on SAS® software with values of at least P<0.05 being considered significant.

2.4 Results

DHA negatively regulates HIF-1α-associated metabolism

To determine the effect of DHA on cancer metabolism, characterization of metabolism regulated by HIF-1α in response to DHA treatment was assessed. Interestingly, HIF-1α protein levels were decreased in both BT-474 and MDA-MB-231 cells in response to DHA treatment compared to control (Figure 17A). Densitometry analysis showed significant decreases in HIF-1α protein levels (Figure 17B). To determine the consequence of decreased HIF-1α protein, HIF-1α transcriptional activity was assessed. Cobalt Chloride (CoCl₂), a known chemical inducer of HIF-1α, was used. DHA treatment significantly decreased HIF-1α transcriptional activity as well as prevented the induction of HIF-1α with CoCl₂ in both BT-474 and MDA-MB-231 cell lines (Figure 18). Further investigation found downregulation of downstream transcriptional targets of HIF-1α, glucose transporter 1 (GLUT 1) and lactate dehydrogenase (LDH), which are key components of the Warburg phenotype (Figure 19A). Densitometry analysis revealed significant DHA induced downregulation of GLUT 1 and LDH protein levels in the BT-474 cell line (Figure 19B). High basal expression of GLUT 1, independent of HIF-1α activity, prevented any significant DHA-induced changes in the MDA-MB-231 cell line. Quantitative Real-Time PCR (qPCR) results showed decreased HIF-1α mRNA levels, however no significant changes were observed.
in mRNA levels of GLUT 1 or LDH following DHA supplementation (Table 7). Taken together, these data suggest that DHA is altering HIF-1α-associated metabolism and the Warburg phenotype.
Figure 17. DHA decreases HIF-1α protein levels. Treatment of 100 μM DHA (n-3 PUFA), LA (n-6 PUFA) or an equal volume of EtOH for 48 hours before harvesting cells for immunoblot analysis. (A) Immunoblot analysis showing HIF-1α expression levels in BT-474 and MDA-MB-231 cell lines. Total β-actin was used as a loading control. (B) Percentage densitometry analysis of HIF-1α in BT-474 and MDA-MB-231 cell lines standardized to β-actin and relative to control. ** P<0.001 vs. indicated treatment. All experiments represent at least n=3 and densitometry analysis represents mean ± SD. Abbreviations: LA (linoleic acid), DHA (docosahexaenoic acid), HIF-1α (hypoxia-inducible factor 1alpha).
A. **Figure 18.** DHA modifies HIF-1α activity in breast cancer cells. (A) BT-474 and (B) MDA-MB-231 cells were transfected with 100 ng of HIF Cignal™ reporter construct along with a negative and positive control. After 6 hours of transfection, medium was changed with the addition of 100 μM DHA (n-3 PUFA), LA (n-6 PUFA) or an equal volume of EtOH for 48 hours before harvesting the cells for reporter gene assays. Dual Luciferase assay was performed and promoter activity values are expressed as relative units standardized to XTT. The percentage relative luciferase activity is represented as mean ± SD. **P<0.001 vs. indicated treatment. All experiments represent at least n=3. Abbreviations: DHA (docosahexaenoic acid), HIF-1α (hypoxia-inducible factor 1alpha).**
**Table 7.** Changes in mRNA of HIF-1α and downstream targets in response to PUFA treatment. 100 μM DHA (n-3 PUFA), LA (n-6 PUFA) or an equal volume of EtOH for 48 hours before harvesting the cells for mRNA isolation and purification. mRNA expression of HIF-1α, GLUT 1, and LDH were assessed by quantitative RT-PCR relative to EtOH control and corrected for β-actin expression in each indicated cell line. The values are means ± SD of at least n=3 independent experiments. Abbreviations: LA (linoleic acid), DHA (docosahexaenoic acid), HIF-1α (hypoxia-inducible factor 1alpha), GLUT 1 (Glucose Transporter 1), LDH (Lactate Dehydrogenase).

<table>
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<th>Cell Line</th>
<th>HIF-1α mRNA expression (relative to control)</th>
<th>GLUT 1 mRNA expression (relative to control)</th>
<th>LDH mRNA expression (relative to control)</th>
</tr>
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<td></td>
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<td>DHA</td>
</tr>
<tr>
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<td>0.92 ± 0.02</td>
<td>0.65 ± 0.20</td>
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<tr>
<td>MCF-10A</td>
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<td>0.93 ± 0.10</td>
<td>0.85 ± 0.10</td>
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A. Figure 19. Changes in protein levels of HIF-1α downstream targets in response to PUFA treatment. Treatment of 100 μM DHA (n-3 PUFA), LA (n-6 PUFA) or an equal volume of EtOH for 48 hours before harvesting cells for immunoblot analysis. (A) Immunoblot analysis showing GLUT 1 and LDH expression levels in BT-474 and MDA-MB-231 cell lines. Total β-actin was used as a loading control. (B) Percentage densitometry analysis GLUT 1 and LDH in BT-474 and MDA-MB-231 cell lines standardized to β-actin and relative to control. ** P<0.001 vs. indicated treatment. All experiments represent at least n=3. Abbreviations: LA (linoleic acid), DHA (docosahexaenoic acid), GLUT 1 (Glucose Transporter 1), LDH (Lactate Dehydrogenase).
Decreases in glucose metabolism and the Pasteur Effect in cancer cells in response to DHA treatment

To determine the extent of the effect of DHA on HIF-1α-associated metabolism, total glucose metabolism was assessed. Glucose uptake was measured by uptake of 2-deoxy-D-[^3]H-glucose in the BT-474, MDA-MB-231 and MCF-10A cell lines. Dose-dependent decreases in[^3]H-2-DG uptake was seen in response to DHA supplementation in both cancer cell lines, but not in the non-transformed cell line (Figure 20). Additionally, total intracellular glucose measurements confirmed[^3]H-2-DG uptake (data not shown). Interestingly, although it appears that high basal levels of GLUT 1 protein, independent of HIF-1α regulation, in the MDA-MB-231 cell line prevented any significant changes in response to DHA supplementation, however glucose uptake was significantly decreased (Figure 20).

Next, total glycolytic rate was examined by measuring the conversion of 5[^3]H-glucose to[^3]H2O in response to DHA supplementation. DHA treatment in BT-474 and MDA-MB-231 cells led to rapid dose-dependent decreases in glucose metabolism compared to control (Figure 21). Identical treatments in the non-transformed MCF-10A cell line showed no significant changes of glucose usage at high concentrations of DHA, but low concentrations significantly increased glucose usage in MCF-10A cells (Figure 21). Total glucose oxidation was then evaluated by measuring total ^14CO2 produced from U-[^14]C-glucose in cells treated with DHA. Similarly, decreases in total glucose oxidation in response to DHA treatment were observed (Figure 22). Notably, basal lactate production was much greater in the MDA-MB-231 cell line, suggesting that the MDA-
MB-231 cells have a greater glycolytic phenotype than the other cell lines. Moreover, DHA treatment significantly decreased total lactate production in the BT-474 and MDA-MB-231 cell lines, indicating that DHA downregulates total Warburg metabolism in cancer cells (Figure 23).

**Figure 20. Decreases in glucose uptake in response to DHA.** Assessment of $^3$H-2-deoxy-D-glucose uptake. Indicated cell lines were treated with 12.5, 25, 50, 75, 100 μM DHA or a control volume of EtOH for 48 hours before 5 min incubation with $^3$H-2-DG. + P<0.05 compared to control. ** P<0.001 compared to control. All experiments were done in triplicate and represent at least n=3 independent experiments ± SD. Abbreviations: CPM (counts per minute), DHA (docosahexaenoic acid), 2-DG (2-deoxy-D-glucose).
Figure 21. **Glucose flux is decreased in breast cancer with DHA supplementation.** $5^{3}\text{H}$-glucose analysis showing rate of diffused $^3\text{H}_2\text{O}$ (rate of glycolysis). Indicated cell lines were treated with 12.5, 25, 50, 75, 100 μM DHA or an equal volume of EtOH for 48 hours before incubation with $5^{3}\text{H}$-Glucose. **P<0.001 compared to control in individual cell lines. All experiments were done in triplicate and represent at least n=3 independent experiments ± SD. Abbreviations: DHA (docosahexaenoic acid).
Figure 22. DHA decreases total glucose oxidation in breast cancer cells. U-$^{14}$C-Glucose analysis showing percentage of $^{14}$CO$_2$ production (glucose oxidation) relative to control. Indicated cell lines were treated with 100 μM DHA (n-3 PUFA), LA (n-6 PUFA) or an equal volume of EtOH for 48 hours before incubation with U-$^{14}$C-Glucose. * P<0.01 vs. indicated treatment. ** P<0.001 vs. indicated treatment. All experiments were done in triplicate and represent at least n=3 independent experiments ± SD. Abbreviations: LA (linoleic acid), DHA (docosahexaenoic acid).
Figure 23. Lactate production analysis. BT-474, MDA-MB-231, and MCF-10A cell lines were treated with 100 μM DHA (n-3 PUFA), LA (n-6 PUFA) or an equal volume of EtOH for 48 hours before total lactate concentration was analyzed by absorbance method and data represents lactate production (μM). ** P<0.001 compared to control. All experiments were done in triplicate and represent at least n=3 independent experiments ± SD. Abbreviations: LA (linoleic acid), DHA (docosahexaenoic acid).
DHA supplementation attenuates the bioenergetic signature of cancer cells

Based on the significant decreases in HIF-1α-associated metabolism, the effects of polyunsaturated fatty acids (PUFAs) on glycolytic and mitochondrial metabolism were explored. Previous reports have shown changes in mitochondrial function in response to dietary fish oil in colon cancer cells [151]. Two metabolically distinct breast cancer cell lines BT-474 and MDA-MB-231 and non-transformed MCF-10A cells were used to examine time- and dose-dependent metabolic assessments in response to PUFA supplementation using Seahorse XF-96 analyzer (Billerica, MA, USA). Basal extracellular acidification rate (ECAR) and basal mitochondrial respiration were calculated simultaneously by subtracting the remaining oxygen consumption rate after addition of the electron transport complex III inhibitor antimycin A (Figure 24). Significant decreases in OCR were observed in a dose-dependent manner in response to DHA supplementation in both BT-474 (Figure 25) and MDA-MB-231 (Figure 26) cell lines, but not in MCF-10A (Figure 27) cells. In the MDA-MB-231, a low oxygen consumer and more glycolytically active cell line, DHA significantly decreased basal ECAR by over 60% (Figure 28) and basal OCR was decreased by 80% compared to control (Figure 29B). Furthermore, in the BT-474 cell line, a higher oxygen consumer and more mitochondrially active, DHA also significantly decreased basal OCR by 70% versus control (Figure 29B). The rate of coupled respiration, ATP production, was calculated by subtracting the OCR after addition of the F1F0-ATPase inhibitor, oligomycin, from the basal respiration (Figure 24) and was significantly decreased in
both cancer cell lines, inhibited over 70% by 100 μM DHA compared to control (Figure 30B).

To further identify the impact by DHA on the bioenergetic capacity of the mitochondria, maximal respiration was calculated by subtracting the non-mitochondrial OCR after the addition of antimycin A from the OCR after addition of the uncoupler FCCP (Figure 24). Similarly, the spare respiratory was calculated by subtracting basal OCR prior to the addition of 2-DG from the OCR after addition of FCCP (Figure 24). Again, large decreases in the respiratory capacity of the mitochondria in cancer cells were observed with decreases over 80% in cells treated with 100 μM DHA (Figure 31B and 32B, respectively). No differences were observed in non-mitochondrial respiration after the addition of antimycin A, which is expected (Figure 33B). All cell line-specific, BT-474, MDA-MB-231 and MCF-10A, data are summarized in Tables 8, 9 and 10, respectively. Combined, these data suggest that DHA induces mitochondrial injury supported by the significant changes in oxidative metabolism. Interestingly, the bioenergetic profile changes induced by DHA seem to be specific to cancer cells.
Figure 24. OCR model used for integrated extracellular metabolism assays. Successive injections of oligomycin, FCCP, and antimycin A. Abbreviations: 2-DG (2-deoxy-glucose), FCCP (carbonyl cyanide 4-trifluorocarbonyl cyanide phenylhydrazone), OCR (oxygen consumption rate), ECAR (extracellular acidification rate).
Figure 25.  Assessment of the bioenergetic profiles of BT-474 cells in response to DHA treatment. Specified concentrations of DHA (n-3 PUFA) were treated for 48 hours on OCR in the BT-474 cell line. All experiments were done in quadruplicate and represent at least n=3 ± SD. Abbreviations: DHA (docosahexaenoic acid), 2-DG (2-deoxy-glucose), FCCP (carbonyl cyanide 4-trifluorocarbonyl cyanide phenylhydrazone), OCR (oxygen consumption rate).
Figure 26. **Assessment of the bioenergetic profiles of MDA-MB-231 cells in response to DHA treatment.** Specified concentrations of DHA (n-3 PUFA) were treated for 48 hours on OCR in the MDA-MB-231 cell line. All experiments were done in quadruplicate and represent at least n=3 ± SD. Abbreviations: DHA (docosahexaenoic acid), 2-DG (2-deoxy-glucose), FCCP (carbonyl cyanide 4-trifluorocarbonyl cyanide phenylhydrazone), OCR (oxygen consumption rate).
Figure 27. Assessment of the bioenergetic profiles of MCF-10A cells in response to DHA treatment. Specified concentrations of DHA (n-3 PUFA) were treated for 48 hours on OCR in the MCF-10A cell line. All experiments were done in quadruplicate and represent at least n=3 ± SD. Abbreviations: DHA (docosahexaenoic acid), 2-DG (2-deoxy-glucose), FCCP (carbonyl cyanide 4-trifluorocarbonyl cyanide phenylhydrazone), OCR (oxygen consumption rate).
Figure 28. Assessment of basal ECAR in response to DHA treatment. Estimate of cellular basal ECAR. Indicated cell lines were treated with 25, 50, 75, 100 μM DHA or an equal volume of EtOH for 48 hours before basal ECAR was assessed in the BT-474, MDA-MB-231 and MCF-10A cell lines. Data represents ECAR (pmoles/min/mg protein). + P<0.05 compared to control. All experiments were done in quadruplicate and represent at least n=3 ± SD. Abbreviations: DHA (docosahexaenoic acid), FCCP (carbonyl cyanide 4-trifluorocarbonylcyanide phenylhydrazone), ECAR (extracellular acidification rate).
A. Basal OCR assessment. Estimate of cellular basal oxygen consumption. (A) Basal oxygen consumption rate (OCR) was calculated by subtracting OCR of non-mitochondrial respiration from OCR before addition of 2-DG. (B) Indicated cell lines were treated with 25, 50, 75, 100 μM DHA or an equal volume of EtOH for 48 hours before basal OCR was assessed in the BT-474, MDA-MB-231 and MCF-10A cell lines. + P<0.05 compared to control. * P<0.01 compared to control. All experiments were done in quadruplicate and represent at least n=3 ± SD. Abbreviations: DHA (docosahexaenoic acid), OCR (oxygen consumption rate), 2-DG (2-deoxy-D-glucose), FCCP (carbonyl cyanide 4-trifluoromethoxy-phenylhydrazone).

B. Basal OCR

Figure 29. Basal OCR assessment. Estimate of cellular basal oxygen consumption. (A) Basal oxygen consumption rate (OCR) was calculated by subtracting OCR of non-mitochondrial respiration from OCR before addition of 2-DG. (B) Indicated cell lines were treated with 25, 50, 75, 100 μM DHA or an equal volume of EtOH for 48 hours before basal OCR was assessed in the BT-474, MDA-MB-231 and MCF-10A cell lines. + P<0.05 compared to control. * P<0.01 compared to control. All experiments were done in quadruplicate and represent at least n=3 ± SD. Abbreviations: DHA (docosahexaenoic acid), OCR (oxygen consumption rate), 2-DG (2-deoxy-D-glucose), FCCP (carbonyl cyanide 4-trifluoromethoxy-phenylhydrazone).
A. Figure 30. **Coupled resp. assessment.** Estimate of the proportion of basal OCR coupled to ATP synthesis. (A) Analysis of coupled respiration was calculated by subtraction of OCR after the addition of oligomycin from OCR before addition of 2-DG. (B) Indicated cell lines were treated with 25, 50, 75, 100 μM DHA or an equal volume of EtOH for 48 hours before coupled respiration was assessed in the BT-474, MDA-MB-231 and MCF-10A cell lines. + P<0.05 compared to control. All experiments were done in quadruplicate and represent at least n=3 ± SD. Abbreviations: DHA (docosahexaenoic acid), OCR (oxygen consumption rate), 2-DG (2-deoxy-D-glucose), FCCP (carbonyl cyanide 4-trifluoromethoxy-phenylhydrazone), ATP (Adenosine-5'-triphosphate).
**Figure 31. Max. resp. assessment.** Estimate of the potential maximal respiratory capacity. (A) Analysis of maximal respiration was calculated by subtracting OCR of non-mitochondrial respiration from OCR after addition of FCCP. (B) Indicated cell lines were treated with 25, 50, 75, 100 μM DHA or an equal volume of EtOH for 48 hours before maximal respiration was assessed in the BT-474, MDA-MB-231 and MCF-10A cell lines. + P<0.05 compared to control. * P<0.01 compared to control. All experiments were done in quadruplicate and represent at least n=3 ± SD. Abbreviations: DHA (docosahexaenoic acid), OCR (oxygen consumption rate), 2-DG (2-deoxy-D-glucose), FCCP (carbonyl cyanide 4-trifluoromethoxy-phenylhydrazone).
Figure 32. **Spare resp. capacity assessment.** Estimate of available respiratory capacity to cells. (A) Analysis of the spare respiratory capacity was calculated by subtraction of basal OCR before the addition of 2-DG from OCR before addition of FCCP. (B) Indicated cell lines were treated with 25, 50, 75, 100 μM DHA or an equal volume of EtOH for 48 hours before spare respiratory capacity was assessed in the BT-474, MDA-MB-231 and MCF-10A cell lines. + P<0.05 compared to control. * P<0.01 compared to control. All experiments were done in quadruplicate and represent at least n=3 ± SD. Abbreviations: DHA (docosahexaenoic acid), OCR (oxygen consumption rate), 2-DG (2-deoxy-D-glucose), FCCP (carbonyl cyanide 4-trifluoromethoxy-phenylhydrazone).
Non-Mitochondrial Respiration

(A) Analysis of non-mitochondrial respiration was calculated by determination of OCR after the addition of Antimycin A. (B) Indicated cell lines were treated with 25, 50, 75, 100 μM DHA or an equal volume of EtOH for 48 hours before non-mitochondrial respiration was assessed in the BT-474, MDA-MB-231 and MCF-10A cell lines. + P<0.05 compared to control. All experiments were done in quadruplicate and represent at least n=3 ± SD. Abbreviations: DHA (docosahexanoic acid), OCR (oxygen consumption rate), 2-DG (2-deoxy-D-glucose), FCCP (carbonyl cyanide 4-trifluoromethoxy-phenylhydrazone).
### Table 8. Assessment of metabolic changes in BT-474 breast cancer cells in response to PUFA supplementation.

BT-474 bioenergetic profiles and oxygen consumption rates (OCR) were determined using a Seahorse Bioscience XF96 Flux Analyzer by successive injections of oligomycin, FCCP, and Antimycin A. Dose-dependent analysis of DHA (n-3 PUFA), LA (n-6 PUFA), ALA (n-3 PUFA), and EPA (n-3 PUFA) for 48 hours on basal OCR, coupled respiration (ATP production), uncoupled respiration (proton leak), maximal respiration, spare-respiratory capacity, non-mitochondrial respiration, and ECAR were assessed in BT-474 cells. All experiments were done in quadruplicate and represent at least n=3 ± SD. Abbreviations: LA (linoleic acid), DHA (docosahexaenoic acid), ALA, (alpha-linolenic acid), EPA (eicosapentaenoic acid), OCR (oxygen consumption rate), ECAR (extracellular acidification rate).

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Table 9. Assessment of metabolic changes in MDA-MB-231 breast cancer cells in response to PUFA supplementation. MDA-MB-231 bioenergetic profiles and oxygen consumption rates (OCR) were determined using a Seahorse Bioscience XF96 Flux Analyzer by successive injections of oligomycin, FCCP, and Antimycin A. Dose-dependent analysis of DHA (n-3 PUFA), LA (n-6 PUFA), ALA (n-3 PUFA), and EPA (n-3 PUFA) for 48 hours on basal OCR, coupled respiration (ATP production), uncoupled respiration (proton leak), maximal respiration, spare-respiratory capacity, non-mitochondrial respiration, and ECAR were assessed in MDA-MB-231 cells. All experiments were done in quadruplicate and represent at least n=3 ± SD. Abbreviations: LA (linoleic acid), DHA (docosahexaenoic acid), ALA, (alpha-linolenic acid), EPA (eicosapentaenoic acid), OCR (oxygen consumption rate), ECAR (extracellular acidification rate).

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Table 10. Assessment of metabolic changes in MCF-10A non-transformed cell line in response to PUFA supplementation. MCF-10A bioenergetic profiles and oxygen consumption rates (OCR) were determined using a Seahorse Bioscience XF96 Flux Analyzer by successive injections of oligomycin, FCCP, and Antimycin A. Dose-dependent analysis of DHA (n-3 PUFA), LA (n-6 PUFA), ALA (n-3 PUFA), and EPA (n-3 PUFA) for 48 hours on basal OCR, coupled respiration (ATP production), uncoupled respiration (proton leak), maximal respiration, spare-respiratory capacity, non-mitochondrial respiration, and ECAR were assessed in MCF-10A cells. All experiments were done in quadruplicate and represent at least n=3 ± SD. Abbreviations: LA (linoleic acid), DHA (docosahexaenoic acid), ALA, (alpha-linolenic acid), EPA (eicosapentaenoic acid), OCR (oxygen consumption rate), ECAR (extracellular acidification rate).

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Inhibition of cancer metabolism leads to decreased intracellular ATP levels and increased metabolic stress upon supplementation with DHA in mammary carcinoma

In combination with decreased cellular metabolism, cells treated with DHA exhibited significant decreased cell proliferation and increased cell death in a dose-dependent manner, confirming reports by others [166]. Follow-up experiments to determine the effect of DHA treatment on total ATP production and metabolic stress were conducted. Significant decreases in total intracellular ATP levels in response to DHA supplementation in the cancer lines BT-474 and MDA-MB-231 were observed, but not in the MCF-10A cell line (Figure 34). Other reports have shown increased autophagy in cells harboring wild-type p53 and increased phosphorylated AMP-activated protein kinase (AMPK) in response to n-3 PUFAs [167]. To determine if the DHA-induced decreased levels of ATP were adequate to induce metabolic stress, phospho-Thr172-AMPKα levels were examined. Indeed, increased levels of phospho-Thr172-AMPKα in both BT-474 and MDA-MB-231 cells were observed. However, no changes were observed in total AMPKα levels (Figure 35A). Densitometry analysis indicated a significant increase in phospho-(Thr172)-AMPKα levels, thus the depletion of intracellular ATP levels by DHA led to increased metabolic stress in human breast cancer (Figure 35B).
Figure 34. DHA decreases total intracellular ATP levels in breast cancer. BT-474, MDA-MB-231 and MCF-10A cells were seeded in black 96-well plates and duplicate standard 96-well plates before being treated with 100 µM of DHA (n-3 PUFA), LA (n-6 PUFA) or an equal volume of ethanol (EtOH) for 48 hours. Cells in the black-well plates were analyzed using the ATPLite kit by adding first a cell lysis solution followed by a luminescent substrate and read on a SpectraMax V5 Microplate reader for luminescence. The duplicate standard-plates were analyzed for relative cell proliferation by XTT and the luminescence readings were standardized to reflect changes. ** P<0.001 compared to control. All experiments were done in triplicate and represent at least n=3 ± SD. Abbreviations: LA (linoleic acid), DHA (docosahexaenoic acid), ATP (adenosine-5’-triphosphate).
Figure 35. **DHA increases metabolic stress in breast cancer.** (A) Immunoblot analysis of BT-474, MDA-MB-231, and MCF-10A cells that were treated with 100 µM of DHA (n-3 PUFA), LA (n-6 PUFA) or an equal volume of EtOH for 48 hours assessing AMPKα expression and AMPKα phosphorylation at Thr172, a marker indicating metabolic stress. Total AMPKα and β-actin were used as loading controls. (B) Percentage densitometry analysis of p-Thr172-AMPKα in BT-474, MDA-MB-231, and MCF-10A cell lines standardized to total AMPKα and relative to control. All experiments were done in triplicate and represents at least n=3** P<0.001 compared to control. All experiments represent at least n=3 and densitometry analysis represents mean ± SD. Abbreviations: LA (linoleic acid), DHA (docosahexaenoic acid), AMPK (AMP-activated protein kinase).
2.5 Discussion

Since cancer cells display increased glycolytic activity, the 8th hallmark of cancer, the fate of cancer cell metabolism is primarily regulated by the HIF pathway, specifically hypoxia-inducible factor 1 (HIF-1), which has been postulated to be responsible for the metabolic reprogramming of cancer cells [12]. The metabolic reprogramming by the HIF pathway aids in the survival of cells undergoing extreme conditions [168], [169]. More specifically, the transition from oxidative to glycolytic metabolism in breast cancer is stabilized by the alpha isoform of HIF-1 (HIF-1α) [170]. Although the HIFs are highly regulated, increases in mutations often lead to HIF-1α accumulation, which have correlated with poor prognosis [171]. Recently, HIF-1α has been shown to be modified by peroxisome proliferator-activated receptor (PPAR) isoform α [172]. Several reports have demonstrated that docosahexaenoic acid (DHA) can modify PPAR levels of isoforms α [152], [173] and γ [174], [175]. However, the effect of n-3 PUFAs on HIF-1α expression is still not well characterized. The studies reported herein demonstrate for the first time that DHA, a dietary fatty acid, can alter the bioenergetic function in cancer cells, whereby the inhibition of the transcriptional activity of HIF-1α attenuates the metabolic reprogramming of cancer cells. Importantly, the DHA-induced changes in metabolism were matched in two metabolically distinct cancer cell lines with no observed changes in non-malignant cells (Figure 36).

The complex actions of n-3 PUFAs are numerous [16] and the effects reported here and by others in cancer prevention and carcinogenesis are positive [176]. Previously documented mechanisms of n-3 PUFAs have identified alterations at the transcriptional
level, specifically with peroxisome proliferator-activated receptors (PPARs). It has only been recently proposed that PPARα activation was shown to suppress HIF-1α signaling by increasing degradation of HIF-1α protein [172]. Although in this study significant decreases in HIF-1α protein levels were detected, the possibility of DHA inducing degradation of HIF-1α protein through activation of PPARα expression cannot be ruled out [152], [172]. However, unpublished data from our laboratory has found that DHA supplementation decreases HSP90 function, a molecular chaperone responsible for the proper folding of HIF-1α [177]. The observed decreases in HIF-1α protein levels may be through HSP90 complex dysfunction, resulting from large decreases of intracellular ATP levels, since depletion of ATP levels have been shown to disrupt HSP90 function for proper folding and stabilization of its client proteins [178].

Metabolic stress is caused from the imbalance of the AMP/ATP ratio, which correlates to total cellular energy available. Increases in AMP/ATP ratio, usually by environmental stresses, result in the activation of AMPKα through post-translational modification by phosphorylation at Thr172 [179]. The results reported herein demonstrate that DHA-induced reduction of ATP levels led to large increases in the phosphorylation levels of AMPKα at Thr172, which suggest that cancer cells are undergoing metabolic stress in response to DHA supplementation.

As noted previously, dietary fish oil has been shown to induce uncoupled respiration (proton leak) in colon cancer cells [151]. Other reports have shown increased mitochondrial damage and cytochrome c release leading to apoptosis [180], [181] through the accumulation of DHA into mitochondrial cardiolipin (CL) [182], [183]. Although significant changes in uncoupled respiration in the breast cancer lines examined
were not observed, significant decreases in mitochondrial bioenergetic function in combination with decreased glycolytic activity were.

Decreases in the extracellular pH of tumor cells have been shown to increase tumor invasion and metastasis into the acidic microenvironment [59]. Results presented here show DHA decreased LDH protein levels and lactate production suggesting a possible protective effect to the surrounding tissue and may allow DHA to be used as an anti-invasive therapy, a hypothesis that has not been previously described. These data as well as the metabolic data support the hypothesis that DHA coordinates integrated targeting of major signal-transduction pathways and metabolism in parallel (Figure 37).

Additionally, DHA has been shown to enhance efficacy in combination with chemotherapies [131], further analysis revealed that combination treatments with 2-DG and DHA enhanced metabolic injury to the cancer cells (unpublished data). These data represent a potential new innovative strategy of combination therapies to target cancer cell metabolism. Combination therapies employing 2-DG are currently underway. Taken together, this study identifies possible cancer prevention or anti-carcinogenic therapies targeting cancer cell metabolism through altered HIF-1α signaling and provide new important metabolic insights into how dietary DHA modifies breast cancer survival and progression.
Figure 36. Summary of DHA-induced alterations in cancer metabolism. DHA induces changes through HIF-1α decreasing downstream target protein levels. Consequently, the bioenergetic capacity, specific to glucose and mitochondrial metabolism, were dose-dependently inhibited in response to DHA treatment. Abbreviations: DHA (docosahexaenoic acid), HIF-1α (hypoxia-inducible factor 1α), GLUT 1 (glucose transporter 1), PK (pyruvate kinase), HK (hexokinase), LDH (lactate dehydrogenase).
DHA attenuates bioenergetic function and Warburg metabolism through HIF-1α. Proposed mechanism of the effect of DHA on malignant cells. DHA induces degradation of HIF-1α protein through PPARα or through hypothesized HSP90 deregulation (dashed line). DHA-induced downregulation of HIF-1α transcriptional activity leads to diminished bioenergetic function in breast cancer cells. Abbreviations: DHA (docosahexaenoic acid), PPARα (peroxisome proliferator-activated receptor α), HSP90 (heat shock protein 90), HIF-1α (hypoxia-inducible factor 1α), GLUT 1 (glucose transporter 1), PDK (pyruvate dehydrogenase kinase), LDH (lactate dehydrogenase), ATP (adenosine-5’-triphosphate).
2.6 Acknowledgements

This project was supported by the MCW Cancer Center Bioenergetics shared resource and Advancing a Healthier Wisconsin. All of the radioisotope experiments were made possible by Dr. Cynthia Mastick, who provided her laboratory and expertise to all experiments. We would also like to recognize and thank Eastern Star, the Women’s Auxiliary of the Veterans of Foreign Wars, and the Stout Foundation for their generous contributions and continuous support of this research.
Chapter 3 - Modification to Myc activity induces oxidative stress and apoptosis in subtype-specific breast cancer in response to docosahexaenoic acid

Michael Mouradian, Haviva Kobany, Christopher R. Douglas, Keith D. Kikawa, and Ronald S. Pardini

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3.1 Abstract

Dietary factors like long-chain fatty acids (LCFA) have been shown to change the activity and expression levels of potent oncogenes involved in cancer in cell culture and animal models. The present study evaluated whether Myc expression or activity is required for the induction of apoptosis in response to docosahexaenoic acid (DHA, C22:6) enrichment in breast cancer. Myc mRNA and protein levels were determined in vitro in the breast cancer BT-474 and MDA-MB-231 cell lines. qPCR results showed increases in Myc mRNA expression upon PUFA treatment in both BT-474 and MDA-MB-231 cancer lines. Protein analysis revealed no changes in total Myc expression, however total phosphorylation at Thr58 and Ser62 were significantly upregulated upon DHA supplementation in the BT-474 cell line. Conversely, Myc phosphorylation was downregulated in the MDA-MB-231 cell line following DHA supplementation. Significant changes in the transcriptional activity of Myc paralleled the same phosphorylation trend in each cell line, suggesting that Myc activity changes in response to DHA treatment. Combination of Myc siRNA knockdown experiments showed a reversal of apoptosis in the BT-474 cell line, which suggests that Myc activation is
required for DHA mediated apoptosis. Increases in apoptosis were also seen with MDA-MB-231 cell line; however knockdown experiments had no effect and suggests that Myc is not required for MDA-MB-231 cellular response to DHA-induced apoptosis. Evaluation of intracellular apoptotic indicators showed that cleaved PARP and caspase 3 were upregulated in response to DHA, confirming both proliferation and apoptosis experiments. Oxidative stress measurements were increased in both cell lines in response to DHA suggesting that DHA induced cellular apoptosis through an oxidative stress mechanism. Taken together, these data suggest that some of the anti-cancer properties of DHA are mediated by Myc activity, which provides opportunities for new treatment strategies combing DHA with current therapies.

### 3.2 Introduction

Breast cancer is the leading diagnosed cancer for women and is the second highest cause of cancer-related death, behind lung cancer, in the United States [27]. A major contributor to tumorigenesis and progression of this disease is myelocytomatosis oncogene (Myc). Myc is a potent protooncogene that transcriptionally regulates 15% of the genome and is found to be deregulated in approximately 70% of cancers, including breast cancer [86], [87]. Numerous studies have shown that Myc is a critical factor in breast tumorigenesis and progression [19], [92], [184].

Myc protein belongs to a family of transcription factors with basic/helix-loop-helix/ leucine zipper (bHLH/LZ) domain. Phosphorylation of Myc at Serine 62 and Threonine 58 induces binding between its required partners, Myc-associated factor X (Max) or Max dimerization protein (Mad) to E-boxes with the consensus core sequence
CACGTG or with noncanonical sequences. Myc phosphorylation activates regulation of pro-survival and pro-apoptotic pathways, as well as Myc ubiquitylation and proteasomal degradation [75], [79], [80]. Although, Myc has been under investigation for approximately 30 years, the regulation of Myc is still poorly understood. Heterogeneity of breast cancer adds to the complexity of Myc regulation and several signaling pathways, transcription factors, and other regulatory factors that facilitate the regulation of Myc transcription and protein stability (Figure 38) [76]. Studies have shown that downregulation of Myc impairs growth and induces cell cycle arrest in cancer cells, which makes Myc inhibition a good therapeutic target [82], [90], [93]. However, overexpression of Myc activity has also been shown to induce programmed cell death (PCD) and apoptosis (Figure 38) [94]. Moreover, Myc has proven to be a difficult molecular target of inhibition because of its ability to promote cell differentiation in a non-transcriptional manner through a cytoplasmic cleaved product called Myc-nick [185]. To add to the complexity, Myc targets modify effects on metabolism, proliferation, cell growth, differentiation and apoptosis [82], [83], [186]. Myc expression has also been shown to induce epithelial-to-mesenchymal transition (EMT) and increase metastasis [187].

Dietary lipids, like omega-3 (n-3) and omega-6 (n-6) polyunsaturated fatty acids (PUFAs), have been shown to modify several of the pathways involved in Myc deregulation shown in Figure 38 and increase the therapeutic index of current therapies [131], [188]. Epidemiological studies have found an inverse relationship between dietary n-3 PUFA intake, specifically docosahexaenoic acid (DHA, C22:6) and eicosapentaenoic acid (EPA, C20:5), and incidence of breast cancer, which is consistent with reduced
growth rates and/or inducing apoptosis in cancer cells [110], [121], [176]. Recent reports indicate a positive effect of n-3 PUFAs on Myc expression and regulation, however the mechanisms behind Myc activity and n-3 downregulation remain unclear [189], [190].

In this study, the effect of DHA on Myc activity in the human breast cancer cell lines BT-474 (Luminal B/HER2+) and MDA-MB-231 (triple-negative/Basal-like) was addressed. Interestingly, Myc expression and response to DHA was dependent on the cell-line-specific genotype, although both cell lines displayed reduced cellular proliferation and induced apoptosis in response to DHA treatment. These distinct effects by which DHA exerts, through modifying Myc activity, may contribute uniquely to current cancer therapies in the reduction of growth and induction of apoptosis in breast cancer.

**Figure 38.** Myc network in breast cancer adapted from Xu et al., 2010 [19]. Repressors of Myc, BRCA1 and TGF-β, are usually inactivated in breast cancer. Major signaling pathways like, Ras, Wnt, Notch, ER-α and EGFR/Her2 and loss of tumor suppressors increase activation of Myc expression and/or stability. Deregulation of Myc can promote tumor growth, invasion, and metastasis. Abbreviations: Myc (myelocytomatosis oncogene), BRCA1 (breast cancer susceptibility gene 1), TGF-β (transforming growth factor β), Ras (rat sarcoma), Wnt (wingless-type murine mammary tumor virus integration site family), ER-α (estrogen receptor α), EGFR (epidermal growth factor receptor), HER2 (Human epidermal growth factor receptor 2).
3.3 Materials & Methods

**Cell Lines & Reagents** – BT-474 mammary ductal carcinoma cells and MDA-MB-231 mammary adenocarcinoma cells were purchased from ATCC (Manassas, VA). BT-474 (Luminal B and Myc negative) and MDA-MB-231 (Basal-like/triple negative and Myc positive) were compared for all experiments. Myc siRNA was purchased from Qiagen (Germantown, MD), resuspended in H$_2$O and frozen at -20°C until use. Fatty acid methyl esters (FAME) (Sigma, St. Louis, MO) were dissolved in ethanol (EtOH), flushed with nitrogen gas, protected from light and stored at -20°C for no more than 60 days.

**Cell Culture** – BT-474 mammary ductal carcinoma cells were maintained in HybriCare (ATCC, Manassas, VA) supplemented with 10% FBS (Hyclone, Logan, UT). MDA-MB-231 mammary adenocarcinoma cells were maintained in RPMI-1640 (Thermo Scientific, Rochester, NY) supplemented with 10% FBS (Hyclone, Logan, UT). MCF-10A non-tumorigenic mammary epithelial cells were maintained in DMEM/F12 (Thermo Scientific, Rochester, NY) supplemented with 5% FBS (Hyclone, Logan, UT). Cells were grown as monolayers at 37°C in a humidified environment with 5% CO$_2$.

**Cell Viability Assays** – Cells were trypsinized and counted using trypan blue staining and a hemocytometer. Unstained cells were counted as viable.

**XTT Assay** – The Cell Proliferation Kit II from Roche (Indianapolis, IN) was used following the manufacture’s instruction. Briefly, an equal number of cells were seeded in 96-well plates before specific treatments. At the end of the incubation times,
XTT reagents (XTT labeling reagent and electron coupling reagent) were mixed and added to each well for 4-24 hours before being read at 450 nm (with a 650 nm reference wavelength) on a SpectraMax M5 machine (Molecular Devices, Sunnyvale, CA).

**siRNA Experiments and p-c-Myc Activation** – Equal numbers of cells were seeded and allowed to sit for 24 hours prior to transfection with Myc siRNA (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) with HiPerFect reagent (Qiagen, Valencia, CA) for an additional 24 hours. Either 100 µM of DHA, LA or an equal volume of EtOH was added and cells were incubated for 48 hours prior to collection for specified assays. H₂O₂ was used to activate phosphorylation of c-Myc because exposure to H₂O₂ has been reported to activate c-Myc phosphorylation at S62/T58, but does not change protein expression levels [191].

**Luciferase Assays** – Cells were transfected with 100 ng of Myc Cignal™ reporter construct along with a negative and positive control (Qiagen, Valencia, CA). After 6 hours of transfection, medium was changed with the addition of either 100 µM of DHA, LA or an equal volume of EtOH and cells were incubated for 24 hours. Cells were then treated with 250 µM H₂O₂ for 1 hour and then harvested for reporter gene assays. Dual Luciferase assay (Promega, Madison, WI) was performed following manufacturer protocol, and promoter activity values are expressed as relative units normalized to the XTT assay (Roche, Indianapolis, IN).
**Immunoblotting** – As described previously [127], cells were washed with ice-cold PBS and lysed using GTP-lysis buffer [50 mM HEPES (pH 7.5), 15 mM NaCl, 6 mM sodium deoxycholate, 1% NP-40, 10% glycerol, 10 mM MgCl₂, 1 mM EDTA] containing freshly added protease and phosphatase inhibitors. Tumors were homogenized in the same buffer. Samples were centrifuged at 16,000 × g for 10 min at 4°C. Supernatants were analyzed for protein concentration using BIO RAD’s DC assay (Hercules, CA). Samples were resolved by SDS-PAGE and transferred to either nitrocellulose or (Polyvinylidene fluoride) PVDF membranes (BIO RAD, Hercules, CA) and probed with specific antibodies. Detection was performed using HRP-conjugated secondary antibodies and visualized with ECL (GE Healthcare, Buckinghamshire, UK). Specific antibodies were purchased for total phospho-c-Myc (S62, T58) (1:1000), Mad (1:1000), Max (1:2000), Bax (1:1000), Cleaved PARP (1:2000), Caspase 3 (1:1000), β-actin (1:5000) (Abcam, Cambridge, MA), c-Myc (1:1000) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

**Annexin V-FITC Binding Assay** – Annexin V binding assays were performed using Calbiochem® (EMD Millipore Corporation, Billerica, MA) following manufacturer’s instruction. Briefly, conditioned media was retained to include any dead or dying cells that had become detached during treatment. 5x10⁵ cells were washed twice in PBS and pelleted by centrifugation at 1000 rpm. Samples were resuspended in 500 µL of conditioned media to analyze late apoptotic or necrotic cells that have detached and 500 µL of 1X binding buffer. 5 µL of Annexin V-FITC was added to 195 µL of cell suspension and incubated for 10 min at room temperature in the dark. Cells
were washed with 1X binding buffer and pelleted by centrifugation at 1000 rpm. The cell pellet was resuspended in 190 µL 1X binding buffer and 10 µL of propidium iodide (PI) was added. Apoptotic cells were identified by flow cytometry.

**Quantitative RT-PCR** – Cells were treated with either 100 µM of LA, DHA or an equal volume of EtOH for 48 hours. Total RNA was isolated and purified using RNeasy mini kit (Qiagen, Valencia, CA) following the manufacturer’s protocol. cDNA was then synthesized using cDNA Synthesis VILO kit (Invitrogen, Grand Island, NY). Quantities of cDNA were measured by quantitative real-time PCR on a CFX96 Real-Time PCR System (Bio-Rad, Hercules, CA). The reaction used SYBR green FAST master mix and conditions followed manufacture’s recommendations (Applied Biosystems, Carlsbad, CA). Published primer sets were used for cancer Myc - c-Myc [192] (Sense: TCAAGAGGTGCCACGTCTCC; Anti-sense: TCTTGGCAGCAGGATAGTCCTT) and β-actin [164] (Sense: CGTCTTCCCCTCCATCG; Anti-sense: CTCCTTAATGTCAAGC). The relative abundance of mRNA was determined by comparative Ct method [165]. Data represents at least n=3 independent experiments ± SD.

**Statistical Analysis** – All experimental results were independently repeated at least three times. All quantitative data shown represent the compiled data as percentages versus control treatments with error bars representing standard deviation and statistical analyses were performed using the Student’s t test and/or ANOVA with the Tukey
method for pairwise comparison on SAS® software with values of at least P<0.05 being considered significant.

3.4 Results

DHA treatment post-translationally modifies c-Myc and does not change protein levels

It has been well characterized that Myc activity is controlled through several factors [84]. Transcriptional activity of Myc is regulated by phosphorylation at Serine 62 (Ser62) and Threonine 58 (Thr58) and the fate of Myc is determined by the site at which is being post-translationally modified, however these post-translational mechanisms remain unclear [193–195]. To determine the effect of DHA on c-Myc activity, c-Myc phosphorylation status was assessed. Remarkably, observations of the phosphorylation status in the BT-474 cell line were different than the MDA-MB-231 cell line. Large increases in total phosphorylation state of c-Myc were observed in the BT-474 cell line, whereas decreases were seen in the MDA-MB-231 cell line in response to DHA supplementation (Figure 39A). Interestingly, total c-Myc protein levels did not change, suggesting that DHA does not induce the c-Myc ubiquitylation and proteasomal degradation pathway at Thr58, in both cell lines. Densitometry analysis showed that DHA significantly modified the phosphorylation levels of c-Myc in breast cancer cells (Figure 39B). Assessment of c-Myc specific isoform mRNA analysis revealed increased mRNA levels in both BT-474 and MDA-MB-231 cell lines, although these data are not consistent with protein levels (Table 11).
Figure 39. Changes in c-Myc phosphorylation status in response to DHA supplementation. (A) Immunoblot analysis of BT-474 and MDA-MB-231 cells that were treated with 100 µM DHA (n-3 PUFA), LA (n-6 PUFA) or an equal volume of EtOH for 48 hours assessing total c-Myc expression and c-Myc phosphorylation at S62/T58 combined. Total c-Myc and β-actin were used as loading controls. (B) Percentage densitometry analysis of p-S62/T58-c-Myc in BT-474 and MDA-MB-231 cell lines normalized to c-Myc and relative to control. + P<0.05 vs. indicated treatment. * P<0.01 vs. indicated treatment. ** P<0.001 vs. indicated treatment. All experiments represent at least n=3 and densitometry analysis represents mean ± SD. Abbreviations: LA (linoleic acid), DHA (docosahexaenoic acid), Myc (myelocytomatosis oncogene).
Table 11. **DHA increases c-Myc mRNA expression in cancer cells.** Treatment of 100 μM DHA (n-3 PUFA), LA (n-6 PUFA) or an equal volume of EtOH for 48 hours before mRNA isolation and purification. mRNA expression of c-Myc isoform status were assessed by quantitative RT-PCR relative to EtOH control and corrected for β-actin expression in each indicated cell line. The values are means ± SD of at least n=3 independent experiments. ** P<0.001 vs. control. Abbreviations: LA (linoleic acid), DHA (docosahexaenoic acid), Myc (myelocytomatosis oncogene).

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<th>Cell Line</th>
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<td>MDA-MB-231</td>
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**Changes to Myc transcriptional activity is mediated by DHA supplementation**

Phosphorylation of c-Myc at S62 has been reported to promote its association with its binding partner(s) to induce transcription on several target genes [196]. Since increased phosphorylation levels were observed, Myc transcriptional activity was assessed in BT-474 and MDA-MB-231 cells treated with DHA. Indeed, Myc transcriptional activation was directly associated with phosphorylation status in both cell lines (Figure 40). These data suggest that DHA directly modified Myc activity in cancer cells.
**Figure 40.** DHA differentially regulates Myc transcriptional activity in cancer cells. BT-474 and MDA-MB-231 cells were transfected with 100 ng of Myc Cignal™ reporter construct along with a negative and positive control. After 6 hours of transfection, medium was changed with the addition of 100 μM DHA (n-3 PUFA), LA (n-6 PUFA) or an equal volume of EtOH for 48 hours before harvesting the cells for reporter gene assays. Dual Luciferase assay was performed and promoter activity values are expressed as relative units normalized to XTT. The percentage relative luciferase activity is represented as mean ± SD. + P<0.05 vs. indicated treatment. ** P<0.001 vs. indicated treatment. Abbreviations: DHA (docosahexaenoic acid), H$_2$O$_2$ (hydrogen peroxide), Myc (myelocytomatosis oncogene).
DHA changes Myc transcriptional activity even in Myc knockdown cells

Myc siRNA knockdown experiments were conducted to evaluate the role of Myc protein levels in the effects induced by DHA. Myc siRNA concentrations were assessed in BT-474 and MDA-MB-231 cells by immunoblot analysis (Figure 41A). Based on the densitometry results, an optimal concentration of 25 nM Myc siRNA was selected to use in both BT-474 and MDA-MB-231 cell lines (Figure 41B). Remarkably, the transcriptional activity was still increased in the BT-474 cell line in response to DHA, even in the presence of Myc siRNA, suggesting that luciferase activity in the BT-474 cell line may not correlate with Myc transcriptional activity. siRNA knockdown or other isoforms of Myc or mutant Myc could be potentially be responsible for upregulated Myc luciferase activity (Figure 42). Additionally, Myc knockdown experiments in the MDA-MB-231 cell line showed a significant decrease in the control treatment (P<0.01), suggesting that Myc transcriptional activity is dependent on functional Myc protein. Moreover, confirming previous results, Myc transcriptional activity was significantly decreased in DHA-treated cells, although DHA did not increase the effect of c-Myc knockdown (Figure 42).
A. Optimization of Myc siRNA concentration. (A) Immunoblot analyses of BT-474 and MDA-MB-231 cells were treated with specified concentrations of Myc siRNA for 24 hours prior to the addition of 100 µM DHA (n-3 PUFA), LA (n-6 PUFA) or a control volume of EtOH for 48 hours assessing total c-Myc expression. β-actin was used as a loading control. (B) Percentage densitometry analysis of c-Myc in BT-474 and MDA-MB-231 cell lines normalized to β-actin and relative to control. All experiments represent at least n=3 and densitometry analysis represents mean ± SD. Abbreviations: Myc (myelocytomatosis oncogene), siRNA (small interfering RNA), EC (effective concentration).
Figure 42. Myc transcriptional activity assessment in response to Myc siRNA knockdown. BT-474 and MDA-MB-231 cells were transfected with 100 ng of Myc Cignal™ reporter construct along with a negative and positive control and 25 nM Myc siRNA. After 6 hours of transfection, medium was changed with the addition of 100 μM DHA (n-3 PUFA), LA (n-6 PUFA) or an equal volume of EtOH for 48 hours before harvesting the cells for reporter gene assays. Dual luciferase assay was performed and promoter activity values are expressed as relative units normalized to XTT. The percentage relative luciferase activity is represented as mean ± SD. * P<0.01 vs. indicated treatment. ** P<0.001 vs. indicated treatment. All experiments represent at least n=3. Abbreviations: DHA (docosahexaenoic acid), H2O2 (hydrogen peroxide), Myc (myelocytomatosis oncogene).
DHA supplementation induces a dose-dependent decrease in cellular proliferation in cancer cells

Myc is very a complex transcriptional regulator because it targets genes responsible for survival and death [87]. Upregulation of Myc has been shown to destabilize the mitochondria and induce cytochrome c release and apoptosis in cancer cells [197–199]. To determine the effects of DHA in breast cancer cells, cellular proliferation assays were assessed in vitro. Dose-dependent decreases in cell proliferation were seen with increasing concentrations of DHA in both BT-474 and MDA-MB-231 cell lines (Figure 43). Similarly, cell viability assessment of BT-474 and MDA-MB-231 cells in response to DHA treatment was performed and a DHA-induced dose-dependent decrease in cell viability of both cell lines was observed (data not shown).

To determine the role of Myc activity in response to PUFA supplementation in regulation of survival and proliferation Myc knockdown experiments were conducted. Myc siRNA knockdown treatments enhanced cellular proliferation upon PUFA treatment (Figure 44). LA and LA+Myc siRNA treatments significantly increased proliferation compared to control, P<0.05 and P<0.001, respectively. However, DHA and DHA+Myc siRNA treatments significantly decreased cell proliferation in the BT-474 cell line compared to control (P<0.001). Interestingly, Myc siRNA knockdown significantly reversed the effect of DHA treatment, which suggests that DHA mediates decreased BT-474 cellular proliferation in-part by a Myc-dependent mechanism. Although no changes were observed upon LA treatment in the MDA-MB-231 cell line, DHA treatment significantly decreased cell proliferation compared to control (P<0.001) (Figure 44). No phenotypic changes were observed in the Myc siRNA experiments, suggesting that c-
Myc depletion does not affect cell proliferation or survival in the MDA-MB-231 cell line, which confirms observations seen by others [191], [200].

**Figure 43.** DHA decreases cell proliferation in cancer cells. BT-474 and MDA-MB-231 cells were seeded for 24 hours prior to supplementation with concentrations of DHA (5, 10, 25, 50, 75, 90, 100, 125, and 150 μM) or an equal volume of EtOH for 48 hours. XTT (Roche, Indianapolis, IN) reagents were then mixed and added to each well for 4 – 24 hours before being read at 450 nm with a 650 nm reference wavelength on a SpectraMax M5 machine (Molecular Devices, Sunnyvale, CA). The percentage absorbance is represented as mean ± SD. All experiments represents at least n=3 independent experiments ± SD. Abbreviations: DHA (docosahexaenoic acid).
Figure 44. Myc siRNA knockdown partially rescues BT-474 cells, but Myc is not required for decreases in cell proliferation in the MDA-MB-231 cell line. BT-474, MDA-MB-231 and MCF-10A cells were seeded for 24 hours prior to transfection with 25 nM Myc siRNA for 24 hours. 100 µM DHA (n-3 PUFA), LA (n-6 PUFA) or a control volume of EtOH for 48 hours was added and XTT (Roche, Indianapolis, IN) reagents were then mixed and added to each well for 4 – 24 hours before being read at 450 nm with a 650 nm reference wavelength on a SpectraMax M5 machine (Molecular Devices, Sunnyvale, CA). The percentage absorbance is represented as mean ± SD. All experiments represents at least n=3 independent experiments ± SD. + P<0.05 vs. control. ** P<0.001 vs. control or indicated treatment. Abbreviations: MK (mock), NT (non-treated), LA (linoleic acid), DHA (docosahexaenoic acid), Myc (myelocytomatosis oncogene).
**DHA decreases cellular viability and increases apoptosis in breast cancer cells**

The relationship between PUFA treatment and Myc knockdown on the apoptotic response in breast cancer cells was evaluated with the Annexin V-FITC assay. Consistent with the decreased proliferation observed in the XTT experiments in the BT-474 cell line, DHA significantly decreased cell viability and increased both early and late apoptotic percent cell populations (P<0.001) compared to control (Figure 45). Moreover, Myc siRNA treatment significantly reversed the effect of DHA, supporting the hypothesis that DHA is exerting some of its anti-proliferative and pro-apoptotic effects through Myc.

DHA treatment in the MDA-MB-231 cell line revealed decreases in cell viability and increases in early apoptotic percent cell populations compared to control, similar to the observations seen in the BT-474 cell line (Figure 46). However, Myc siRNA treatments did not change the induction of apoptosis in response to DHA. Taken together these data suggest that DHA decreases cellular proliferation and increases apoptotic responses in breast cancer, however the role of Myc-mediated apoptosis is dependent on the genotype of the cell line.
**Figure 45.** DHA induces apoptosis in BT-474 cells. Annexin-V FITC (EMD Millipore Corporation, Billerica, MA) analysis of BT-474 cells that were treated with 100 μM of DHA (n-3 PUFA), LA (n-6 PUFA) or a control volume of EtOH with or without 25 nM Myc siRNA. Myc siRNA treatment was added 24 hours prior to 48 hours PUFA treatment or control. Data represents percentage population distribution in response to treatment. All experiments represent at least n=3 ± SD. **P<0.001 vs. indicated treatment. Abbreviations: LA (linoleic acid), DHA (docosahexaenoic acid), Myc (myelocytomatosis oncogene).
**Figure 46.** DHA induces apoptosis in MDA-MB-231 cells. Annexin-V FITC (EMD Millipore Corporation, Billerica, MA) analysis of MDA-MB-231 cells that were treated with 100 μM DHA (n-3 PUFA), LA (n-6 PUFA) or a control volume of EtOH with or without 25 nM Myc siRNA. Myc siRNA treatment was added 24 hours prior to 48 hours PUFA treatment or control. Data represents percentage population distribution in response to treatment. All experiments represent at least n=3 ± SD. * P<0.01 vs. control. ** P<0.001 vs. control. Abbreviations: LA (linoleic acid), DHA (docosahexaenoic acid), Myc (myelocytomatosis oncogene).
Induced oxidative stress and apoptotic pathways by DHA treatment in breast cancer

Since DHA has been shown to induce oxidative damage and mediate cell death in cancer cells [16], oxidative stress was assessed in BT-474MDA-MB-231 cell lines following PUFA treatment. DHA treatment significantly induced oxidative stress in both cancer cell lines (Figure 47). Interestingly, Myc siRNA knockdown experiments in the MDA-MB-231 cell line resulted in an increase in oxidative stress in response to PUFA treatment, which shows that the combination treatment of PUFA and Myc siRNA increased the effect of oxidative stress compared to either treatment alone.

Apoptotic markers were assessed in response to Myc knockdown and PUFA treatment by immunoblot analysis. In both cancer cell lines, DHA treatment increased cleaved poly (ADP-ribose) polymerase (PARP), a marker of programmed cell death, and caspase 3, a marker of cellular apoptosis (Figure 48 and 49). Interestingly, cleaved PARP and caspase 3 expression levels were decreased in response to Myc siRNA treatment in the BT-474 cell line, which again confirms that the pro-apoptotic effects observed in response to DHA treatment may be partially dependent on Myc.

Myc-induced apoptosis has been shown to have a specific requirement for Bcl-2-associated X protein (Bax) [14], [96], [98]. Indeed, Bax expression in both cancer cell lines was increased in response to DHA treatment, suggesting that DHA induces a mitochondrial apoptotic response in breast cancer (Figure 48 and 49). Furthermore, Myc siRNA knockdown in the BT-474 cell line partially decreased Bax expression levels in response to DHA, which suggests that Bax-mediated apoptosis may be partly controlled
by c-Myc activity (Figure 48). No decrease in Bax levels were observed in the MDA-MB-231 cells treated Myc siRNA (Figure 49).

In addition, Myc-associated factor X (Max) and Max dimerization protein (Mad) expression levels were not changed in the BT-474 cell line in response to PUFA treatment (Figure 48). However, Max expression was decreased in the MDA-MB-231 cell line in response to DHA treatment, suggesting another possible explanation to DHA-induced changes in the Myc pathway (Figure 49). Altogether, these data suggest that although there are two different DHA-induced responses on Myc, the end result is the same causing decreased cell viability and increased cellular apoptosis.
Figure 47. DHA increases oxidative stress in breast cancer cells. (A) BT-474 and MDA-MB-231 cells were seeded into 6-well plates and treated with 100 μM DHA (n-3 PUFA), LA (n-6 PUFA) or an equal volume of EtOH with or without the addition of 25 nM Myc siRNA. After treatment, cells were incubated with DCF and fluorescence was measured on a SpectraMax M5 (Molecular Devices, Sunnyvale, CA). Excitation was set at 485 nm and emission was set at 530 nm. The percentage relative to DCF represents mean ± SD. All experiments were done in triplicate and represent at least n=3. + P<0.05 vs. control or indicated treatment. * P<0.01 vs. control. ** P<0.001 vs. control. Abbreviations: LA (linoleic acid), DHA (docosahexaenoic acid), Myc (myelocytomatosis oncogene).
Figure 48. Apoptotic induction with DHA supplementation in BT-474 cell line. Immunoblot analysis of BT-474 cells that were treated with 25 nM Myc siRNA for 24 hours prior to the addition of 100 µM DHA (n-3 PUFA), LA (n-6 PUFA) or an equal volume of EtOH for 48 hours assessing total c-Myc expression. β-actin was used as a loading control. All experiments represent at least n=3. Abbreviations: LA (linoleic acid), DHA (docosahexaenoic acid), Max (Myc-associated factor X), Mad (Max dimerization protein), Bax (Bcl-2-associated X protein), PARP (poly (ADP-ribose) polymerase), Myc (myelocytomatosis oncogene).
### MDA-MB-231

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**Figure 49.** Apoptotic induction with DHA supplementation in MDA-MB-231 cell line. Immunoblot analysis of MDA-MB-231 cells that were treated with 25 nM Myc siRNA for 24 hours prior to the addition of 100 µM DHA (n-3 PUFA), LA (n-6 PUFA) or an equal volume of EtOH for 48 hours assessing total c-Myc expression. β-actin was used as a loading control. All experiments represent at least n=3. Abbreviations: LA (linoleic acid), DHA (docosahexaenoic acid), Max (Myc-associated factor X), Mad (Max dimerization protein), Bax (Bcl-2-associated X protein), PARP (poly (ADP-ribose) polymerase), Myc (myelocytomatosis oncogene).
3.5 Discussion

Several cancer cells with deregulated Myc are associated with poor prognosis [19]. Deregulated Myc has shown to promote growth, cell proliferation, differentiation, apoptosis and altered metabolism [82], [83], [186]. Indeed, overexpression of Myc in the MDA-MB-231 cell line has been shown to promote invasion and metastasis into lung and brain [201]. However, overexpression of Myc has also been shown to induce apoptosis and suppression of metastasis [83], [198], [199], [201]. The cell lines reported herein display this apparent anomaly. The effects of DHA in the BT-474 cell line substantially increased total Myc phosphorylation at S62/T58 and transcriptional activity (Figure 39 and Figure 40, respectively). Whereas, in the MDA-MB-231 cell line the reverse response was observed, decreasing Myc total phosphorylation as well as Myc transcriptional activity (Figure 39 and Figure 40, respectively).

DHA has been shown to alter the transcriptional activities of key transcriptional factors that regulate inflammation, adhesion and metabolism, such as peroxisome proliferator-activated receptor (PPAR) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) [16]. Unexpectedly, DHA altered the transcriptional activity in BT-474 cells even in the presence of a Myc siRNA (Figure 42). However, Myc knockdown experiments in the BT-474 cell line did not correlate to a decrease in Myc transcriptional activity in control or DHA treatments. Recent evidence has indicated a possible feedback loop between Myc activity/translocation and certain miRNAs [202]. Unexpectedly, observations in the BT-474 cell line suggest that Myc activity may be mediated by or through a specific miRNA pattern that could be responsible for the differential effect on Myc transcriptional activity and gene expression. Alternatively,
DHA-induced activation of Myc transcriptional activity could be driven by another isoform or a mutant Myc in the BT-474 cell line and further assessment is needed. However, to our knowledge, this is the first time that DHA has been reported to alter Myc transcriptional activity in cancer cells. The effect of DHA on Myc transcriptional activity will have to be further analyzed by chromatin immunoprecipitation (ChIP) assays to determine Myc translocation and promoter-specific regulation in response to DHA. Furthermore, a more specific inducer of Myc, other than H$_2$O$_2$, in the MDA-MB-231 cell line may be needed because H$_2$O$_2$ could be inducing other non-specific cellular effects. Moreover, the involvement between the association of Myc dimerization partners, Max and Mad, upon DHA supplementation is still unclear. Co-immunoprecipitation analyses of these associations, as well as phosphorylation analyses of Myc at the individual S62 and T58 sites are currently underway. However, the data reported herein show a functional consequence on Myc activity with DHA treatment that supports current studies [14], [98], [201], [203].

Although the changes in Myc transcriptional activity did not correlate with Myc protein phosphorylation in Myc knockdown experiments, the DHA-induced changes in cell proliferation and apoptosis were reversed with Myc siRNA in the BT-474 cell line, demonstrating that decreases in Myc protein levels were sufficient in reversing the DHA-induced effects in cell proliferation and apoptosis. Although the same trend was not seen with the MDA-MB-231 cell line (Figure 46), no changes were seen in cell proliferation and apoptosis with Myc siRNA in the control or DHA treated cells. These data suggest that Myc may not be required for cell proliferation or apoptosis in the MDA-MB-231 cell
line. Nonetheless, DHA treatment significantly decreased cell proliferation and cell viability and increased early apoptosis populations in both cancer cell lines.

The anti-proliferative effects by DHA in cancer cells have been extensively studied [16]. The results demonstrated here, show a dose-dependent decrease in cell viability and cellular proliferation in response to DHA (Figure 43). The decreases in cellular proliferation in response to DHA supplementation was confirmed in Myc knockdown experiments as well (Figure 44). In contrast, the n-6 PUFA LA, demonstrated an opposite response in the phosphorylation status of Myc. Reports have shown that n-6 PUFAs may enhance tumorigenic progression and metastasis [204–206]. Unpublished data from our laboratory have demonstrated increases in cell proliferation and invasion upon LA supplementation. The data demonstrated here may point to another possible mechanism of tumor progression by n-6 PUFAs.

PUFAs have been shown to decrease cell viability through increasing lipid peroxidation products and increasing oxidative stress in cancer cells. Induction of oxidative stress and increases in lipid peroxidation products with DHA treatment have been shown to be partially reversed with Vitamin E (VE) [207]. Indeed DHA treatment demonstrated significant increases in oxidative stress in both cell lines, suggesting that DHA may be inducing apoptosis and decreased cell proliferation through increases in oxidative stress (Figure 47). In MDA-MB-231, Myc siRNA silencing resulted in increases in oxidative stress induced by DHA, which suggests that Myc may be required for oxidative stress in the MDA-MB-231 cell line. These data support findings that Myc is required for cellular responses to oxidative stress by inducing glutathione (GSH) biosynthesis [85]. Previous research from our laboratory showed decreased GSH:GSSG
ratio in response to dietary fish oil intake in mammary carcinoma (MX-1) cells xenografted into mice [208]. Analysis of GSH, GSH:GSSH ratio, glutathione peroxidase (GPx), and glutathione S-transferase (GST) are currently underway to further elucidate this hypothesis. Both cell lines seem to be dependent on the expression levels and activity of Myc, however Myc may not be required for cellular proliferation and induction of apoptosis in the MDA-MB-231 cell line. The results presented here demonstrate paradoxical effects induced by DHA, although the end result was the same for both cell lines. DHA treatment in the BT-474 cells induced Myc activity, thereby increasing Myc-mediated pro-apoptotic responses. DHA treatment in the MDA-MB-231 cell line reduced Myc activity and decreased pro-survival pathways possibly through GSH biosynthesis and increases in oxidative stress. These results implicate Myc in the survival and progression of breast cancers and demonstrate that Myc is a difficult therapeutic target (Figure 50). The movement for more personalized medicine and individualized genotypic analysis is necessary to elucidate the molecular effects of Myc activity. However, dietary factors like PUFAs may enhance sensitivity and efficacy of current therapies [131], [188]. Overall, these data provide justification and rationale for evaluation of current therapies in combination with DHA for the treatment of cancer.
Figure 50. DHA differentially regulates Myc activity in breast cancer cells. Proposed mechanism of the effect of DHA on malignant cells. DHA treatment induces Myc activation and decreases survival through decreasing proliferation and increasing oxidative stress and apoptosis in the BT-474 cell line. In the MDA-MB-231 cell line, DHA inhibits Myc activity and decreases survival through decreasing proliferation and GSH biosynthesis, ultimately increasing oxidative stress and apoptosis. Abbreviations: DHA (docosahexaenoic acid), Myc (myelocytomatosis viral-related oncogene).

3.6 Acknowledgements

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Chapter 4 – Conclusions and Outlook

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4.1 Dissertation summary and outlook

The objective of this thesis was to identify mechanisms by which n-3 and n-6 PUFAs affect mammary carcinoma survival and progression. This was achieved through studying the bioenergetic signatures of the cancer cell lines BT-474, MDA-MB-231 and the non-transformed MCF-10A cell line. Using a comprehensive multi-experimental approach, with the combination of radiotracer experiments and integrated cellular metabolic flux assays, data sets were generated and used to characterize the metabolic phenotypic changes in breast cancer in response to n-3 and n-6 PUFAs. Other analytical approaches were carried out to determine responses in Myc oncogenic activity in assessing the effects of n-3 and n-6 PUFAs in breast cancer survival and progression. Comprehensive analysis of these biological systems was necessary to gain insight into the abundance of interactions cancer cells display.

In Chapter 1, an introduction to the thesis was presented discussing the diverse systems cancer cells display. It was clear that analysis of the effects of PUFA treatment in these systems was necessary and the mechanisms involved were unclear. The work contained in Chapter 2 proposed novel insights into negative regulation of cancer cell metabolism by n-3 PUFAs through the attenuation of HIF-1α protein transcriptional
activity. Metabolic profiling of each cell line provided novel opportunities to measure real-time respiration, which lead to the finding that the BT-474 and the MDA-MB-231 cell lines were metabolically distinct. However, regardless of the metabolic phenotype displayed by cancer cells, surprisingly DHA concomitantly decreased glycolytic and oxidative metabolism in a dose-dependent fashion. Moreover, the marked decrease in metabolism lead to increases in metabolic stress and cell death. All of these data confirm scattered reports of the involvement of DHA in metabolism and stress [151], [153], [209], [210]. Current laboratory studies, not included here, have identified other targets of glycolytic inhibition by DHA, such as HKII and PFK1. Furthermore, metabolic targeting by DHA in combination with current therapies, like 2-DG, are currently being evaluated for therapeutic potential as well.

The effects of n-3 and n-6 PUFAs on Myc activity and oxidative stress were reviewed in Chapter 3. Unexpectedly, opposite effects on Myc activity were observed in the BT-474 and MDA-MB-231 cell lines. It has been well established that Myc stability and overexpression usually correlate with high malignancy. However, in the case of the BT-474 cell line, DHA-induced increases in total Myc phosphorylation and transcriptional activity related to increases in Bax-mediated apoptosis. Interestingly, Myc knockdown experiments reversed the effects by DHA in the BT-474 cell line, suggesting that DHA is inducing cellular apoptosis in the BT-474 cell line through a Myc-dependent mechanism.

Specific subtype characterization MDA-MB-231 cell line was needed in order to define the observations reported in Chapter 3. Indeed the basal-like MDA-MB-231 cells
had decreased cell viability and proliferation in response to DHA supplementation, like the Luminal B, BT-474 cell line. Increases in oxidative stress and apoptosis were also observed in response to treatment of DHA in both cell lines, which suggests that Myc activity contributes to cellular responses for survival and progression. Further studies are currently underway to determine Myc involvement in GSH in response to DHA treatment in both cell lines with the hypothesis that DHA may be decreasing oxidative stress control in cancer cells at least in part through a Myc-dependent mechanism.

We were able to demonstrate for the first time a metabolic consequence of DHA incorporation through the attenuation of HIF-1α and cancer cell metabolism with subsequent increases in metabolic stress. It was also shown that DHA reduced cancer cell growth, increased oxidative stress, and induced apoptosis in cancer cells specifically. The results reported herein are summarized in Figures 51 and 52. Although new therapeutic strategies are constantly being developed, the current standard of care for breast cancer treatment is extremely harmful to the patients. Hopefully, the work presented herein can provide rationale for further clinical testing of DHA. Although the effects of DHA alone may not cure cancer, therapies that can be combined with DHA to work together synergistically, may.
Figure 51. The effect of DHA on HIF-1α-associated metabolism and Myc-mediated survival and progression adapted from Keijer et al., 2011 [20]. DHA inhibits several mechanisms indicated by the red lines, resulting in metabolic stress, oxidative damage and apoptosis in cancer cells.
Figure 52. Proposed model on the effect of DHA on cancer cells adapted from Gordan et al., 2007 [15]. Blue indicates HIF targets. Red indicates Myc targets. Green indicates both HIF and Myc targets. HIF and Myc network in order to stabilize the malignant state. DHA inhibits several aspects of metabolism, redox activity, and Myc function. Altogether, these data support the notion that DHA inhibits mammary carcinogenesis survival and progression.
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